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Taurine 11

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Preface

The 21st International Taurine Meeting was held from May 20 to 26, 2018, in Liaoning Province, China. Unique to this taurine meeting, the first half of the conference was held in the historical, 2600-year-old, industrial city of Shenyang and the second half in the modern, coastal city of Dalian. The participants of the meeting, who came from 14 different countries, were exposed to the rich culture, ancient history, and stunning beauty of this special region of China. The meeting featured approximately 100 presentations covering 8 different taurine-related topics (metabolism, nutrition, organ dysfunction, heart health, cancer, antioxidation and antimicrobial activities, neuroprotection, and anti-inflammation). The aims of the meeting were to spur interest in recent advances in the taurine field and to provide impetus for further research and growth. Novel findings reported during the meeting supported new actions for taurine, including antianxiety and antitumor activity, modulation of miRNA activity, promotion of thioredoxin-interacting protein expression, potential use as a laxative, and potential benefit against immunosuppression. Also, the identity of novel taurine containing derivatives with unique or undiscovered activities opened up promising new avenues to study taurine.

Several presentations during the meeting focused on the importance of taurine in minimizing the severity of various diseases, including alcohol liver disease, fragile X, dementia, Down syndrome, diabetes, neurodegenerative diseases, and heart disease. Although taurine was approved several years ago for the treatment of congestive heart failure, we await further research showing that taurine possesses clinical value as a therapeutic agent in the treatment of several other diseases.

Another important application of taurine is its inclusion in nutritional supplements. Because endurance exercise can damage muscle, protein and nutritional supplementation have been commonly utilized to improve the outcome of exercise training. One of the presentations during the meeting focused on the effect of chronic taurine treatment on muscle mass and physical performance in subjects receiving exercise training. Taurine supplementation was also found to improve cognitive function in elderly women with dementia, a finding that may prove useful in treating intractable diseases, such as Alzheimer's disease. Because taurine plays a fundamental role within cells, perinatal taurine supplementation is important for

the development of normal cellular and organ function, proper cellular differentiation, and cell viability.

One of the hosts of the meeting was the Shenyang Agricultural University. It was therefore timely that one of the presentations discussed a potential use of taurine in agriculture. According to the Department for Environment, Food, and Rural Affairs in London, England, heat stress is a major cause of reduced production and actual loss of broilers during summer months. A study presented in the meeting reported that taurine treatment protects broilers against the adverse effects of heat stress. In future meetings, we await further studies about potential uses of taurine in aiding the poultry industry. Additional studies should also clarify the value of taurine and taurine derivatives from seafood sources in nutritional supplements.

We are deeply indebted to the sponsors of this meeting for their generous support. These include Red Bull GmbH4, Austria and Europe; Dong-A Group, Korea;Suzhou Dong-A Beverage Corp, China (苏州东亚饮料有限公司, 中国);Becton, Dickinson, and Company, USA; Shenyang Dequan VS Leica, China (沈阳德泉仪器设备销售有限公司, 中国); and Chengdu Taimeng Software Co., Ltd, China (成都泰盟软件有限公司, 中国). We would also like to thank all the participants of the meeting and the authors contributing to this volume.

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Contents

Part II Taurine and Nutrition

Part I
Taurine and Metabolism

Effect of Aging on Taurine Transporter (TauT) Expression in the Mouse Brain Cortex

Manoj Kumar Neog, Hyunju Chung, Min Joo Jang, Dong Jin Kim, Sang Ho Lee, and Kyoung Soo Kim

Abstract Taurine content in an older brain is decreased compared to a younger brain and is associated with cognitive deficits. It is not yet known whether the decrease in taurine content is associated with decreased expression of taurine inflow mediating transporters during the aging process. In this study, we investigated whether aging affects taurine transporter and glycine transporter 1 expression in the brain cortex of the mouse. Taurine and glycine transporter expression was compared in the brain cortex of C57BL/6 mice at different ages (2, 12, and 24 months) and to age-matched NLRP3 inflammasome knockout mice. In wild type mice, taurine transporter (TauT) expression in the brain cortex of 12- or 24-month-old mice did not significantly differ from TauT expression in 2-month-old mice. Moreover, TauT expression in the brain cortex of 12- or 24-month-old mice did not significantly differ from age-matched NLRP3 KO mice. This result indirectly suggests that TauT expression may be not affected by aging or age-induced inflammation. In addition, glycine transporter expression was similar to the TauT expression pattern. In conclusion, aging and age-related inflammation might not significantly affect taurine

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and glycine transporter expression in aged mice. Thus, the decrease of taurine content in an older brain, which is associated with cognitive deficits, may not be significantly related to altered taurine and glycine transporter expression.

Keywords Taurine Transporter (TauT) · Glycine Transporter (GLYT) · NLRP3 · Age-related diseases

1 Introduction

Aging is an obligatory process commonly described as the aggregation of various inimical changes within cells and tissues that leads to disease manifestation and eventually death (Tosato et al. 2007). Aging is often synonymous with muscle deterioration, disease (for example, cardiovascular disease, stroke, cancer, and dementia), and mortality (Hayflick 2000a; Tosato et al. 2007). Even potential cures for age-related diseases can only lead to a 15-year extension in human life expectancy (Hayflick 2000a, b). Consequently, aging is the basis for human mortality.

Cognitive impairment or postoperative cognitive dysfunction (POCD) results in multiple disabilities in elderly individuals, which present major challenges for the individual and their family. Lack of concentration and attention, as well as memory loss, in an older individual has been associated with "inflammaging" of the central nervous system (Harada et al. 2013). Although aging has never been defined as a disease, many studies have attempted to provide insight into its etiology (Harada et al. 2013; Hayflick 2000a). The respective theories include the free radical theory, the immunologic theory, the inflammation theory, and mitochondrial theory (Beckman and Ames 1998; Franceschi et al. 2000; Harman 1972). However, aging occurrences associated with a single cause or theory have been contradictory and obscure in many aspects. Therefore, aging is considered a synergistic effect of the above-mentioned theories.

Cognitive deficiency in older individuals has been dictated by the chronic inflammatory status of microglia in the brain (Wang et al. 2018). Thus, ablation of NLRP3-mediated inflammation insulates cognitive function from age-related inflammation, such as in Alzheimer's disease (Wang et al. 2018). In addition, genetic depletion of glycine transporter 1 (GLYT1) in forebrain neurons facilitates multiple pro-cognitive phenotypes (Zafra and Gimenez 2008). Glycine is a well known inhibitory neurotransmitter that is associated with coordination of the reflex response and with pain sensation. GLYT1 is thermodynamically coupled to sodium ions and maintains a transmembrane gradient of glycine. An efflux of sodium ions leads to glycine release from the synaptic cleft and normal GLYT1 activity. It has been posited that the glycine released during this process participates in the activation of NMDARs (N-methyl-D-aspartate receptors) and GlyRs (glycine receptors) (Dubroqua et al. 2010; Zafra and Gimenez 2008).

Moreover, studies have shown that taurine, a free sulfur amino acid, is decreased in the brain of an older individual compared to the developing brain and may have a vital role in brain development (Gebara et al. 2015). Also, chronic administration of taurine to an Alzheimer's mouse model reduces anxiety and depression and enhances hippocampus-dependent learning and retention (Kim et al. 2014; Wenting et al. 2014). Notably, taurine does not undergo metabolization or incorporate into protein synthesis; taurine remains free within the cell and serves as an "idiogenic osmolyte" in the brain where taurine transporter regulates the concentration of taurine via blood-brain barrier crossover (Benrabh et al. 1995). A taurine transporter knockout mouse model presented with a small brain, progressive retinal degeneration, and shrunken kidneys (Pow et al. 2002). Studies have speculated that taurine release and uptake represents one of the communication systems between neurons and glial cells (Gebara et al. 2015; Kim et al. 2014; Toyoda et al. 2015). Moreover, taurine deficiency in older adults has been found to be associated with brain disorders, which include Alzheimer's and Parkinson diseases, and suggests a role as a potential contributor to disease and a possible target for treatment (Kim et al. 2014; Zhang et al. 2016a). However, the mechanism by which taurine increases procognitive abilities is not known. Specifically, it is not known whether a decrease in taurine content is associated with a decrease in expression of the taurine inflow mediating transporters during the aging process.

2 Material and Methods

2.1 Animals

Two-month, 1-year, and 2-year-old C57BL/6 mice were purchased from Orient Bio and housed in a specific pathogen-free (SPF) facility with a 12 h light/dark cycle. Mice were given ad libitum access to food and water until sacrifice. NLRP3 knockout mice were provided by Dr. Lee Sang Ho from Kyung Hee University Hospital at Gangdong.

2.2 Mice Brain Cortexes

Brain cortexes were harvested from sacrificed mice, instantly frozen in liquid nitrogen, and kept at −80 °C until analysis. Total RNA was extracted from the brain cortex using Trizol (Thermo Fisher Scientific Korea, Seoul). Total protein from the brain cortex was extracted with homogenization in protein lysis buffer (PRO-PREP, iNTRON BIOTECHNOLOGY, Sungnam, Kyungki, Korea).

2.3 Quantitative Real-Time RT-PCR

cDNA was synthesized from RNA using a commercial cDNA synthesis kit (Thermo Fisher Scientific Korea, Seoul) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using an Applied Biosystem™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with the primer sequences listed in Table 1. The quantity of mRNA of the target gene was calculated using the ΔΔCt method and was normalized to 18S rRNA as an internal control.

2.4 Western Blotting

Samples were separated using 12% SDS-PAGE and were then transferred to Hybond-ECL (enhanced chemiluminescence) membranes (Amersham, now part of GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were first blocked with 6% nonfat milk dissolved in TBST buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20). The membranes were then probed with various rabbit polyclonal antibodies against taurine transporter (Bioworld Technology, Inc., Minneapolis, MN) and β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX). The antibodies were diluted 1:1000 in Tris-buffered saline at 4 °C overnight and incubated with 1:1000 dilutions of goat anti-rabbit IgG secondary antibody or anti-mouse IgG secondary antibody coupled with horseradish peroxidase. The blots were developed using the ECL method (GE Healthcare).

2.5 Statistical Analysis

Experimental data are expressed as mean ± standard error of the mean (SEM). Differences between three groups were analyzed using the nonparametric Kruskal-Wallis test. If a statistical difference was detected ($p < 0.05$), post-hoc pairwise group comparisons were performed using Dunn's test with Bonferroni multiple-testing correction (Dunn 1964). Differences between two groups was compared by the Mann-Whitney test. Prism software v.5 (Graphpad Software, San Diego, CA)

Table 1 Primer sequences

Taurine transporter	Forward	GCGTTTCCCGTACCTCTGC
	Backward	ATGGATGCGTAGCCAATGCC
Glycine transporter1	Forward	CCCACAATGGGACAAGATCC
	Backward	CCGGTAGCAGTTGTTGTGAA
18s RNA	Forward	GTAACCCGTTGAACCCCATT
	Backward	CCATCCAATCGGTAGTAGCG

was used for statistical analysis and graphing. Differences were considered statistically significant at $P < 0.05$.

3 Results

To determine if aging affects taurine transporter (TauT) expression in the mouse brain cortex, brain cortexes from C57BL/6 mice of three different ages (2, 12, and 24 months) were used. As shown in Fig. 1a, the taurine transporter (TauT) and glycine transporter 1 transcriptional levels in the brain cortex of 12-month-old mice were slightly higher than the levels in 2-month-old mice, although the differences were not significant. In addition, the transcriptional levels in 12-month-old mice were compared to the levels in 24-month-old mice. Expression levels in the 12-month-old mice did not differ from the levels in 24-month-old mice. In addition, the glycine transporter 1 expression pattern was similar to the TauT expression pattern. The decreased transcriptional level at 12 months was slightly recovered at 24 months. The protein level of TauT was also determined (Fig. 1b). Consistent with the TauT transcription result, protein levels did not change with age. These results suggest that taurine and glycine transporter expression is not affected by aging.

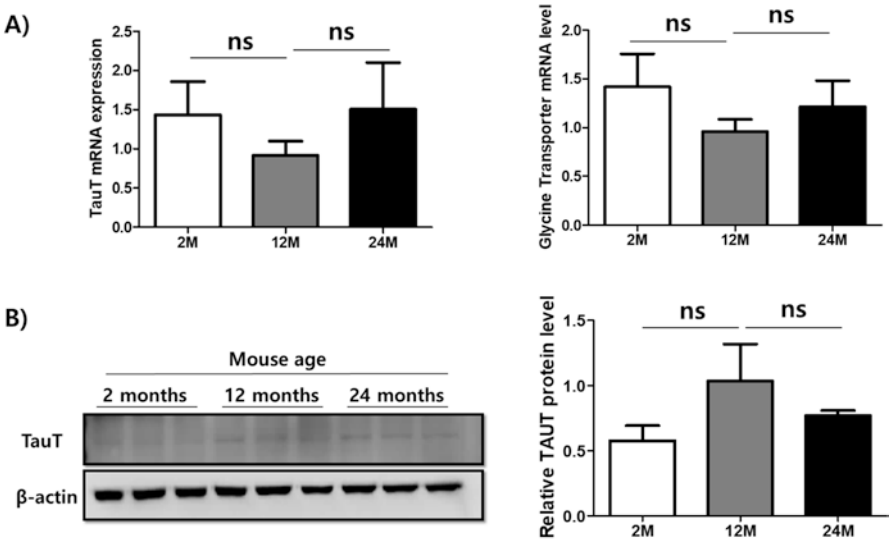

Fig. 1 Effect of aging on taurine and glycine transporter expression in the mouse brain cortex. Total RNA was extracted from brain cortexes of 2-, 12-, and 24-month-old mice. (**a**) Taurine and glycine transporter transcriptional expression levels were compared in the age groups. Five mice of each age group were used. (**b**) The protein level of taurine transporter (TauT) from each age was compared by Western blot. The intensity of band was measured by imageJ program. *ns* not significant

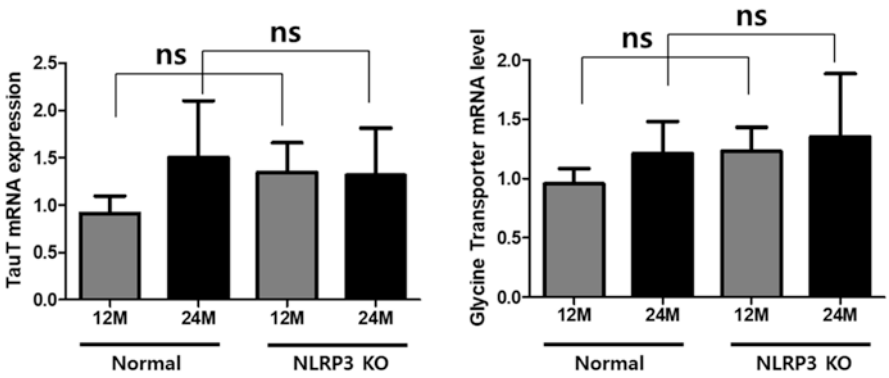

Fig. 2 Effect of age-induced inflammation on taurine and glycine transporter expression. The NLRP3 knockout mouse is IL-1β deficient. The transcriptional levels of the transporters from wild type mice at 12 and 24 months were compared to those of NLRP3 knockout (KO) mice. Five mice of each age group were used. *ns* not significant

To evaluate whether increased inflammation during aging affects the transporter expression, gene expression levels were determined in age-matched NLPR3 knockout mice, which are deficient for IL-1β (Fig. 2). Taurine and glycine transporter expression levels in normal mouse did not differ from the expression levels in NLRP3 knockout mouse. This result indirectly suggests that increased inflammation during aging may not affect the expression of these two transporters.

4 Discussion

Taurine, a free amino acid, is one of the most abundant amino acids and has been associated with fundamental biological and physiological functions (Ferguson and Audesirk 1990). It has been broadly evaluated as a prospective therapeutic agent and is a potential contributor to disease pathophysiology. When evidence had been found that taurine concentration was decreased in an older brain compared to developing brains, researchers have been keen to understand the underlying mechanism (Gebara et al. 2015; Kim et al. 2014; Toyoda et al. 2015). Consequently, taurine transporter knockout mice presented with a small brain, degenerated retina, and shrunken kidneys. Taurine deficiency arrested normal neuronal development, resulting in weakened brain growth. In addition, human studies have shown that Parkinson and Alzheimer's patients have diminished taurine level in the plasma (Kim et al. 2014; Wenting et al. 2014; Zhang et al. 2016a). Interestingly, damaged neurites due to chemical stress and toxins in older individuals were found to be restored after taurine supplementation. These neurites enhance electrical impulse flow, which correspondingly supports memory, cognition, emotions, and thinking (Ferguson and Audesirk 1990).

Cognitive deficits are often proportionally associated with the elderly population: cognitive abilities decline as a person ages (Ferguson and Audesirk 1990). Although the exact mechanism for this phenomenon has not been determined, the decrease in taurine in elderly individuals may underlie cognitive impairment. Taurine is abundant in the brain of young individuals and is reported to increase adult neurogenesis. Moreover, it has been reported that isoflurane-induced cognitive impairment was retrogressed upon pre-treatment with taurine in aged rats (Zhang et al. 2016b). Although the decreased taurine content was suggested to be associated with the increase in cognitive deficits during the process of ageing, it has become imperative to understand the exact cause of taurine decline in older individuals.

The level of taurine (Tau) in mouse brain seems to be primarily determined by taurine transporter (TauT) and taurine synthesizing enzymes. It is known that some species of mammalian brain do have the limited capacity to synthesize Tau by a specific enzyme called L-cysteic and cysteine sulfinic acids decarboxylase (CADCase/CSADCase) (Wu 1982). In this study, it would be highly desirable to check the level of CSAD/CAD in the brain at different ages in addition to TauT. However, we could not check it at this experiment. The decrease of Tau level in the aged animal brain could be partially due to decrease in the expression of Tau synthesizing enzymes, CADCase/CSADCase, resulting in decreased level of Tau. However, studies are lacking that explain whether a decrease in taurine is associated with decreased expression of the taurine transporter (TauT) during the aging process. Our study showed an increase in the expression of TauT and glycine transporter 1 in the brain cortex of 12-month-old mice compared to 2-month-old mice; the change in expression was not significant though. Moreover, no change in taurine expression was observed in 12-month-old mice compared to 24-month-old mice. Our results were somewhat consistent with the finding that showed an increase in TauT expression in the spinal motor neurons of patients with ALS (Jung et al. 2013).

Aging is associated with increased inflammation. Studies have reported that, during the aging process, canonical NLRP3 inflammasome activation is associated with the decline in cognitive function underlying inflammation. Importantly, it has been shown that isoflurane induces age-related cognitive dysfunction via NLPR3 activation. Our study with age-matched NLPR3 knockout mice, which are deficient in IL-1β, showed no significant difference in taurine transporter (TauT) or glycine transporter 1 expression compared to normal mice.

5 Conclusion

Aging and age-related inflammation may not significantly affect the expression of taurine and glycine transporters in aged mice. As a result, the decrease in taurine in an aged brain, which is associated with cognitive deficits, may not be due to decreased taurine and glycine transporter expression.

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Effect of Taurine on Intestinal Microbiota and Immune Cells in Peyer's Patches of Immunosuppressive Mice

Hui Fang, Fanpeng Meng, Fengyuan Piao, Bo Jin, Ming Li, and Wenzhe Li

Abstract Taurine is a sulfur-containing amino acid which has strong activities in enhancing immunity. Gut microbiota is closely interrelated with intestinal mucosal immunity, but the effects and mechanisms of taurine on intestinal microbiota and mucosal immune cells under an immunosuppressive condition remain unclear. This study was conducted to investigate the effect of taurine on gut microbiota and immune cells in Peyer's patches (PPs) of dexamethasone (Dex)-induced immunosuppressive mice. Mice (4-week-old, Male) were randomly divided into three groups: the Control group (n = 12), the Dex-induced immunosuppressive model group (n = 12) and the taurine intervention group (n = 12). The model was established by Dex injection for 7 days and the taurine intervention group was gavaged 100 mg/kg soluble taurine for 30 days. The changes of intestinal microbiota and immune cells in PPs were tested by denaturing gradient gel electrophoresis (DGGE) and flow cytometry, respectively. Results showed that the microbiota in immunosuppressive mice was obvious different compared with control group, in which, the *Lachnospiraceae* and *Ruminococcaceae* groups were significantly reduced, and their reduction were reversed after taurine intervention. Compared to the control group, the total cell number in PPs, as well as the subsets of $CD3^+$ cells (T cells), $CD19^+$ cells (B cells) in model groups were significantly lower, and they were dramatically improved after taurine treatment. Our results suggested that taurine has a positive effect on intestinal homeostasis of the immunosuppressive mice.

Keywords Taurine · Microbiota · Peyer's patches · Immune cells

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1 Introduction

Taurine is a 2-aminoethanesulfonic acid. It is one of the most abundant amino acids existing in retina, muscle, brain and entire body, which mainly relies on the intake of food (Ripps and Shen 2012). An increasing tendency of its beneficial effects is reported in recent years, such as anti-inflammatory (Kim and Cha 2014), antioxidant (Di Leo et al. 2002, 2003), promoting the development of brain and nervous system (Murakami 2014), alleviating the condition of atherosclerosis (De Luca et al. 2015; Wassef et al. 2017), skeletal muscle disorders (Sarkar et al. 2017; Spriet and Whitfield 2015), type 1 and type 2 diabetes (Agca et al. 2014; Sirdah 2015) and so on. However, the exact ways how taurine plays such an important role in organism are still ambiguous.

The mammalian gastrointestinal tract is home to a large community of bacteria, defined as microbiome, which includes all of the microbial inhabitants and their collective genomes (Chase 2018; Kato et al. 2014; Vereecke et al. 2011). It is well known that intestine is the body's most important and powerful immune organ. A close interaction between gut microbes and intestinal mucosal immune system, especially Peyer's patches (PPs), was discovered by many studies. PPs are the most recognizable inductive sites for the priming of lymphocytes generating mature lymphocytes that then migrate to effective sites, such as the lamina propria, to respond to microbial stimulation (Newberry and Lorenz 2005). It has been reported that the numbers and size of PPs in Germ-free (GF) mice are smaller than conventional mice (Pollard and Sharon 1970), they are less active and resulted in the reduced number of IgA^+ plasma cells in GF mice (Machado et al. 1985), which exhibits an immunosuppressive status. Certain gut microbiota can stimulate the development of PPs. For example, Tsai et al. confirmed that feeding of *Lactobacillus* was able to increase the percentage of $CD4^+$ T cells in PPs (Tsai et al. 2010). These kinds of bacteria could also improve the PPs cell-mediated IgA expression in a destructed gut microbial environment (Kim et al. 2016). Changes of gut microbiota and immune cells in PPs are always associated with each other, especially during the onset and the development of diseases (Qiao et al. 2014). Hence, the cross-talk between microbiota and PPs is critical in the maintenance of the gut homeostasis.

Several studies have suggested that taurine has the potential to improve body's immunity in immunosuppressive models induced by dexamethasone (Dex) (Jeklova et al. 2008), or by infection of pathogens such as *Pneumocystis carinii* and *Cryptosporidium parvum* (Miller and Schaefer 2007). Treatment of taurine was found to improve the ratio of T cell or B cell of spleen in these mice. However, given the crucial role of microbiota-PPs interaction in mucosal immunity, no research has focused on the regulatory effect of taurine on the intestinal microflora and immune cells in PPs of immunosuppressive mice. To this end, our study was conducted to investigate the effect of taurine on gut microbiota and immune cells in PPs of Dex-induced immunosuppressive mice.

2 Materials and Methods

2.1 Materials

Taurine (MW, 125.15) was purchased from Sigma Chemical Company (St. Louis, USA). When used, it was dissolved in water at a concentration of 0.1 g/ml. Dexamethasone was purchased from laboratory of Dr. Ehrenstorfer in Augsburg-Germany. It was dissolved in phosphate buffered solution (PBS) at a concentration of 4 mg/ml.

2.2 Mice

Thirty-six Kunming male mice (18–22 g) were gained from the Major Gene Engineering and Disease Model Animal Research Institute of Dalian Medical University, China. They were maintained in a room illuminated for 12 h (08:00–20:00) and kept at 24 ± 1 °C, a relative humidity of 65 ± 15%, with free access to food and water under the specific pathogen-free (SPF) condition. All the animal experiments were approved by the Ethics Committee at the Dalian Medical University, China. Mice were randomly divided into three groups: Control group (Control, n = 12), the Dex-induced immunosuppressive model group (Dex, n = 12) and the taurine intervention group (Taurine, n = 12). The model was established by Dex injection for 7 days and the taurine intervention group was gavaged 100 mg/kg soluble taurine for 30 days.

2.3 Antibodies

The monoclonal-antibodies (mAbs) used in this study are anti-mouse CD16/CD32 (2.4G2), PE-labeled anti-mouse CD4 (GK1.5), PE-labeled anti-mouse CD3 (2C11), PE-Cy5-anti-mouse CD19 (6D5), all from BD Biosciences.

2.4 Flow Cytometric Analysis

PPs were picked up and grinded in PBS buffer, 1×10^6 cells were used for Flow Cytometry (FACS). Cells were incubated with anti-mouse CD16/CD32 (2.4G2) mAb to block fragment crystallizable (Fc) γ receptors for 1 h at room temperature and then stained on ice for 45 min with combinations of mAbs (CD3, CD19, CD4). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, Mountain View, CA), and the data were analyzed with CFlow (Becton Dickinson).

2.5 Feces Collection and Total DNA Isolation

Fecal samples of mice were collected every week and stored at −80 °C before analysis. Total metogenomic DNA was extracted from fecal samples using stool DNA kit (Omega).

2.6 PCR Amplification and Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

PCR amplification and DGGE analysis of gut bacteria and the identification of bands of interest cut from the documented stained polyacrylamide gel were performed according to Mrázek et al (2008). The 16S rDNA fragment was PCR amplified by using the microbial universal primer (338F-GC and 518R) as described in (Klammer et al. 2008; Wu et al. 2016). PCR reaction conditions are as follows: 94 °C pre-denaturation for 5 min, 94 °C denaturation for 30 s, 54.5 °C annealing for 30 s, 72 °C extension for 45 s, 35 cycles. The DGGE results were analyzed by Quantity-One software (Bio-Rad). Phoretix 1 D (Phoretix International, UK) was used to analyze the abundance and relative intensity of bands in the DGGE gel (Gafan et al. 2005). Similarities were displayed graphically as a dendrogram. The Shannon-Wiener index of diversity (H′) (Boon et al. 2002) was used to determine the diversity in fecal samples collected from the control group, Dex group and taurine group. This index was calculated by $H' = -\sum (pi)(Inpi)$, where Pi was the proportion of the bands in the track and was calculated as follows: $Pi = ni/\sum ni$, where ni was the average density of peak in the densitometric curve. The evenness (E) that reflected uniformity of bacterial species distribution also was computed. This index was calculated by $E = H'/InS$, where S was the number of bands.

2.7 Statistical Analysis

Statistical analyses were carried out using the ANOVA test by GraphPad prism 6. When the interaction was significant, Student's *t* test was performed. All statistical analyses were performed using the general linear model procedure. P value of less than 0.05 was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3 Results

3.1 Structure of Taurine and the Experimental Design

Taurine is a 2-aminoethanesulfonic acid (Fig. 1a). Mice were divided into control group, Dex group and the taurine group, n = 12 (Fig. 1b). For the first 1 week, we treated mice with 20 mg/kg Dex except mice in control group. The control group was injected with PBS. In the next 4 weeks, the former two groups were gavaged with PBS, and the taurine group was gavaged with 100 mg/kg taurine every day.

3.2 Taurine Improved the Size and Cell Number of PPs in Dex-Induced Immunosuppressive Mice

Upon necropsy, we observed that, although all mice in the Dex and Taurine groups had a similar number of PPs as the control mice, the sizes of PPs in Dex group were much reduced compared to control (Fig. 2a, b). The sizes of PPs in the Dex group were smaller because they had fewer domes than the PPs in control mice. Surprisingly, post administration of taurine, the size of PPs in taurine-treated group was similar to that of the control group. Indeed, Dex caused immunosuppression in mice, the cell number of PPs in immunosuppressive mice was significantly decreased

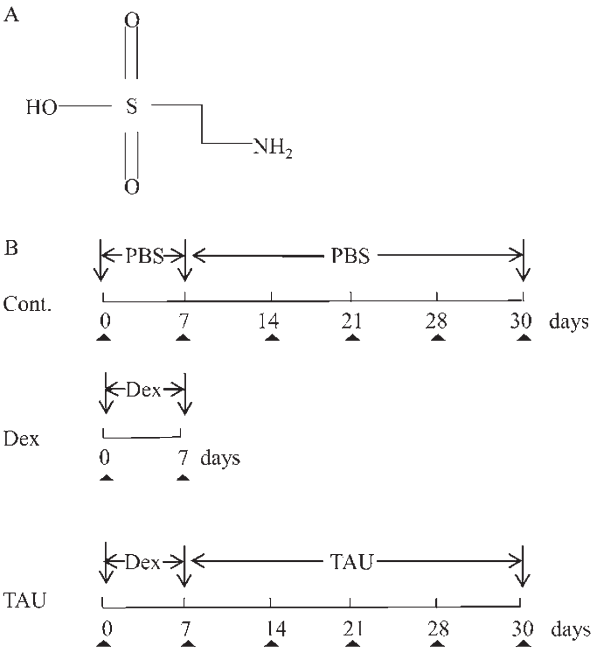

Fig. 1 Structure of taurine and experimental design. (**a**) Structure of taurine. (**b**) Experiment plan: mice were divided into control group, Dex group and the taurine group (n = 12 per group). The control group was injected with PBS while the other groups were injected with 20 mg/kg Dex for the first 1 week. In the next 4 weeks, the former two groups were gavaged with PBS, and the taurine group was gavaged with 100 mg/kg taurine every day. Fecal samples were collected per 7 days

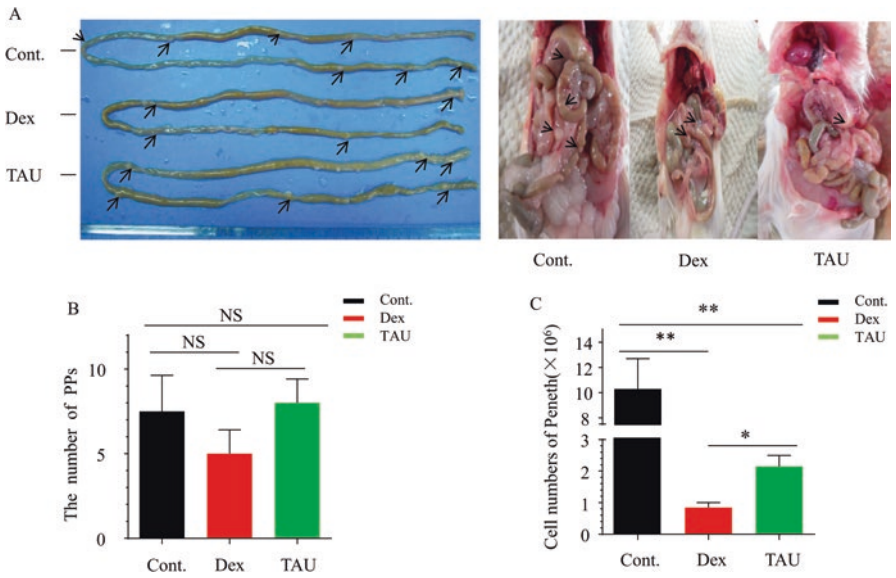

Fig. 2 Taurine recovers the number and cell number of PPs in immunosuppressed mice. (**a**) Dissecting the mice from following groups concluded control group, Dex group and the taurine group. Dissect the small intestine, and the black arrows marked the location of PPs. (**b**) The number of PPs showed by bar graph (n = 12 per group). Data are expressed by mean ± SEM, there was no significant difference between any two groups. (**c**) The cell numbers of PPs showed by bar graph (n = 12 per group). Data are expressed by mean ± SEM, $^{*}P < 0.1$; $^{**}P < 0.01$

compared with that of the control group ($P < 0.01$), while the cell number of PPs was remarkably increased after taurine intervention ($P < 0.1$) (Fig. 2c).

3.3 Taurine Increased Lymphocytes Proportion in PPs of the Immunosuppressive Mice

By using flow cytometry, we analyzed the proportion of lymphocytes in PPs of mice and evaluated the effects of taurine on the generation of different lymphocytes. Our results revealed that the percent of $CD3^+$ cells in the Dex group were significantly reduced to 18.8 ± 0.2%, compared with the control group with a proportion of 23 ± 0.2%, and the elevation of $CD3^+$ cell population was observed after taurine administration, it was 26 ± 0.2% (Fig. 3a, b). The total $CD3^+$ cell count dropped substantially after dexamethasone treatment from an average of $(2.26 \pm 0.53) \times 10^6$ to $(0.16 \pm 0.027) \times 10^6$, after treatment of taurine, this total count increased gradually to $(0.41 \pm 0.084) \times 10^6$ (Fig. 3c). In contrast to the changing of $CD3^+$ cell populations, no significant difference was found in the frequency of $CD19^+$ cell between Dex group and taurine group. $CD19^+$ cells accounted for 11.7 ± 0.3% in control group while it dropped to 10.1 ± 0.1% in Dex group, while the proportion of $CD19^+$

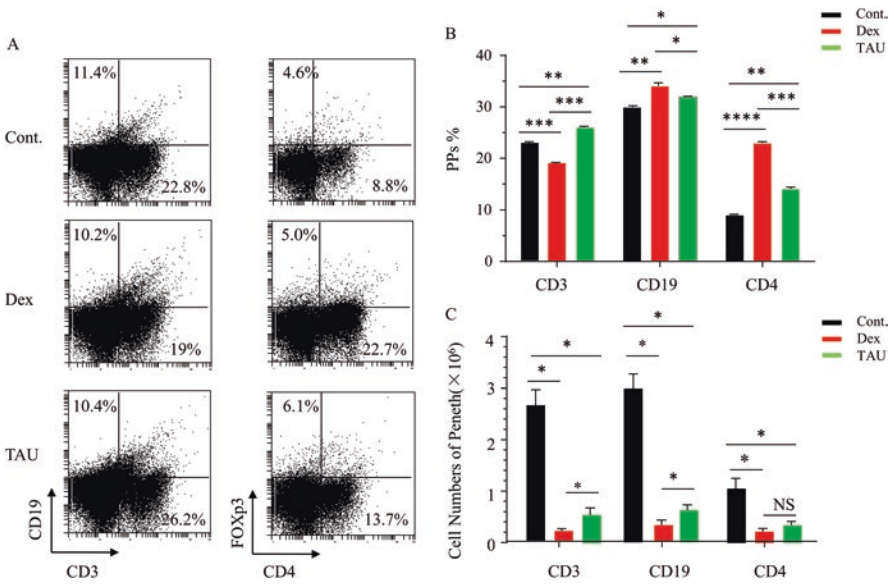

Fig. 3 Taurine recovers the absolute cell number as well as proportion of $CD3^+$ T cell in PPs of immunosuppressed mice. (**a**) The proportion of lymphocytes in PPs analysis by flow cytometry. (**b**) The percent of leukomonocytes in PPs (n = 12 per group). Data are expressed by mean ± SEM, $^*P < 0.1$;$^{**}P < 0.01$; $^{***}P < 0.001$. (**c**) The absolute cell number of PPs leukomonocytes (n = 12 per group). Data are expressed by mean ± SEM. $^*P < 0.1$; $^{**}P < 0.01$; $^{***}P < 0.001$

cells was 10.5 ± 0.1% in taurine group. When calculated the total cell number, we found that the total $CD19^+$ cell number of PPs was (1.19 ± 0.30) × 10^6 in control group, and this number reduced dramatically in Dex group (0.15 ± 0.31) × 10^6 and in taurine group (0.21 ± 0.20) × 10^6. As for the proportion of $CD4^+$ cells, in the Dex group, they were significantly increased to 22.95 ± 0.15% compared with the control group 9 ± 0.2%, and down-regulation of $CD4^+$ cell population was observed after taurine administration, it was 14.05 ± 0.35%. The total $CD4^+$ cell count dropped substantially after dexamethasone treatment, falling from an average of (0.9 ± 0.27) × 10^6 to (0.18 ± 0.06) × 10^6, taurine treatment increased this number to (0.25 ± 0.08) × 10^6.

3.4 Effect of Taurine on Gut Microbiota of Immunosuppressive Mice

The PCR-DGGE analysis was used to determine the bacterial communities in fecal samples of mice. The fingerprint profiles of the control group, the Dex group and the taurine group are shown in Fig. 4a. When comparing the control group with the Dex group and taurine group, we found a considerable variation in the band patterns, which reflected the response of fecal microbiota to the Dex and taurine application.

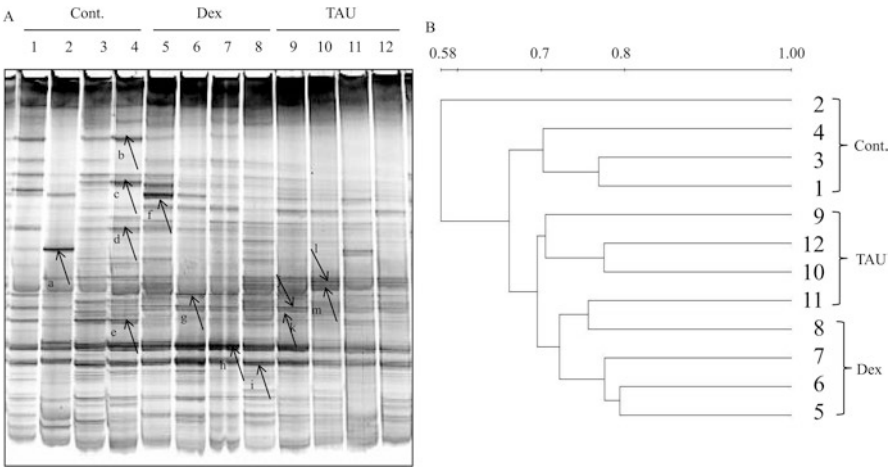

Fig. 4 Taurine increased the abundance of *Ruminvococcaceae* and *Lachnospiraceae* in immunosuppressed mice. (**a**) PCR-DGGE profile representing the fecal bacterial population of mice during the application of control diet, Dex, and taurine. For a description of bands: a~m, see Table 1. (**b**) The UPMGA clustering analysis of gut bacterial community

Table 1 Identification of bands extracted from DGGE gels

Band	Closest genus	Similarities (%)
a	*Parasuttella*	98%
b	*Ruminvococcaceae*	99%
c	*Lachnospiraceae*	99%
d	*Staphylococcus*	97%
e	*Lactobacillus*	84%
f	*Bacteroides rodentium*	98%
g	*Prevotellaccae*	97%
h	*Bacteroidetes*	95%
i	*Prevotellaceae*	95%
j	*Lachnospiraceae*	85%
k	*Gram-positive bacterium*	85%
l	*Alistipes*	98%
m	*Ruminococcus*	77%

a, b, c, d, e were from control group; f, g, h, i, were from Dex group; j,k, l, m, were from taurine group

Some fragments intensified, such as band f, g, h, i in Dex group, and band j, k, l, m, in taurine group. Some were less intensive, such as band b, c, d, e in control group. The fragments of interest were cut out of the gel and consequently characterized by sequencing analysis, after that, their sequences were compared with the EMBL (GenBank) database (Table 1) to identify the bacteria genera. Compared with the control group and the taurine intervention group, the abundance of *Ruminvococcaceae*

Table 2 Shannon-weaver index (H), Richness (E), and Evenness (Eh) of tested fecal samples

Group	H	E	Eh
Control group	5.363 ± 2.005	33.5 ± 1.555	1.509 ± 0.5396
Dex group	5.167 ± 1.841	33.0 ± 2.273	1.485 ± 0.5331
Taurine group	3.408 ± 0.047	33.5 ± 1.708	0.972 ± 0.0061

Values are mean ± SEM; n = 4 per group; all $P > 0.05$

and *Lachnospiraceae* in the Dex group was significantly reduced or disappeared. What's more, the complexity of microbiota in the Dex group has no difference from the control group (Table 2), as the mean number of bands was 33 ± 2.237 in Dex group and 33.5 ± 1.555 in control group, respectively. Whereafter, the evenness index, richness index and diversity index of gut microbiota in each group were not significantly different from each other (Table 2, all $P > 0.05$). The dendrogram constructed based on analysis of the DGGE profile supported that they joined in different clusters. Figure 4b displayed that there were three main clusters in the dendrogram. Lanes 1, 2, 3,4 from the control group, 5,6,7, 8 from the Dex group, and 9,10, 11, 12 from the taurine group joined in single cluster, respectively.

4 Discussion

Many studies have reported that taurine has immunomodulatory effects on human body. The high taurine levels in phagocytes and inflammatory lesions suggests its role in innate immunity (Schuller-Levis and Park 2004). As taurine is present at high concentrations in leukocytes, one may hypothesize that taurine deficiency will affect functions of immune cells. Indeed, prolonged taurine deficiency in cats leads to profound abnormalities in the immune system including significant leukopenia, a decreased respiratory burst in neutrophils and depletion of cells from B cell areas of lymph nodes and spleen (Schuller-Levis et al. 1990). However, there is no clear evidence concerning the association between taurine deficiency and a defect of the immune system in humans.

On the other hand, it is commonly accepted that taurine plays an important role in promoting immunity (Ito et al. 2009; Wang et al. 2009). Intestine is not only a digestive organ but also the body's most important and powerful immune organ. Hundreds of millions of microbes live in intestine, collectively referred to as the gut microbiota (Gensollen et al. 2016). There is a close relationship between microbiota and intestinal mucosal immunity. GF mice are lacking intestinal symbiotic bacteria, in these mice, the numbers and sizes of PPs and cecal patches, which are thought to be sites of induction of the intestinal immune system, are smaller than in conventional mice (Griebel and Hein 1996). On the other hand, the intestinal immune system can maintain bacterial homeostasis and prevent dysbiosis (Alegre et al. 2014). However, the effect of taurine on intestinal flora and intestinal mucosal immunity in immunocompromised models is yet unknown.

The innovation of this research lies in that we detected changes in certain intestinal flora and the proportion of immune cells, such as $CD3^+$ and $CD4^+$ cells, in PPs of immunosuppressive mice, and evaluate the effects of taurine treatment on these parameters simultaneously. Our results from PCR-DGGE suggested that some pathogenic bacteria are more abundant in Dex-treated mice, in contrast to the decreased abundance of probiotics. Whereas the abundance of some beneficial bacteria recovered after taurine administration. For example, the *Lachnospiraceae* and *Ruminococcaceae* groups were significantly re-increased after taurine treatment. This is the first time we found that taurine can improve the *Lachnospiraceae* and *Ruminococcaceae* in gut. Jasmohan et al. found that the reduction of these two bacterial families is related to the occurrence of hepatic encephalopathy (Bajaj 2014); Nakanishi et al. found that the decrease of *Lachnospiraceae* is related to the severity of colitis (Nakanishi et al. 2015). Lagkouvardos et al. found that enterolactone production was linked to the abundance of two bacterial species identified as *Ruminococcus bromii* and *Ruminococcus lactaris*. Moreover, they also investigated that the relative abundance of one *Gemmiger* species (*Ruminococcaceae*) and of *Coprococcus comes* (*Lachnospiraceae*) correlated positively with blood levels of low-density lipoprotein (LDL) cholesterol and triglycerides, respectively (Lagkouvardos et al. 2015). Li et al. found that enhanced T helper type 1 (interferon-gamma) and anti-inflammatory (interleukin-10) cytokines in the ileum co-occured with the increasing of unclassified *Lachnospiraceae*, which suggested that some of the *Lachnospiraceae* groups may responsible for activation of Th 1 cells (Li et al. 2014). Christina et al. found that the relative abundance of *Ruminococcus* was inversely associated with IL-6 and TNF-α, therefore, this genus may contribute to the improvement of inflammation status in the body (West et al. 2015). Coincide with the results of our experiment, we also found that the changes of $CD3^+$ cells in PPs co-occured with changing of *Lachnospiraceae* and *Ruminococcus*, and these changes were closely related to taurine treatment. *Prevotellaceae*, *Ruminococcaceae*, *Lachnospiraceae* and *Veillonellaceae* were recognized as the central nodes of luminal microbial network (Zhang et al. 2018). They were positively correlated with gene functions related to amino acids, energy, cofactors and vitamins metabolism, which are indispensable for the hosts (Biddle et al. 2013). Our data suggested that *Ruminococcaceae* and *Lachnospiraceae* may use taurine as the substrate for their growth, and in turn resulted in re-establishment of gut microbial network, which is beneficial for immune improvement of host. Further detail studies to explore the mechanism of taurine improve the abundance of these two bacterial families in gut are necessary.

5 Conclusion

In conclusion, our results revealed that taurine has an important regulatory effect on the intestinal flora and the proportion of immune cells of PPs in immunosuppressed mice, changing of gut microbiome may partly contribute to the immune-modulating effects of taurine.

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Effects of Chronic Intake of a Low Concentration of Taurine on Physical Strength and Body Composition in Mice

Kyung Suk Cho, Manoj Kumar Neog, Joo Young Kim, Hyung-In Yang, and Kyoung Soo Kim

Abstract Most studies of taurine on athletic performance have been conducted at acute and high doses in rodents. These doses and duration of administration are not reasonable for normal human life. Thus, it is not valid to extrapolate these animal results to people. Dose and duration that mimic human use of taurine in normal life can help to clarify the taurine effect in humans. This study investigated whether long-term, low-dose taurine (2% taurine drinking water for 25 weeks), similar to normal taurine intake in humans, can affect endurance exercise and body composition. Twenty ICR mice were divided into two groups. The control group received normal drinking water, and the taurine treated group received 2% taurine drinking water for 25 weeks. The mice were evaluated for body composition by mass and for physical strength by treadmill exhaustion and suspension tests. The supply of chronic 2% taurine drinking water has a slight effect on weight gain. In body composition analysis, a slight increase in body weight was due to an increase in muscle mass, not an increase in body fat. However, taurine ingestion did not increase endurance exercise. In conclusion, these results indirectly suggest that acute, high-dose taurine treatment is better than long-term, low-dose treatment to increase athletic performance.

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Keywords Treadmill test · Four-limb hanging test · Athletic performance · Body composition

1 Introduction

Taurine, which is synthesized from hypotaurine through methionine and cysteine metabolism in hepatocytes, is often classified as "a conditional amino acid" (Reymond et al. 1996; Schaffer and Kim 2018). It is the second most abundant amino acid in the muscles, and is essential for fundamental biological and physiological functions (Lombardini 1983). However, the endogenous synthesis of taurine in the body has been reported to be insufficient, so exogenous taurine intake has been recommended as an ergogenic resource for a better quality of life (Lombardini 1983). In addition, studies have indicated that taurine could be beneficial as a regular dietary supplement to increase muscle force and insulin sensitivity, improve energy expenditure and lipid metabolism, and prevent oxidative stress (Schaffer and Kim 2018). Interestingly, several experimental studies and epidemiological reports have suggested that taurine could be the anti-aging agent that enhances the life expectancy of the Japanese (Yamori et al. 2009). The multifaceted roles of taurine, including enhanced energy metabolism and muscle contraction strength, have led to its positive reception among athletes and the pharmaceutical industry.

In spite of these essential functions, taurine does not participate directly in protein synthesis or muscle building (Ripps and Shen 2012). Therefore, many investigators have investigated the relationship of this amino acid with physio-biochemical activities. Taurine has been found to exert anti-inflammatory activity, but its direct involvement in the attenuation of inflammatory mediators needs further confirmation (Marcinkiewicz and Kontny 2014). Due to its sulfonic acid content, taurine is most accepted as a potent antioxidant that promotes the conversion of chlorides and hypochlorous acid (an extremely toxic oxidant) into chloramine (a relatively stable and less toxic molecule) (Aruoma et al. 1988; Marcinkiewicz and Kontny 2014). Apparently, taurine acts on HOCl and HOBr to generate the biologically active compounds of taurine chloramine (TauCl) and taurine bromamine (TauBr). It has also been suggested that taurine complements the expression and metabolic activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Moreover, hypotaurine, a taurine precursor, can readily scavenge the hydroxyl radical, thus preventing the peroxidation of lipids and the self-oxidation of iron (Fe^{2+}). Accordingly, taurine has been demonstrated to ameliorate inflammation-associated disorders, including acute and chronic inflammation, cancer and aging (Marcinkiewicz and Kontny 2014; Schaffer and Kim 2018).

Although more evidence is needed to determine the detailed mechanisms whereby taurine affects human endurance, many experimental studies have demonstrated that taurine levels in the blood and skeletal muscles are reduced after exercise, irrespective of whether an intense or brief workout was performed (Matsuzaki et al. 2002). Several strictly examined reports have indicated that taurine depletion stimulates the

heartbeat, hypertension and oxidative stress and reduces muscle contractions, which may compromise athletes' performance (Matsuzaki et al. 2002; Zhang et al. 2004). Therefore, athletes who use taurine supplements have optimized blood pressure, improved maximal aerobic, anaerobic and mental performance, and enhanced feelings of well-being (Geiss et al. 1994; Matsuzaki et al. 2002; Zhang et al. 2004). Due to its vital role in regulating calcium homeostasis in skeletal muscle and cardiac tissue, taurine has proven to be a holy grail for athletes (Chen et al. 2012; Lombardini 1983). Taurine ingestion elevates the level of calsequestrin 1, a calcium binding protein, which ensures the availability of calcium in the sarcoplasmic reticulum for muscle contractions. Hence, acute daily taurine supplementation for 7 days significantly increased the time to exhaustion, maximized the workload capacity and enhanced oxygen uptake. In addition, in validated studies, taurine supplementation improved the running and cycling performance of athletes (Chen et al. 2012; Yatabe et al. 2003; Zhang et al. 2004).

An energy drink containing taurine, caffeine and glucuronolactone (namely, Red Bull) has been shown to improve athletes' moods and aerobic, anaerobic and mental performance (Ballard et al. 2010). However, these drinks contain a large amount of sugar, which is not desirable for the maintenance of a low-calorie diet. Moreover, the effective combination of these ingredients and dosage of taurine are still not clear. Previous studies have suggested 500–3000 mg of taurine per day as the standard dosage, because taurine administered above 3000 mg did not benefit athletes' performance (Fujita et al. 1987; Shao and Hathcock 2008). However, six-week supplementation with 6000 mg of taurine per day diminished the systolic, diastolic and mean blood pressures of patients with hypertension (Fujita et al. 1987). Importantly, most of the reports pertaining to taurine usage by athletes have demonstrated its effects for short periods of time, while there is limited information on taurine efficacy in the long run.

This study investigated whether long-term low-dose taurine intake (2% taurine drinking water for 25 weeks), mimicking common human supplementation patterns of taurine, would improve endurance exercise performance and body composition in mice.

2 Materials and Methods

2.1 Animals

Twenty male, 4-week-old ICR mice were randomly divided into two groups, housed in a specific pathogen-free (SPF) facility with a 12-h light/dark cycle, and given *ad libitum* access to food and water. The first group was fed a normal chow diet and given purified drinking water (Normal, n = 10), while the second group was fed a normal chow diet and given drinking water supplemented with 2% taurine (Tau, n = 10). All animal protocols were approved by the Committee on Animals of Kyung Hee University Hospital at Gangdong (KHNMC AP 2016-009). The normal

diet was purchased from Orient Bio (YongIn, Kyungki, Korea). Taurine was obtained from the Institute of Dong-A Pharmaceuticals (YongIn, Kyungki, Korea).

2.2 Body Weight and Composition

Body weight was monitored every 2 or 4 weeks from the age of 5 weeks. Body composition was analyzed by nuclear magnetic resonance (LF90 Minispec, Bruker Corp., TX, USA).

2.3 Treadmill Exhaustion Test

A treadmill exhaustion test was conducted to measure the physical performance of the skeletal muscles. Each mouse was placed on the belt of a six-lane motorized treadmill supplied with an electric shocker plate (Columbus Instruments, Columbus, OH, USA) and subjected to treadmill running for 10 min at 10 m/min. For the exhaustion test, the treadmill was run at an inclination of 0° at 20 m/min until the mice remained on the shocker plate for more than 10 s without attempting to run, and the time to exhaustion was determined.

As a measure of exercise tolerance, mice were run to exhaustion at 21 m/min at a 10% grade on a rodent-specific treadmill (Columbus Instruments). Exhaustion was defined as the inability/refusal to continue when encouraged with a bottlebrush or a small puff of air.

2.4 Four-Limb Hanging Test

For the start position of this test, a mouse was placed with all four limbs on a grid. The grid was then turned upside-down above a cage filled with bedding. The session ended after a hanging time of 10 min was achieved, or otherwise after three sessions. The maximum hanging time was used for further analysis (van Putten et al. 2012).

3 Results

3.1 Effect of Long-Term Low-Dose Taurine Supplementation on Body Weight

As a test of whether long-term low-dose taurine supplementation in the drinking water would affect body weight and body composition in mice, purified water containing 2% taurine was provided as the drinking water for the Taurine (TAU) group, while unsupplemented purified water was provided to the Control (CON) group. Chronic supplementation with 2% taurine resulted in a slight weight gain, but the difference was not significant (Fig. 1). The body weights (mean ± SEM) of the CON and TAU groups after 6 weeks of feeding were 36.69 ± 0.75 g and 39.69 ± 1.31 g, respectively. At 25 weeks, the mean body weights of the CON and TAU groups were 47.13±1.80 g and 49.71 ± 1.80 g, respectively. The slight increase in body weight persisted from 4 weeks to 25 weeks of taurine supplementation.

The source of the increase in body weight was then determined by body composition analysis (Fig. 2). The fat composition and fluid mass did not differ between the CON group and the TAU group. However, the lean mass (representing muscle mass) was slightly greater in the TAU group than in the CON group, although the difference was not significant. All these results suggest that 2% taurine supplementation in the drinking water may help to increase body weight, particularly muscle mass.

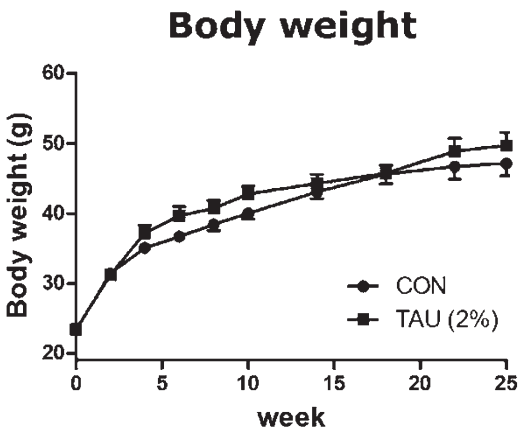

Fig. 1 Effect of long-term low-dose taurine supplementation on body weight. For long-term low-dose taurine supplementation, purified water containing 2% taurine was provided to the Taurine (TAU) group (n = 10) as the drinking water for 25 weeks, while the Control (CON) group (n = 10) received purified water. Body weight was monitored every 2 or 4 weeks from 5 weeks of age

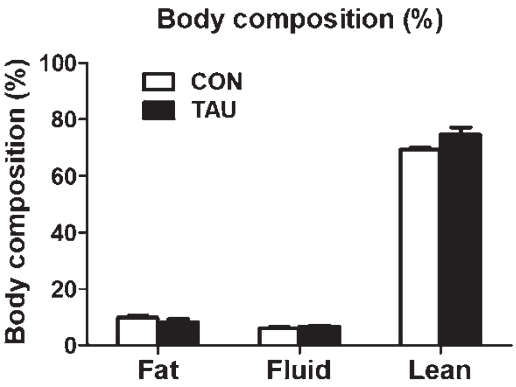

Fig. 2 Effect of long-term low-dose taurine supplementation on body composition. After taurine supplementation for 25 weeks as described in Fig. 1, the changes in body composition of mice in the Taurine (TAU) (n = 4) and Control (CON) (n = 4) groups were measured by nuclear magnetic resonance (LF90 Minispec)

3.2 Effect of Long–Term Low–Dose Taurine Supplementation on Athletic Performance

As a test of whether long-term low-dose taurine supplementation in the drinking water would increase athletic performance, the two groups of mice were tested for their treadmill and hanging performance. In the treadmill test, the two groups did not differ significantly in performance. The running times (mean ± SEM) of mice in the CON (n = 9) and TAU (n = 8) groups were 21.67 ± 1.05 min and 21.88 ± 1.28 min, respectively. The running distances of the two groups were 304.7 ± 22.9 m and 291.0 ± 28.5 m, respectively. The end speeds of the two groups were 23.5 ± 0.9 m/min and 22.8.0 ± 1.2 m/min, respectively. In addition, in the hanging test, the suspension times did not differ significantly between the TAU and CON groups. All these data demonstrate that long-term low-dose taurine supplementation did not effectively increase athletic performance, suggesting indirectly that acute high-dose treatment is better than long-term low-dose treatment with taurine to increase athletic performance (Fig. 3).

4 Discussion

Taurine, a sulfur-containing amino acid, is an important ingredient that is added to most popular energy drinks (Ballard et al. 2010; Geiss et al. 1994; Reymond et al. 1996). It appears to facilitate a diversity of bio-physiological processes that enhance performance and support endurance in athletes. It is well documented that taurine, alone or in combination with caffeine, can improve maximal exercise performance (Yatabe et al. 2003). In taurine transporter knockout mice, the arrest of taurine uptake reduced the exhaustion time (Ito et al. 2014). Furthermore, as an antioxidant, taurine can regulate the mitochondrial matrix to improve the turnover of ATP in muscle cells (Lombardini 1983).

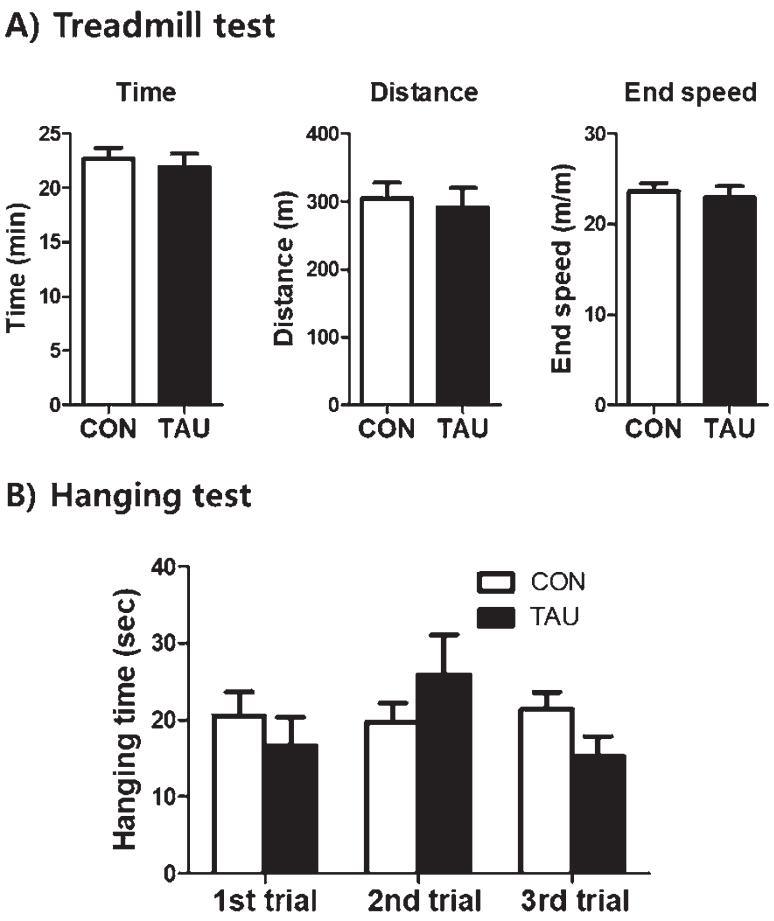

Fig. 3 Effect of long-term low-dose taurine supplementation on athletic performance. After taurine supplementation for 25 weeks as described in Fig. 1, athletic performance was measured in the Taurine (TAU) (n = 4) and (CON) (n = 4) groups by the Treadmill test (**a**) and Hanging test (**b**)

Previous research demonstrated that seven-week taurine supplementation of overweight (non-diabetic) subjects reduced the serum concentrations of triacylglycerol, total cholesterol and LDL-C and increased the concentration of HDL-C (Mochizuki et al. 1998; Yokogoshi et al. 1999). However, studies on the effects of taurine on body weight and fat accumulation have had contradictory results. A few studies have demonstrated that taurine supplementation significantly reduced the body weights of hyperglycemic mice (Mizushima et al. 1996). On the other hand, in other studies, no effect of taurine on body weight has been observed in mice (Mizushima et al. 1996; Murakami et al. 1999). These contradictory findings could be the result of the various study durations and taurine dosages. In contrast to earlier studies, our study revealed that 2% taurine supplementation for 25 weeks slightly

induced body weight gain in mice. The increase in body weight was found to be independent of fat accumulation and fluid mass in the CON (non-treated) and TAU (taurine-treated) groups. The slight increase in lean body mass upon taurine treatment may have been responsible for this body weight gain (Muller et al. 2014).

In terms of weight loss, it is known that a greater fat mass is associated with a lower proportion of lean mass with weight loss. Significantly, high fat mass in ageing adults was found to decrease lean body mass, which predominantly encompasses muscle mass and skeletal mass. As taurine supplementation may induce fat mass loss in hyperglycemic or obese subjects, this would increase lean body mass and body weight correspondingly.

Increases in lean body mass are known to enhance the performance of athletes, so taurine administration has undoubtedly been linked with the performance and endurance of athletes. This non-essential amino acid has been reported to efflux from the muscles cells after exercise, leading to a deficient taurine concentration in the skeletal muscles (Pierno et al. 1998). On the other hand, the taurine concentration was found to be elevated in urine collected from athletes immediately after a marathon (Cuisinier et al. 2001). Excessive exercise is often associated with severe muscle damage, resulting in the spontaneous release of taurine and its excretion via the urinary system. Therefore, taurine supplementation could replenish the deficient taurine concentration in the skeletal muscles, thus improving performance and increasing the time to exhaustion (Cuisinier et al. 2001; Yatabe et al. 2003).

However, in the present study, 25-week taurine supplementation of mice had no effect on their performance or endurance. Of note, most of the earlier studies evaluating the effects of taurine on body composition, endurance and performance were carried out for a short period of time. Consequently, our study and previous reports seem to support the hypothesis that acute taurine supplementation enhances the immediate performance and endurance of athletes (Waldron et al. 2018).

5 Conclusion

Our study demonstrated that long-term supplementation of mice with 2% taurine in the drinking water increased the lean body mass and body weight but did not significantly increase athletic performance. These results indirectly suggest that acute high-dose taurine treatment is better than long-term low-dose treatment to increase athletic performance.

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Influences of Taurine Deficiency on Bile Acids of the Bile in the Cat Model

Teruo Miyazaki, Sei-ich Sasaki, Atsushi Toyoda, Mutsumi Shirai, Tadashi Ikegami, Yasushi Matsuzaki, and Akira Honda

Abstract Taurine content in the body is maintained by both biosynthesis from sulfur-contained amino acids in the liver and ingestion from usual foods, mainly seafoods and meat. Contrary to the rodents, the maintenance of taurine content in the body depends on the oral taurine ingestion in cats as well as humans because of the low ability of the biosynthesis. Therefore, insufficient of dietary taurine intake increases the risks of various diseases such as blind and expanded cardiomyopathy in the cats. One of the most established physiological roles of taurine is the conjugation with bile acid in the liver. In addition, taurine has effect to increase the expression and activity of bile acid synthesis rate-limiting enzyme CYP7A1. Present study purposed to evaluate the influence of taurine deficiency on bile acids in the cats fed taurine-lacking diet. Adult cats were fed the soybean protein-based diet with 0.15% taurine or without taurine for 30 weeks. Taurine concentration in serum and liver was undetectable, and bile acids in the bile were significantly decreased in the taurine-deficient cats. Taurine-conjugated bile acids in the bile were significantly decreased, and instead, unconjugated bile acids were significantly increased in the taurine-deficient cats. Present results suggested that the taurine may play an important role in the synthesis of bile acids in the liver.

Keywords Bile acid · Cats · LC-MS/MS · Liver · Taurine deficiency

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Abbreviations

APDS	3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate
BACS	ATP-dependent microsomal bile acid-CoA synthetase
BAT	bile acid-CoA:amino acid *N*-acetyltransferase
BW	body weight
CA	cholic acid
CDCA	chenodeoxycholic acid
CDO	cysteine dioxygenase
CSD	cysteine sulfinate decarboxylase
CYP7A1	cytochrome P450 7a1
DCA	deoxycholic acid
DIA	days in age
ESI	electrospray ionization
FXR	farnesoid X receptor
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GLCA	glycolithocholic acid
GUDCA	glycoursodeoxycholic acid
HDCA	hyodeoxycholic acid
LCA	lithocholic acid
MCA	muricholic acid
SRM	selected reaction monitoring
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TLCA	taurolithocholic acid
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UFLC	ultra-fast liquid chromatography

1 Introduction

Taurine (2-aminoethanesulphonic acid) is the most abundant free amino acid-like compound found in mammalian tissues including liver. A lot of physiological and pharmacological effects of taurine have been confirmed in many studies, and one of the established actions is the conjugation with bile acids (Danielsson 1963; Sjovall 1959).

Taurine is an end product in sulfur-containing amino acid catabolism through the methionine and cysteine pathways in the liver (De La Rosa and Stipanuk 1985; Bella et al. 1999a, b). In this taurine biosynthesis pathway, cysteine dioxygenase

(CDO) (Hosokawa et al. 1990) and cysteine sulfinate decarboxylase (CSD) (Reymond et al. 1996; Tappaz et al. 1999; Kaisaki et al. 1995) are key limited enzymes. Taurine is actively biosynthesized in the rodents, but it is an essential amino acid for cats because the activity of CSD is very low (Sturman and Hayes 1980). Furthermore, cats have low ability of renal regulation of urinary excretion against body taurine fluctuation (Rentschler et al. 1986). Therefore, the taurine deficiency is caused in the cats by the insufficient dietary taurine intake (Knopf et al. 1978). In the taurine deficient cats, various diseases including blind (Hayes et al. 1975) and expanded cardiomyopathy (Pion et al. 1987) have been observed to be developed.

Bile acids are biosynthesized from cholesterol in the liver, most of which are conjugated with taurine or glycine. Most of bile acids are conjugated with taurine in the rodents, while glycine conjugated bile acids are the predominance in humans; glycine: taurine conjugation ratio is 3:1 (Bruusgaard and Thaysen 1970). The roles of conjugation with taurine/glycine are the enhancement of water solubility of bile acids, the alleviation of its cytotoxicity, the promotion of its excretion to the bile, the formation of micelles in the bile and the facilitation of lipid absorption in the intestinal tract. In addition, previous studies reported that taurine promoted the expression and activity of cholesterol 7α-hydroxylase (also known as cytochrome P450 7a1; CYP7A1) that is the rate-limiting enzyme in the bile acid synthesis (Chen et al. 2012). Therefore, there is a possibility that the taurine deficiency might influence bile acid metabolism.

Present study evaluated the influence of taurine deficiency on the concentration, composition, and conjugation rate of bile acids in the bile in the taurine deficient cat model induced by feeding of taurine-lacking diet.

2 Methods

2.1 Taurine Deficient Model in Cats

Taurine deficient model cats were prepared by feeding of taurine deficient diet according to the methods previously reported by Hickman et al. (1992) and Park et al. (1999). Eleven cats were divided into the taurine-contained control diet supplemented group (Control group; N = 4 [male 2, female 2], body weight [BW]; 2.9 ± 0.8 kg, days in age [DIA]; 479 ± 100 days; mean ± SEM) and the taurine-deficient diet supplemented group (Deficient group: N = 7 [male 3, female 4], BW; 3.1 ± 0.4 kg, DIA; 414 ± 52 days).

Control and Deficient groups were fed with soy protein based experimental diet with 0.15% taurine and without taurine, respectively, for 30 weeks (Table 1). Soy protein isolate was obtained from Fuji Oil Co., Ltd. (Osaka, Japan). Taurine, sucrose, and dicalcium phosphate were purchased from (Wako Pure Chemical Industries, Osaka, Japan). Corn starch and beef tallow were obtained from CLEA

Table 1 Composition of the soy protein based experimental diet

Contents	Taurine contained diet (Control group)	Taurine deficient diet (Deficient group)
Soy protein isolate	409 g	411 g
Corn starch	202 g	202 g
Sucrose	200 g	200 g
Beef tallow	100 g	100 g
AIN 93G mineral mix	50 g	50 g
AIN 93 vitamin mix	10 g	10 g
Dicalcium phosphate	24 g	24 g
Choline chloride	3 g	3 g
Taurine	1.5 g	*Absent*
Total	1000 g	1000 g

Nutritional composition of the experimental diet was 41.2% carbohydrates, 37.2% proteins, and 9.9% lipids (4.02 kcal/g)

Japan (Tokyo, Japan). AIN 93G mineral mix, AIN 93 vitamin mix, and choline chloride were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All of contents were mixed, and pellet was softly dried after forming by adding of tap water. Daily feeding of the experimental diet was limited to 100 g (dry weight), and all cats ate all of it every day.

One female cat in the Deficient group died at the 30th week. After the experimental period, cats were euthanatized by overdose injection of pentobarbital. Blood was collected from the inferior vena cava, and serum was separated for quantification of taurine. Liver tissue was also collected for taurine quantification. Bile was collected from gall bladder by syringe with needle, and bile acids in the bile were measured by HPLC-MS/MS system. The animal experiment was conducted with the approval of the Animal Care Committee in Ibaraki Prefectural University of Health Sciences.

2.2 Taurine Analysis

Taurine in serum and liver quantified by a derivatization method with 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) using the HPLC-ESI-MS/MS system (Shimbo et al. 2009; Miyazaki et al. 2019). Liver tissue was homogenized with ten-times volume of PBS solution (w/v), and the supernatant following the centrifugation at 3,500×g, 4 °C, 10 min, was used for quantification of taurine. The HPLC-ESI-MS/MS system consisted of a TSQ Vantage triple stage quadruple mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography system (Shimadzu, Kyoto, Japan). Fifty μL of serum and 5 μL of liver homogenized sample were mixed with 50 μL of APDSTAG® Wako Amino Acids Internal Standard Mixture solution (Wako Pure Chemical Industries, Osaka, Japan) and 100 μL of acetonitrile

and centrifuged at 20,000×g for 10 min. Then, 20 μL of APDS-acetonitrile solution (20 mg/mL; Wako Pure Chemical Industries Ltd) and 60 μL of 0.2 M sodium borate buffer, at *pH* 8.8, were mixed with 20 μL of the supernatant, and incubated at 55 °C for 10 min. Thereafter, the reaction mixture was added to 100 μL of 0.1% formic acid-water solution, and 5 μL was injected into the HPLC-P-ESI-MS/MS system. Amino Acids Mixture Standard solutions of Type B and AN-2 (Wako Pure Chemical Industries) were used for quantification. The APDS-derivatized amino acids were separated with a 100 × 2.0 mm i.d. Wakosil-II 3C8-100HG column (particle size 3 μm) for the analytical column and a 10 × 1.5 mm i.d. Wakosil-II 3C8-100HG column for the guard column (particle size 3 μm) (Wako Pure Chemical Industries Ltd), 0.3 mL/min at 40 °C by gradient flow. The general HPLC and MS/MS conditions were carried out using the previous method (Shimbo et al. 2009).

2.3 Bile Acids Analysis

Analysis of bile acids was carried out using HPLC-ESI-MS/MS system according to the method by Murakami et al. (2018). Cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), lithocholic acid (LCA), glycolithocholic acid (GLCA), taurolithocholic acid (TLCA), ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) were obtained from Steraloids Inc. (Newport, RI, USA). [2,2,4,4-$^2H_4$]CA, [2,2,4,4-$^2H_4$]DCA, [2,2,4,4-$^2H_4$]LCA were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Canada), and [2,2,4,4-$^2H_4$]TCA were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). [11,11,12,12-$^2H_4$]CDCA was supplied by the Research Laboratory of Nippon Kayaku Co. (Tokyo, Japan), and tauroursodeoxycholic acid (TUDCA), [11,11,12,12-$^2H_4$]UDCA and [11,11,12,12-$^2H_4$]TUDCA were supplied by Tokyo Tanabe Co. (Tokyo, Japan).

A mixture of internal standards consisting of 41.6 ng of [$^2H_4$]CA, 57.5 ng of [$^2H_4$]CDCA, 32.8 ng of [$^2H_4$]DCA, 22.4 ng of [$^2H_4$]LCA, 34.4 ng of [$^2H_4$]UDCA, 34.4 ng of [$^2H_4$]TUDCA, 100 ng of [$^2H_3$]TCA, and 20 μL of ethanol-water (9:1, *v/v*) was added to 20 μL of bile diluted with distilled water by 1,000-times. Samples were diluted with 2 mL of 0.5 M potassium phosphate buffer (*pH* 7.4) and passed through Bond Elut C18 cartridges (200 mg, Agilent Technologies, Santa Clara, CA) (Shoda et al. 1988). After washing the cartridge with 1.6 mL of water, bile acids were eluted in 3 mL of ethanol-water (9:1, *v/v*). The eluate was evaporated to dryness at 100 °C under a nitrogen stream and redissolved in 20 mM ammonium acetate buffer (*pH* 7.5)-methanol (1:1, *v/v*). After centrifugation at 12,000 ×*g* for 1 min, an aliquot of the supernatant was injected into the HPLC-MS/MS system for analysis. Chromatographic separation was performed using a Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) at 40 °C. The following gradient system was used at a flow rate of 200 μL/min: initially, the mobile phase consisted

of 20 mM ammonium acetate buffer (*pH* 7.5)–acetonitrile–methanol (70:15:15, *v/v/v*); then it was programmed in a linear manner to 20 mM ammonium acetate buffer (*pH* 7.5)–acetonitrile–methanol (30:35:35, *v/v/v*) over 20 min. The final mobile phase was kept constant for an additional 10 min. The general MS/MS conditions were as follows: spray voltage, −2500 V; vaporizer temperature, 450 °C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 15 arbitrary units; ion transfer capillary temperature, 220 °C; collision gas (argon) pressure, 1.0 mTorr; and ion polarity, negative. The general HPLC and MS/MS conditions were carried out using the previous method (Murakami et al. 2018).

Concentrations of bile acids in the bile were shown as the sum of all types of bile acids conjugated with and without taurine and glycine. Amino acid-conjugation of bile acids were shown as unconjugated, taurine-conjugated, and glycine-conjugated forms in the sum of all types of bile acids. Compositions of bile acids were shown as the ratio of CA, CDCA, DCA, LCA, and UDCA in sum of unconjugated and conjugated forms.

2.4 Statistic Analysis

Statistical significance was determined by Student's *t*-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated *p*-value was less than 0.05.

3 Results

3.1 The Conjugation Ratio, Composition and Concentration in the Bile Acids in the Bile of the Taurine Deficient Cats

In the Deficient group, taurine concentrations in serum and liver were undetectable after the supplementation of taurine deficient diet for 30 weeks. In the Control group, 99.95 ± 0.02% of the bile acids in the bile were conjugated with taurine. On the other hand, unconjugated and glycine-conjugated forms were 0.05 ± 0.02% and 0.001 ± 0.0001%, respectively. In the Deficient group, the taurine conjugated form was significantly reduced to 35.2 ± 2.5% ($P < 0.0001$ compared to that in the Control), on the other hand, the unconjugated and the glycine conjugated forms were significantly increased to 62.9 ± 2.3% ($P < 0.0001$) and 1.9 ± 0.5% ($P < 0.05$), respectively.

Bile acids in the bile of the Control group were 83.4 ± 3.6%, 11.8 ± 3.5% and 4.8 ± 1.3% in CA, DCA and CDCA, respectively (Fig. 1). LCA and UDCA were less than 0.01%. In the cats, there were no other types of bile acids such as muricholic acid (MCA) and hyodeoxycholic acid (HDCA) found in the rodents and other ani-

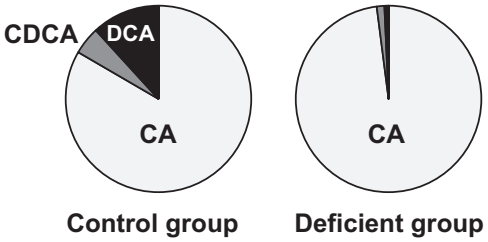

Fig. 1 Composition of bile acids in the bile of the Control and Deficient groups. Bile acids are the sum of unconjugated and amino acids-conjugated forms. *Abbreviations*: *CA* cholic acid, *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid

mals. In the Deficient group, CA was increased to 98.0 ± 1.2% ($P < 0.01$ compared to that in the Control), CDCA and DCA decreased to 1.3 ± 0.6% ($P < 0.01$) and 0.7 ± 0.6% ($P < 0.01$), respectively (Fig. 1). LCA and UDCA were also less than 0.01% in the Deficient group.

Biliary concentration of total bile acids (the sum of unconjugated and amino acid-conjugated forms) were significantly decreased in the Deficient group (172.3 ± 59.4 mM) compared to that in the Control group (404.8 ± 58.6 mM, $P < 0.05$).

4 Discussion

Taurine as well as glycine have been well known to be conjugated with bile acids to increase their polarity, which results in increased excretion into the bile and reduced toxicity (Danielsson 1963; Sjovall 1959). Bile acids conjugated with taurine or glycine also more efficiently promote absorption of lipids in the intestine. In addition, it has been also well known that the ability of taurine biosynthesis is absent in cats (Jacobsen and Smith 1968), and therefore, cats become in the taurine deficient state if taurine was not supplied through oral intake. In the present study, the concentration, composition, and amino acid conjugation ratio in the bile were evaluated in the taurine deficient cats induced by taurine-lacking diet.

There are species differences in the conjugation ratio of amino acids with bile acids and the composition of bile acids. In the rodents, bile acids are mostly conjugated by taurine (Hellstrom and Strand 1963; Bremer 1956), while the bile acids in human are conjugated by glycine and taurine as ratio at 3:1 (Bruusgaard and Thaysen 1970). Present study confirmed that most of bile acids were conjugated with taurine in the healthy control cats. On the other hand, the conjugation ratio of bile acids with taurine was decreased to 35%, and instead, unconjugated bile acids were increased to 63% in the taurine deficient cats. Although taurine concentration in serum and liver was completely deficient in the taurine deficient cats, 35% of taurine-conjugated bile acids were retained. Taurine and glycine are conjugated with bile acids following the biosynthesis of bile acids in the liver, and the amino

acid-conjugated bile acids that excreted in the intestine are transported into the portal vain at the terminal of ileum. In addition, a part of the amino acid-conjugated bile acids is deconjugated by intestinal bacteria, and are re-conjugated with taurine or glycine in the liver after circulation from intestine. Therefore, the retained taurine-conjugated bile acids in the taurine deficient cats might be due to preservation in the enterohepatic circulation without deconjugation by the intestinal bacteria before the deficiency of taurine.

On the other hand, the increased unconjugated bile acids in the taurine deficient cats might be resulted from both de novo biosynthesis in the liver and enterohepatic circulation following deconjugation, because it was impossible to conjugate bile acids with taurine due to taurine deficiency. Furthermore, glycine-conjugated bile acids were mostly unfound (0.001%) in the healthy normal cats, but were increased to only 2%. This observation that the unconjugated bile acids, but not the glycine-conjugated bile acids, were replaced to the taurine-conjugated bile acids in case of taurine decreases was consistent with the report in humans (Sjovall 1959).

Conjugation of bile acids with amino acids in the liver involves two sequential enzyme reactions mediated by ATP-dependent microsomal bile acid-CoA synthetase (BACS), which converts a bile acid to an acyl-CoA thioester; and bile acid-CoA:amino acid *N*-acetyltransferase (BAT), which transfers the acyl-CoA thioester to taurine or glycine. Although it has been known that the affinity of BAT is higher to taurine rather than glycine (Kwakye et al. 1991), it is unclear why the taurine-conjugated bile acids were not replaced to the glycine-conjugated form in the taurine deficiency. There is a possibility that the expressions of BAT and BACS might be decreased in the taurine deficient cats, because the genes of both enzymes are the target of farnesoid X receptor (FXR; NR1H4) (Pircher et al. 2003). Bile acids are an endogenous ligand of FXR that is a member of the nuclear hormone receptor superfamily and a bile acid-responsive transcription factor, plays key roles on the regulation of bile acid synthesis (Makishima et al. 1999). The ligand activity for FXR is higher in the primary bile acids, especially CDCA. Through FXR activation, bile acids regulate the conjugation with amino acids as well as the biosynthesis by themselves (Miyazaki et al. 2014). In the present study, the concentration of total bile acids in the bile was significantly reduced to about 40% in taurine-deficient cats compared to that in the healthy control cats. In addition, there was a significant difference in the composition of bile acids between the control and taurine deficient cats, and the ratio of CDCA in the taurine deficient cats was decreased to 1/10 compared to that in the healthy controls. Therefore, the ability of conjugation of bile acids with taurine and glycine might be decreased in the taurine deficient cats through the inactivation of FXR.

However, next question why the amount of bile acids in the bile was significantly decreased in the taurine deficient cats is arisen. Because the gene of CYP7A1 is also the target of FXR (Makishima et al. 1999), the CYP7A1 is down-regulated by FXR when the content of bile acids are increased. Therefore, it is suggested that the CYP7A1 might be oppositely increased in the taurine deficient cats. On the other hand, it has been reported that taurine could promote the expression and activity of CYP7A1 (Chen et al. 2012), and therefore, there is a possibility that the expression

or activity of CYP7A1 might be decreased in the taurine deficient cats. Thus, the mechanism is still unclear why the amounts of bile acids were decreased in taurine deficient state.

5 Conclusion

In the present result, significantly decreases of total bile acid concentration, taurine-conjugated bile acids, and rates of CDCA and DCA in the bile were observed in the taurine deficient cats. Instead, unconjugated bile acids and rate of CA were significantly increased in the taurine deficient cats. The bile acid biosynthesis and amino acid-conjugation are regulated by bile acids themselves through FXR, but it is unclear the mechanism how taurine regulates the biosynthesis and conjugation of bile acids.

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Inhibition of Renin-Angiotensin System from Conception to Young Mature Life Induces Salt-Sensitive Hypertension via Angiotensin II-Induced Sympathetic Overactivity in Adult Male Rats

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Abstract Previous studies indicate that perinatal compromise of taurine causes cardiovascular disorders in adults via the influence of taurine on renin-angiotensin system (RAS). This study tested whether perinatal inhibition of the RAS would itself alter the adult cardiovascular system in a similar way. Female Sprague-Dawley rats were fed normal rat chow and given water alone (Control) or water containing captopril (400 mg/l) from conception until weaning. Then, the male offspring drank water or water containing captopril until 5 weeks of age followed by normal rat chow and water alone until 7 weeks of age. Thereafter, they drank water alone (Control, Captopril) or 1% NaCl solution (Control+1%, Captopril+1%). At 9 weeks of age, all animals were implanted with femoral arterial and venous catheters. Forty-eight hours later, blood chemistry, glucose tolerance, and hemodynamic parameters were determined in freely moving conscious rats. Then, the same experiments were repeated 2 days after captopril treatment. Body weights, kidney and heart to body weight ratios, fasting and non-fasting blood sugar, glucose tolerance, and heart rates were not significantly different among groups. Further, plasma sodium, mean arterial pressure, and sympathetic activity significantly increased whereas baroreflex sensitivity decreased in Captopril+1% compared to other groups. These changes were normalized by acute captopril treatment and the arterial pressure differences

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also by acute ganglionic and central adrenergic blockade. The present study suggests that inhibition of the RAS in the early life induces RAS overactivity, leading to salt-sensitive hypertension via sympathetic nervous system overactivity and depressed baroreflex sensitivity in adult male rats.

Keywords Baroreflex · Captopril · Hypertension · Renin-angiotensin system · Salt sensitivity · Sympathetic activity

Abbreviations

ACE	angiotensin-converting enzyme
AT1 receptor	angiotensin II subtype 1 receptor
AT2 receptor	angiotensin II subtype 2 receptor
Captopril+1%	captopril-treated rat with 1% NaCl
Control+1%	control rat with 1% NaCl
mRNA	messenger ribonucleic acid
RAS	renin-angiotensin system
SHR	spontaneously hypertensive rat
WKY	Wistar-Kyoto

1 Introduction

Perinatal environment determines not only the fetal and newborn growth and development, but also programs adult function and disease. Among several factors, differential exposure to taurine (Roysommuti and Wyss 2014) and the RAS (Mao et al. 2009) have been reported to contribute to these long-term effects. While the linkage of perinatal taurine effects appear to be related to the RAS, they may not be due to taurine's inhibition of perinatal RAS activity (Roysommuti et al. 2015), since taurine possesses many physiologic activities other than direct inhibition of the RAS (Roysommuti and Wyss 2014). In this study, we inhibited the RAS by administering the angiotensin-converting enzyme inhibitor to test the serial linkage of taurine to the RAS action.

During pregnancy, the maternal RAS changes dramatically (Irani and Xia 2011). High estrogen stimulates hepatic angiotensinogen synthesis, while plasma renin levels increase mainly from extra-renal tissues, including the ovary. Plasma angiotensin II and aldosterone also increase, though plasma angiotensin-converting enzyme level decreases during pregnancy. The maternal RAS has been shown to play an important role for fetal formation. Further, all components of the RAS begin functioning in the fetus (Irani and Xia 2011; Mao et al. 2009). Thus, alterations of the RAS can affect many human functions from conception onward, and may program adult function and disease. For example, high salt diets decrease plasma

angiotensin II and increase cardiac angiotensin II in the fetus. Angiotensin II-increased S-phase in the fetal cardiac cells is mediated primarily via AT1 receptor mechanisms, as supported by increasing AT1 receptor protein, AT1a, and AT1b mRNA, while AT2 receptor mRNA and protein in the fetal heart is not affected (Ding et al. 2010). In contrast, low birth weight from maternal salt restriction is not due to alterations of placental and peripheral RAS (Leandro et al. 2008).

Perinatal environmental alterations can program the RAS activity in adults. Maternal diabetes induces hypertension, renal injury, and glucose intolerance in the offspring via the activation of the intrarenal RAS and transforming growth factor-β1 gene expression (Chen et al. 2010). Metabolic disorders during lactation stimulates renal Na^+ transport in adult offspring via renal RAS (Luzardo et al. 2011). In the mouse, antenatal maternal low protein diets induce hypertension in the female offspring, probably via decreased pulmonary ACE2 (Goyal et al. 2015). Further, neonatal high oxygen exposure causes cardiac dysfunction in adults via the RAS overactivity (Bertagnolli et al. 2014). In rats, early life stress increases angiotensin II-induced hypertension and vascular inflammation in adult life (Loria et al. 2010). In addition, both perinatal taurine excess and deficit alter the interplay of RAS on arterial pressure regulation and renal function (Roysommuti and Wyss 2014).

Perinatal RAS inhibition alters arterial pressure control and disorder in adults. Prenatal RAS inhibition induces newborn anomalies, while its early postnatal inhibition causes renal damage in adult life (Sekine et al. 2009). However, other studies indicate that ACE inhibitors and angiotensin II receptor antagonists are not major teratogens when used in the first trimester (Diav-Citrin et al. 2011). Chronic losartan treatment is reported to up-regulate AT1 receptors and increase vulnerability to acute onset of cardiac ischemia/reperfusion injury in male rats (Song et al. 2015). Further, perinatally administered losartan augments renal ACE2 expression, but does not increase cardiac or renal Mas receptor in SHRs (Klimas et al. 2015). In addition, the RAS inhibition decreases pregnancy-associated hypertension in mice and ameliorates placental histological changes and severe intrauterine growth restriction effect (Ishimaru et al. 2012). In contrast, cardiac remodeling during the advanced stages of pregnancy-associated hypertension is minimally affected. These lines of evidence indicate the current understanding of RAS inhibition on the offspring function and disorder.

The unique effect of perinatal RAS exposure on adult function and disease was first recognized in SHRs. Compared to control SHR, the SHR that has been continuously treated with captopril from conception onward is normotensive, but more sensitive to NaCl-sensitive induced hypertension (Wyss et al. 1994). Further, the salt-sensitive hypertension of the lifetime captopril-treated SHR is abolished by sympathetic inhibition (Wyss et al. 1995). On a normal salt diet, SHR hypertension is eliminated by central or ganglionic blockade of the sympathetic nervous system and by the RAS inhibition. These lines of evidence suggest the important contribution of the RAS and sympathetic nervous system to the development of essential hypertension. Wild-type WKY rats are salt resistant, i.e., a high NaCl diet does not affect both nighttime and daytime arterial pressure, whereas the lifetime captopril-treated WKY rats are salt sensitive as indicated by the high NaCl diet induced a

rapid rise in arterial pressure (Fang et al. 1999). Discontinuation of the lifetime captopril treatment in the WKY rats for 2 weeks did not diminish this NaCl-induced hypertension, even though baseline mean arterial pressure is similar between control and captopril-treated WKY rats. The present study tests the hypothesis that inhibition of the RAS from conception to young mature life induces salt-sensitive increased arterial pressure via angiotensin II-induced sympathetic overactivity in adult male rats, thus supporting the potential for the RAS to contribute to the cardiovascular effects of perinatal taurine depletion in rats.

2 Methods

2.1 Animal Preparation

Sprague-Dawley rats were bred at the animal unit of Faculty of Medicine, Khon Kaen University and maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (0600–1800 h). Female Sprague-Dawley rats were fed normal rat chow and given water alone (Control) or water containing captopril (an ACE inhibitor, 400 mg/l; Captopril) from conception until weaning. After weaning, the male offspring drank water or water containing captopril until 5 weeks of age followed by normal rat chow and water alone for all rats until 7 weeks of age. Thereafter, they were given water alone (Control and Captopril groups) or 1% NaCl solution (Control+1% and Captopril+1% group) until the end of experiment.

All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 9 weeks of age, all male rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body weight, intraperitoneal) and implanted with femoral arterial and venous catheters. Forty-eight hours later, their arterial catheters were connected to the pressure transducer for continuous monitoring of arterial pressure and heart rate (BIOPAC Systems, Goleta, CA, USA) and venous ones to an infusion pump for drug infusion (Model No.975, Harvard Apparatus, USA) in freely moving rats. Then, arterial blood samples (0.2 ml each) were collected for measurements of non-fasting blood glucose, plasma sodium, and plasma potassium. Blood loses were replaced with equal volumes of saline. After 15–20 min resting, their baroreflex sensitivity controls of heart rate were determined by the intravenous infusion of phenylephrine (a specific alpha-adrenergic agonist; 100 mg/ml in saline; 20 μl/min

for 2 min or until mean arterial pressure increment about 30 mm Hg) and subsequently sodium nitroprusside (25 mg/ml in saline; 20 μl/min for 2 min or until mean arterial pressure decrement about 30 mm Hg). On the next day after an overnight fasting, fasting blood glucose and blood glucose levels after an intravenous glucose injection (2 g/kg body weight; glucose tolerance test) were measured. Then, all animals were fed with the normal rat chow and captopril in drinking water (400 mg/l) until the end of experiment and the above experiments (except glucose tolerance test) were repeated 2 days after the initiated captopril treatment.

To study the role of RAS on sympathetic nerve activity, Control+1% and Captopril+1% rats were subjected to ganglionic and central adrenergic blockade before and after short-term captopril treatment. Briefly, 2 days after arterial and venous catheterization as mentioned above, arterial pressures and heart rates were continuously recorded with the BIOPAC Systems in freely moving rats. After 15–20 min baseline recording, hexamethonium hydrochloride in saline (a ganglionic blocker; 100 mg/ml) was intravenously infused at a rate of 10 μl/kg/min for 10 min (Wyss et al. 1995). Ten minutes later, phenylephrine in saline (a specific alpha-adrenergic agonist; 100 mg/ml) was intravenously infused (20 μl/min for 2 min) to confirm the complete adrenergic blockade. Twenty-four hours later, a central adrenergic blockade was similarly studied by an intravenous clonidine hydrochloride infusion (a α_2-adrenergic receptor agonist; 50 μg/ml in saline) at a rate of 10 μl/kg/min for 20 min. Twenty minutes later, the complete adrenergic blockade was also confirmed by phenylephrine infusion. Then, the animals were allowed to rest and treated with captopril in drinking water until the end of experiment. Two days after initiated captopril treatment, the ganglionic and central adrenergic blockade studies were repeated.

At the end of experiment, all animals were sacrificed by a high dose of anesthesia followed by heart and kidney weights collection.

2.3 Data Analyses

Mean arterial pressure, heart rate, baroreflex sensitivity following phenylephrine or sodium nitroprusside, and power spectral density of arterial pressure were determined offline by using Acknowledge software (BIOPAC Systems, Goleta, CA, USA). Plasma Na^+ and K^+ were measured by a flame photometer (Hitachi, Tokyo, Japan) and blood sugar by a glucometer and glucostrips (One Touch Basic, Lifescan, USA). The autonomic nervous system control of arterial pressure was estimated from the power spectral density of baseline arterial pressure at low frequency (LF 0.3–0.5 Hz; sympathetic nerve activity) and high frequency (HF 0.5–4.0 Hz; parasympathetic nerve activity) components by using the Fourier analysis. Each power spectral density was normalized by the summation of LF and HF spectral density (Roysommuti et al. 2009).

2.4 Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons among groups were performed by using one-way ANOVA and *post hoc* Duncan's multiple range test and within the same group by Student's paired t-test (StatMost32 version 3.6, Dataxiom, CA, USA). The significant criterion was p-value less than 0.05.

3 Results

3.1 General Characteristics

Body weights, kidney to body weight ratios, heart to body weight ratios (Table 1), fasting and non-fasting blood glucose, and glucose tolerance (Table 2) were not significantly different among the four groups. Plasma sodium significantly increased only in Captopril+1% compared to the other three groups, while plasma potassium levels significantly decreased in Control+1% and Captopril compared to Control groups (Table 1).

After 2-day captopril treatment, the plasma sodium levels were not significantly different among groups (Control 121.7 ± 0.1 mEq/l; Control+1% 124.4 ± 0.8 mEq/l; Captopril 124.4 ± 0.7 mEq/l; Captopril+1% 126.2 ± 0.4 mEq/l; $p > 0.05$), while the plasma potassium significantly increased in all the three treated groups compared to Control (Control 3.6 ± 0.1 mEq/l; Control+1% 3.9 ± 0.1 mEq/l; Captopril 4.2 ±

Table 1 Basic characteristics of adult male rats

Treatment	BW (g)	HW/BW (%)	KW/BW (%)	Na^+ (mEq/l)	K^+ (mEq/l)
Control (n = 13)	260 ± 6	0.31 ± 0.02	0.83 ± 0.02	123.2 ± 0.1	4.4 ± 0.1
Control+1% (n = 15)	262 ± 5	0.32 ± 0.01	0.85 ± 0.01	123.9 ± 0.4	3.6 ± 0.1[a]
Captopril (n = 13)	265 ± 10	0.32 ± 0.01	0.84 ± 0.02	123.9 ± 0.4	3.1 ± 0.1[a]
Captopril+1% (n = 15)	280 ± 6	0.33 ± 0.01	0.80 ± 0.01	132.0 ± 0.6[a,b,c]	3.9 ± 0.1

Data are mean ± SEM

BW body weight, *HW* heart weight, *KW* kidney weight, Na^+ plasma sodium, K^+ plasma potassium

[a,b,c]$P < 0.05$ compared to Control, Control+1%, and Captopril, respectively

Table 2 Non-fasting blood glucose (NFBG), fasting blood glucose (FBG), and blood glucose after intravenous glucose injection (2 g/kg body weight) in adult male rats

	Blood glucose (mg/dl)				
Treatment	NFBG	FBG	30 min	60 min	120 min
Control (n = 13)	99.8 ± 2.5	82.5 ± 2.6	131.9 ± 7.3*	101.6 ± 4.1*	88.5 ± 3.1
Control+1% (n = 15)	100.8 ± 1.8	85.3 ± 2.1	132.7 ± 5.8*	101.2 ± 4.4*	91.0 ± 3.9
Captopril (n = 13)	97.2 ± 2.9	76.1 ± 3.3	134.2 ± 8.3*	102.0 ± 3.7*	94.4 ± 3.2*
Captopril+1% (n = 15)	95.8 ± 1.6	79.9 ± 1.9	146.4 ± 5.2*	103.0 ± 3.1*	99.9 ± 2.0*

Data are mean ± SEM

*$P < 0.05$ compared to FBG of the same group

0.1 mEq/l; Captopril+1% 4.3 ± 0.1 mEq/l; $p < 0.05$), but not among the three treated groups. The 2-day captopril treatment did not affect the hematocrit among and within groups.

3.2 Hemodynamic Parameters

Resting mean arterial pressure significantly increased in Captopril+1% compared to the other groups, while heart rates were not significantly different among groups both before and after 2-day captopril treatment (Fig. 1). After 2-day captopril

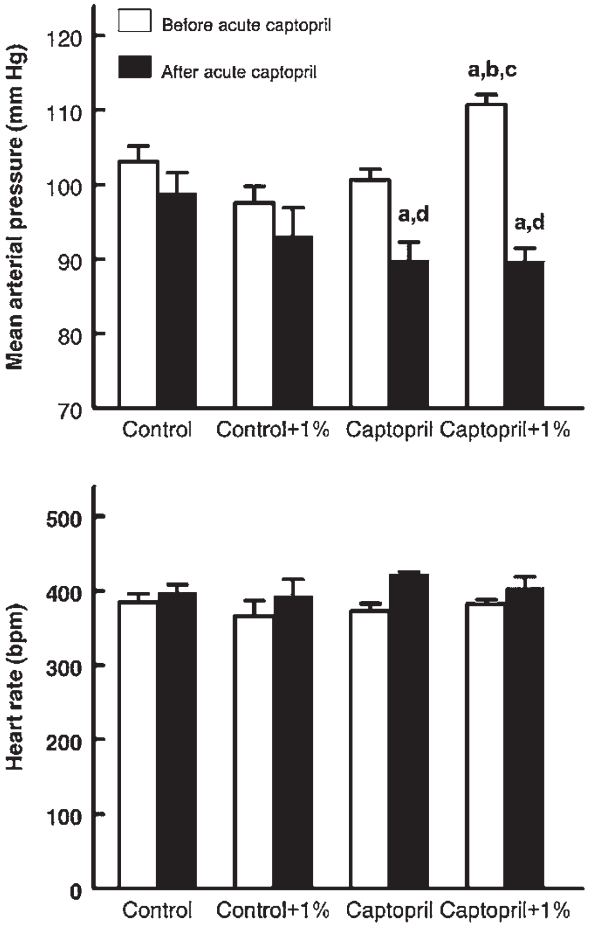

Fig. 1 Mean arterial pressure (upper) and heart rate (lower) before (white bar) and after (black bar) 2-day captopril treatment ($^{a,b,c,d}P < 0.05$ compared to Control, Control+1%, Captopril, and between Before and After 2-day captopril treatment, respectively)

treatment, the arterial pressure of Captopril+1% were not significantly different from those of Control+1% and Captopril, but lower than that of Control group ($p < 0.05$). Baroreflex sensitivity was blunted in the Captopril+1% compared to the other groups, and this change was significantly improved to the levels of Control+1% and Captopril groups by 2-day captopril treatment (Fig. 2 upper panel). In addition, the 2-day captopril treatment significantly increased baroreflex sensitivity in the Control group ($p < 0.05$).

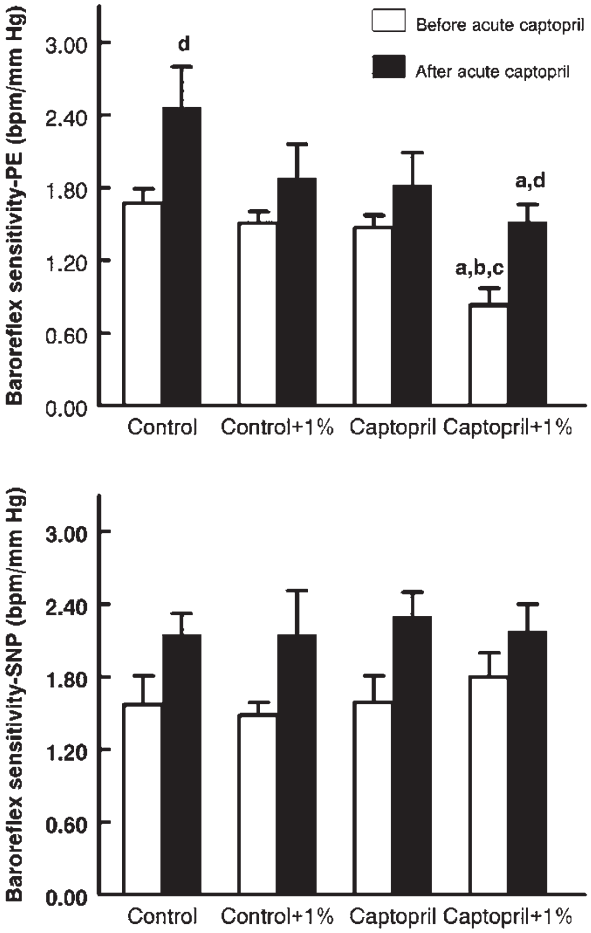

Fig. 2 Baroreflex sensitivity tested using phenylephrine (upper) and sodium nitroprusside (lower) before (white bar) and after (black bar) 2-day captopril treatment ($^{a,b,c,d}P < 0.05$ compared to Control, Control+1%, Captopril, and between Before and After 2-day captopril treatment, respectively)

3.3 Autonomic Nervous Control of Arterial Pressure

Estimated sympathetic nerve activity significantly increased and parasympathetic nerve activity decreased in Captopril+1% compared to the other three groups (Fig. 3). After 2-day captopril treatment, this autonomic dysfunction was improved to the levels of Control+1% and Captopril, and the sympathetic nerve activity (but not parasympathetic nerve activity) was slightly and significantly lower than the Control ($p < 0.05$). The mean arterial pressure differences between Control+1% and Captopril+1% groups were completely abolished by either acute ganglionic blockade (hexamethonium; Fig. 4 upper panel) or acute central sympathetic

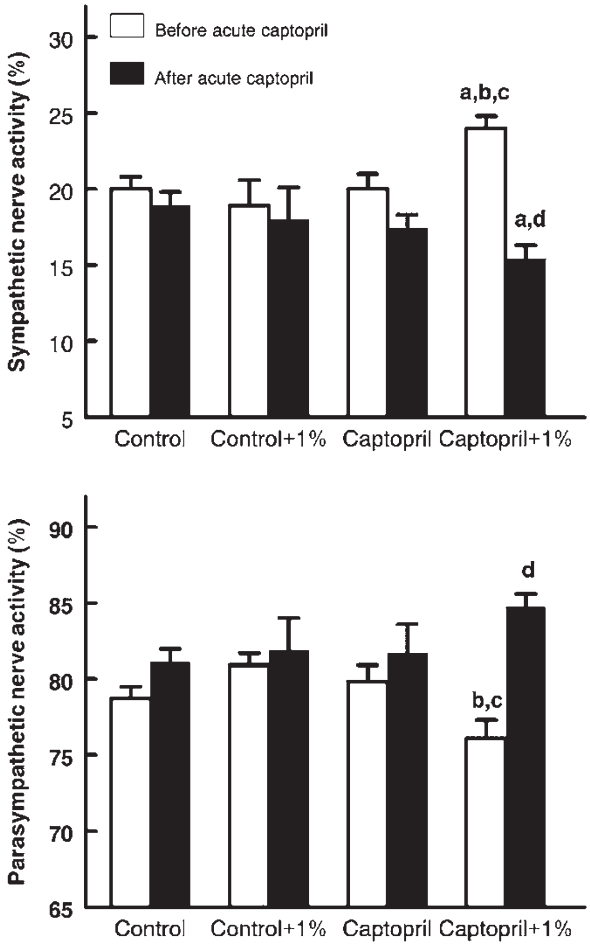

Fig. 3 Sympathetic (upper) and parasympathetic nerve activity (lower) before (white bar) and after (black bar) 2-day captopril treatment ($^{a,b,c,d}P < 0.05$ compared to Control, Control+1%, Captopril, and between Before and After 2-day captopril treatment, respectively)

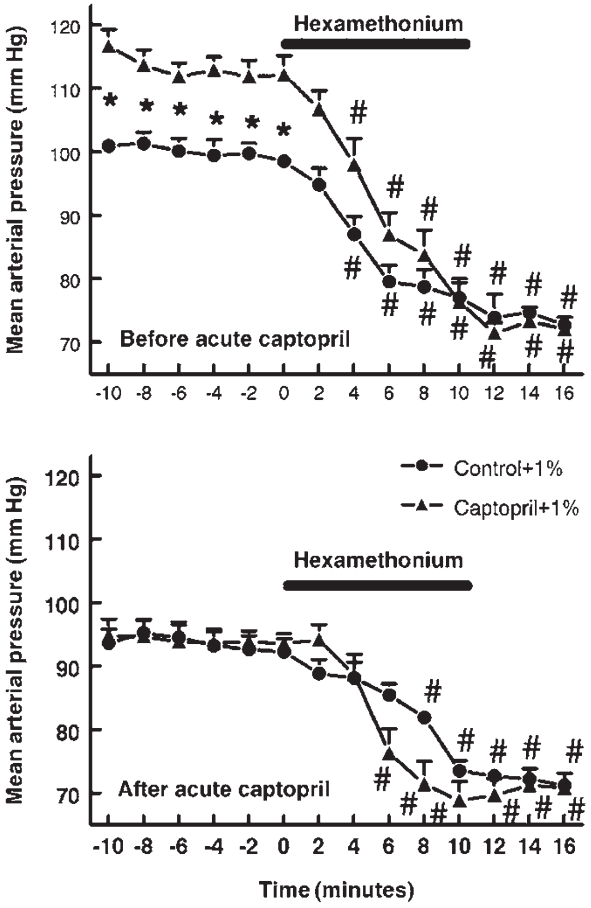

Fig. 4 Mean arterial pressures of Control+1% and Captopril+1% at rest and after a ganglionic blockade with hexamethonium infusion before (upper panel) and after (lower panel) 2-day captopril treatment ($^{*,\#}P < 0.05$ compared between the two groups and to the 0 min value of each group, respectively)

inhibition (clonidine; Fig. 5 upper panel). Two-day captopril treatment eliminated the mean arterial pressure difference between Control+1% and Captopril+1%, groups and the differential hypotensive effect of hexamethonium and clonidine (Fig. 4 lower panel; Fig. 5 lower panel).

4 Discussion

Lifetime inhibition of the RAS attenuates hypertension but does not decrease salt-induced hypertension in SHRs (Wyss et al. 1994). Further, salt-induced hypertension in the SHRs is eliminated by both ganglionic and central adrenergic inhibition

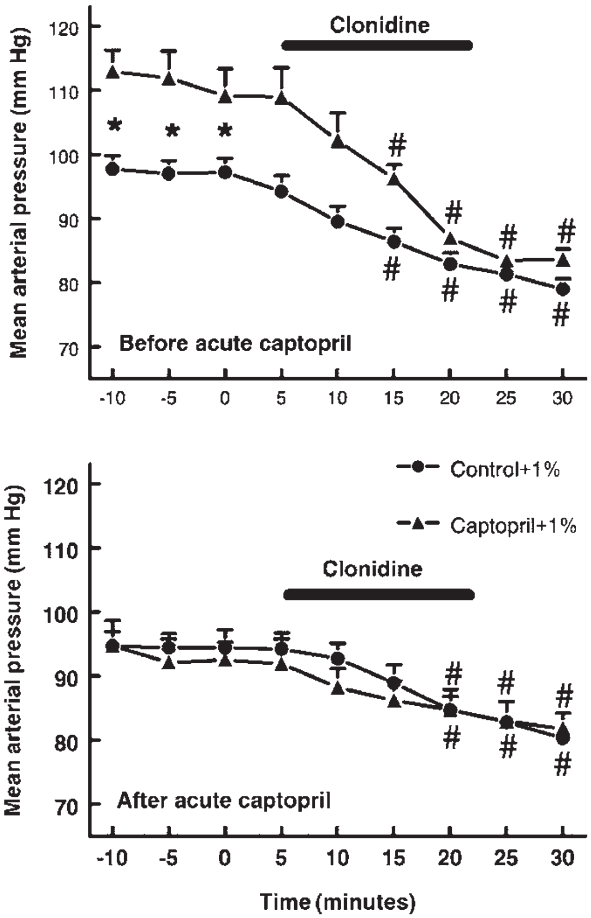

Fig. 5 Mean arterial pressures of Control+1% and Captopril+1% at rest and after a central adrenergic blockade with clonidine infusion before (upper panel) and after (lower panel) 2-day captopril treatment ($^{*,\#}P < 0.05$ compared between the two groups and to the 0 min value of each group, respectively)

(Wyss et al. 1995). In contrast, early life inhibition of the RAS induces renal damage in adult normotensive rats (Woods and Rasch 1998), and these animals develop salt-sensitive hypertension (Fang et al. 1999), that is caused by sympathetic nervous system overactivity. The present study indicates that in Sprague-Dawley male rats, a 2-day inhibition of the RAS normalized autonomic nerve activity, mean arterial pressure, baroreflex sensitivity, and hypernatremia in the Captopril+1% compared to other groups. These data suggest that the inhibition of the RAS from conception to young mature life induces salt-induced hypertension later in life via the RAS overactivity-induced sympathetic overactivity.

Compared to WKY rats, SHRs have a higher plasma concentration of angiotensin II but similar cardiac angiotensin II levels (Okamoto et al. 2017; Silva et al.

2017), while renal renin, AT1 receptor, and ACE expression are lower (Klimas et al. 2015). This suggests that the reduction of the renal RAS components in the SHR is related to a down-regulation of high plasma angiotensin II. In the SHR, perinatal RAS inhibition by losartan results in temporary hypotension; e.g., 4 weeks after discontinuation of losartan at 4 weeks of age, the arterial pressures of treated SHR become as hypertensive as untreated SHR (Klimas et al. 2015). These data indicate a rapid rise in arterial pressure a few weeks after losartan discontinuation, even when the rats are on a normal diet. This may be a consequence of up-regulation of the RAS activity after perinatal losartan discontinuation, as supported by the present data. Inhibition of the RAS from conception to 5 weeks of age induced salt-sensitive hypertension in 9 weeks old male rats, and this adverse effect could be eliminated by 2-days of captopril treatment.

Local and systemic RAS plays very important roles in arterial pressure regulation. The RAS components are present in many brain areas from prenatal to adult life and involve brain growth and development (Mao et al. 2009). Angiotensin II receptors are prominent in hypothalamic, thalamic, cerebellar, and cortical neurons by late gestation (Mungall et al. 1995). Alterations of fetal hypothalamic angiotensin II receptors can alter fetal tissue maturation affecting adult nervous system function (Chen et al. 2005). Further, perinatal RAS inhibition by captopril alters brain function and behaviors in the adult offspring, particularly leading to decreased water and sodium intake in response to deficit challenges (Mecawi et al. 2010) and increased anxiety and nociceptive behavioral responses (Mecawi et al. 2009). However, although the increased water and sodium intake after intracerebroventricular angiotensin I and angiotensin II injection is not altered by perinatal RAS inhibition, acute captopril treatment increases water and salt intake to a lesser extent in the adult rats with perinatal RAS inhibition than control (Mecawi et al. 2010). These lines of evidence suggest that the brain RAS may malfunction in the adult rats that are perinatally RAS inhibited. The salt sensitivity of SHR has been reported to result from brain changes, particularly in the hypothalamus (Carlson et al. 2001). It is possible that the salt-sensitive increase in arterial pressure in the present model originates from brain RAS overactivity induced by the perinatal RAS inhibition. Brain RAS overactivity may then increase renal sympathetic nerve activity to stimulate renal renin release and subsequently, the systemic RAS is activated. Further, increases in plasma angiotensin II and sympathetic nerve activity increase peripheral resistance and arterial pressure, respectively. This hypothesis is supported by the normalization of arterial pressure, autonomic nerve activity, baroreflex sensitivity, and hypernatremia after 2-day captopril treatment in the Captopril+1% rats. However, measurements of plasma and brain angiotensin II and other components of the RAS are necessary to support this hypothesis.

It is well known that angiotensin II can act on several brain areas related to autonomic control of arterial pressure, especially the anterior hypothalamus and rostral ventrolateral medulla. This angiotensin II action results in depressed baroreceptor reflex sensitivity and increased peripheral sympathetic outflow (Allen et al. 2009;

DiBona 2001). The present study also supports this notation. In addition, the action of RAS on the baroreflex function and autonomic control of arterial pressure as observed in the present study is also reported in the adult rats perinatally depleted of taurine followed by high sugar intake from weaning onward (Roysommuti and Wyss 2014). Both taurine and the RAS influence early life growth and development, and program adult arterial pressure control. It is possible that, during perinatal life, both compromise the same epigenetic factors that control the expression of RAS components in adult life. Thus, in adult rats perinatally depleted of taurine, both high salt and high sugar intake can induce RAS overactivity and contribute to hypertension.

High salt intake is a risk factor of hypertension in salt-sensitive humans and animals. In rats, plasma sodium concentrations increase during the daytime and decrease during the nighttime (Fang et al. 2000). In contrast, arterial pressure increases during the nighttime and decreases during the daytime. These diurnal variations occur irrespective of low or high salt intake. The high plasma sodium level, as in the Captopril+1%, can increase intracerebroventricular Na^+ (Mozaffari et al. 1990), which can act on Na^+-sensitive neurons in some brain areas, including anterior hypothalamus and rostral ventrolateral medulla, to stimulate the peripheral sympathetic outflow to heart, blood vessel, and kidney (Peng et al. 1996). However, this may not be the case in the present study since a 2-day captopril treatment abolishes hypernatremia and sympathetic overactivity in the Captopril+1% rats. Nevertheless, hepatic sensory nerve activation by isotonic saline infusion reflexively causes a decrease in renal nerve activity and probably renal renin release (Jirakulsomchok et al. 2012).

5 Conclusion

Perinatal taurine exposure and RAS activity affect adult arterial pressure control. Abnormal expression of the autonomic nervous system and the RAS components appear to underlie this adverse effect in adult offspring. The present study indicates that in adult normotensive rats, inhibition of the RAS from conception to young mature life induces RAS overactivity and thus could lead to salt-sensitive increases in arterial pressure via sympathetic nerve stimulation and depressed baroreflex sensitivity in adult male rats. In addition, the present results provide a potential scientific basis for the perinatal taurine effects on adult arterial pressure control and cardiovascular regulation.

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Taurine Chloramine Inhibits Osteoclastic Differentiation and Osteoclast Marker Expression in RAW 264.7 Cells

In Soon Kang and Chaekyun Kim

Abstract Taurine is an abundant sulfur-containing amino acid in myeloid cells. It undergoes halogenation in activated phagocytes and is converted to taurine chloramine (TauCl) and taurine bromamine. Bone homeostasis is mediated by the balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts are bone-resorbing multinucleated cells differentiated from monocyte/macrophage precursor cells in response to receptor activator of NF-κB ligand (RANKL). In this study, we investigated the effect of TauCl on RANKL-induced osteoclastogenesis from RAW 264.7 macrophages. TauCl inhibited the formation of multi-nucleated osteoclast and the activity of tartrate-resistant acid phosphatase (TRAP). TauCl decreased the mRNA expression of osteoclast markers, such as TRAP, cathepsin K, and calcitonin receptor. TauCl also inhibited expression of the transcription factors, c-Fos and nuclear factor of activated T cells, which are important for osteoclast differentiation. These results suggest that TauCl might be used as a therapeutic agent to treat bone diseases associated with excessive bone resorption.

Keywords Taurine chloramine · Osteoclast · Macrophage · Receptor activator of NF-κB ligand (RANKL) · Tartrate-resistant acid phosphatase (TRAP) · Nuclear factor of activated T cells (NFATc)

Abbreviations

BMMs	Bone marrow-derived monocyte/macrophage precursor cells
CTR	Calcitonin receptor
CTSK	Cathepsin K
M-CSF	Macrophage colony-stimulating factor
NFATc1	Nuclear factor of activated T cell 1

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NF-κB	Nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
TauCl	Taurine chloramine
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase

1 Introduction

Taurine (2-aminoethanesufonic acid) is an abundant free amino acid in mammals and plays important roles in many biological processes (Learn et al. 1990; Huxtable 1992; Marcinkiewicz and Kontny 2014). Taurine reduces oxidative stress by scavenging halogenated oxidants. Taurine react with hypochlorous acid (HOCl) by the halide-dependent myeloperoxidase system to form the long-lived taurine chloramine (TauCl) in neutrophils. This process protects cells from the cytotoxic action of hypochlorous acid. TauCl also serves as an anti-inflammatory agent by inhibiting many inflammatory mediators, such as the superoxide anion (O_2^-), nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukins, prostaglandins, and matrix metalloproteinases (Barua et al. 2001; Kanayama et al. 2002; Kim and Cha 2014; Marcinkiewicz and Kontny 2014), and by activating antioxidant enzymes, such as heme oxygenase and peroxiredoxin (Sun Jang et al. 2009; Piao et al. 2011; Kim and Cha 2014). Thus, TauCl has been recognized as an anti-inflammatory signaling molecule that is generated during the inflammatory process by activated neutrophils.

Osteoclasts are multinucleated cells that are essential for bone resorption and bone remodeling. Osteoclast differentiation is regulated by two essential cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL). M-CSF induces monocyte/macrophage precursor cells and the expression of RANK in early-stage osteoclastogenesis. M-CSF also activates osteoclast precursors during early phases of osteoclast differentiation (Nakanishi et al. 2013). RANKL induces differentiation of osteoclast precursors into osteoclasts and suppresses osteoclast apoptosis through NF-κB and nuclear factor of activated T cell 1 (NFATc1) signaling (Boyce et al. 2009). Binding of RANKL to RANK activates the TNF receptor-associated factor 6 (TRAF6) and c-Fos pathways, which lead to the osteoclast-specific event, auto-amplification of NFATc1. NFATc1 is a master regulator of osteoclast differentiation and regulates a number of osteoclast-associated genes such as tartrate-resistant acid phosphatase (TRAP, acid phosphatase 5), cathepsin K (CTSK), calcitonin receptor (CTR), and osteoclast-associated receptor through cooperation with the microphthalmia-associated transcription factor and c-Fos (Takayanagi et al. 2002; Matsumoto et al. 2004; Kim et al. 2005a, b).

In our previous study, TauCl significantly reduced RANKL-induced osteoclastogenesis from bone marrow-derived monocyte/macrophage precursor cells (BMMs) (Kim and Kang 2015). In this study, we investigated the effect of TauCl on RANKL-induced osteoclastogenesis in the murine macrophage cell line, RAW 264.7 cell. These cells are a valuable tool to study osteoclast differentiation and activity due to its expression of RANK and differentiation to osteoclast in response to RANKL (Hsu et al. 1999). TauCl inhibited osteoclastic differentiation of RAW 264.7 cells in a concentration-dependent manner, and inhibited the expression of osteoclast markers including TRAP, CTSK, and CTR. In addition, TauCl decreased TRAP activity and the induction of osteoclast-associated transcription factors, such as c-Fos and NFATc1.

2 Materials and Methods

2.1 Synthesis of TauCl

TauCl was synthesized immediately before experiments by adding equimolar amounts of sodium hypochlorite to taurine in 1.8% NaCl (Kim et al. 2010). Regents were purchased from Sigma-Aldrich, unless otherwise specified.

2.2 RAW 264.7 Cell Culture

The murine macrophage cell line, RAW 264.7 cells (American Type Culture Collection) were grown in Dulbecco's modified eagle medium (Hyclone) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Hyclone) at 37°C in 5% CO_2.

2.3 Osteoclast Differentiation

RAW 264.7 cells were placed in 96-well (6×10^3 cells/well) or 24-well (2×10^4 cells/well) plates and cultured for 4 days in α-minimal essential medium (Hyclone) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 100 ng/ml RANKL (Peprotech) in the presence of TauCl (0, 0.05, 0.1, and 0.2 mM). The differentiation medium was changed on day 3. RANKL-derived osteoclastic differentiation was assessed by counting TRAP-positive cells containing more than three nuclei.

2.4 TRAP Staining

The presence of TRAP activity is regarded as an important cytochemical marker for osteoclastogenesis. After 4 days in culture, the medium was removed and the cells were gently washed with phosphate buffered saline. Cells were fixed with 3.7% formaldehyde for 5 min, incubated with 0.1% Triton X-100 for 5 min, and stained with the Leukocyte Acid Phosphatase Assay kit. In brief, cells were incubated in a mixture containing Fast Garnet GBC base, sodium nitrite, naphthol AS-BI phosphoric acid, acetate, and tartrate for 1 h at 37°C in the dark. Cells were washed with water and counterstained with hematoxylin solution Gill No. 3 for 2 min at room temperature. TRAP-positive multinucleated cells were counted as osteoclasts.

2.5 TRAP Activity

Cells were incubated in 50 mM citrate buffer (pH 4.5) containing 10 mM sodium tartrate and 5 mM 4-nitrophenylphosphate for 30 min. The reaction was stopped by adding an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm by using a Versamax microplate reader (Molecular Devices) equipped with SoftMax software.

2.6 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of 200 ng of total RNA was performed according to the instructions by Takara. PCR amplification of mRNA was carried out on a Bio-Rad CFX using SYBR Green Master Mix (Toyobo) and the following primers (Table 1).

2.7 Western Blot Analysis

Cell lysates were prepared from osteoclasts as described previously (Kim and Dinauer 2001) and 20 μg of total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Resolved proteins were transferred onto polyvinylidene fluoride membranes (Millipore) and probed with specific antibodies against c-Fos (Cell Signaling Technology, 1:1000), NFATc1 (Santa Cruz Biotechnology, 1:1000), and β-actin (1:10,000). Membranes were incubated with

Table 1 Primers used in qRT-PCR

Gene	Primers (5′→3′)
TRAP	F: ACG GCT ACT TGC GGT TTC A
	R: TCC TTG GGA GGC TGG TCT T
CTSK	F: GAA GAA GAC TCA CCA GAA GCA G
	R: TCC AGG TTA TGG GCA GAG ATT
CTR	F: TGC TGG CTG AGT GCA GAA ACC
	R: GGC CTT CAC AGC CTT CAG GTA C
GAPDH	F: CCT TCC GTC CTA CCC C
	R: CCC AAG ATG CCC TTC ATG

the appropriate secondary antibodies and then signals were developed with an enhanced chemiluminescence solution (Thermo Fisher).

2.8 Statistical Analysis

The two-tailed paired Student's t-test was performed using Microsoft Excel software. Results are expressed as means ± standard deviation (SD), and p values <0.05 are considered statistically significant.

3 Results

3.1 TauCl Inhibited RANKL-Induced Osteoclastic Differentiation and TRAP Activity of RAW 264.7 Cells

RANKL is expressed on osteoblasts and induces the signaling essential for osteoclastogenesis (Yasuda et al. 1998; Theill et al. 2002). To investigate the effect of TauCl on macrophage osteoclastogenesis, RAW 264.7 cells were treated with 0.05–0.2 mM TauCl in the presence of RANKL. Unlike with BMMs, RAW 264.7 cells secrete M-CSF. Thus, treatment with M-CSF is unnecessary (Takahashi et al. 2007). The number of multinucleated TRAP-positive cells and TRAP activity were determined. TauCl decreased RANKL-induced TRAP-positive osteoclast numbers (Fig. 1a, b) and TRAP activity (Fig. 1c) concentration-dependently. TauCl concentrations lower than 0.5 mM showed no cytotoxic effect on RAW 264.7 cells (data not shown).

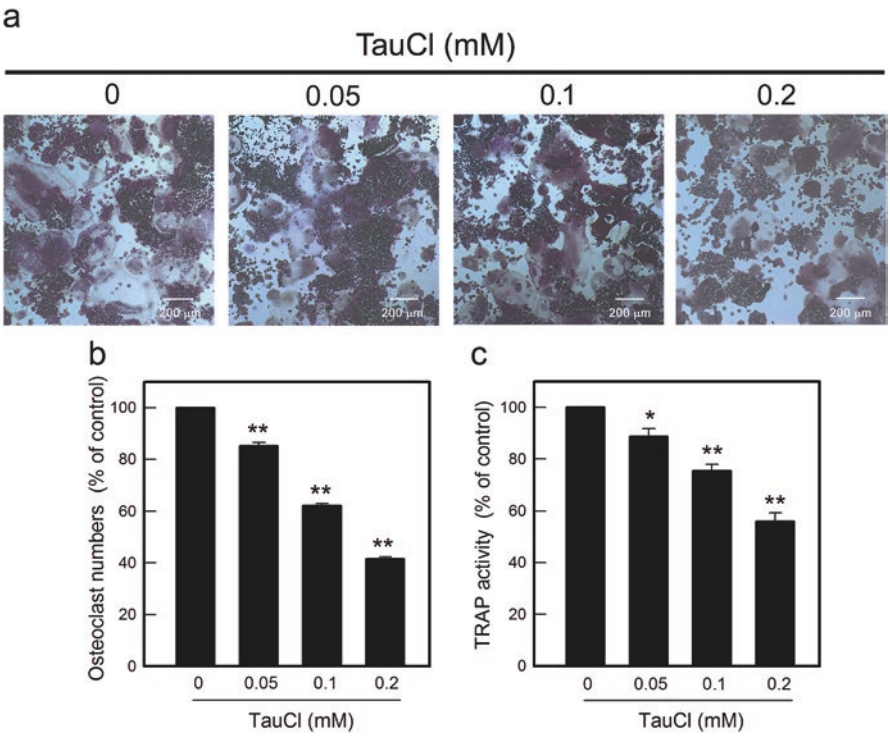

Fig. 1 TauCl inhibits RANKL-induced osteoclastic differentiation of RAW 264.7 cells. (**a**) Osteoclastic differentiation of RAW 264.7 cells in response to 100 ng/ml RANKL was visualized. (**b**) The number of multinuclear (nuclei ≥3) TRAP-positive cells was counted. (**c**) TRAP activity of mature osteoclasts was determined. Data are presented as means ± SD (n = 3), $*p < 0.05$ and $**p < 0.01$

3.2 TauCl Inhibited the mRNA Expression of Osteoclast-Associated Markers

Along with TRAP, CTSK, and CTR serve as histochemical markers of osteoclast. CTSK is a cysteine proteinase secreted by osteoclasts and plays a role in bone resorption and remodeling (Fuller et al. 2008). CTR is a G-protein-coupled receptor that is expressed on osteoclasts but not osteoblasts (Nicholson et al. 1986) and regulates maintenance of calcium homeostasis during bone formation and metabolism (Dacquin et al. 2004; Naot and Cornish 2008).

We investigated the effect of TauCl on the expression of genes associated with osteoclasts. Consistent with the TRAP-positive cell numbers, TauCl significantly inhibited the expressions of TRAP, CTSK, and CTR mRNAs in osteoclasts in a concentration-dependent manner (Fig. 2).

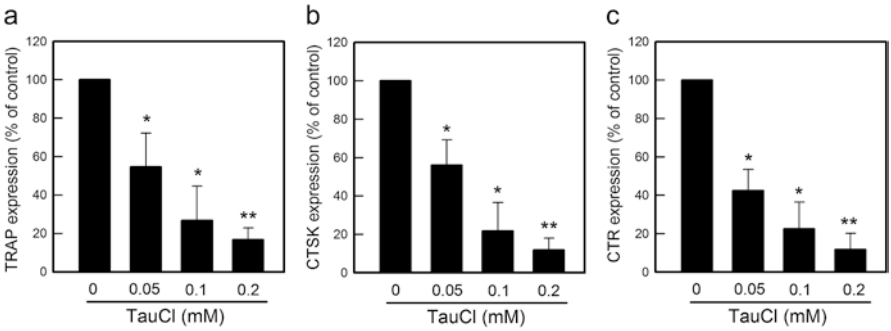

Fig. 2 TauCl inhibits RANKL-induced mRNA expression of osteoclast-associated markers. mRNA expression of (**a**) TRAP, (**b**) CTSK, and (**c**) CTR in osteoclasts was measured by qRT-PCR. Relative expression values were obtained after normalization to GAPDH. Data are presented as means ± SD (n = 3), ∗p < 0.05 and ∗∗p < 0.01

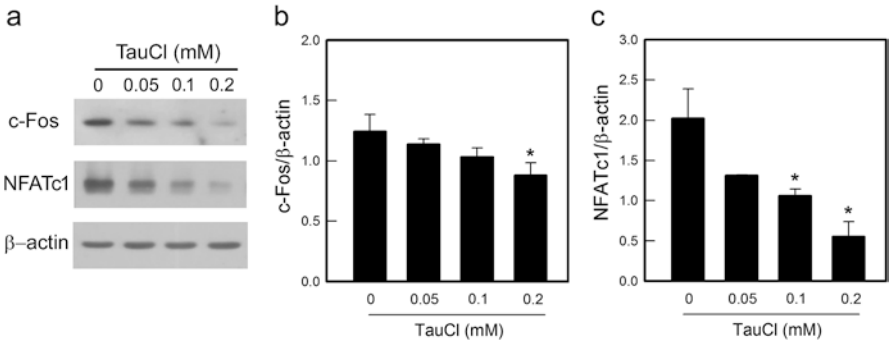

Fig. 3 TauCl inhibits the RANKL-induced expression of c-Fos and NFATc1. (**a**) Western blot analysis of c-Fos and NFATc1 proteins after 4 days of RANKL stimulation. (**b**) The levels of c-Fos and NFATc1 proteins were determined by densitometry of immunoblot signals and normalized to β-actin. Data are presented as means ± SD (n = 3), ∗p < 0.05

3.3 TauCl Inhibited RANKL-Induced Expression of c-Fos and NFATc1

A number of transcription factors, such as PU.1, c-Fos, NF-κB, and NFATc1, play critical roles during osteoclastogenesis (Grigoriadis et al. 1994; Teitelbaum 2000). To investigate the effect of TauCl on RANKL-induced transcription factors, c-Fos and NFATc1 expression was determined. TauCl inhibited RANKL-induced expression of c-Fos and NFATc1 in osteoclasts (Fig. 3). This suggests that the inhibitory effect of TauCl on osteoclastic differentiation of RAW 264.7 cells is mediated, at least in part, by the down-regulation of c-Fos and NFATc1.

4 Discussion

TauCl is generated by the halide-dependent myeloperoxidase system in activated neutrophils during inflammation and is released from neutrophils. In inflamed bone, excessive activation of osteoclasts causes pathological conditions associated with bone resorption including rheumatoid arthritis and osteoporosis (Boyle et al. 2003). Accordingly, down-regulation of osteoclast differentiation or functions is a promising approach for treating such bone diseases.

RAW 264.7 cells are widely used as osteoclast precursors due to the expression of RANK and differentiation into osteoclasts in response to RANKL. In this study, we found that TauCl significantly inhibited RANKL-induced osteoclast differentiation and TRAP activity of RAW 264.7 cells (Fig. 1). Osteoclast differentiation is also associated with the upregulation of TRAP in response to RANKL. TRAP is highly expressed in osteoclasts, activated macrophages, and neurons, and its expression is increased in certain disease conditions including osteoclastoma, osteoporosis, and metabolic bone diseases. In bone resorption sites, TRAP is involved in initiating osteoclast differentiation, activation, and proliferation. TRAP-deficient mice exhibit reduced osteoclast activity and mild osteopetrosis (Hayman et al. 1996). Thus, our results suggest that TauCl inhibits osteoclastic differentiation and activity, thus preventing inflammatory bone resorption.

RANKL binding to RANK recruits c-Fos and subsequently regulates osteoclastogenesis via NFATc1 (Grigoriadis et al. 1994; Wang et al. 1992; Zhao et al. 2010). c-Fos is known as a key regulator of osteoclast-macrophage lineage determination and bone remodeling. NFATc1 is a master transcription factor that is induced by RANKL stimulation and its expression is auto-amplified by TRAF6 and c-Fos (Takayanagi et al. 2002). NFATc1 regulates proteins that are important for bone resorption and formation, such as TRAP, CTSK, and CTR. In this study, TauCl inhibited expression of the osteoclast-associated transcription factors, c-Fos and NFATc1, and osteoclast specific markers (Figs. 2 and 3). These data suggest that TauCl inhibits osteoclastogenesis of RAW 264.7 cells through suppression of c-Fos and NFATc1.

5 Conclusion

In summary, TauCl inhibited RANKL-induced osteoclastic differentiation of RAW 264.7 cells and the expression of osteoclast markers, such as TRAP, CTSK, and CTR. TauCl also inhibited expression of the osteoclast-associated transcription factors, c-Fos and NFATc1. Thus, TauCl produced endogenously by the oxidative burst of activated neutrophils during the inflammatory response inhibits RANKL-induced osteoclastic differentiation of RAW 264.7 cells via regulation of c-Fos and NFATc1. Our results may contribute to further investigations examining the utilization of TauCl as a therapeutic candidate for various bone diseases associated with inflammatory bone resorption.

Acknowledgements This work was supported by the Inha University Research Fund.

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Taurine Enhances the Protective Actions of Fish Oil Against D-Galactosamine-Induced Metabolic Changes and Hepatic Lipid Accumulation and Injury in the Rat

Yuanjun Shen and Cesar A. Lau-Cam

Abstract This study has evaluated the effects of a supplementation with taurine (TAU) on the actions of fish oil (FO) against the hypoglycemia, hypoproteinemia, and hepatic accumulation of lipids and liver damage caused by D-galactosamine (GAL) in the rat. To this end, male Sprague-Dawley rats (200–225 g), in groups of 6, were orally treated with physiological saline (2.5 mL, control group), FO (60 mg/kg), TAU (2.4 mmol/kg) or FO-TAU for three consecutive days and before a single oral dose of GAL (400 mg/kg) given on day 3. In parallel, rats receiving only GAL on day 3 or N-acetylcysteine (NAC, 2.4 mmol/kg) for 3 days before GAL served as controls. On day 4 blood samples were collected by cardiac puncture and used to either measure glucose (GLC) or to obtain plasma fractions. Immediately thereafter, the livers were excised, made into a homogenate in phosphate buffered saline pH 7.4, and centrifuged to obtain clear supernatant. Plasma samples were assayed for their total protein (TP), triglycerides (TG), cholesterol (CHOL), phospholipids (PLP), free fatty acids (FFA) and total bilirubin (TB) and direct bilirubin (DB) contents, and for the activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). The liver homogenates were used to measure TG, CHOL, PLP and total lipids (TL) contents. Without exceptions, GAL was found to markedly affect ($p < 0.001$) all of the experimental parameters examined, with increases occurring in all instances except for the values of the plasma GLC, TP and PLP which were decreased. A pretreatment with either FO or TAU led to significant attenuation of the effects of GAL and which, in most cases, were of similar magnitude. On the other hand, a combined pretreatment with FO plus TAU usually resulted in a greater protection than with either agent alone ($p \leq 0.05$). NAC, serving as a reference treatment, was, in most instances, equipotent with FO alone and. in addition, was the only agent that significantly attenuated the increases in both liver weight and liver weight to body weight ratio caused by GAL.

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Keywords Galactosamine · Fish oil · Taurine · Plasma glucose · Total protein · Bilirubin · Alanine aminotransferase · Aspartate aminotransferase · Alkaline phosphatase · Plasma and liver lipids

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CHOL	Cholesterol
DB	Direct bilirubin
FFA	Free fatty acids
FO	Fish oil
GAL	Galactosamine
GLC	Glucose
NAC	N-acetylcysteine
PLP	Phospholipids
TAU	Taurine
TB	Total bilirubin
TL	Total lipids
TP	Total protein

1 Introduction

D-Galactosamine (GAL) is an experimental toxin capable of causing biochemical, functional and histological changes consistent with liver cell injury and resembling those present in human viral hepatitis (Keppler and Decker 1969). In addition to progressively increasing the serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) serving as indices of liver damage (Shanmugarajan et al. 2008), it also causes hypoglycemia (Al-Tuwaijri et al. 1981), hyperbilirubinemia (Saracyn et al. 2015), hepatic lipid accumulation, hyperplasia of the smooth endoplasmic reticulum and organelle injury in the liver (Koff et al. 1971), and liver cell death (El-Mofty et al. 1975).

Omega-3 polyunsaturated fatty acids (PUFA) present in fish oil (FO) have been found to lower the elevation of the plasma total bilirubin that follows the injection of GAL to mice (Roy et al. 2007). Furthermore, there is also evidence that FO with a high omega-3 PUFA content can protect against the lipid peroxidation of hepatic phospholipids of naive rats (Ando et al. 2000; Kikugawa et al. 2003), and the elevation of serum triacylglycerols of psychologically stressed mice (Oarada et al. 2008).

Several reports have described the protective actions of taurine (TAU) against hepatotoxicity by a wide variety of toxicants (Heidari et al. 2016), but, in compari-

son to other amino acids, only a limited number of studies have been devoted to investigating its ability to completely reverse GAL-induced hepatic failure (Asha and Devadasan 2013) and necrosis (MacDonald et al. 1985). In contrast, there are studies that have investigated the effects of TAU against the hepatic accumulation of lipids caused by a variety of pathologies such as nonalcoholic fatty liver disease (Gentile et al. 2011) and ethanol-induced hepatic steatosis and lipid peroxidation (Kerai et al. 1999; Pushpakiran et al. 2005).

Taken into account the known protective effects of both FO and TAU, the present study was undertaken to investigate if these agents can protect against hepatic carbohydrate and lipid dysfunction caused by GAL in the rat.

2 Materials and Methods

2.1 Chemicals

D-(+)-Galactosamine hydrochloride (GAL) was from Santa Cruz Biotechnology, Santa Cruz, CA, USA. FO as 1200 mg liquid softgels was from Nature Made Products, Mission Hills, CA, USA. N-Acetyl-L-cysteine (NAC) and TAU were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Animals

Male Sprague-Dawley rats, 200–225 g in weight, obtained from Taconic Farms Inc., Germantown, NY, USA, housed in a temperature- (23 ± 1 °C) and humidity controlled room on a 12 h light-12 h dark cycle, and used after a 7 day acclimation period, during which they had free access to a commercial rodent diet (LabDiet® 5001, PMI Nutrition International, Brentwood, MO, USA) and filtered tap water. The rats were randomly assigned to groups of 6 each, and were cared in accordance with guidelines described in the Animal Care Blue Book from the United States Department of Agriculture (2013).

2.3 Treatments

Animals in the control groups received a single dose of physiological saline (2.5 mL), of FO (60 mg/kg) or of TAU (2.4 mmol/kg) each day for three consecutive days. Rats in the GAL received a singed dose of physiological saline every day for 2 consecutive days and a single, 400 mg/kg, dose of GAL on day 3. Animals in a treatment group received a single dose of a treatment agent (FO, TAU or NAC) for 2 days and

GAL plus a treatment agent on day 3; and in the case of a treatment combination, FO plus TAU for 2 days and GAL plus FO and TAU on day 3. All treatment solutions, but that of FO, were prepared in physiological saline. In the case of FO, the dissolution was facilitated by adding Tween 20 (1 mL per 2 softgels) and heating to 60°C on a sonicator. All the treatments were made by the intraperitoneal route.

2.4 Sample Collection and Preparation

At 24 h after a treatment with physiological saline, GAL or GAL plus a treatment agent, the rats were anesthetized with isoflurane and a sample of tall vein blood was collected for the measurement of glucose using a commercial glucometer (TRUEtrack™ from Nipro Diagnostics, Fort Lauderdale, FL, USA).

Immediately thereafter, a blood sample was collected from each rat by cardiac puncture into heparinized tubes, and processed for its plasma fraction. Without delay, the abdomen of each rat was cut open to expose the liver, which was removed, weighed and immediately stored in liquid nitrogen until needed. A 0.5 g portion of each liver was place in glass tube, mixed with ice-cold phosphate buffered saline (PBS) solution of pH 7.4, and homogenized with a hand held electric tissue homogenizer (Tissue-Tearor™, Bio Spec Products Inc., Bartlesville, OK, USA) to obtain 10 mL of suspension, which was centrifuged at 3000 rpm and 0°C for 30 min to obtain a clear supernatant.

2.5 Assays

2.5.1 Assays on Plasma and Liver Samples

The concentrations of plasma and liver CHOL, TG and TP, plasma TB and DB, and activities of plasma ALP, ALT and AST were measured using commercial assay kits from for Stanbio Laboratories Boerne, TX, USA. The concentration of plasma and liver PLP was measured using a commercial kit from BioAssay Systems, Hayward, CA, USA. Plasma free fatty acids (FFA) were measured using commercial microplate kit from BioVision Inc., Mountain View, CA, USA. The concentration of liver TL represented the sum of the liver CHOL, PLP and TG.

2.6 Statistics

Results are expressed as mean±SD for n = 6. They were statistically analyzed for significance using unpaired Student's t-test followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$.

3 Results

3.1 Body Weights, Liver Weights and Liver Weight to Body Weight Ratios

No marked differences were noted between the body weights of control animals and those of animals receiving GAL alone or GAL plus a treatment agent (Table 1). In contrast, the liver of GAL-treated rats was heavier than that of control rats (by 33%, $p < 0.01$), an effect that was reduced significantly by NAC (19% less, $p < 0.05$ vs. GAL) and FO plus NAC (13% less, $p < 0.05$ vs. GAL) but not by FO, TAU and FO pus TAU (reductions ≤8% of the values seen with GAL alone). A similar situation was apparent regarding the effect on the various treatments on the liver weight to body weight ratios. Thus, while GAL raised the value of the ratio by 28% ($p < 0.05$ vs. control), NAC reduced it to a value similar to that of control animals (only 8% over) and FO and TAU reduced it to an insignificant (~18% over) extent. A treatment with FO plus NAC was found insignificantly more effective than one with FO plus NAC in antagonizing the elevating effect of GAL on the liver weight to body weight ratio (+16% increase vs. 25% increase, $p < 0.05$ vs. control). By themselves, none of the treatment agents had an obvious effect on the body weight, liver weight and liver weight to body weight ratio of control animals.

3.2 Effect of the Treatment Agents on the Blood GLC and Plasma TP Levels

As shown in Table 2, GAL lowered the control blood GLC by 53% ($p < 0.001$), an effect that was reduced significantly not only by both FO (−36%, $p < 0.01$) and TAU (−34%, $p < 0.01$) but also by NAC (−27%, $p < 0.05$). The counteracting effect of

Table 1 Body weights, liver weights and liver weight to body weight ratios of rats receiving GAL and GAL plus a treatment agent[a]

Treatment group	Body weight, g	Liver weight, g	Liver weight to body weight ratio, $\times 10^{-2}$
Control	250.00 ± 2.99	$6.89 \pm 0.61^{+,\bullet}$	$2.76 \pm 0.20^{+}$
GAL	257.50 ± 3.65	$9.07 \pm 0.87^{**}$	$3.52 \pm 0.23^{*}$
FO	249.20 ± 3.56	$6.90 \pm 0.82^{+,\bullet}$	$2.77 \pm 0.23^{+}$
TAU	259.20 ± 3.58	$7.25 \pm 0.69^{+,\bullet}$	$2.80 \pm 0.19^{+}$
NAC	245.00 ± 2.89	$6.87 \pm 0.79^{+,\bullet}$	$2.80 \pm 0.27^{+}$
FO + GAL	265.80 ± 3.95	$8.59 \pm 0.84^{*}$	$3.23 \pm 0.21^{*}$
TAU + GAL	250.80 ± 3.94	$8.21 \pm 0.91^{*}$	$3.27 \pm 0.23^{*}$
NAC + GAL	248.30 ± 3.59	$7.38 \pm 0.81^{+}$	$2.97 \pm 0.23^{+}$
FO + TAU + GAL	246.70 ± 3.48	$8.52 \pm 0.83*$	$3.46 \pm 0.24^{*}$
FO + NAC + GAL	240.00 ± 4.05	7.71 ± 1.19	$3.21 \pm 0.29^{*}$

[a]Differences were statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. Control group; at $^{+}p < 0.05$ vs. GAL group; and at $^{\bullet}p < 0.05$ vs. FO + GAL group

Table 2 Circulating levels of GLC and TP of rats receiving GAL and GAL plus a treatment agent[a]

Treatment group	Blood GLC, mg/dL	Plasma TP, g/dL
Control	$109.00 \pm 3.25^{+++,▪▪▪}$	$5.65 \pm 0.15^{+++}$
GAL	$52.20 \pm 4.10^{***,*}$	$2.14 \pm 0.52^{***,▪▪▪}$
FO	$96.00 \pm 2.90^{+++,▪▪}$	$5.72 \pm 0.49^{+++,▪}$
TAU	$93.21 \pm 3.44^{+++,▪▪}$	$5.79 \pm 0.28^{+++,▪}$
NAC	$102.03 \pm 4.02^{+++,▪▪}$	$5.58 \pm 0.39^{+++,▪}$
FO + GAL	$69.90 \pm 3.14^{**,++}$	$4.48 \pm 0.60^{*,+++}$
TAU + GAL	$72.00 \pm 4.06^{**,++}$	$4.40 \pm 0.47^{*,+++}$
NAC + GAL	$79.80 \pm 3.36^{*,+++}$	$4.36 \pm 0.39^{*,+++}$
FO + TAU + GAL	$83.70 \pm 3.13^{*,+++,▪}$	$4.87 \pm 0.51^{+++}$
FO + NAC + GAL	$95.30 \pm 4.25^{+++,▪▪}$	$5.01 \pm 0.30^{+++}$

[a]Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$ vs. Control group; at $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. GAL group; and vs. FO + GAL at $^{▪▪}p < 0.01$ and $^{▪▪▪}p < 0.001$

FO was enhanced when it was given along with either TAU (−23%, $p < 0.05$) or NAC (−13%). In naive rats none of the test compounds showed a significant effect on the blood GLC level.

In parallel with its decreasing effect on the blood GLC, GAL also caused a significant reduction of the plasma TP value (by 63%, $p < 0.001$ vs. control, Table 2. This effect was markedly attenuated by all the test agents, with their effect being rather similar (by 21–23%, $p < 0.01$ vs. control). The protection was further enhanced when FO was co-administered with either TAU (only −14%) or NAC (only −11%). In naive rats none of the treatments agents had any effect on the plasma TP value.

3.3 Effect of the Treatment Agents on the Plasma ALT, AST and ALP Activities

As an indirect evidence of the extent of liver injury caused by GAL, the activities of ALT, AST and ALP were measured in the plasma. Based on the marked increases in the plasma values of ALT, AST and ALP (332%, 129% and 76%, respectively, all at $p < 0.001$ vs. corresponding control values) shown in Table 3, it can be seen that GAL caused an extensive liver injury. These effects were significantly attenuated by all the treatment agents, with the individual potencies varying within a narrow range. Thus, in the case of the plasma ALT, TAU was the most potent, followed by NAC and FO (decreases of 40%, 28% and 23%, respectively, of the values recorded for GAL alone) These protective effects were enhanced further when FO was given together with NAC (35% decrease) or, better, with TAU (47% decrease) relative to GAL alone. Likewise, all the treatment agents attenuated the increase in plasma AST cause by GAL, with TAU and NAC being about equipotent (~20% decrease,

Table 3 Plasma ALT, AST and ALP activities of rats receiving GAL and GAL plus a treatment agent[a]

Treatment group	Plasma ALT, U/L	Plasma AST, U/L	Plasma ALP, U/L
Control	$29.10 \pm 3.39^{+++}$	$69.26 \pm 4.31^{+++}$	$145.87 \pm 4.99^{++}$
GAL	$125.62 \pm 12.16^{***}$	$158.41 \pm 11.43^{***}$	$256.22 \pm 9.04^{***}$
FO	$29.97 \pm 2.20^{+++,\bullet\bullet\bullet}$	$78.42 \pm 4.09^{+++,\bullet\bullet}$	$131.39 \pm 4.68^{++,\bullet\bullet}$
TAU	$38.41 \pm 4.34^{**,+++,\bullet\bullet\bullet}$	$71.45 \pm 5.27^{+++,\bullet\bullet}$	$136.19 \pm 4.28^{+++,\bullet}$
NAC	$25.17 \pm 3.40^{+++,\bullet\bullet\bullet}$	$71.15 \pm 9.42^{+++,\bullet\bullet}$	$125.71 \pm 4.87^{+++,\bullet\bullet}$
FO + GAL	$96.27 \pm 5.46^{***,+}$	$112.13 \pm 9.42^{***,+}$	$193.91 \pm 6.12^{**,+}$
TAU + GAL	$75.83 \pm 6.14^{***,++,\bullet}$	$127.00 \pm 11.87^{***,+}$	$198.23 \pm 5.10^{**,+}$
NAC + GAL	$89.98 \pm 3.17^{***,++}$	$128.04 \pm 8.56^{***,+}$	$174.26 \pm 4.79^{*,++}$
FO + TAU + GAL	$67.10 \pm 7.71^{***,++,\bullet\bullet}$	$71.77 \pm 6.56^{+++,\bullet\bullet}$	$172.54 \pm 5.18^{*,++}$
FO + NAC + GAL	$81.31 \pm 9.35^{***,++,\bullet}$	$68.12 \pm 6.41^{**,+++}$	$140.70 \pm 7.46^{++,\bullet}$

[a]Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$ vs. Control group; at $^{+}p < 0.045$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. GAL group; and at $^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$ and $^{\bullet\bullet\bullet}p < 0.001$ vs. FO + GAL

$p < 0.05$ vs. GAL) and FO providing a slightly greater effect (−29%, $p < 0.05$ vs. GAL). In contrast, providing FO with either TAU or NAC brought the AST value to within normal. In parallel with the results seen with the transaminase activities, all of the treatment agents were found to reduce the ALP elevation caused by GAL, with TAU and FO providing about equal protection (36% and 33% increases, respectively, $p < 0.01$ vs. control) and NAC lesser protection (20% increase, $p < 0.05$). A combined treatment with FO and TAU reduce the increase of the ALP further (18%) and one with FO plus NAC brought the value to within the control value. None of the treatment agents showed a significant effect on the basal activities of ALT, AST and ALP (increases ≤14%).

3.4 Effect of the Treatment Agents on the Plasma TB and DB

As shown in Table 4, GAL raised the plasma levels of TB and DB by 6.1-fold and 46-fold, respectively, over the corresponding control values (both at $p < 0.001$). In the presence of TAU the TB rose by only 3.5-fold and the DB by only 16-fold. On the other hand, increases in TB and DB in rats receiving GAL plus FO rose by ~4.8-fold and 13.9-fold, respectively (both at $p < 0.001$ vs. control values); and providing FO plus TAU kept the increases in TB and DB caused by GAL to only 1.9-fold and 6.8-fold respectively. By comparison, values for TB and DB were fourfold and 13.7-fold greater than control values, and which further decreased to only 1.77-fold and 6.3-fold when given together with FO. None of the test agents affected the baseline values of both TB and DB to a significant extent.

Table 4 Circulating levels of TB and DB of rats receiving GAL and GAL plus a treatment agent[a]

Treatment group	Plasma TB, mg/L	Plasma DB, mg/L
Control	$345.31 \pm 9.87^{+++,***}$	$22.00 \pm 4.46^{+++,***}$
GAL	$2172.23 \pm 32.79^{***}$	$1011.06 \pm 15.62^{***,▪▪▪}$
FO	$356.64 \pm 8.92^{+++,▪▪▪}$	$19.70 \pm 4.68^{+++,▪▪▪}$
TAU	$385.22 \pm 22.89^{+++,▪▪▪}$	$27.32 \pm 6.74^{*,+++,▪▪▪}$
NAC	$381.58 \pm 23.15^{+++,▪▪▪}$	$26.22 \pm 4.59^{*,+++,▪▪▪}$
FO + GAL	$1656.76 \pm 33.65^{***,+++}$	$306.38 \pm 14.07^{***,+++}$
TAU + GAL	$1243.20 \pm 36.49^{***,+++}$	$354.15 \pm 14.67^{***,+++,▪}$
NAC + GAL	$1400.29 \pm 36.30^{***,+++}$	$302.44 \pm 15.65^{***,+++}$
FO + TAU + GAL	$658.20 \pm 44.40^{***,+++}$	$150.09 \pm 14.26^{***,+++,▪▪▪}$
FO + NAC + GAL	$612.04 \pm 22.29^{***,+++}$	$138.00 \pm 6.26^{***,+++,▪▪▪}$

[a]Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$ vs. Control group; at $^{+++}p < 0.001$ vs. GAL group; and at $^{▪}p < 0.05$ and $^{▪▪▪}p < 0.001$ vs. FO + GAL

3.5 Plasma CHOL, TG, PLP and FFA

GAL raised the plasma levels of CHOL (by 59%), TG (by 163%) and FFA (by 4.35-fold) significantly over corresponding control values (all differences at $p < 0.001$). In contrast, the corresponding plasma PLP was decreased (by 76%, $p < 0.001$) (Table 5). Increases in plasma CHOL were lower in rats receiving GAL plus FO (+18%, $p < 0.05$), TAU (+15%, $p < 0.05$) and especially NAC (+13%); and were returned to baseline values when FO was administered together with either TAU or NAC. Likewise, all the treatment agents were found to drastically reduce the increase in plasma TG cause by GAL, with NAC (+75%) providing a slightly greater effect than either TAU (+79%) or FO (+88%). These attenuating effects increased further when FO was given together with either TAU (+53%) or NAC (+47%). Furthermore, GAL caused a massive increase in plasma FFA (4.35-fold, $p < 0.001$ vs. control). This effect was significantly reduced by all the treatment agent ($p < 0.001$ vs. GAL), with the increase being smaller with NAC (+2.2-fold) than with either TAU (+2.4-fold) or FO (+2.5-fold). A still greater attenuation was attained when FO was available alongside either TAU or NAC (only 1.75-fold increase, $p < 0.001$ vs. GAL). None of the treatment agents was found to significantly affect the baselines values of CHOL, TG and PLP; but FO and TAU had a small, although significant, lowering effect on the baseline plasma FFA level (by 16% and 18%, respectively, both at $p < 0.05$ vs. control).

3.6 Liver CHOL, TG, PLP and TL

As seen in Table 6, GAL raised the liver CHOL (by 269%), TG (by 79%) and PLP (by 89%) relative to corresponding control values (all at $p < 0.001$). As a result, the liver TL was also significantly elevated (by 125%, $p < 0.001$). By comparison,

Table 5 Plasma CHOL, TG, PLP and FFA of rats receiving GalN and GalN plus a treatment agent[a]

Treatment group	CHOL, mg/dL	TG, mg/dL	PLP, μmol/L	FFA, μmol/L
Control	$49.19 \pm 3.36^{++,\bullet}$	$30.11 \pm 1.84^{+++,\bullet\bullet}$	$442.65 \pm 7.92^{+++}$	$11.05 \pm 1.52^{+++,\bullet\bullet\bullet}$
GalN	$78.44 \pm 4.64^{***,\bullet\bullet}$	$79.22 \pm 6.76^{***,\bullet\bullet}$	$107.32 \pm 5.83^{***}$	$48.06 \pm 3.71^{***,\bullet\bullet\bullet}$
FO	$52.21 \pm 3.54^{++}$	$29.80 \pm 3.76^{+++,\bullet\bullet}$	$521.07 \pm 7.08^{*,+++,\bullet\bullet\bullet}$	$9.09 \pm 1.79^{*,+++,\bullet\bullet\bullet}$
TAU	$51.70 \pm 4.38^{++}$	$28.15 \pm 3.51^{+++,\bullet\bullet\bullet}$	$451.35 \pm 13.11^{+++,\bullet\bullet\bullet}$	$9.27 \pm 1.07^{*,+++,\bullet\bullet\bullet}$
NAC	$47.82 \pm 4.13^{++,\bullet}$	$34.44 \pm 3.93^{+++,\bullet\bullet}$	$477.23 \pm 10.70^{+++,\bullet\bullet\bullet}$	$1.41 \pm 2.11^{+++,\bullet\bullet\bullet}$
FO + GalN	$58.08 \pm 1.84^{*,+}$	$56.67 \pm 5.08^{***,+}$	$150.28 \pm 3.85^{***,++}$	$28.01 \pm 3.76^{***,++}$
TAU + GalN	$56.80 \pm 4.87^{*,+}$	$54.03 \pm 5.73^{***,++}$	$165.17 \pm 7.52^{***,+++}$	$26.84 \pm 3.67^{***,++}$
NAC + GalN	$55.73 \pm 4.40^{+}$	$52.59 \pm 5.31^{***,++}$	$153.93 \pm 9.63^{***,++}$	$24.37 \pm 3.33^{***,++}$
FO + TAU + GalN	$50.77 + 5.61^{++}$	$46.13 \pm 5.76^{***,++,\bullet}$	$268.02 \pm 7.18^{**,+++,\bullet\bullet\bullet}$	$19.20 \pm 1.10^{***,+++,\bullet\bullet}$
FO + NAC + GalN	$51.56 \pm 4.25^{++}$	$44.26 \pm 4.67^{**,++,\bullet}$	$259.60 \pm 10.29^{**,+++,\bullet\bullet\bullet}$	$19.50 \pm 2.87^{***,+++,\bullet\bullet}$

[a]Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$ vs. Control group; at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. GalN group; and at $^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$ and $^{\bullet\bullet\bullet}p < 0.001$ vs. FO + GalN

Table 6 Liver CHOL, TG, PLP and TLP levels of rats receiving GalN and GalN plus a treatment agent[a]

Treatment group	CHOL, mg/g	TG, mg/g	PLP, mg/g	TLP, mg/g
Control	$4.28 \pm 1.51^{+++,\bullet}$	$11.17 \pm 2.01^{++}$	$2.78 \pm 0.16^{++,\bullet\bullet}$	$18.23 \pm 2.28^{+++}$
GalN	$15.81 \pm 3.18^{***,\bullet\bullet\bullet}$	$19.99 \pm 2.23^{***}$	$5.25 \pm 0.49^{***,\bullet}$	$41.05 \pm 4.15^{***,\bullet\bullet\bullet}$
FO	$5.19 \pm 1.42^{*,+++}$	$12.10 \pm 0.38^{++}$	$2.81 \pm 0.31^{*,+++,\bullet\bullet}$	$19.48 \pm 1.75^{+++}$
TAU	$7.75 \pm 3.59^{**,+++,\bullet}$	$7.75 \pm 3.59^{**,+++,\bullet\bullet}$	$3.06 \pm 0.35^{++,\bullet}$	$15.09 \pm 3.81^{*,+++,\bullet}$
NAC	$5.35 \pm 1.34^{*,+++,\bullet\bullet}$	$9.24 \pm 2.02^{*,+++,\bullet}$	$3.06 \pm 0.35^{++,\bullet}$	$17.65 \pm 2.64^{+++,\bullet}$
FO + GalN	$5.26 \pm 1.46^{*,+++}$	$11.39 \pm 0.86^{++}$	$4.24 \pm 0.38^{***,+}$	$20.88 \pm 2.04^{++}$
TAU + GalN	$9.00 \pm 2.78^{***,++}$	$11.12 \pm 1.88^{++}$	$3.72 \pm 0.35^{**,+}$	$23.84 \pm .51^{**,++}$
NAC + GalN	$8.13 \pm 1.12^{***,++,\bullet\bullet\bullet}$	$9.94 \pm 1.08^{++}$	$4.58 \pm 0.37^{***}$	$22.65 \pm 1.91^{*,++}$
FO + TAU + GalN	$5.64 \pm 1.92^{**,+++}$	$7.95 \pm 2.73^{*,+++,\bullet\bullet}$	$3.13 \pm 0.32^{++}$	$16.72 \pm 3.47^{+++}$
FO + NAC + GalN	$5.67 \pm 1.38^{**,+++}$	$9.87 \pm 2.07^{+++}$	$3.98 \pm 0.31^{**,++}$	$19.52 \pm 2.66^{+++}$

[a]Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Control group; at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. GalN group; and at $^{\bullet}p < 0.05$, $^{\bullet\bullet}p < 0.01$ and $^{\bullet\bullet\bullet}p < 0.01$ from FO + GalN

animals receiving FO, TAU or NAC showed liver CHOL levels that were markedly lower, with FO (only 23% increase, $p < 0.05$) showing a greater attenuating effect than either NAC (90% increase, $p < 0.001$) or TAU (110% increase, $p < 0.001$). Furthermore, a pretreatment with either FO plus TAU or FO plus NAC led to a liver CHOL value that was much lower than that seen with either NAC or TAU (only 32% increase, $p < 0.01$). In naive rats, TAU did not alter the basal liver CHOL, and both FO and NAC had a small but significant elevating effect (+21% and +25%, respectively, $p < 0.05$ vs. control). All of the pretreatment agents returned the liver TG to values that were equal to or slightly below the control value. This effect was also seen when FO was co-administered with either TAU or NAC. In naive rats, the liver TG was comparable to (FO) or below (TAU, NAC) the control value. In common with the effects on the liver CHOL and TG, all the treatment agents also lower the increase in liver PLP, with TAU showing a greater potency (+34%, $p < 0.01$) than either FO (+53%, $p < 0.001$) or NAC (+65%, $p < 0.001$). On the on the hard a combined treatment with FO plus TAU (+13%) or with FO plus NAC (+43%, $p < 0.001$) had a greater lowering effect than with TAU or NAC alone. None of the treatment agents had a significant effect on the basal PLP levels. Taking into account the effects of the various pretreatment agents on the various types of liver lipids, it is not surprising that they also prevented the increase in liver TP caused by GAL. In this regard, FO was more potent (only 15% increase, $p < 0.05$ vs. control) than either NAC (24% increase, $p < 0.05$) or TAU (31% increase, $p < 0.01$). Adding TAU or NAC to a pretreatment with FO returned the liver TP to a value comparable to that of control animals.

4 Discussion

The statistically significant reduction of the plasma GLC and TP coupled with an increase in the levels of circulating TB and the activities of ALT, AST and ALP in the plasma are taken as an indication of the development of acute liver failure as a result of the necrotizing action of GAL on the liver (Okada et al. 2011; Saracyn et al. 2015; Sugiyama et al. 1999). Although it has been suggested that the morphologic and functional alterations caused by GAL on the liver resemble human viral hepatitis (Keppler and Decker 1969), the accumulation of triglycerides, hyperplasia of the smooth endoplasmic reticulum add cell necrosis set GAL apart (Stramentinoli et al. 1978).

Several lines of evidence suggest the use of TAU in the treatment of GAL-related liver injury. For example, low levels of liver TAU have been correlated with increased susceptibility to the damaging effects of a hepatotoxicant (Timbrell et al. 1995; Waterfield et al. 1991). Moreover, increases in the urinary levels of TAU has been found to be associated with the extent of the hepatic cell necrosis and steatosis caused by toxins such as GAL, possibly as a result of changes in protein synthesis and, hence, in sulfur amino acid metabolism caused by the hepatotoxin (Timbrell and Waterfield 1996; Waterfield et al. 1993). There is also evidence that TAU can protect against GAL-induced hepatic necrosis without preventing the subcellular accumulation of calcium (MacDonald et al. 1985). On the other hand, omega-3 fatty acids like those found in FO have shown the ability to alleviate GAL-induced inflammation, to lower serum ALT and AST elevations, to decrease the accumulation of liver fat, and to lessen the severity of histologically-demonstrable liver damage (Dossi et al. 2014; Pettinelli et al. 2009; Schmöcker et al. 2007). In addition, supplementation of a normal diet with omega-3 long chain polyunsaturated fatty acids (LPUFA) has been found to trigger an antioxidant response, to alleviate inflammation and to prevent liver steatosis in mice fed a high fat diet (Tapia et al. 2014; Valenzuela et al. 2012). Several mechanisms have been put forward to account for the antisteatotic action of omega-3 LPUFA, including diminution of fatty acids and glycerol mobilization from peripheral tissue to the liver for subsequent lipolysis, and enhancement of hepatic fatty acid oxidation since EPA and DHA are intracellular signals regulating lipid metabolism in the liver (Tapia et al. 2014).

In agreement with the results of other laboratories (Shin et al. 2014; Sugiyama et al. 1998) rats receiving GAL alone were heavier than livers from control rats even though the body weights among the various experimental groups were rather similar. Following a pretreatment with FO, TAU, NAC or FO plus TAU, the liver weights were significantly lower than those of rats receiving only GAL but still above the control weights. A pretreatment with FO plus NAC reduced the liver weights to a value not significantly different from control weights. From the results on liver lipids, the higher weight of livers from rats receiving only GAL compared to control livers is probably the result of a higher TL content.

The statistically significant reduction of the plasma GLC and TP coupled with an increase in the levels of circulating TB and the activities of ALT, AST and ALP in the plasma following treatment with GAL are taken as an indication of the develop-

ment of acute liver due to the necrotizing action of GAL in the liver (Okada et al. 2011; Saracyn et al. 2015; Sugiyama et al. 1988, 1999). Hypoglycemia, a common clinical feature of fulminant hepatic necrosis, has been ascribed to a loss of gluconeogenesis, reduced hepatic glycogen stores, reduced hepatic insulin clearance (Diaz-Buxo et al. 1997) or reduced rate of glycogen synthesis (Keppler and Decker 1969). In the present study all of the pretreatment agents were able to attenuate the hypoglycemic action of GAL, especially with FO-TAU and FO-NAC, and to a greater extent with NAC and FO-NAC. On the other hand, the reduction of the plasma TP by GAL is considered to be a reflection of the inhibitory action of GAL on of liver protein synthesis as a result of a reduction of the cellular UTP content (Decker and Keppler 1974) or interference by a glucosamine-glycogen complex, designated as aminoglycogen I and -II, on the protein synthesizing apparatus (Mészáros et al. 1976). Like in the case of the blood GLC, all of the pretreatment agents were able to reduce the hypoproteinemia caused by GAL, which was greater when TAU or, better, NAC, was co-administered with FO.

The intracellular aminotransferases ALT and AST and the phosphatase ALP are commonly used as indices of biliary tree inflammation (MacDonald et al. 1985) and hepatocellular injury (Lee et al. 2000; Shin et al. 2014; Sugiyama et al. 1998). As verified elsewhere (Aristatile et al. 2009), the present study found the plasma activities of the three enzymes grossly elevated by GAL, and to be significantly attenuated by all the test agents, especially when available as a combination with FO. These findings are interpreted as being the result of a decreased disruption of hepatocellular membranes and of biliary flow, and hence, of leakage of intracellular enzymes into the circulation (Ravikumar et al. 2005).

The increased levels of plasma TB and DB caused by GAL are suggestive of hepatocellular damage and biliary obstruction, respectively, with the corresponding ratio being about 2:1. Without exceptions, all the pretreatment agents were able to lower these increases, with TAU providing a greater effect on the TB value than NAC and FO, in that order, and with FO and NAC being somewhat more effective than TAU in lowering the DB value. In contrast, a combination of FO with either TAU or NAC was much more effective than any of the individual agents, especially on the plasma TB which was about lowered by at least one-half by the pretreatment combination compared to an individual treatment.

GAL was found to raise the plasma levels of CHOL, TG and FFA and to lower those of PLP. These findings are consisted with the plasma lipid changes seen in other laboratories when assessing parenchymal liver damage by GAL (McIntyre 1978) and GAL-lipopolysaccharide (Ravikumar et al. 2005) although there are also reports indicating an increase in plasma and tissue PLP (Cartwright et al. 1982; Radhiga et al. 2016). The increase in the total CHOL level by GAL is only due to the free CHOL fraction since the accompanying esterified CHOL level is decreased (Sathivel et al. 2008). The trend for changes in liver lipid by GAL were identical to those observed for plasma samples and agreed with the trend observed elsewhere (Ravikumar et al. 2005). In general, the potency of all of the test compounds was rather similar when provided singly, but increased when paired with FO.

In conclusion, the present study has determined that both FO and TAU can effectively lower the changes in circulating GLC and TP, the plasma and hepatic levels of CHOL, TG, and PLP, and the increase in hepatic TL caused by an acute treatment with GAL. In addition, FO and TAU can protect against the damaging effects of GAL on the liver. These effects can be enhanced when TAU and FO are given together. The cysteine precursor NAC, serving as a reference antiinflammatory and antioxidant compound, was found to provide a protection similar to that by FO and TAU, and which increased in the presence of FO.

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Taurine Improves the Actions of Metformin and Lovastatin on Plasma Markers of Carbohydrate and Lipid Dysfunction of Diabetic Rats

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Abstract The present study has investigated the effect of adding taurine (TAU) to a treatment of diabetes with metformin (MET), a hypoglycemic, and lovastatin (LOV), an antihyperlipidemic. To this end, male Sprague-Dawley rats, agent, 250–275 g in weight, were made diabetic with a single 60 mg/kg intraperitoneal (i.p.) dose of streptozocin (STZ) in 10 mM citrate buffer pH 4.5, and, after 14 days, treated daily with oral doses of MET (2.4 mM/kg), LOV (0.075 mM/kg) or TAU (2.4 mM/kg), and with binary and ternary combinations of these agents. Rats receiving only 10 mM citrate buffer pH 4.5 or only STZ served as negative and positive controls, respectively. In addition, rats receiving insulin (INS, 4 units/kg) by the subcutaneous route served as a reference treatment. All the rats were sacrificed on day 57 and their bloods collected into heparinized tubes. The corresponding plasma samples were analyzed for their glucose (GLC), insulin (INS), glycated hemoglobin (HbA_{1c}), cholesterol (CHOL) and triglycerides (TG) contents. In comparison to normal rats, diabetic ones showed marked increases in GLC (+313%), HbA_{1c} (+207%), CHOL (+66%) and TG (+188) and a profound decrease of INS levels (−76%) ($p < 0.001$ vs. control values). Among the various treatments, one with INS produced the greatest lowering effect on the plasm a GLC (+23%, $p < 0.05$), INS (+23%, $p < 0.05$) and TG (+3%), with the remaining changes being similar to those seen with MET. A treatment with MET reduced all the diabetic changes by at least threefold; and one with LOV had a significant ($p < 0.001$) lowering effect on the plasma CHOL and TG but was without an effect on the plasma GLC, INS and HbA_{1c}. In common with LOV, TAU reduced the diabetic levels of both CHOL and TG and, in addition, reduced the diabetic plasma GLC and raised the corresponding INS level. Among binary combinations, one with LOV-MET provided a greater effect than MET alone only in terms of the plasma CHOL and TG; and one with LOV-TAU was only significantly better than TAU alone in lowering the TG levels. However, a treatment with LOV-MET-TAU led to reductions in all the plasma parameters examined that were much greater than those achieved with any of the individual agents or with their binary combinations (at $p \leq 0.05$).

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Abbreviations

CHOL	Cholesterol
DM	Diabetes mellitus
GLC	Glucose
HbA_{1c}	Glycated hemoglobin
INS	Insulin
LOV	Lovastatin
MET	Metformin
STZ	Streptozotocin
TAU	Taurine
TG	Triglycerides

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from a defect in INS secretion, action or a combination of both (Luippold et al. 2016). Additional biochemical abnormalities of DM are hyperlipidemia due to more often by hypretriglyceridemia than by hypercholesterolemia and reflected in increased levels of very low density lipoprotein (VLDL) TG and low density lipoprotein (LDL) CHOL and decreased or unchanged high density lipoprotein (HDL) CHOL (Brownlee and Cerami 1981).

The biguanide MET is an established antidiabetic medication that can improve glucose tolerance in persons with type 2 DM by a combination of effects that include decreased intestinal absorption of glucose accompanied by increased intestinal GLC utilization (Bailey et al. 1994), decreased hepatic GLC production (Periello et al. 1994; Rena et al. 2017), enhanced peripheral INS sensitivity (Hother-Nielsen et al. 1989) and increased peripheral GLC uptake and utilization but without the risk of hyperglycemia (Owen et al. 2000).

Taurine (TAU) is a free sulfur-containing amino acid that in animal models has demonstrated beneficial effects in diabetes and it complications independently of a hypoglycemic effect (Ito et al. 2012). For example Alvarado-Vásquez et al. (2003) reported that in rats made diabetic with STZ and receiving TAU showed lower concentrations of serum GLC, HbA_{1c} CHOL and TG than untreated diabetic rats. These findings were confirmed and extended by Saleh (2012), who found that TAU also lowered the diabetic LDL levels, and by Koh et al. (2014) who determined that TAU can increase INS secretion.

Earlier work from this laboratory has verified that adding TAU to a treatment of DM with MET can lead to a gain in protective potency against changes in indices of

GLC metabolism and of renal functional impairment and oxidative stress (Pandya et al. 2015). On this basis, the present study was undertaken to determine if adding TAU to a treatment of DM with both MET and LOV, a hypocholesterolemic agent, can enhance the actions of both MET and LOV on the hyperglycemia and hyperlipidemia resulting from DM. An additional reason for undertaking this study was to clarify the role of supplementing DM therapy with TAU on diabetic hypercholesterolemia since there is at least one report indicating that is has no effect on either the plasma CHOL or TG levels of rats made diabetic with STZ (Stowe et al. 1999).

2 Materials and Methods

2.1 Treatment Compounds

The treatment agents LOV, MET and TAU were obtained from Sigma-Aldrich, St. Louis, MO, USA. NPH human INS isophane suspension (Humulin N®) was from Eli Lilly and Co., Indianapolis, IN, USA. Streptozotocin (STZ) was purchased from A.G. Scientific, San Diego, CA, USA. All other chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Animals

Each experimental group consisted of six male Sprague-Dawley rats, 200–225 g in weight, obtained from Taconic Farms, Germantown, NY, USA. All the animals were subjected to an acclimatization period of 7 days in a room kept at a constant temperature (23 ± 1°C) and humidity and on a normal 12 h light-12 h dark cycle. During such time, the animals had free access to a standard rodent diet and filtered tap water.

2.3 Treatment Solutions and Treatments

STZ was dissolved in 10 mM citrate buffer pH 4.5 to provide a 0.23 mM solution. Solutions of the treatment compounds (LOV, MET, TAU) were freshly made each day, with LOV being dissolved in 0.5% carboxymethylcellulose and MET and TAU in physiological saline. The solution of INS, with a declared content of 100 units/mL, was directly withdrawn from a commercial vial and injected subcutaneously at a dose of 4 units/kg/day. DM was induced with a single, 60 mg/kg/mL, intraperitoneal dose of STZ. After 14 days, the diabetic rats started to receive a daily dose of a

treatment agent (0.075 mM/kg of LOV, 2.4 mM/kg of MET or TAU, singly or as binary or ternary combination) by oral gavage. When more than one treatment agent was involved, they were provided at 15 min intervals. Control (normal) rats received a 2 mL volume of physiological saline by the oral route in place of a treatment agent solution. Body weights and tail vein blood GLC levels were monitored on a weekly basis for a total of 56 days.

2.4 Sampling and Samples

A drop of blood was collected each week from the tail vein and used to measure the blood GLC level with the help of a commercial glucometer (TRUEtrack™ and test strips, both from Nipro Diagnostics, Fort Lauderdale, FL, USA). Only rats exhibiting a blood GLC level above 250 mg/dL were used in the study. On day 57, the rats were sacrificed by decapitation and their bloods were collected in heparinized tubes. A portion of each blood sample was used to measure the concentration of HbA_{1c} and the other portion was centrifuged at 700 × g for 10 min to obtain the plasma fraction.

2.5 Assay of Plasma GLC

The plasma GLC was measured using a commercial assay kit (Procedure No. 510, Sigma-Aldrich, St. Louis, MO, USA) based on the glucose oxidase-peroxidase colorimetric method of Raabo and Terkildsen (1960). The results were expressed in mg/dL.

2.6 Assay of Plasma INS

The concentration of circulating INS was measured with a commercial immunoassay kit (ELISA kit, item No. IS130D, Calbiotech Inc., Spring Valley, CA, USA) and an ELISA plate reader set at 450 nm. The results were expressed in μIU/mL.

2.7 Assay of Blood HbA_{1c}

This parameter of long term glycemic control was measured with the help of a commercial assay kit (Glycohemoglobin Test, Procedure No. 0350–060, Stanbio Laboratory, Boerne, TX, USA) based on an ion-exchange resin procedure. The results were expressed as a percentage of the total Hb concentration.

2.8 Assay of Plasma CHOL

The total plasma CHOL was measured by means of a commercial endpoint/enzymatic-colorimetric assay kit (Cholesterol LiquiColor® Test, Procedure No. 1010, Stanbio Laboratory, Boerne, TX, USA) based on the method of Allain et al. (1974). The results were expressed as mg/dL.

2.9 Assay of Plasma TG

The levels of plasma TG were measured using a commercial enzymatic-colorimetric assay kit (Enzymatic Triglycerides, Procedure No. 2150, from Stanbio Laboratory, Boerne, TX, USA). The concentrations were derived from a calibration curve made with a glycerol standard equivalent to 200 mg/dL of triolein, and the results were expressed in mg/dL.

2.10 Statistical Analyses

The experimental results are reported as mean ± SEM for groups of six rats each. Differences between groups were analyzed for statistical significance using unpaired Student's t-test and a commercial computer-based statistics program (GraphPad Prism® Version 4.0 from GaphPad Software, Inc., San Diego, CA, and SigmaStat® from Systat Sofware, Inc., San Jose, CA, USA), followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$).

3 Results

3.1 Body Weight Gains

On day 1 of the study and before the start of any treatment body weight differences among the various animal groups did not exceed 4%; but at 14 days after a particular treatment, marked differences were noted among the diabetic group, the control group and the treatment groups (Table 1). Thus, untreated diabetic rats were only ~14% heavier than on day 0, control animals gained 68% weight ($p < 0.001$ vs. day 1), and the weight of diabetic rats receiving a treatment agent or combination of treatment agents varied between 35% and 59% of the original weights ($p \leq 0.01\%$ vs. day 1, with diabetic rats receiving TAU showing the lowest gain and diabetic rats receiving a ternary treatment showing the highest gain. At 56 days, untreated

Table 1 Body weight gains of the various animal groups over a period of 56 days

Treatment group	Day 0, g	Day 28, g	Day 56, g
Control	225.83 ± 2.39	378.83 ± 3.16^{+++}	438.33 ± 5.88^{+++}
DM	235.83 ± 3.96	267.67 ± 9.19**	319.17 ± 9.61**
DM + LOV	230.83 ± 7.90	337.50 ± 7.04^{++}	404.17 ± 4.36^{++}
DM + MET	228.33 ± 5.58	324.17 ± 6.25^{+}	417.50 ± 5.12^{++}
DM + TAU	235.00 ± 5.63	320.83 ± 6.51$*$,$^{+}$	394.17 ± 3.74^{+}
DM + LOV + MET	230.30 ± 3.80	350.42 ± 6.42^{++}	430.90 ± 6.23^{++}
DM + LOV + TAU	230.00 ± 3.65	341.67 ± 5.11^{++}	415.00 ± 5.32^{++}
DM + MET +TAU	230.10 ± 4.47	334.17 ± 6.11^{+}	431.67 ± 8.33^{++}
DM + MET + LOV + TAU	225.00 ± 5.32	358.33 ± 6.28^{++}	429.17 ± 7.90^{++}

Differences were statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. Control group; and at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. DM group

diabetic rats were 35% heavier than on day 0 ($p < 0.01$), a value that was 59% lower than corresponding body weight gain for control rats. Among diabetic rats receiving a treatment, the body weight gains were 68–91% higher (all at $p < 0.001$ of initial) than corresponding body weights on day 0, with the lowest gain seen again in animals receiving TAU (68% gain), the highest gain in animals receiving LOV-MET-AU (91% gain), and with the binary treatments contributing to body weight gains that were intermediate (80–88% above starting weights). By comparison, rats receiving a daily dose of INS showed body weight gains that were comparable to those of the control group, with the intergroup difference at 56 days being only 4% lower.

3.2 Effects on the Circulating Levels of GLC, INS and HbA_{1c}

Table 2 shows the values for blood GLC, plasma INS and blood HbA_{1c} of the various experimental groups. Diabetic rats showed a blood GLC level that exceeded the control value by 310% ($p < 0.001$). While LOV had no effect on such an increase, MET reduce it by about 55% ($p < 0.001$ vs. DM) and TAU by 22% ($p < 0.05$ vs. DM). On the other hand, while the effect of a combined treatment with LOV-MET or with MET-TAU did not change the effect of MET alone, one with LOV-TAU was insignificantly less potent than one with TAU alone (+18%, $p < 0.05$ vs. DM). In contrast, a treatment of diabetic rats with LOV-MET-TAU was insignificantly more potent in lowering the diabetic GLC level than MET alone (by 10%). Rats receiving INS on a daily basis had a blood GLC level that, although higher than the control value (+23%, $p < 0.05$), was still much lower than the blood GLC attained with any of the other treatment agents.

In parallel with the increasing effect on the blood GLC, diabetic rats showed a marked decreased in their plasma INS levels (by 76%, $p < 0.001$ vs. control). This effect was effectively counteracted by MET (only 26% decrease, $p < 0.05$ vs. control), to a much lesser extent, by TAU (52% decrease, $p < 0.001$), with LOV

Table 2 Circulating levels of GLC, INS and HbA_{1c} of the various experimental groups

Group	Blood GLC, mg/dL	Plasma INS, μIU/mL	Blood HbA_{1c}, %
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$	$6.89 \pm 0.11^{+++}$
DM	$428.08 \pm 21.74^{***}$	$10.58 \pm 0.52^{***}$	$21.14 \pm 1.19^{***}$
DM + LOV	$429.85 \pm 16.15^{+++}$	$12.99 \pm 0.49^{*,+++}$	$18.58 \pm 1.15^{***}$
DM + MET	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 0.28^{**,+++}$	$7.77 \pm 0.41^{+++}$
DM + TAU	$333.27 \pm 6.60^{***,+}$	$20.94 \pm 0.39^{***,++}$	$10.96 \pm 0.96^{***,+++}$
DM + LOV + MET	$197.51 \pm 2.80^{***,+++}$	$33.70 \pm 1.80^{**,+++}$	$9.81 \pm 1.02^{***,+++}$
DM + LOV + TAU	$353.00 \pm 12.11^{***,+++}$	$17.51 \pm 2.68^{***,+++}$	$16.71 \pm 1.43^{***,+}$
DM + MET + TAU	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$	$7.65 \pm 1.04^{+++}$
DM + LOV + MET + TAU	$178.42 \pm 18.54^{***,+++}$	$36.91 \pm 0.60^{*,+++}$	$9.61 \pm 0.65^{***,+++}$

Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$ vs. Control group; and at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. DM group

showing no effect. While a combined treatment with LOV-MET did not change the effect achieved with MET alone, one with MET-TAU was slightly better than MET alone (18% decrease vs. control) and one with LOV-TAU was less effective than one with TAU alone (60% decrease, $p < 0.001$ vs. control). Providing the diabetic rats with LOV-MET-TAU did not change the results seen with MET-TAU (16% decrease, $p < 0.05$ vs. control).

In common with the GLC and INS changes, diabetic rats showed very high levels of HbA_{1c} when compared to control values (+207%, $p < 0.001$) (Table 1). Monotherapy with TAU (59% increase, $p < 0.001$) or, better, with MET (+13%) was very effective in attenuating the effect of DM; but one with LOV was much less effective (+170%, $p < 0.001$). Combining MET with TAU provided nearly equivalent action as MET alone (+11%), but combining MET with LOV led to a lowering of the effect of MET (+59%, $p < 0.001$ vs. control). In contrast, providing LOV plus TAU improved the lowering effect of LOV (+145%, $p < 0.001$ vs. control); and one with LOV-MET-TAU was no different from one with MET alone or with MET-TAU (+10% increase). In diabetic rats receiving INS, the value of the blood HbA_{1c} was rather similar to that of rats receiving TAU (+52%, $p < 0.001$ vs control).

3.3 Effects on the Circulating Levels of CHOL and TG

Relative to the control value, the plasma CHOL of diabetic rats was ~65% higher ($p < 0.001$. Table 3). A treatment with any of the test agents was highly protective against this increase, with LOV reducing it to a much greater extent (to −8%) than either MET or TAU (+16% and +19% above control, respectively, $p < 0.05$). This effect was enhanced further when the test agents were made available as pairs, in which case the value of the plasma CHOL was, in all instances, not significantly different from the control value, with MET-TAU (−11%) and LOV-TAU (−6%) bringing the plasma CHOL to just below the control value and LOV-MET to just

Table 3 Circulating levels of CHOL and TG of the various experimental groups

Treatment group	Plasma CHOL, mg/dL	Plasma TG, mg/dL
Control	$59.53 \pm 1.93^{+++}$	$141.15 \pm 10.51^{+++}$
DM	$99.06 \pm 5.04^{***}$	$405.97 \pm 22.98^{***}$
DM + LOV	$55.12 \pm 1.40^{+++}$	$171.19 \pm 18.85^{*,+++}$
DM + MET	$69.10 \pm 5.50^{*,++}$	$222.40 \pm 2.96^{***,+++}$
DM+ TAU	$70.81 \pm 2.90^{*,++}$	$206.33 \pm 9.42^{+++}$
DM + LOV + MET	$65.90 \pm 4.09^{+++}$	$189.20 \pm 14.72^{**,+++}$
DM + LOV + TAU	$55.86 \pm 4.91^{+++}$	$155.21 \pm 4.72^{+++}$
DM + MET +TAU	$61.62 \pm 5.00^{+++}$	$186.44 \pm 13.09^{**,+++}$
DM + LOV + MET + TAU	$51.54 \pm 1.14^{+++}$	$143.05 \pm 17.34^{+++}$

Differences were statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Control group; and at $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. DM group.

above control (+3.5%). A treatment with LOV-MET-TAU reduced the diabetic plasma CHOL to a value similar to that seen with LOV-TAU (−8%). Similarly, all of the treatment agents were found to attenuate the massive increase in plasma TG (188%, $p < 0.001$) caused by DM, with LOV causing a much greater reduction (only +21%, $p < 0.05$ vs. control) than either TAU (−46%, $p < 0.001$) or MET (+58%, $p < 0.001$) (Table 3). A treatment with LOV-TAU (+10%) was found more effective than one with either LOV-MET (+32%, $p < 0.01$) or LOV-TAU (+34%, $p < 0.01$); and one with LOV-MET-TAU returned the value to one similar to control. In diabetic rats treated with INS, the plasma CHOL and TG levels were significantly lower than those of untreated diabetic rats (by 28%, $p < 0.05$, and 64%, $p < 0.001$, respectively).

4 Discussion

In comparison to the significantly lower body weight gains of untreated diabetic rats over a period of 56 days, diabetic ones receiving a single treatment agent or combination thereof showed body weights that were only 2–10% lower than those in the control group, and with the lowest gains occurring in rats receiving only TAU. From the results of experiments in which diabetic rats were given INS, the body weight gains of treated diabetic rats is probably the result of a greater accumulation of body fat (Tobin et al. 2000), but an alternative explanation, possibly related to the less effective control of the glycemic status and lower plasma INS levels, may be in place for rats receiving LOV, TAU and LOV-TAU and whose body weights were lower than those of diabetic rats receiving MET. In contrast, adding TAU to a treatment with either LOV or MET raised the effects of either of these compounds further. On the other hand, the results seen here with MET contrast markedly with those reported by Pournaghi et al. (2012), who found that the body weights of diabetic rats receiving MET for a 8 weeks period were not different than those of untreated diabetic ones.

Not unexpectedly, the hypoglycemic drug MET lowered the diabetic blood GLC by more than one-half, an effect that was maintained when it was co-administered with LOV, and it was insignificantly improved in the presence of TAU. By itself LOV had no effect on the diabetic blood GLC level in the face of evidence that statins can dowregulate GLC transporters (Chogtu et al. 2015), and, in agreement with earlier results from other laboratories (Kulakowski and Maturo 1984; Tokunaga et al. 1983) TAU reduced it to a significant extent. Furthermore, in STZ-treated rats the lowering effect of TAU was found to be accompanied by a decrease in pancreatic immunoreactive INS content and a reduction of morphological changes in the islets of Langerhans (Tokunaga et al. 1983); and work with mice pancreatic islet cell showed that TAU was able to increase INS release, GLC metabolism, peripheral INS sensitivity, and GLC tolerance in mice (Carneiro et al. 2009; Ribeiro et al. 2009). A more recent study in rats has demonstrated that TAU may contribute to lower circulating GLC levels by suppressing the activity of the GLC transporter SGLT1 in the jejunum (Tsuchiya and Kawamata 2017). However, there are also reports indicating that TAU lacks an effect on both diabetic hyperglycemia (Nishimura et al. 2002; Wang et al. 2008) and INS secretion and sensitivity (Brøns et al. 2004). In agreement with a previous finding from this laboratory (Pandya et al. 2015), adding TAU to a treatment with MET or, better, with LOV-MET, can enhance the hypoglycemic action of MET further although not to a significant extent.

In parallel with the trend of results seen for the blood GLC, the various treatment agents differed in their ability to counteract the elevation of the blood HbA_{1c} caused by DM. Thus, while MET and, to a lesser extent, TAU, lowered the diabetic HbA_{1c} to a significant extent, LOV exerted only a modest effect. Furthermore, adding TAU to a treatment with MET had no obvious effect on the potency of MET, but combining TAU with LOV raised the potency of LOV to an insignificantly extent. In general, LOV was found to lower the potency of MET significantly when administered either as a binary combination or along with TAU as a ternary combination. On the other hand the present results do not agree with the trends reported in certain human studies and indicating an elevating effect on the blood levels of both GLC and HbA_{1c} by a treatment with a statin type of drug (Thongtang et al. 2011).

Evaluation of the plasma levels of both CHOL and TG is of clinical interest since the dyslipidemia associated with type 2 DM is a major contributor to an increased risk of cardiovascular disease. Diabetic dyslipidemia includes quantitative lipoprotein abnormalities which are reflected in increased circulating TG and CHOL levels and decreased HDL-CHOL levels (Vergès 2015). In reviewing the effects of the three treatment agents used in the present study, it is evident that they share as well as differ on their effects on the various types of circulating lipid and lipoproteins. In the case of the biguanide compound MET, recognized as a first-line hypoglycemic treatment for type 2 DM, it is found to be an effective supplement to LOV, diet and lifestyle advice for non-diabetic male patients with coronary heart disease to further improve the lipid pattern but without affecting the fasting GLC level and reducing body weight in overweight individuals (Carlsen et al. 1996). Furthermore, in noninsulin dependent diabetics, MET has shown the ability to lower both the plasma

total CHOL and LDL-CHOL but without affecting the levels of TG and of HDL-CHOL or of the body weights (Robinson et al. 1998). In the present study, MET lowered the levels of both the plasma CHOL and plasma TG in diabetic rats to a significant extent, especially when co-administered with TAU.

LOV, an example of a statin drug, is commonly indicated for the treatment of the dyslipidemia of patients with non-INS-dependent DM and a therapeutic approach to reducing the risk coronary heart disease thanks to its ability to lower elevated plasma CHOL, LDL-CHOL and LDL apolipoprotein B. Additional benefits derived from this drug are a reduction of the plasma TG and of the VLDL-CHOL levels but without altering those of the plasma HDL-CHOL (Garg and Grundy 1988). In the present study, adding LOV to a treatment with MET-TAU did not change the effect of the binary combination on the plasma CHOL even though LOV proved more potent than MET when they were given singly. Similarly, LOV was more potent than either TAU or MET, in that order, in reducing the diabetic plasma TG. This effect was further enhanced when LOV was given alongside with MET or, better, with TAU. A treatment with MET plus TAU was about equipotent with one using LOV-MET. A further, although insignificant, gain in potency was attained with a combination of all three treatment agents.

TAU has demonstrated several pharmacological actions that make it an attractive candidate for investigation in the treatment of diabetes and its dyslipidemia. Using STZ-treated diabetic rats, it has been verified that a 6-month daily treatment with TAU was able to lower the total CHOL and TG levels along with those of the serum glucose and HbA_{1c} (Alvarado-Vásquez et al. 2003). In addition, a study conducted with Otsuka Long-Evans Tokushima fatty rats with long-term duration of diabetes, feeding an experimental animal diet with 2% added TAU reduced the blood GLC level over 12 weeks, and improved the outcome of an oral glucose tolerance at 6 weeks after TAU supplementation. The same study verified that TAU significantly reduced INS resistance but without improving pancreatic β-cell function or islet mass. After 12 weeks, TAU was also found to significantly lower the serum levels of CHOL, TG, HDL-CHOL and LDL-CHOL (Kim et al. 2012); and in overweight or obese young adults a daily intake of 3 g of TAU for 7 weeks led to significant decreases of the serum TG and the atherogenic coefficient calculate as (Total CHOL-HDL-CHOL)/HDL-CHOL, an effect that was taken as an indication of a beneficial effect on lipid metabolism and on cardiovascular disease prevention in overweight or obese subjects (Zhang et al. 2004). Providing TAU in the drinking water (0.5%) for 6 weeks to rabbits made diabetic with alloxan was able to significantly decrease the elevation of the plasma TG but not of the plasma CHOL (Tenner et al. 2003). The present results confirm the benefits of TAU in lowering the increases of both the plasma CHOL and plasma TG elicited by DM, with the effect on the former being equivalent to and that on the latter being somewhat greater than that of MET. In comparison with LOV, the decreasing effects of TAU on the diabetic plasma CHOL and TG were significantly lower than those of LOV. The effect of TAU on the plasma CHOL has been related to an enhancement of CHOL biotransformation to bile acid by cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme of bile acid synthesis, for subsequent excretion in the bile and eventual fecal disposal (Guo et al.

2017; Murakami et al. 2016; Yokogoshi et al. 1999). In contrast, the mechanism accounting for the ability of TAU to lower the circulating levels of TG remain unsettle, and proposals such as an increase of the activity of acyl-CoA oxidase, the rate-limiting enzyme of peroxisomal fatty acid β-oxidation (Mikami et al. 2012), a reduction of the incorporation of fatty acids into TG, and a decreased synthesis of TG through an inhibitory effect on the hepatic secretion of apolipoprotein B100, a major protein component of plasma VLDL and LDL (Yanagita et al. 2008) by TAU await further verification.

In conclusion, the present work finds that, except for the effects on both the blood GLC and plasma INS levels, the treatment combinations TAU-MET and TAU-LOV-MET are as effective or better than INS in promoting body growth and in correcting for DM lipid-related biochemical alterations.

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Taurine Supplementation Inhibits Cardiac and Systemic Renin-Angiotensin System Overactivity After Cardiac Ischemia/Reperfusion in Adult Female Rats Perinatally Depleted of Taurine Followed by High Sugar Intake

Supaporn Kulthinee, James Michael Wyss, and Sanya Roysommuti

Abstract Perinatal taurine depletion and high sugar intake from weaning onward worsen cardiac damage and arterial pressure control after ischemia/reperfusion (IR) in adult male and female rats, which can be ameliorated by high taurine diets or inhibition of renin-angiotensin system. This study tests if taurine supplementation ameliorates cardiac damage and arterial pressure control in adult female rats via alterations of both cardiac and systemic renin-angiotensin system. Female Sprague-Dawley rats were fed normal rat chow and drank water alone (control, C) or water containing 3% beta-alanine (taurine depletion, TD) from conception to weaning, and female offspring were subjected to high sugar intake (normal rat chow and 5% glucose in water; CG and TDG) or the normal rat diet (CW and TDW). At 7 weeks of age, half of the rats in each group received 3% taurine in water (CW+T, CG+T, TDW+T, and TDG+T). One week later, rats were subjected to IR or Sham procedures followed by renal nerve recording, plasma and cardiac angiotensin II measurements. Cardiac angiotensin II levels significantly elevated in CG, TDW, and TDG. Further, plasma angiotensin II concentrations were significantly elevated only in the TDG, in consistent with a significant increase in renal nerve activity to juxtaglomerular cells, but not renal vessels and tubules. These abnormalities were

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ameliorated by short-term taurine supplementation. Thus, in adult female rats that are perinatally depleted of taurine followed by high sugar intake after weaning, taurine supplementation decreases the adverse effects of cardiac IR via inhibition of both cardiac and systemic renin-angiotensin system overactivity.

Keywords Angiotensin II · Arterial pressure control · Cardiac injury · High sugar intake · Perinatal taurine depletion · Taurine supplementation · Sympathetic activity

Abbreviations

IR	ischemia/reperfusion
Sham	normal rats without cardiac ischemia/reperfusion induction
CW	control with water intake alone
CW+T	control with water intake alone plus taurine
CG	control with high sugar intake
CG+T	control with high sugar intake plus taurine
TDW	perinatal taurine depletion with water intake alone
TDW+T	perinatal taurine depletion with water intake alone plus taurine
TDG	perinatal taurine depletion with high sugar intake
TDG+T	perinatal taurine depletion with high sugar intake plus taurine

1 Introduction

Many women in their childbearing years are on a vegetarian/vegan diet. Since taurine is only contained in animal byproducts, this results in their fetuses being depleted of taurine (Roysommuti and Wyss 2014). Further, children now consume relatively high levels of sugar, due in part to the excess amounts of sugar in processed foods (Fidler et al. 2017; Patel et al. 2018). Together, this could place the child at risk for adverse cardiovascular effects later in life. Our previous studies and those of others indicate that the combination of perinatal taurine depletion and a post-weaning high sugar diet leads to adverse cardiovascular effects following cardiac IR in young adult rats (Kulthinee et al. 2010, 2015, 2017).

Following cardiac ischemia, the oxygen supply to the heart decreases leading to low ATP production by mitochondrial respiratory chain reactions, while anaerobic glycolysis not only produces a very low ATP level, but it also increases cytosolic H^+ (Roysommuti and Wyss 2017). The low energy state of the cardiac cell induces cell electrolyte and volume imbalance. IR also induces increased reactive oxygen species and low taurine content. The severity/infarct size of cardiac injury is dependent on the severity and duration of ischemia and the subsequent reperfusion parameters. On reperfusion, the high oxygenation causes the mitochondria to generate more

reactive oxygen species by cytochrome C oxidase in complex IV of the electron transport chain. Thus, the cardiac injury is exacerbated during the reperfusion phase. Taurine supplementation and inhibition of the renin-angiotensin system are reported to ameliorate the cardiac IR injury (Schaffer et al. 2014).

Myocardial infarction patients experience increased sympathetic nerve activity beginning a few days after ischemia and lasting up to 6 months (Graham et al. 2002). Increased sympathetic and decreased parasympathetic activity may facilitate cardiac death in myocardial ischemic patients (La Rovere et al. 1998). This autonomic dysfunction may initially result from an ischemic hypotension induced baroreceptor reflex response and cardiac sensory c-fiber activation (Morrey et al. 2010). The later autonomic responses may be due to central autonomic resetting induced by neurohormonal factors, particularly the renin-angiotensin system.

Perinatal taurine exposure affects adult function and disease, particularly the cardiovascular disease (Roysommuti and Wyss 2014). Taurine transporter knockout (TauTKO) animals display cardiac damage and dysfunction (Ito et al. 2010). Thus, it is not surprising that cardiac injury and dysregulation after cardiac IR are more severe in these rats compared to wild-type control rats. Following IR, adult male rats perinatally depleted of taurine display increased cardiac injury and autonomic dysfunction compared to rats perinatally treated with normal or high taurine diets (Kulthinee et al. 2010). Further, these adverse effects are exacerbated by high sugar intake after weaning, suggesting a synergistic effect of high sugar intake and perinatal taurine depletion on IR-induced cardiac injury. The more severe cardiac IR responses in the perinatally taurine depleted rats (compared to controls) may be a consequence of their higher sympathetic and renin-angiotensin system responses. These effects are also observed in female rats, although compared to young adult males, in young adult females, IR causes significantly less severe cardiac damage (Kulthinee et al. 2010, 2017). In addition, these abnormalities are, at least in part, ameliorated by adult taurine supplementation. The present study clarified the contribution of cardiac and systemic renin-angiotensin system after cardiac IR in adult female rats perinatally depleted of taurine followed by high sugar intake after weaning.

2 Methods

2.1 Animals and Experiments

All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee (AEKKU 15/2557) and were conducted in accordance with the National Institutes of Health guidelines.

Mature male and female Sprague-Dawley rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand and treated at the Animal Unit of the Faculty of Medicine, Khon Kaen University. After confirmed conception, female Sprague-Dawley rats were fed normal rat chow

(C.P. Mice Feed 082) and drank water containing β-alanine until weaning (taurine depletion, TD). Their female offspring were then fed normal rat chow and water containing 5% glucose (TDG group) or water alone (TDW group) throughout the experiment. A week before cardiac IR induction, half of the rats in each group were treated with 3% taurine in the drinking water (TDW with taurine, TDW+T; TDG with taurine, TDG+T) continuously to the end of experiment. The female control rats were similarly treated with (CG group) or without (CW group) high sugar intake followed by taurine supplementation (CW+T and CG+T groups).

The experimental protocol and experimental groups in the present study were similar to previous studies (Kulthinee et al. 2015, 2017), except a sham IR control (CW without cardiac IR induction) was also performed. In brief, at 7–8 weeks of age, all rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, intraperitoneal), catheterized with the femoral arterial and venous cannulas, and then, induced the cardiac IR by tracheal tube occlusion. Three days later, rats were anesthetized with pentobarbital and tracheostomized; then, arterial pressure was continuously recorded. Further, right renal nerve was exposed, and its activity was continuously recorded. Multiunit recordings of renal sympathetic nerve activity were confirmed by increases in nerve firing frequency following sodium nitroprusside-induced hypotension. Body temperature was servo-controlled at 37 ± 0.5 °C by a temperature regulator. At the end of experiment, blood volumes (about 5.0 ml) were collected from abdominal aortas for determination of plasma angiotensin II. Finally, all animals were terminated by a high dose of anesthesia and their hearts were collected for cardiac angiotensin II measurement.

2.2 Renal Nerve Activity Analysis

The renal sympathetic nerve-firing rate and power spectral densities were determined by using the Acknowledge software 3.9.1 (BIOPAC system), as previously reported (Rakmanee et al. 2017).

2.3 Plasma Angiotensin II Measurement

The concentration of plasma angiotensin II was measured by using angiotensin II enzyme immunoassay (EIA) kit (Merck KGaA, Darmstadt, Germany). This assay is based on a principle of a competitive enzyme immunoassay. In brief: (1) add 100 μl/well of anti-angiotensin II antibody to each well and incubate overnight at 4 °C, (2) discard the solution and wash wells four times with 1× Wash buffer, (3) add 100 μl/well of each standard, positive control and sample into the appropriate well and incubate 2.5 h at room temperature with gentle shaking, (4) aspirate well and wash three times with Wash buffer, (5) add 100 μl/well of prepared-HRP streptavidin solution to each well and incubate 45 min at room temperature with gentle shaking,

(6) discard the solution and wash wells four times with 1× Wash buffer, (7) add 100 µl/well of TMB One-Strep Substrate Reagent to each well and incubate for 30 min at room temperature in the dark with gentle shaking, and (8) add 50 µl/well of stop solution to each well and read immediately by the ELISA Reader (Tecan GmbH., Grodig, Australia) at 450 nm. The standard curve was prepared by plotting absorbance against standard angiotensin II concentration and used to calculate angiotensin II concentrations of the samples.

2.4 Cardiac Angiotensin II Measurement

After the frozen heart was allowed to warm at room temperature, the following procedure was performed. Briefly: (1) a piece of cardiac tissue was added to 1000 µl complete extraction buffer (cell lysate buffer and protease inhibitor cocktail) and homogenize with an electric homogenizer, (2) centrifuged for 20 min at 13,000 rpm at 4 °C, and (3) placed on ice. The supernatant was removed and the assay immediately performed. Angiotensin II was measured using angiotensin II enzyme immunoassay (EIA) kit (Merck KGaA, Darmstadt, Germany), as mentioned in Sect. 2.3, and expressed as pg/ml lysate.

2.5 Statistical Analysis

All data are expressed as mean ± SEM. One-way ANOVA followed by the *post hoc* Tukey's test (StatMost32 version 3.6, Dataxiom, CA, USA) was used to statically compare among groups with a significant criterion of p-value less than 0.05.

3 Results

Animals' general characteristics (body weight, heart weight, plasma electrolytes, blood urea nitrogen, plasma creatinine, mean arterial pressure, and heart rate), plasma cardiac injury markers, baroreflex sensitivity control of heart rate and renal nerve activity, and estimated autonomic control of arterial pressure were previously reported (Kulthinee et al. 2015, 2017). The present data further report the possible role of cardiac and systemic renin-angiotensin system activity.

Other than TDG, plasma angiotensin II concentrations after cardiac IR induction were not significantly different among the IR and Sham control groups, irrespective of taurine supplementation (Fig. 1). Cardiac IR induced a marked and significant increase in plasma angiotensin II in the TDG compared to all other groups, and this adverse effect of perinatal taurine depletion followed by a high sugar diet from weaning onward was eliminated by short-term taurine supplementation (TDG+T).

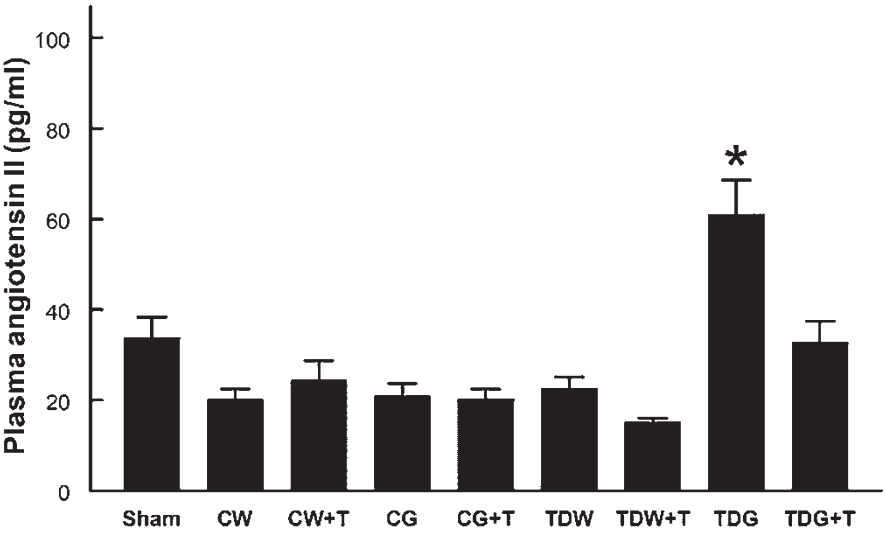

Fig. 1 Plasma angiotensin II concentrations in adult female rats (*P < 0.05 compared to all other groups; *Sham* normal rats without cardiac ischemia/reperfusion induction, *CW* control with water intake alone, *CW+T* control with water intake alone plus taurine, *CG* control with high sugar intake, *CG+T* control with high sugar intake plus taurine, *TDW* perinatal taurine depletion with water intake alone, *TDW+T* perinatal taurine depletion with water intake alone plus taurine, *TDG* perinatal taurine depletion with high sugar intake, *TDG+T* perinatal taurine depletion with high sugar intake plus taurine; n = 5–7 each group)

Cardiac angiotensin II concentration was significantly increased in CG, TDW, and TDG compared to Sham and CW groups (Fig. 2). Cardiac angiotensin II was much higher in TDG compared to all other groups. Taurine supplementation did not affect the cardiac angiotensin II level in CW+T, but it normalized cardiac angiotensin II levels in CG+T and TDW+T (compared to CW and CW+T) and significantly lowered the level in the TDG+T (compared to TDG) group.

After cardiac IR, multiunit recording of renal nerve activity indicates that high glucose intake (CG group) or perinatal taurine depletion alone (TDW group) significantly increased renal nerve firing rates compared to Sham and CW groups (Fig. 3). Further, the perinatal taurine depletion followed by high sugar intake from weaning onward (TDG group) significantly increased the renal nerve firing rates, compared to all other groups. Taurine supplementation significantly decreased the renal firing rates in CG+T, TDW+T and TDG+T compared to their comparable non-taurine groups, but renal nerve activities of TDW+T and TDG+T (but not the CG+T) groups were significantly higher than that of the CW group.

Power spectral analysis of renal nerve activity indicates that each group displayed different patterns of the power spectral density (Fig. 4). After cardiac IR, the power spectral densities of renin release-related frequency component (0.5–1.5 Hz) significantly increased in TDG compared to all other groups (including the Sham control) and in CG compared to the Sham group (Table 1). The sodium excretion-

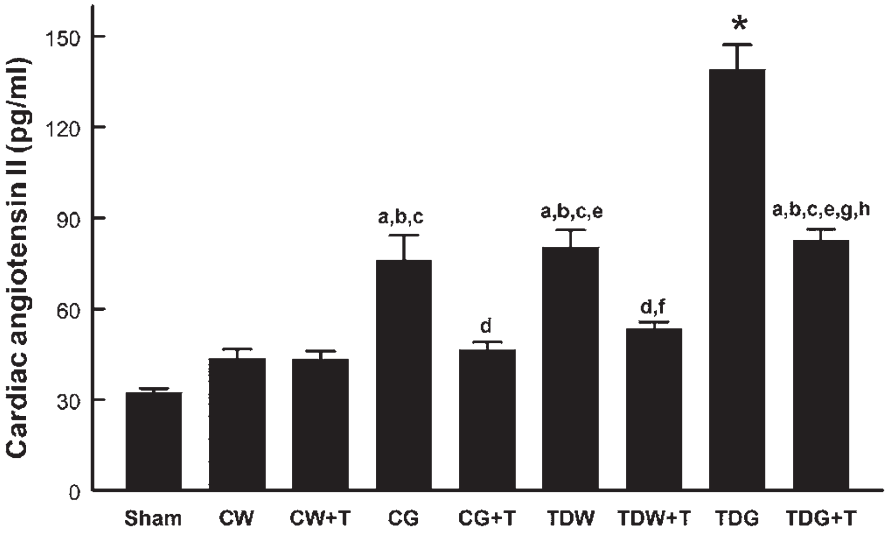

Fig. 2 Cardiac angiotensin II concentrations in adult female rats ($^{a,b,c,d,e,f,g,h,*}P < 0.05$ compared to Sham[a], CW[b], CW+T[c], CG[d], CG+T[e], TDW[f], TDW+T[g], TDG[h], and all other groups[*]; *Sham* normal rats without cardiac ischemia/reperfusion induction, *CW* control with water intake alone, *CW+T* control with water intake alone plus taurine, *CG* control with high sugar intake, *CG+T* control with high sugar intake plus taurine, *TDW* perinatal taurine depletion with water intake alone, *TDW+T* perinatal taurine depletion with water intake alone plus taurine, *TDG* perinatal taurine depletion with high sugar intake, *TDG+T* perinatal taurine depletion with high sugar intake plus taurine; n = 6 each group)

related frequency component (1.0–2.5 Hz) in the TDG is lower than all other groups and significantly decreased compared to the CG+T group. In addition, the renal blood flow-related frequency component (2.0–5.0 Hz) in the TDW group is higher than other groups and significantly increased compared to CG and CG+T groups. Furthermore, these renal sympathetic nerve effects of cardiac IR were completely abolished by short-term taurine supplementation (Table 1).

4 Discussion

Taurine depletion in the early life affects cardiovascular control and disorder in adult life. Our previous studies indicate that the perinatal taurine depletion increases cardiac IR injury and arterial pressure dysregulation in adult male more than female rats (Kulthinee et al. 2010, 2017). These adverse effects are exacerbated by high sugar intake from weaning onward. Further, short-term taurine supplementation a week before IR until the end of experiment ameliorates these adverse effects in the adult female rats (Kulthinee et al. 2017). The present data indicate that the perinatal taurine depletion increased cardiac angiotensin II content in CG, TDW, and TDG,

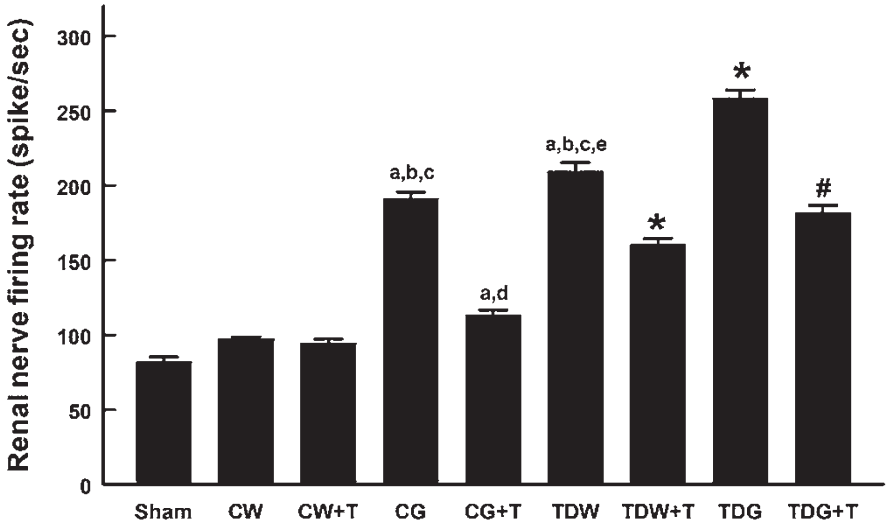

Fig. 3 Renal nerve firing rates at rest in adult female rats ($^{a,b,c,d,e,*,\#}P < 0.05$ compared to Sham[a], CW[b], CW+T[c], CG[d], CG+T[e], all other groups[*], and all other groups except CG[#]; *Sham* normal rats without cardiac ischemia/reperfusion induction, *CW* control with water intake alone, *CW+T* control with water intake alone plus taurine, *CG* control with high sugar intake, *CG+T* control with high sugar intake plus taurine, *TDW* perinatal taurine depletion with water intake alone, *TDW+T* perinatal taurine depletion with water intake alone plus taurine, *TDG* perinatal taurine depletion with high sugar intake, *TDG+T* perinatal taurine depletion with high sugar intake plus taurine; n = 6–8 each group)

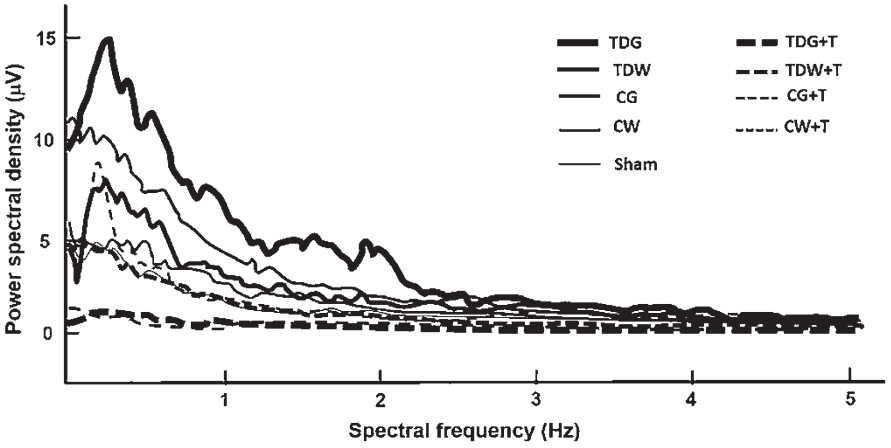

Fig. 4 Patterns of renal nerve activity in adult female rats (*Sham* normal rats without cardiac ischemia/reperfusion induction, *CW* control with water intake alone, *CW+T* control with water intake alone plus taurine, *CG* control with high sugar intake, *CG+T* control with high sugar intake plus taurine, *TDW* perinatal taurine depletion with water intake alone, *TDW+T* perinatal taurine depletion with water intake alone plus taurine, *TDG* perinatal taurine depletion with high sugar intake, *TDG+T* perinatal taurine depletion with high sugar intake plus taurine; n = 6–8 each group)

Table 1 Renal nerve firing rates and power spectral densities of renal nerve activity at the frequencies related to renin release (0.5–1.5 Hz), sodium excretion (1.0–2.5 Hz), and renal blood flow (2.0–5.0 Hz) in adult female rats

Group	Firing rates (spike/sec)	Power spectral densities (%)		
		0.5–1.5 Hz	1.0–2.5 Hz	2.0–5.0 Hz
Sham	81.9 ± 3.1	35.2 ± 0.4	33.4 ± 2.6	31.4 ± 2.3
CW	97.4 ± 1.3	37.3 ± 1.1	32.0 ± 1.6	30.7 ± 1.9
CW+T	94.8 ± 2.3	36.4 ± 1.0	31.3 ± 1.4	32.3 ± 1.3
CG	191.4 ± 4.0[a,b,c]	41.4 ± 2.1[a]	33.6 ± 2.9	25.0 ± 1.2
CG+T	113.0 ± 3.7[a,d]	37.5 ± 0.8	35.4 ± 1.8	27.1 ± 1.6
TDW	209.5 ± 5.5[a,b,c,e]	40.1 ± 1.3	24.4 ± 1.4[e]	35.5 ± 2.4[d,e]
TDW+T	160.3 ± 4.0[a,b,c,d,e,f]	37.6 ± 0.9	32.4 ± 2.1	30.0 ± 1.8
TDG	258.4 ± 5.4[a,b,c,d,e,f,g]	46.7 ± 1.1[a,b,c,e,f,g]	26.3 ± 1.5	27.1 ± 1.4[f]
TDG+T	181.7 ± 4.7[a,b,c,e,f,g,h]	41.1 ± 1.2	31.1 ± 1.4	27.9 ± 1.7

Values are mean ± SEM ([a,b,c,d,e,f,g,h]$P < 0.05$ compared to Sham[a], CW[b], CW+T[c], CG[d], CG+T[e], TDW[f], TDW+T[g], and TDG[h]; *Sham* normal rats without cardiac ischemia/reperfusion induction, *CW* control with water intake alone, *CW+T* control with water intake alone plus taurine, *CG* control with high sugar intake, *CG+T* control with high sugar intake plus taurine, *TDW* perinatal taurine depletion with water intake alone, *TDW+T* perinatal taurine depletion with water intake alone plus taurine, *TDG* perinatal taurine depletion with high sugar intake, *TDG+T* perinatal taurine depletion with high sugar intake plus taurine; n = 6–8 each group)

and this was correlated with the severity of cardiac IR injury and arterial pressure dysregulation (Kulthinee et al. 2017), but taurine depletion increased plasma angiotensin II level only in the TDG. Further, taurine supplementation, at least in part, normalized cardiac angiotensin II content in these groups, and this was proportionate to improvement of cardiac IR injury and arterial pressure dysregulation (Kulthinee et al. 2017), while it completely normalized the plasma angiotensin II level in the TDG+T group. The present study suggests that in the adult female rats, perinatal depletion of taurine followed by a high sugar diet from weaning onward, taurine supplementation ameliorates IR-induced cardiac damage and arterial pressure dysregulation via inhibition of both cardiac and systemic renin-angiotensin system overactivity. In addition, the present data also suggest that the systemic renin-angiotensin system overactivity was mediated via renal sympathetic nerve regulated renal renin release or juxtraglomerular cells.

Cardiac renin-angiotensin system overactivity is reported to increase cardiac IR oxidative stress and myocardial injury (Roysommuti and Wyss 2017). This adverse effect is abolished by taurine treatment before, during, and/or after cardiac IR induction. The present study indicates that after cardiac IR, cardiac angiotensin II markedly increased in CG, TDW, and TDG, while cardiac injury markers markedly increased only in TDG rats (Kulthinee et al. 2017). Although taurine supplementation improved the cardiac IR injury of these three groups, the TDG+T group still displayed higher cardiac IR injury than the other groups (Kulthinee et al. 2015, 2017). Further, the partially normalized cardiac angiotensin II level was also observed in the TDG+T group. In addition, the taurine supplementation abolished a rise in plasma angiotensin II in the TDG+T group. Together, the present data sup-

port the major role of cardiac rather than systemic renin-angiotensin system on cardiac IR injury, particularly in rats perinatally depleted of taurine followed by a post-weaning high sugar diet.

Although taurine supplementation improved cardiac IR injury in the CW+T compared to CW groups (Kulthinee et al. 2015), both plasma and cardiac angiotensin II levels did not change. These data suggest that taurine may act via other mechanisms, particularly anti-oxidant and cardiac calcium balance (Roysommuti and Wyss 2017). Taurine can react with hydrochlorous acid to form taurochloramine, a lesser oxidant compared to hydrochlorous acid, and taurine and taurochloramine possess anti-inflammatory and anti-apoptosis activities that normally underlie cardiac IR damage. Further, cardiac angiotensin II content increased in CG and TDW, but increased much more (additional ~75%) in TDG, suggesting that the effect of a post-weaning high sugar diet and perinatal taurine depletion may be additive. However, cardiac injury individually induced by the high sugar intake or the perinatal taurine depletion might, at least in part, differ from that caused by the perinatal taurine depletion plus high sugar intake. Although the cardiac angiotensin II content increased in CG, TDW, and TDG, the plasma angiotensin II increased only in the TDG. In addition, the effects of taurine supplementation on cardiac and plasma angiotensin II of these groups also support this notation.

Renal renin release is well known to initiate the renin-angiotensin system activity. The present study indicates that after cardiac IR, plasma angiotensin II increased only in TDG, while renal nerve firing rates increased in CG, TDW, and TDG groups. However, in the TDG+T group, circulating angiotensin II was completely normalized by short-term taurine supplementation, and its IR-induced renal nerve frequencies (Table 1) and the baroreflex dysfunction (Kulthinee et al. 2017) were partially improved. These data suggest that the systemic renin-angiotensin system overactivity may partially contribute to cardiac IR-induced baroreflex and autonomic dysfunction in TDG rats, but not other groups.

The present study supports the specific innervation of renal sympathetic nerve on three main renal structures (DiBona 2005) and might explain why after cardiac IR, the renal firing frequencies significantly increased in CG, TDW, and TDG, but plasma angiotensin II significantly increased only in TDG. After cardiac IR, the TDG increased both renal sympathetic firing rate and power spectral density related to renin release, suggesting that the sympathetic nerve firing change increased renin release, while the CG and TDW groups increased renal firing rate without affecting the power spectral density related to renin release. In addition, in the TDG+T (compared to the TDG) group, taurine supplementation eliminated the IR-induced increase in plasma angiotensin II and renin release, but partially normalized the renal sympathetic firing rate.

Taurine decreases the effect of angiotensin II on cell Ca^{2+} transport, protein synthesis, and angiotensin II signaling (Schaffer et al. 2000). Taurine may act centrally by interfering the action of glutamate. In the nervous system, the main transmitter released by primary baroreceptor afferent fiber within the tractus solitary nucleus is glutamate. Second-order baroreceptor-sensitive neurons that terminate in the caudal

ventrolateral medulla also release glutamate on the rostral ventrolateral medulla, which controls sympathetic activity to the heart and kidney (Okada and Bunag 1994; Pricher et al. 2008). In addition, taurine can act on peripheral sympathetic nerve terminals to suppress norepinephrine release (Hano et al. 2009). Thus, taurine supplementation in the present study may affect both central and peripheral sympathetic pathways to selectively modify global and regional renin-angiotensin system activity after cardiac IR in the TDG rats.

5 Conclusion

Taurine and renin-angiotensin system interactively affect autonomic and renal control of arterial pressure in both normal and perinatal taurine-depleted rats, particularly those that receive a high sugar diet (Roysommuti and Wyss 2014). The present study indicates that following cardiac IR, cardiac and systemic RAS overactivity underlie cardiac IR injury and arterial pressure dysregulation in perinatal taurine-depleted rats and these adverse effects can be ameliorated, at least in part, by short-term taurine supplementation. The present findings also suggest that renal sympathetic nerve-induced renal renin release is the key contributor to IR-induced renin-angiotensin system overactivity in rats perinatally depleted of taurine that receive a high sugar diet from weaning onward.

Acknowledgements This study was supported by grants from Faculty of Medicine and Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand.

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The Production of a Rat Model That Inhibits Phosphoenolpyruvate Carboxykinase (PEPCK), a Rate-Limiting Enzyme of Hepatic Gluconeogenesis

Hajime Ohmori, Masataka Matsumura, Shoichi Komine, Haruki Kobayashi, Yusei Kobayashi, Jun Shiromoto, and Sumpei Miyakawa

Abstract We previously showed that taurine administration contributed to the extension of time to exhaustion through exercise-induced hypoglycemia restraint, and we suggested that the activation of hepatic gluconeogenesis was initiated before the exercise with the taurine administration. We hypothesize that the extension effect of exercise duration with the taurine administration is restrained in the rats which inhibited hepatic gluconeogenesis. In this study, we aimed to produce a rat model that inhibited hepatic gluconeogenesis as a first step in testing our hypothesis.

F344 male rats of 8 weeks after birth were purchased. The blood samples were collected via jugular vein catheter to perform the pyruvate tolerance test (PTT) with the intraperitoneal administration, and to determine the optimal time point of blood glucose measurement. 3-mercaptopicolinic acids (3MPA) was used as an inhibitor of PEPCK. The rats were divided into three groups, Non-dosage control (CON) group, 30 mg/kg · BW 3MPA (3MPA 30) group, and 300 mg/kg · BW 3MPA (3MPA 300) group.

The blood glucose level showed a significant peak 15 min after pyruvate administration. The change of the blood glucose level after the PTT in 3MPA 300 group was significantly smaller than that of the CON group at this time point. These results show we could prepare the rat model that inhibited hepatic gluconeogenesis.

Keywords Hepatic glucose production · Prolonged exercise · Running performance

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Abbreviations

PTT	pyruvate tolerance test
3MPA	3-mercaptopicolinic acids
G6Pase	glucose 6-phosphatase
PEPCK	phosphoenolpyruvate carboxykinase

1 Introduction

Taurine was first discovered from the bile of the bovine in 1827. It is biosynthesized from cysteine in various animals. It is plentiful in fish such as oyster and cuttlefish. There is abundant taurine in many organs such as cardiac muscle, skeletal muscle, and liver as a free amino acid-like compound, and it has various physiological functions such as neurotransmission inhibition, osmolarity regulation, cell membrane protection, intracellular Ca^{2+} homeostasis, antioxidation, bile acid conjugation, and blood glucose regulation.

Given these physiological effects of taurine, the influence of taurine on exercise performance should be expected. The increase or decrease of taurine does influence exercise performance such as endurance time. Ito et al. (2008) reported that the taurine transporter knocked-out mice showed shorter swimming time in comparison with the wild type mice. On the other hand, Miyazaki et al. (2004) demonstrated that the rats administered taurine could run longer in a dose-dependent manner.

Various factors affect the exercise endurance time, and one factor is blood glucose level. Supplementation with carbohydrates during prolonged running maintains the human blood glucose level, resulting in longer exercise time (Coggan and Coyle 1987).

We showed that the administration of taurine inhibited the exercise-induced decrease in the blood glucose level and brought about the extension of exercise time (unpublished data). The blood glucose level is determined by dynamic balance between the glucose release from the liver and the glucose uptake by the extrahepatic organs such as muscle, brain, and so forth. It is unknown whether the maintenance of the blood glucose level in the taurine- administrated group is attributable to the increase of glucose release from liver, decrease of the glucose uptake to the extrahepatic organs, or to both.

Figure 1 shows the pathway of hepatic glucose production and release from the liver. We showed that the difference was not seen in liver glycogen level regardless of taurine administration, although liver glycogen was decreased by prolonged exercise (unpublished data). On the other hand, we showed that taurine administration activated G6Pase, the rate-limiting enzyme of gluconeogenesis, and decreased the concentrations of the glucogenic amino acids before exercise (unpublished data). These results are summarized in Fig. 2.

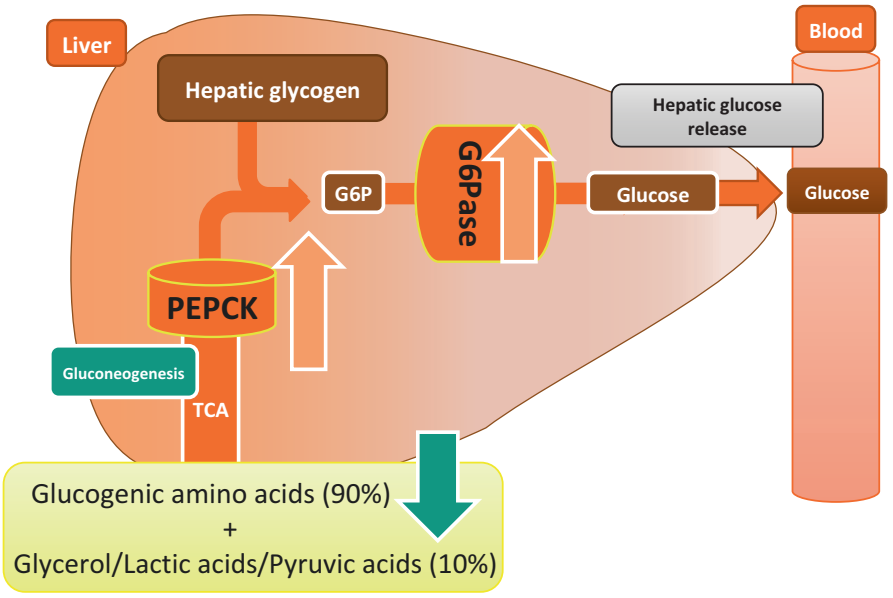

Fig. 1 Intrahepatic glucose production pathway

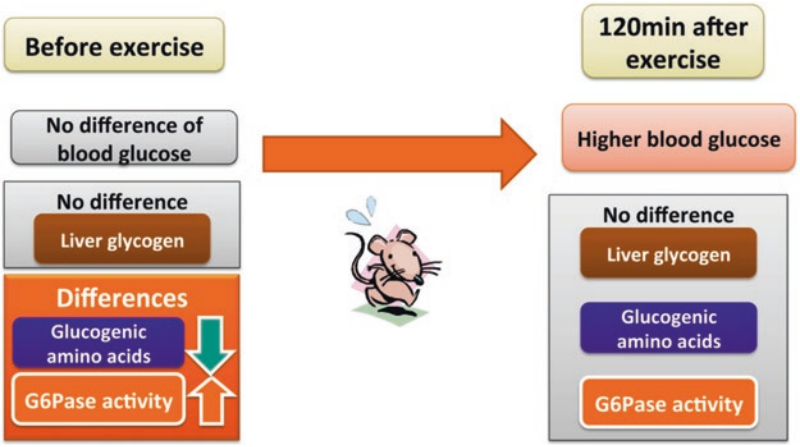

Fig. 2 Is pre status of hepatic gluconeogenesis prepared via G6Pase activation by taurine before exercise?

From these results, we hypothesized the possibility that "the gluconeogenesis-activating situation in the liver was prepared before the start of exercise by taurine administration, and it brought hypoglycemia restraint during prolonged exercise." In order to test this hypothesis, 3-mercaptopicolinic acid (3MPA), an inhibiter of phosphoenolpyruvate carboxykinase (PEPCK: a rate-limiting enzyme of gluconeo-

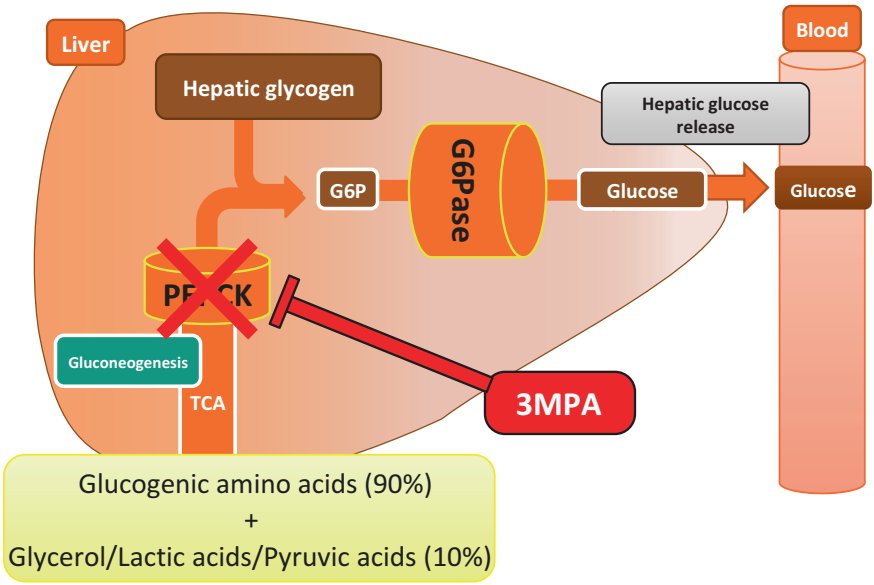

Fig. 3 Inhibit of phosphoenolpyruvate carboxykinase by 3-mercaptopicolinic acid

genesis) was used (Fig. 3). Ditullio et al. (1974) indicated that pyruvate-induced glucose production was suppressed by 3MPA in rat liver perfusate. In addition, Turcotte and Brooks (1990) reported a decrease in the ability to maintain blood glucose levels during prolonged exercise when 3MPA was given to the rats.

It is a goal of this study to examine whether taurine-induced exercise prolongation is suppressed by PEPCK inhibition. As the first step to achieve this goal, we made a rat model that inhibits hepatic gluconeogenesis by 3MPA

2 Methods

F344 male rats 8 weeks old were subject to the pyruvate tolerance test (PTT). The peak of blood glucose was observed 15 min after PTT. Therefore, to determine the appropriate dosage of 3MPA, we evaluated the amount of changes in blood glucose from just before, to 15 min after the PTT (Fig. 4).

Then we examined the influence of 3MPA administration on gluconeogenesis. Dissolved 3MPA in 350 mM sodium bicarbonate solution was given to F344 male rats intraperitoneally just before, and 90 min before the PTT. Sodium bicarbonate of 350 mM, 30 mg/kgBW 3MPA, and 300 mg/kgBW 3MPA were given in the control group, 3MPA(30) group, and 3MPA(300) group, respectively. Blood glucose levels after 3-h fasting were measured just before and 15 min after the PTT.

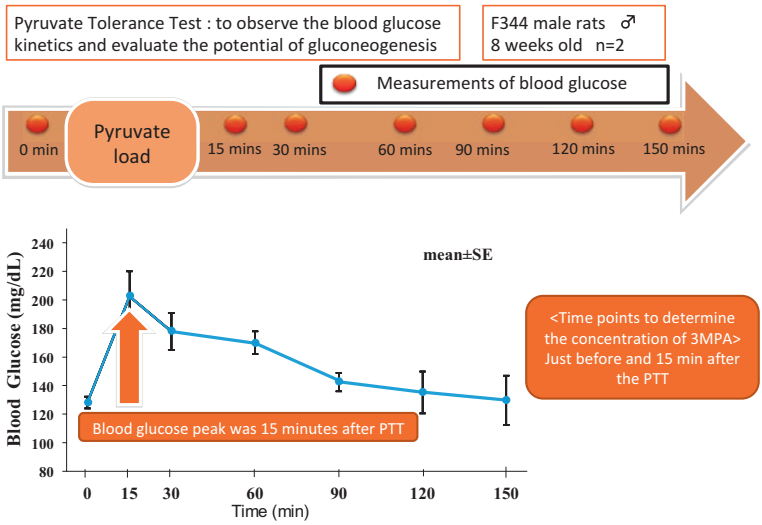

Fig. 4 Blood glucose kinetics in pyruvate tolerance test

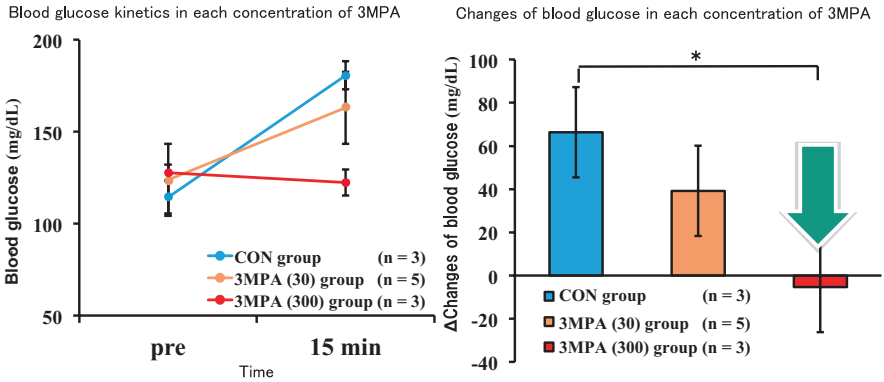

Fig. 5 The effect of 3MPA administration on hepatic gluconeogenesis

3 Results and Discussion

The left graph of Fig. 5 shows changes in the blood glucose levels with each concentration of 3MPA. An upward trend was seen in the CON group and the 3MPA(30) group in 15 min after administration, but no change was observed in the 3MPA(300) group. The right graph indicates the amount changes in the blood glucose levels with each concentration of 3MPA. Significant lower values were obtained in the 3MPA(300) group compared with the CON group. From these results, it is clear that 30 mg/kgBW 3MPA in the rat model could not achieve the complete restraint of the blood glucose increase after PPT, but 300 mg/kgBW 3MPA could (Fig. 5).

4 Conclusion and Prospect

In conclusion, we were able to make a rat model inhibiting hepatic gluconeogenesis by inhibition of PEPCK with the administration of 300 mg/kgBW 3MPA.

Prolonged exercise exhaustion testing should be performed with analysis of blood glucose, lactic acid, insulin, and glucagon in the control group, 3MPA group, taurine group, and taurine/3MPA combination group. It is expected that only the taurine group can maintain the blood glucose level, and other groups cannot maintain it. It is anticipated that the taurine/3MPA combination group cannot maintain blood glucose level in spite of taurine administration. Also, we expect that only the taurine group can increase the endurance time, but the taurine/3MPA combination group can not.

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Preventive or Curative Administration of Taurine Regulates Lipid Metabolism in the Liver of Rats with Alcoholic Liver Disease

Riyi Tang, Qunhui Yang, Shumei Lin, Ying Feng, Jiancheng Yang, Qiufeng Lv, Gaofeng Wu, and Jianmin Hu

Abstract Excessive consumption causes alcoholic liver disease (ALD), which injures hepatocytes and induces imbalance of lipid metabolism. Taurine is known to protect the liver from various liver injuries, and relieve lipid profile. Our previous studies also found that taurine can prevent or cure ALD, reduce fat deposition, but the mechanism remains unclear. In the present study, ALD rat model was established by administration of alcohol, pyrazole and high fat diet. Two percent taurine was administered at the same time or after ALD model establishment. Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), serum and hepatic TC, TG, HDL-C and LDL-C were analyzed. Real-Time RT-PCR was conducted to detect the mRNA expressions of fatty acid synthetase (FAS), acetyl-CoA catboxylase (ACC), carnitine palmitoyl transferase 1 (CPT-1), 3-Hydroxy-3-methyl glutaric acid acyl Coenzyme A reductase (HMGCR), peroxisome proliferators activated receptor α (PPARα) and sterol regulatory element-binding protein 1c (SREBP-1c). The results showed that serum ALT, AST, serum and hepatic TC, TG and LDL-C were higher, while HDL-C in ALD model rats was lower than normal rats, the changes of which can be significantly relieved by taurine administration. mRNA expressions of ACC, FAS, CPT-1, HMGCR, PPARα and SREBP-1c which were significantly changed by ethanol can also be regulated by taurine. The results indicated that taurine can prevent and repair hepatic injury of ALD rats and balance lipid metabolism indexes in the liver, the mechanisms may involves in the regulation of related enzymes and transcriptional regulators participated in lipid metabolism.

Keywords Taurine · Alcoholic liver disease · Lipid metabolism · Rats

Authors Riyi Tang and Qunhui Yang have been equally contributed to this chapter.

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Abbreviations

ALD	alcoholic liver disease
AFLD	alcoholic fatty liver disease
AH	alcoholic hepatitis
AF	alcoholic fibrosis
AC	alcoholic cirrhosis
ADH	acetaldehyde dehydrogenase
ALDH	aldehyde dehydrogenase
TG	triglyceride
TC	total cholesterol
LDL-C	low-density lipoprotein cholesterol
HDL-C	high-density lipoprotein cholesterol
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ACC	acetyl-CoA catboxylase
FAS	fatty acid synthetase
CPT-1	carnitine palmitoyl transferase 1
HMGCR	3-Hydroxy-3-methyl glutaric acid acyl Coenzyme A reductase
PPARα	peroxisome proliferators activated receptor α
SREBP-1c	sterol regulatory element-binding protein 1c

1 Introduction

Excessive consumption of alcohol is related to increasing morbidity, mortality and disability all over the world and causes 3.3 million deaths annually (Livero et al. 2016). Alcoholic liver disease (ALD) is in consequence of alcohol abuse, which is widely prevalent and has been ranked among the world's top 20 causes of death (Rehm and Shield 2013). Alcoholic fatty liver disease (AFLD) is the initial stage of ALD followed by more severe hepatic injuries including alcoholic hepatitis (AH), alcoholic fibrosis (AF) and alcoholic cirrhosis (AC). The liver is the main organ responsible for alcohol metabolism, in which ethanol is primarily metabolized into acetaldehyde by dehydrogenase (ADH), and then into acetate by aldehyde dehydrogenase (ALDH) (Panyod et al. 2016). Large amount of alcohol and acetaldehyde is deposited in the liver under excessive alcohol intake condition and further exert hepatotoxicity to damage hepatocytes which are capable of lipid metabolism. Finally, fat molecules are accumulated in the liver due to the disruption of the lipid metabolism balance which results in the augmentation of triglyceride (TG) formation (Orman et al. 2013). Thus, an ideal prevention or early treatment for ALD was to reduce lipid accumulation in the liver by balancing lipid metabolism.

Taurine is widely distributed in animal tissues and is involved in large variety of physiological functions. It has been known that taurine has significant roles in

maintaining the normal cellular function including hepatic cells (Ito et al. 2014; Jamshidzadeh et al. 2017). Taurine was found to exert protective effect on alcoholic injured rats through decreasing oxidative stress in the liver (Balkan et al. 2002), and interrupting ethanol-induced inflammatory cycle in kidney (Latchoumycandane et al. 2014). Furthermore, dietary taurine intake has been reported to exert effects on plasma total cholesterol (TC) levels in experimental animals given a high-fat diet and in rats chronically fed with alcohol (Kerai et al. 1998; Murakami et al. 2016). Our previous studies also demonstrated that taurine can prevent and ameliorate ALD in rat model, accelerate alcohol metabolism by enhancing the activities of ADH and ALDH, inhibit and reduce lipid accumulation, relieve lipid profile including TC, TG, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) (Wu et al. 2009, 2013, 2015), but the exact mechanism of taurine on the regulation of lipid metabolism in the liver still remains unclear. This study examined the regulatory effects of taurine on hepatic lipid metabolism in ALD rats, in order to provide further interpretation for the mechanism of taurine on the prevention and the treatment of ALD.

2 Materials and Methods

2.1 Animals

Seventy male SPF Wistar rats (180 ± 20 g) obtained from Chang Sheng Biotechnology Co., LTD, Liaoning province, China, were given free access to feed and water, and kept at a temperature of 22 ± 2 °C with a 12 h light and 12 h dark cycle.

2.2 Experimental Design

The experiment includes preventive trial and curative trial. In the preventive trial, rats in the ALD model group (M) and taurine preventive group (M+T) were administered with ethanol and pyrazole intragastrically, and were given high fat diet, tap water or 2% taurine in tap water. Rats in the normal control group (N) and taurine control group (T) were administered with the same volume of water intragastrically, and were given standard diet, tap water or 2% taurine in tap water. In the curative trial, ALD model rats were divided into taurine curative group (Tc) and automatically recovery group (A), in which rats received water or 2% taurine. Hepatic tissues and serum were collected randomly from each group once a week to detect the pathological changes and serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in order to assess the model establishment as was described in our previous studies (Wu et al. 2009, 2015). The preventive and curative trials lasted for 12 weeks and 4 weeks respectively. Serum and liver were collected for further analysis at the end of the experiment.

2.3 Biochemical Analysis

Serum activities of ALT, AST, and serum/hepatic TC, TG, LDL-C and HDL-C were assayed by commercial kits (Nanjing Jiancheng bioengineering institute, China) as per the protocol from the manufacturer.

2.4 Real-Time PCR Analysis

Total RNA were extracted from the liver using Trizol (Takara, Dalian, China, 9109), and reverse transcription was carried out using a commercial kit (Takara, Dalian, China). Real-time PCR was carried out at 94 °C for 5 min, and with 32 cycles as follows: 94 °C, 30 s; 65.6 °C for *acetyl-CoA catboxylase* (*ACC*), 63.7 °C for *fatty acid synthetase* (*FAS*), 63.5 °C for *carnitine palmitoyl transferase 1* (*CPT-1*), 61.7 °C for *3-Hydroxy-3-methyl glutaric acid acyl Coenzyme A reductase* (*HMGCR*), 60.3 °C for *peroxisome proliferators activated receptor α* (*PPARα*), 63.1 °C for *sterol regulatory element-binding protein 1c* (*SREBP-1c*), 30 s; 72 °C, 30 s; and finally terminated at 72 °C, 10 min using SYBR® green kit (ABI, USA, No.4472908) on a Bio-Rad iQTM5 detection system (Bio-Rad, Hercules, USA). The primers were as follows: *ACC* (*ACCCAACCATCAATCCTCGGCACAT* -forward and *CCTTCCACTTCCACAAACCAGCGTC*-reverse), *FAS* (*GACGCCAGTTCCGAGTCATC*-forward and *CACTTTCCCGCTCACTATCA*-reverse), *CPT-1* (*AGGAGAGTGCCAGGAGGTCATAG*-forward and *GGTGAGGCCAAACAAGGTGATAA*-reverse), *HMGCR* (*AACATCGTCACTG CCATCTACA*-forward and *GCTGCCATCAAGGACAACTCAC*-reverse), *PPARα* (*GCCGTTTCCACAAGTGCCT*-forward and *TCTTTCCTGCGAGTATGACC*-reverse), *SREBP-1c* (*TGCGGCTGTCGTCTACCATAAG*-forward and *TGTTCACAGAATAGTCGGGTCA*-reverse), *β-actin* (*TTGTAACCAACTGGGACG*-forward and *GATATTGATCTTCATGGTG*-reverse). Each reaction was repeated three times, and the melting curve was analyzed. Relative gene expression was normalized to the expression of the β-actin that is the housekeeping gene calculated by $2^{-\triangle\triangle Ct}$ method. Data are expressed as the relative fold-change to the control group.

2.5 Statistic Analysis

Data were analyzed by one-way ANOVA, followed by LSD and Duncan's multiple range tests using SPSS 17.0. Graphpad Prism 5.0 was used to create the artwork. All the values are expressed as means ± SEM. A difference at $p < 0.05$ level was considered to be statistically significant, and $p < 0.01$ was considered to be extremely significant different.

3 Results

3.1 *Effects of Taurine on Serum ALT and AST*

Figure 1 showed that serum ALT and AST activities in rats of M group in the preventive trial increased significantly compared with rats in N, M+T and T groups ($p < 0.01$), indicating that ALD model rats had higher ALT and AST activities than normal rats, while taurine preventively administered to ALD rats can inhibit the increase of serum transaminase.

In the curative trial, serum ALT and AST activities in A group elevated significantly compared with N and Tc groups ($p < 0.01$), indicating taurine administered curatively can reverse the increase of serum transaminase.

3.2 *Effects of Taurine on Serum and Hepatic Lipid Metabolism Indexes*

TC, TG, LDL-C and HDL-C are lipid metabolism indexes usually detected in clinical. As was shown in Fig. 2, serum and hepatic TC, TG, LDL-C in M group were much higher than N group ($p < 0.01$). In M+T group, the above levels were all obviously lower than M group ($p < 0.05$ or $p < 0.01$). Levels of HDL-C in M group declined significantly compared with N and M+T groups ($p < 0.01$). All these indexes had no significant differences between T group and N group ($P > 0.05$).

At the end of the curative trial, compared with A group, TC, TG, LDL-C were significantly lower, while HDL-C was obviously higher in Tc group ($p < 0.05$ or $p < 0.01$), which have no significant differences compared with N group ($p > 0.05$).

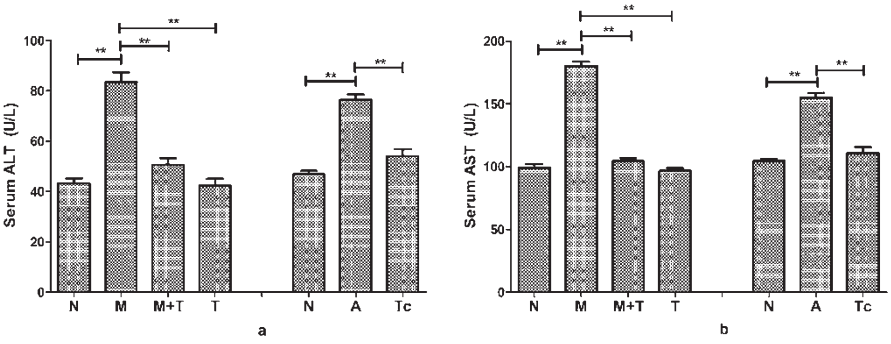

Fig. 1 Effects of taurine on serum ALT and AST. (a) Serum ALT; (b) Serum AST. *N* control group, *M* ALD model group, *M+T* taurine preventive group, *A* automatically recovery group, *Tc* taurine curative group; All data are expressed as means±SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 6). The bars in the figure show means ± SEM of each group. ∗indicates $p < 0.05$, ∗∗indicates $p < 0.01$

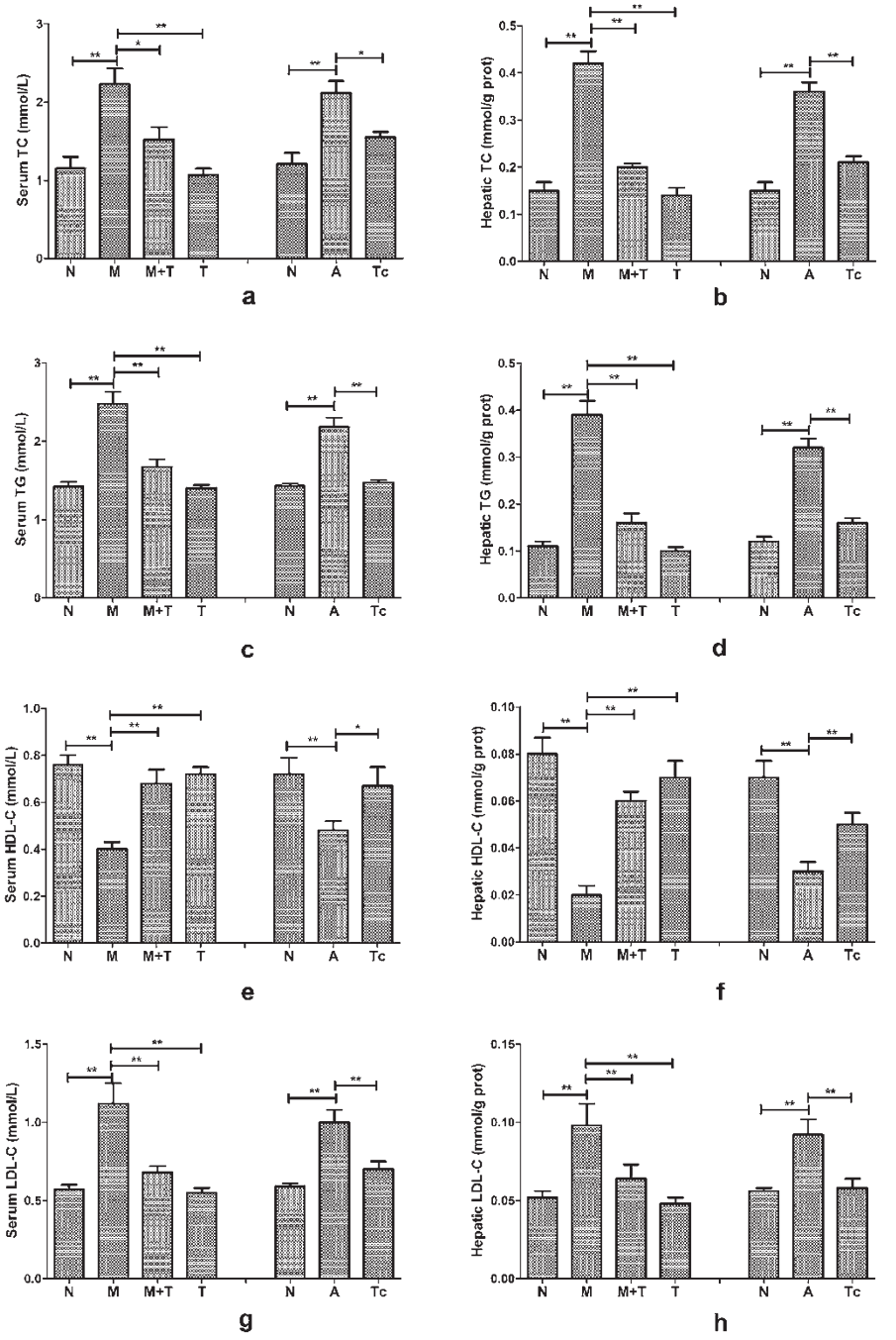

Fig. 2 Effects of taurine on serum and hepatic lipid metabolism indexes. (a) Serum TC; (b) Hepatic TC; (c) Serum TG; (d) Hepatic TG; (e) Serum HDL-C; (f) Hepatic HDL-C; (g) Serum LDL-C; (h) Hepatic LDL-C. *N* control group, *M* ALD model group, *M+T* taurine preventive group, *A* automatically recovery group, *Tc* taurine curative group; All data are expressed as means ± SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 6). The bars in the figure showed SEM of each group. ∗indicates $p < 0.05$, ∗∗indicates $p < 0.01$

The results indicate that preventively or curatively administered of taurine can reverse the changes of lipid metabolism indexes both in the serum and liver.

3.3 *Regulation Effects of Taurine on Hepatic Lipid Metabolism*

Lipid metabolism in the liver includes both lipid anabolism and lipolysis that involves a series of enzyme regulation process. In these experiments, mRNA expression of enzymes and related regulators in the liver were detected. As shown in Fig. 3, compared with N, M+T or T groups in the preventive trial, mRNA expressions of *ACC*, *FAS*, *HMGCR* and *SREBP-1c* in M group increased significantly ($p < 0.01$), while mRNA expressions of *CPT-1* and *PPARα* in the M group decreased significantly ($p < 0.01$). In the curative trial, compared with N group, mRNA expressions of *ACC*, *FAS*, *HMGCR* and *SREBP-1c* in A group increased significantly ($p < 0.01$), which were also significantly higher than Tc group ($p < 0.01$). mRNA expressions of *CPT-1* and *PPARα* in A group decreased obviously compared with N ($p < 0.01$) and Tc groups ($p < 0.01$). The results demonstrate that taurine can down regulate the expressions of *ACC*, *FAS*, *HMGCR* and *SREBP-1c*, while up regulate the expressions of *CPT-1* and *PPARα* independently of how it is administered preventively or curatively.

4 Discussion

ALD is a major chronic liver disease caused by excessive alcohol consumption. Ethanol and its metabolites exert toxic effects, that directly or indirectly injure hepatocytes, the results of which was that aminotransferases release from the liver into blood to cause high serum concentrations of ALT and AST that are the main sensitive indexes of liver injury. Abnormal elevated blood AST and ALT levels were detected both in chronic ethanol consumption patients and animals (Leggio and Lee 2017; Zhang et al. 2017). In this study, high serum ALT and AST were also found in ALD rats, indicating that hepatocytes of ALD rats were damaged. However, 2% taurine in drinking water administered preventively or curatively can significantly decrease serum ALT and AST, demonstrating that taurine exert protective effects on the liver and accelerate the repair of hepatocytes of ALD rats. These results were in accordance with our previous studies (Wu et al. 2009, 2013, 2015) and research on CCl4 or acetaminophen treated rats that taurine administration significantly lowered blood ALT and AST (Das et al. 2010; Abdel-Moneim et al. 2015). However, there are reports that higher doses of taurine combined with alcohol kill the animals (Taranukhin et al. 2013), in which ethanol was administered subcutaneously and taurine was administered intraperitoneally. The discrepancy may be attributed to

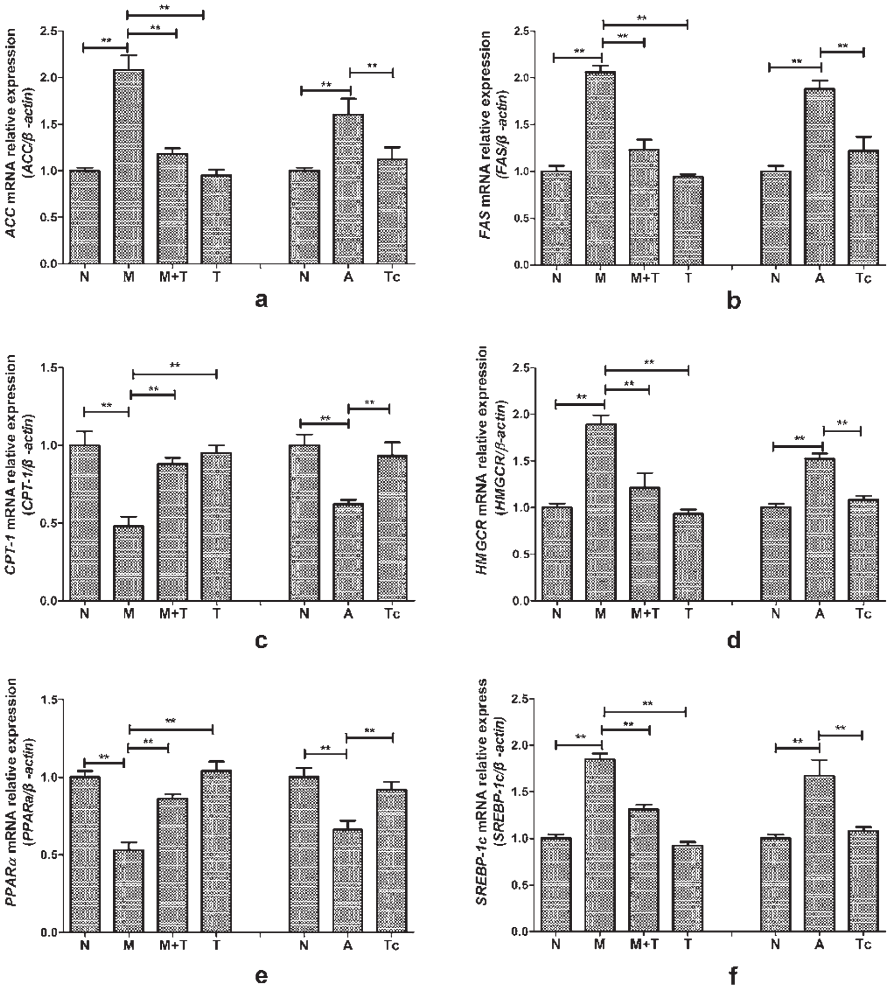

Fig. 3 Effects of taurine on mRNA expressions of lipid metabolism related genes and transcriptional regulators. (a) *ACC mRNA* expression; (b) *FAS mRNA* expression; (c) *CPT-1 mRNA* expression; (d) *HMGCR mRNA* expression; (e) *PPARα mRNA* expression; (f) *SREBP-1c mRNA* expression. *N* control group, *M* ALD model group, *M+T* taurine preventive group, *A* automatically recovery group, *Tc* taurine curative group; All data are expressed as means ± SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 6). The bars in the figure showed SEM of each group. ∗indicates $p < 0.05$, ∗∗indicates $p < 0.01$

two reasons. First, the administration method of ethanol and taurine were different from the present study that ethanol was administered intragastrically to adult rats, and taurine was added in drinking water at the concentration of 2%. In addition, the amount of 2% taurine in drinking water taken by rats in the present study has been recorded by our research group, which equals to 2 g/kg taurine per day. In our

previous study, higher concentration of taurine (5% taurine) was also used in drinking water, the amount of intake equals to 5 g/kg body weight. Both of the two doses of taurine in our study for adult rats were under the lethal doses of 8, 10 and 12 g/kg in adult rats. Moreover, it has also been discussed by Taranukhin et al. (2013) that both taurine and ethanol have significant ability to reduce blood glucose, so the lethality of high doses of taurine and ethanol may be attributed to hypoglycemia induced by dual function of ethanol and taurine at a high dosage (Taranukhin et al. 2013).

Lipid metabolism happens in the liver. Under physiological conditions, the balance of lipid anabolism and lipolysis keeps normal concentrations of TC and TG. When the liver was damaged by alcohol, lipoprotein synthesis and secretion were inhibited, which further hinder TC and TG transportation from the liver. Finally, TG and TC accumulated in the liver to induce ALD. Excessive alcohol and high fat intake also disrupt lipid anabolism in the liver, which further increase hepatic and blood TC and TG (Kerai et al. 1998). Research on rats fed with high fat diet showed higher serum TC, TG, LDL-C and lower serum HDL-C compared with normal rats, while taurine administration could reverse these changes in serum lipid profile (Cheong et al. 2009). Later study also demonstrated that taurine could effectively relieve the changes of serum TG, TC, LDL-C and HDL-C in CCl_4-induced liver damaged rats and STZ-induced diabetic rats (Lin et al. 2010; Abdel-Moneim et al. 2015). These results were in accordance with the present and our previous studies on ALD rats (Wu et al. 2009, 2015), indicating that taurine can regulate hepatic fat metabolism in ALD rats.

The biosynthesis process of fatty acids includes several steps, among which ACC is the rate-limiting enzyme of the first step that catalyze the acetyl-CoA to malonyl-CoA, the substrate for the synthesis of fatty acids catalyzed by FAS which is a multi-enzyme protein catalyzing fatty acid synthesis (Engin 2017). ACC and FAS were regulated by SREBP-1c, which is a PPAR-α-dependent transcription factor that modulates multiple genes involved in lipid production (Edwards et al. 2000). Increased activities of ACC, FAS and higher expressions of SREBP-1c were found in animals given high fat diet (Dossi et al. 2014; Gnoni and Giudetti 2016). In a rat model fed with a high fructose and saturated fat diet, elevated expressions of SREBP-1c, ACC and FAS were observed (Chen et al. 2017). Meanwhile, studies have proved that alcohol caused the increase of SREBP-1c, the accumulation of TG, and the reduction of fatty acid oxidation (Bai et al. 2016; Panyod et al. 2016; Yao et al. 2017), these results were similar to the present results in ALD rats showing that ethanol and high-fat diet enhance the expression of *SREBP-1c*, *ACC* and *FAS* in the liver, while taurine administered to ALD rats can inhibit or reverse the changes of the expressions of *SREBP-1c*, *ACC* and *FAS*, which was in accordance with the studies on macrophages that taurine can reduce SREBP-1C expression (Hoang et al. 2012). The results indicated that on the one hand, taurine can directly affect the transcription of ACC and FAS, decrease fat synthesis and TG accumulation by inhibiting the expressions of ACC and FAS; on the other hand, taurine may indirectly regulate gene transcription of ACC and FAS by lowing the expression of SREBP-1c,

which further decrease the synthesis of fatty acid and lead to the decline of both serum and hepatic TG.

Beside fatty acid, cholesterol is another type of lipid in the liver. HMGCR is the rate-controlling enzyme in the mevalonate pathway, catalyzing the conversion of HMG-CoA to mevalonic acid, which is a necessary step in the production of cholesterol. HMGCR is also the down-stream target of SREBP that regulate HMGCR at the transcriptional level. Although it is argued that regulation of HMGCR activity does not occur exclusively via changes in the quantity of enzyme protein but also includes the phosphorylation/dephosphorylation of the enzyme (Kennelly and Rodwell 1985), several observations do state that expression of HMGCR may reflect cholesterol synthesis (Jung and Kim 2013; Hong et al. 2016; Yang et al. 2017). Meanwhile, the changes in the activities of HMGCR is in accordance to the protein expression of the enzyme, implying that expression of the enzyme may partly reflect its activity (Nan et al. 2013). Increased expression of HMGCR was found in rats fed with liquid diet containing ethanol (Wang et al. 2010) and high fat diet (Nammi et al. 2010), which was similar to the present result that gene expression of *HMGCR* increased significantly in ALD rats, illustrating that cholesterol synthesis may be enhanced in the liver of ALD rats. Down-regulation in the expression of *HMGCR* in the liver of ALD rats administered with taurine was found, suggesting taurine may decrease cholesterol synthesis in the liver by inhibiting *HMGCR* expression. But if taurine can directly act on the activity of HMGCR, further accurate analysis of the enzyme activity needs to be carried out.

The catabolic process of fatty acid is β-oxidation, by which fatty acid broke down in the mitochondria and generate acetyl-CoA, which then enters the citric acid cycle. The CPT system is vital in the process because the diffusion of activated long chain fatty acids through the mitochondrial inner membrane from the outer membrane requires a shuttle system. CPT-1 is the enzyme located on the outer membrane of mitochondria, which converts long-chain fatty acyl-CoA molecules to their corresponding acylcarnitine molecules (Zammit 2008) and is a downstream target of PPARα, a member of peroxisome proliferator-activated receptors (PPARs) that function as transcription factors regulating gene expression. PPARα is highly expressed in the liver, regulating lipid metabolism (Chinetti et al. 2001). Research showed that decreased expressions of PPARα and CPT-1 were observed in HepG2 cells challenged with oleic acid (Liu et al. 2017). Animals fed with liquid diet containing ethanol or high fat diet showed lower expressions of PPARα and CPT-1 in the liver (Dossi et al. 2014; Bang et al. 2016). Meanwhile, in PPARα-null mice, fatty acid β-oxidation was greatly hindered in response to long-term alcohol feeding (Li et al. 2014). The present research also found the same changes in rats given alcohol and high fat diet, suggesting a change in the metabolic status of the liver in ALD rats. Furthermore, as CPT-1 is also a mitochondrial enzyme, the expression of which can reflect mitochondrial function. The decrease of the expression of CPT-1 in ALD rats in the present study manifested serious mitochondria injury, which is in agreement with the studies in non alcoholic fatty liver disease (NFLA) rats that showed mitochondria dysfunction (Liu et al. 2015). Taurine no matter how it is administered, preventively or curatively, can up-regulate *PPARα* and *CPT-1*

expression in this study, which is in accordance with previous studies showing that taurine enhances *PPARα* and *CPT-1* in obese rat and hamster model fed with high fat diet (Chang et al. 2011; Bonfleur et al. 2015). These findings indicated that taurine can directly up-regulate gene transcription of *CPT-1* or indirectly act on the transcription of *PPARα*, which further affects its downstream target, CPT-1, promoting the transportation of fatty acid into the mitochondria for degradation through β-oxidation pathway, and finally decrease the accumulation of TG in the liver. From the other aspect, the metabolic process of ethanol and lipid in the liver produces a large quantity of ROS, which seriously impair the mitochondria, and taurine has already been found to eliminate excessive ROS and exerts its antioxidant ability on various organ injuries (Yildirim et al. 2007; Schaffer et al. 2009; Jamshidzadeh et al. 2017). Hence, the up-regulation of CPT-1 by taurine administration reflects that taurine may protect mitochondria from injury caused by oxidative stress during the process of ethanol or lipid metabolism.

5 Conclusion

Given the effects of taurine on the levels of TC, TG, HDL-C, LDL-C, the mRNA expressions of *ACC*, *FAS*, *CPT-1*, *HMGCR*, *PPARα* and *SREBP-1c*, and combined with our previous studies, we conclude that taurine can prevent and cure ALD, by a mechanism that may involve the regulation of lipid metabolism in the liver of ALD rats through its direct or indirect effects on the expressions of related enzymes and transcriptional regulators.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31772694, 31872441 and 31302051).

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Taurine Improves Lipid Metabolism and Skeletal Muscle Sensitivity to Insulin in Rats Fed with High Sugar and High Fat Diet

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Abstract Metabolic syndrome is a lifestyle-related disease caused by high nutrient condition and lack of exercise. The insulin resistance due to obesity has attracted attention as an underlying mechanism of metabolic syndrome. Insulin resistance refers to reduced insulin sensitivity in insulin target tissues. In this case, in order to maintain normal blood glucose levels, a compensatory large amount of insulin is released, leading to the occurrence of hyperinsulinemia. Taurine is widely distributed in animal tissues. Although it is not involved in protein synthesis, taurine plays an important role in maintaining the body's physiological function. In this experiment, insulin resistance model was induced by high fat and high sugar diet. Two percent taurine was added in drinking water to explore the mechanism of taurine in insulin resistance and to provide theoretical basis for using taurine to improve insulin resistance. The result showed that high-fat and high-sugar diet could decrease insulin sensitivity, and taurine could improve it by oral glucose tolerance test. Moreover, serum TG, TC were higher, while HDL-C in rats fed with high sugar and high fat diet was lower than normal rats, the changes of which can be significantly relieved by 2% taurine administration. mRNA and protein expressions of IRS1, and GLUT4 which were significantly changed by high sugar and high fat diet can also be regulated by 2% taurine. The results indicated that taurine can improve insulin sensitivity through remediating lipid metabolism disorder and regulating the expressions of IRS and GLUT4.

Keywords Taurine · Insulin resistance · Lipid metabolism · Rats

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Abbreviations

FBG	fasting glucose
FINS	fasting serum insulin levels
OGTT	oral glucose tolerance test
HOMA-IR	insulin resistance index
STZ	streptozotocin
IR	Insulin resistance
TC	total cholesterol
TG	triglyceride
HDL-C	high density lipoprotein cholesterol
IR	insulin receptor
IRS1	insulin receptor substrate 1
GLUT4	glucose transporter 4

1 Introduction

Taurine is a conditionally essential amino acid for human beings and most animals, and is an essential amino acid for cats (Sturman and Messing 1991, 1992; Sturman 1993). It has many important physiological, pharmacological and nutritional functions. Taurine is widely distributed in many tissues and organs, although it is not involved in protein synthesis, it plays an important role in maintaining the physiological function of the body. Taurine can regulate a variety of cellular functions, including antioxidant activity (Schaffer. et al. 2009; Jeon et al. 2009; Nakamura et al. 1993; Shivananjappa and Muralidhara 2012), osmotic regulation (Nieminen et al. 1988; Pasantes et al. 1998), neurotransmitter regulation (El Idrissi 2008), bile acid synthesis and membrane stability regulation (Bremer 1956; Petrosian and Haroutounian 1998), intracellular calcium ion regulation, apoptosis inhibition (Zulli et al. 2009), proinflammatory cytokine levels reduction (Schuller-Levis and Park 2006; Schuller-Levis et al. 1995, 2009). We have demonstrated that taurine can inhibit the occurrence and development of type 2 diabetic nephropathy induced by streptozotocin (STZ) combined with high fat and high sugar diet (Shumei Lin et al. 2010), In addition, oral taurine has been shown to improve insulin sensitivity in rats fed with high glucose and high fat diet. In this study, we examined the mechanisms by which taurine improves insulin sensitivity in skeletal muscle of rats fed with high glucose and high fat diet.

2 Materials and Methods

2.1 Experimental Animals and Treatments

Wistar rats were purchased from Liaoning Changsheng biotechnology company, China. All the experimental protocols were approved by Shenyang Agricultural University Ethical Committee and were followed the Declaration of Helsinki.

Four to 5 weeks male SPF Wistar rats weighing 120 ± 10 g were housed in a climate-controlled (temperature; 22–24 °C, humidity; 40–60%) and light-regulated room with 12-h light and dark cycles. These rats were fed with normal chow for 1 week to stabilize their metabolic condition. Forty male Wistar rats were randomly divided into three groups, normal control group (C group, n = 10), taurine intervention group (TI group, n = 10) and the model establishment group (ME group, n = 20). Throughout the test period, the normal control group was fed with standard food and water; taurine intervention group was fed with standard food and water containing 2% taurine; the model establishment group was fed with high sugar and high fat diet to establish insulin resistance model. Fasting glucose (FBG) and fasting serum insulin levels (FINS) were measured and insulin resistance index (HOMA-IR) was calculated after 8 weeks of model establishment. The sign of successful establishment of the model was that HOMA-IR of the model establishment rat was significantly higher than that of the normal control group. From the 9th to the 12th weeks, the model rats (M group, n = 10) were fed with high sugar and high fat diet and normal water. While, taurine reversal rats (TR group, n = 10) were fed with high sugar and high fat diet and water containing 2% taurine.

2.2 Experimental Diets

Normal diet was purchased from Yuhong District test animal feed factory (Shenyang), and the nutrition ingredient of diet was in accordance with the nutritional standards. High sugar and high fat feed was purchased from Yuhong District test animal feed factory (Shenyang) which contains 15% lard, 25% sucrose, 2.5% cholesterol, 1% bile salts and 56.5% normal feed.

2.3 Biochemical Analysis

Blood samples were collected from the jugular vein of eight rats randomly selected from each group at the end of the 8th and the 12th weeks. After standing at room temperature for 4 h, serum was separated by centrifuging at 1500 rpm for 15 min at 4 °C, stored at −20 °C. TC, TG, HDL-C were determined by colorimetry using kits.

2.4 Standardization of Insulin Resistance

Fasting plasma glucose was measured by Roche blood glucose meter. Fasting plasma insulin was measured using radioimmunoassay method. Insulin resistance (IR) was assessed according to the homeostasis model assessment index (HOMA-IR) and calculated using the following formula: (fasting plasma insulin (FINS) [μU/ml] × fasting plasma glucose (FBG) [mmol/l])/22.5, and the natural logarithm of HOMA-IR was calculated.

2.5 Oral Glucose Tolerance Test (OGTT)

Briefly, overnight fasted rats (14 h) were administered 1.5 g of glucose per kg of body weight by perfusion for the OGTT, Blood glucose levels were measured at 0, 15, 30, 60 and 90 min using Roche blood glucose meter.

2.6 RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from skeletal muscle using TRIzol reagent (Takara, Dalian, China) and dissolved in EPC-treated water according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed to cDNA using oligo primers and moloney murine leukemia virus reverse transcriptase (RT) in a final volume of 20 μl under the conditions recommended by the supplier (Takara). The resulting cDNA was amplified using primers specific for IRS1, GLUT4 or GAPDH in a total volume of 10 μl. The expression level of IRS1 and GLUT4 was normalized to that of GAPDH, which was used as a specific endogenous control.

Primer sequences used were as follows: IRS1 forward 5′- AAG ACG CTC CAG TGA GGA TTT A -3′, reverse 5′- AGG ATT GCT GAG GTC ATT TAG G -3′; GLUT4 forward 5′- TTC CTT CTA TTT GCC GTC CTC -3′, reverse 5′- ATT TTG CCC CTC AGT CAT TCT -3′; GAPDH forward 5′- CAA GTT CAA CGG CAC AGT CAA -3′, reverse 5′- CGC CAG TAG ACT CCA CGA CAA -3′. The reaction cycle conditions were: denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s and extension at 60 °C for 40 s. The PCR products were resolved using a 2% agarose gel and visualized with ethidium bromide staining.

2.7 Western Blot Analysis

Protein samples were prepared with lysis buffer. Equal amounts of protein were separated by 10% SDS-PAGE, and electrotransferred to polyvinylidene difluoride membranes, and were then blocked with 5 g/L bovine serum albumin for 2 h at

room temperature. Membranes were incubated with appropriate diluted primary antibodies of anti rabbit Insulin receptor substrate 1 (IRS1), glucose transporter type 4 (GLUT4) and GAPDH (all from Santa Cruz, United states) respectively overnight at 4 °C, and then with the respective secondary antibody for 2 h. Proteins were detected with the enhanced chemiluminescence detection system. GAPDH served as an internal control protein. The experiments were replicated three times.

2.8 Statistic Analysis

Data were analyzed by one-way ANOVA, followed by LSD and Duncan's multiple range tests using SPSS 16.0. All the values are expressed as means ± SE. A difference at $p < 0.05$ level was considered to be statistically significant.

3 Results

3.1 Insulin Resistance Was Formed by Feeding High Sugar and High Fat Diet for 8 Weeks in Rats

FBG, FINS, and HOMA-IR of groups C and PM are shown in Fig. 1. In the present study, we established the model of insulin resistance in rats fed with high sugar and high fat diet. At the end of 8th week, the FBG and FINS were determined, and then the HOMA-IR was calculated. Figure 1 shows that the FBG level of group ME had no significant differences compared with the C group, but The FINS and level of the ME group was significantly higher than that of the C group, moreover the value of HOMA-IR was significantly increased. The results indicates that feeding high fat and high carbohydrate diet for 8 weeks can induce lower insulin sensitivity in rats.

3.2 Levels of Lipid Metabolism Indexes in Each Group

TC, TG, and HDL-C are lipid metabolism indexes usually detected clinically. As shown in Fig. 2, serum concentrations of HDL-C, TG and TC in M group were significantly affected compared with the normal control group ($p < 0.05$), among which, the concentration of HDL-C was significantly decreased, and the concentrations of TG and TC were significantly increased. Serum concentrations of HDL-C, TG and TC in TI and TR group had no significant differences compared with the normal control group. The results indicate that curatively administered taurine can reverse the changes of lipid metabolism in the serum of rats fed with high sugar and high fat diet.

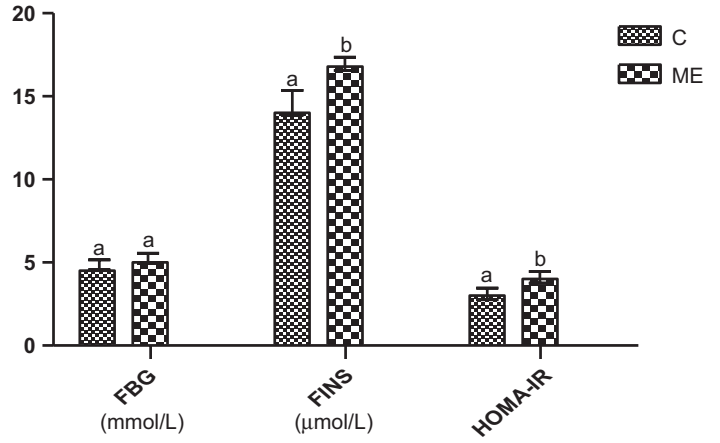

Fig. 1 FBG, FINS and HOMA-IR level in the control and Model group. *C* Control group, *ME* model establishment group; All data are expressed as means±SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (Control group n = 10; model establishment group n = 20). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letters are significantly different ($p < 0.05$)

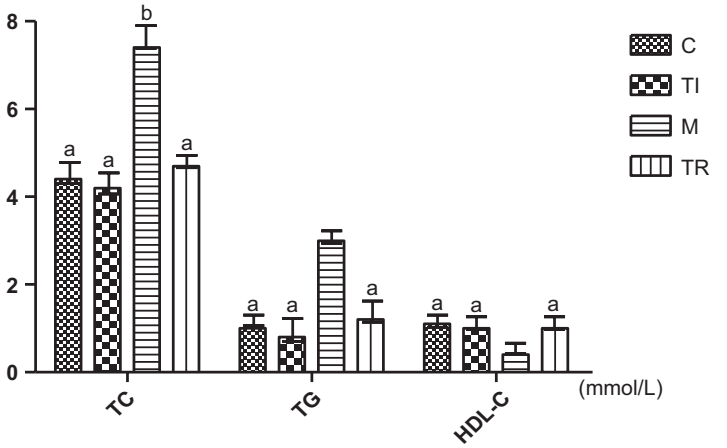

Fig. 2 Levels of lipid metabolism indexes in each group. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means±SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 8). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letter are significantly different ($p < 0.05$). There are three indexes of lipid metabolism, including TC, TG and HDL-C, the indictors of lipid metabolism in serum were determined at the end of the test

3.3 Oral Glucose Tolerance Test

As shown in Fig. 3, for fasting blood glucose level, it had not differ significantly among the each group ($p > 0.05$). In the C group, the blood glucose reached a peak at 30 min, and then gradually dropped, ultimately down to close to the fasting blood glucose value at 120 min. In the M group, the peak delay was observed, the blood glucose reached a peak at 60 min. The trend of oral glucose tolerance curve of the TI group and the TR group was the same as that of the C group, and the blood glucose levels of each time point were reached to the level close to that of the C group ($p > 0.05$).

3.4 IRS1 mRNA Expression in Skeletal Muscle of Rats in Each Group

As shown in Fig. 4, The IRS1 mRNA expression in skeletal muscle of rats in M group was significantly decreased compared with the normal control group ($p < 0.05$), whereas the TI and TR group had no significant differences compared with the normal control group ($p > 0.05$).

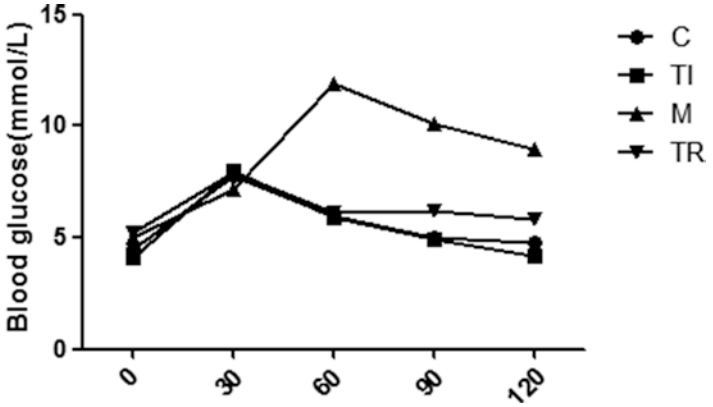

Fig. 3 Oral glucose tolerance test. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means ± SE. Oral glucose tolerance test was performed in three rats from each group. Over night fasted rats (14 h) were administered by perfusion to make 1.5 g of glucose per kg of body weight for the OGTT, Blood glucose levels were measured at 0, 15, 30, 60 and 90 min using Roche blood glucose meter

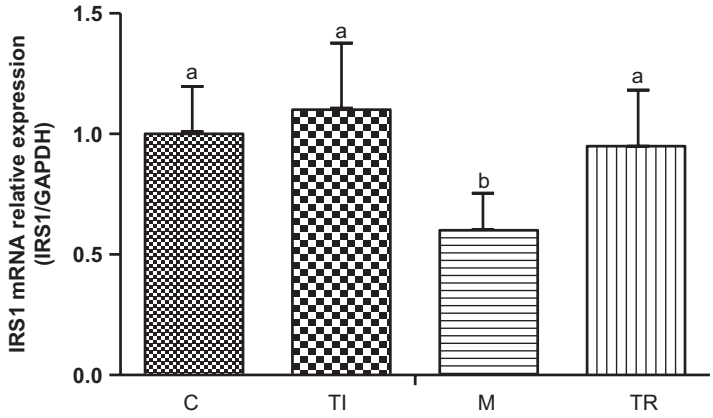

Fig. 4 IRS1 mRNA expression in skeletal muscle of rats in each group. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means ± SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 8). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letter are significantly different ($p < 0.05$). There are three replicates with three rats in each group

3.5 GLUT4 mRNA Expression in Skeletal Muscle of Rats in Each Group

As shown in Fig. 5, The GLUT4 mRNA expression in skeletal muscle of rats in M group was significantly decreased compared with the normal control group ($p < 0.05$), whereas the TI and TR group had no significant differences compared with the normal control group ($p > 0.05$).

3.6 IRS1 Protein Expression in Skeletal Muscle of Rats in Each Group

As shown in Fig. 6, The IRS1 protein expression in skeletal muscle of rats in M group was significantly decreased compared with the normal control group ($p < 0.05$), whereas the TI and TR group had no significant differences compared with the normal control group ($p > 0.05$).

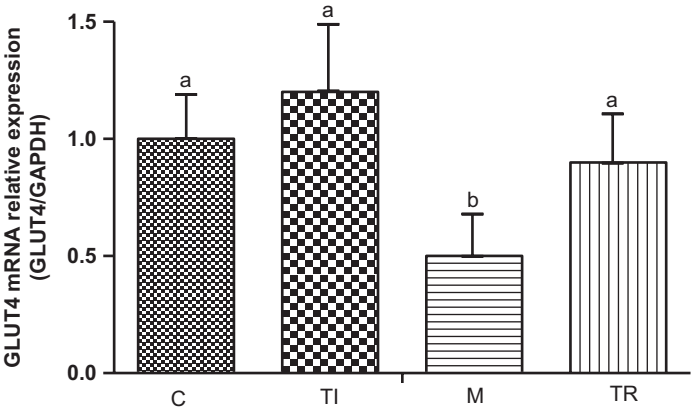

Fig. 5 GLUT4 mRNA expression in skeletal muscle of rats in each group. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means ± SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 8). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letter are significantly different ($p < 0.05$). There are three replicates with three rats in each group

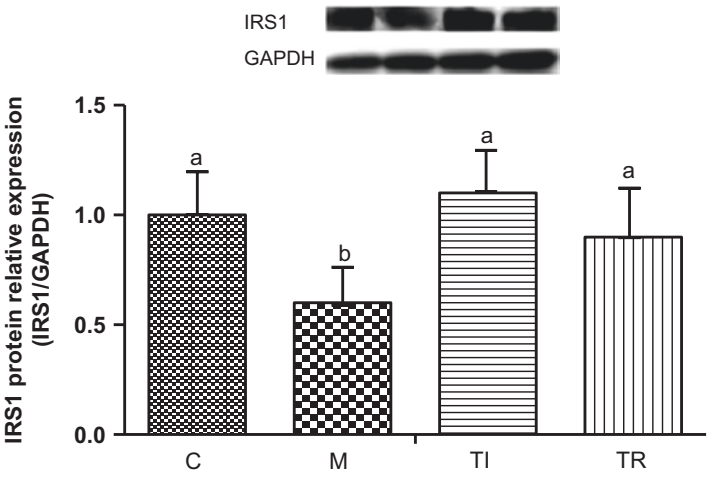

Fig. 6 IRS1 protein expression in skeletal muscle of rats in each group. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means ± SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 8). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letter are significantly different ($p < 0.05$). There are three replicates with three rats in each group

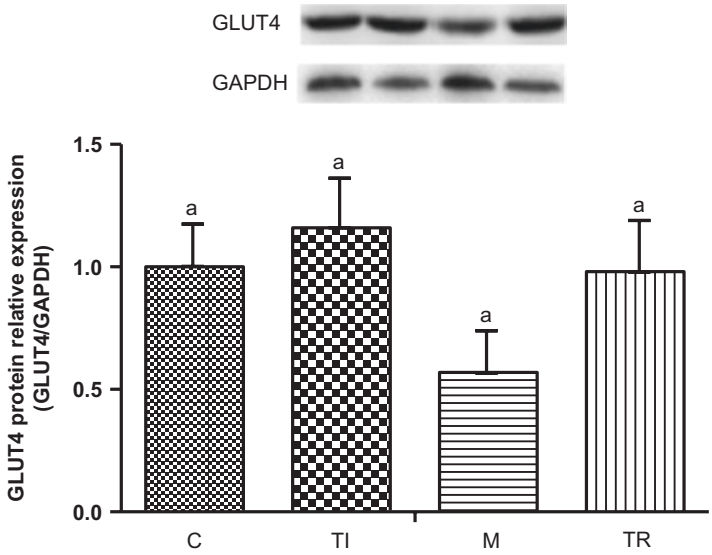

Fig. 7 GLUT4 protein expression in skeletal muscle of rats in each group. *C* control group, *TI* taurine intervention group, *M* model group, *TR* taurine reversal group; All data are expressed as means±SE and were analyzed by one-way ANOVA followed LSD and Duncan's multiple comparisons post hoc test (n = 8). The number in same index have same letter in the superscription are insignificantly different ($p > 0.05$), with different letter are significantly different ($p < 0.05$). There are three replicates with three rats in each group

3.7 GLUT4 Protein Expression in Skeletal Muscle of Rats in Each Group

As shown in Fig. 7, The GLUT4 protein expression in skeletal muscle of rats in M group was significantly decreased compared with the normal control group ($p < 0.05$), whereas the TI and TR group had no significant differences compared with the normal control group ($p > 0.05$).

4 Discussion

Insulin resistance and impaired β cell function are the main characteristics in type 2 diabetes mellitus (T2DM). Insulin resistance is the phenomenon that fat cells, muscle cells and liver cells have a reduced ability to respond to normal concentrations of insulin, which requires higher concentrations of insulin to produce a normal response. In this case, a large amount of insulin is secreted in order to maintain normal blood glucose levels. High concentration of insulin can cause the occurrence of mitochondria and endoplasmic reticulum stress in β cells, then promote apoptosis of β cells, and ultimately lead to the occurrence of type 2 diabetes. Therefore, it is

believed that the control of insulin resistance can prevent the occurrence and development of diabetes to some extent.

This study revealed that taurine could attenuate insulin resistance by improving the metabolism of lipid, as well as the insulin resistance index through increasing the mRNA and protein expressions of IRS1, GLUT4 in rats fed with high sugar and high fat diets.

Metabolic syndrome is a lifestyle-related disease caused by a high nutrient condition or lack of exercise. The development of insulin resistance due to obesity has attracted attention as an underlying mechanism for metabolic syndrome. In our study, insulin resistance model rats were induced via high sugar and high fat diet fed for 8 weeks. The results showed that the FBG level of group PM had no significant differences compared with the C group, but The FINS level of the PM group was significantly higher than that of the C group, moreover, the value of HOMA-IR was significantly increased. The model rats exhibited hyperinsulinemia, decreased insulin sensitivity and other features, which demonstrated that insulin resistance model was successfully established in the present study. Furthermore, after 12 weeks, the insulin resistance model rats exhibited increased levels of serum TG, TC and decreased level of serum HDL-C. Taurine treatment at the same time of model establishment with for 4 weeks could reduce the levels of serum TG, TC and elevate the level of HDL-C compared to model rats, suggesting that taurine can improve dyslipidemia in rats fed with high sugar and high fat diet. It is reported that skeletal muscle express IRS1 and GLUT4 genes, which are involved the insulin signaling pathway (Myers et al. 1992; Nishiyama and Wands 1992; Friedman et al. 1991; Kern et al. 1990). In the present study, taurine treatment enhanced the activity of IRS1 and GLUT4 in skeletal muscle tissue compared to the model rats, indicating that taurine could ameliorate insulin sensitivity through regulating the expression of IRS1 and GLUT4. It is conceived that insulin and insulin-like growth factor 1 bind to insulin receptors located on the cell membrane in skeletal muscle, and then activate the IRS-1/PI3K/Akt signaling pathway, which is a critical step in the regulation of glucose transport in response to insulin, ultimately, transport glucose into cells through GLUT4 to synthesize glycogen or be oxidized in order to maintain a stable glucose level in plasma (Li et al. 2016; Hu et al. 2014). Under physiological conditions, only 10–20% insulin receptor can exert a full physiological effect of insulin, hence, the defects of insulin receptor signal transduction pathway will lead to insulin resistance in liver, adipocyte and skeletal muscle of most type II diabetes and obesity patients (Smith et al. 1996; Obata et al. 2016). In other words, defects at steps between the IRS1 and GLUT4 on the pathway of IRS-1/PI3K/Akt may result in insulin resistance. It is widely believed that defects at IRS1 represent a central feature of this disorder (Ueki et al. 2004; Morino et al. 2005). IRS1 is required for glucose metabolism and myoblast differentiation in skeletal muscle cells (Huang et al. 2005). IRS1 knockout mice showed growth retardation and impaired insulin action, especially in muscle, but the glucose tolerance was not affected. Feeding obese nondiabetic subjects (body mass index, 30–45 kg/m^2) with very low calorie diet (VLCD) could increase IRS1 tyrosine phosphorylation. Researches found that IRS-1 protein levels of obesity rats with insulin resistant

were significantly decreased in skeletal muscle, and the phosphorylation of IR and IRS-l were significantly decreased (Zeng et al. 2000; Kim et al. 2000).

GLUT4 is the major glucose transporter of muscle and adipose tissue. Most of the glucose transporter 4 is intracellular without insulin, insulin stimulation can induce the translocation of GLUT4 from intracellular vesicular compartments to plasma membrane (Huang et al. 2007). Knocking out GLUT4 gene induced insulin resistance in adipose and skeletal muscle tissues of mice, and caused hyperinsulinemia and reduced the glucose uptake (Abel et al. 2001; Zisman et al. 2000). However, over expression GLUT4 gene in adipose and skeletal muscle of diabetic db/db mice tissue improved glucose metabolism, reversed insulin resistance and symptoms of type 2 diabetes mellitus (Brozinick et al. 2001). Our results revealed that the insulin receptor transduction was impaired, inducing insulin-resistance in skeletal muscle under high insulin conditions. The expressions of the IRS-1, GLUT4 in skeletal muscle were significantly decreased in rats fed with high sugar and high fat diet. After treatment with taurine, the expressions of IRS-1 and GLUT4 were partially restored. Here, we showed that the taurine-mediated recovery of insulin action was related to the improvement of the IRS-1/PI 3-kinase signaling pathway.

5 Conclusion

In summary, the present research demonstrated the beneficial effects of taurine on reversing insulin resistance and insulin sensitivity through remediating lipid metabolism disorder and regulating the expressions of IRS and GLUT4. These results may have clinical applications for insulin resistance and the treatment of type-2 diabetes without side effects.

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Taurine Inhibited Uric Acid Uptake in HK-2 Renal Tubular Epithelial Cells

Ying Feng, Shumei Lin, Xiaoyan Zhao, Qunhui Yang, Gaofeng Wu, Qiufeng Lv, Jiancheng Yang, and Jianmin Hu

Abstract It has been confirmed by our laboratory that taurine could decrease uric acid levels in hyperuricemic rats and regulate the expressions of some urate transporters. The present study aims to investigate the effects of taurine on uric acid uptake in human renal proximal tubular epithelial cells (HK-2). The cell growth inhibition rate was measured by MTS assay, which was up to 50% after treatment with 1.5 mmol/L uric acid. After administration of 15 mmol/L taurine, the inhibition rate and uric acid uptake were both significantly decreased. Then the HK-2 cells were grouped as follows: control group (C); model group (M), in which 1.5 mmol/L uric acid was added to the medium; taurine group (MT), in which 1.5 mmol/L uric acid and 15 mmol/L taurine were added to the medium; and taurine control group (T), in which 15 mmol/L taurine was added to the medium. The mRNA and protein expression levels of URAT1 and GLUT9 were measured by real-time PCR and western-blot. The results showed that URAT1 and GLUT9 mRNA/protein expression levels in group M were significantly increased compared with group C, and they were both down-regulated in MT group. In addition, the expression levels of these two transporters in group T were significantly lower than group C. The results indicated that taurine could inhibit uric acid uptake and down-regulate the expressions of URAT1 and GLUT9 in HK-2 cells.

Keywords Taurine · Uric acid · URAT1 · GLUT9 · HK-2 cells

Abbreviations

FBS	Fetal bovine serum
GLUT9	Glucose transporter 9

Ying Feng and Shumei Lin have been contributed the same to the article.

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MTS [3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-etrazolium,inner salt]
TAUT Taurine transporter
URAT1 Urate anion transporter 1

1 Introduction

Uric acid is the main end product of purine metabolism in humans. Too much production and/or under-excretion of uric acid may cause hyperuricemia. In human kidneys, uric acid undergoes simultaneously both reabsorption and secretion in the proximal tubule, and results in net reabsorption of 90–95% of glomerularly filtered urate. This process is complex, and involves many transporters. Urate is extensively reabsorbed from the glomerular ultrafiltrate in the proximal tubule via the brush-border urate-anion exchanger, urate transporter 1 (URAT1, encoded by SLC22A12), which is targeted by uricosuric and antiuricosuric agents (Enomoto et al. 2002). Glucose transporter 9 (GLUT9, encoded by SLC2A9) has been proved to be central to urate reabsorption (Ruiz et al. 2018; Vitart et al. 2008; Anzai et al. 2008), and its genetic inactivation leads to greatly elevated urate excretion and suppressed renal reabsorption in G9KO mice (Preitner et al. 2009). Taurine participates in several renal physiological processes and has been shown as a renoprotective agent. Our previous study showed that taurine could significantly decrease uric acid levels and alleviate the kidney injury in hyperuricemic rats (Feng et al. 2017). In the kidney, the epithelial cells of the proximal tubule play a major role in urate reabsorption and excretion. The present study aims to further investigate the relationships between taurine and uric acid uptake by HK-2 cells, a line of proximal tubular epithelial cells from normal human kidney.

2 Materials and Methods

2.1 Cell Culture

Human renal proximal tubular epithelial cells (HK-2) were purchased from stem cell library in Chinese Academy of Sciences (CAS). The cells were maintained in F12 medium containing 10% fetal bovine serum (FBS) and grown in 5% CO_2 atmosphere at 37 °C.

2.2 MTS Assay

The HK-2 cells were harvested from flasks and plated in 96-well polystyrene plates with approximately 10,000 cells per well. Plates were incubated at 37 °C for 24 h to allow cells to attach. Uric acid and taurine were dissolved in F12 medium without

FBS. The plates were then incubated for an additional 48 h at 37 °C. Cell viability was determined using an MTS (Promega) and 20 μL of which was added to each well. After 4 h, the absorbance at 490 nm was measured by Tecan M200PRO microplate reader (Tecan, Switzerland). Averages from four replicate wells were used for each sample, and each experiment was repeated for three times.

2.3 Uric Acid Uptake Assay

HK-2 cells were cultured in 96-well polystyrene plates with approximately 10,000 cells per well. Taurine was dissolved in F12 medium without FBS, and added to the cells at a final concentration of 0 mmol/L, 1 mmol/L, 5 mmol/L, 10 mmol/L, 15 mmol/L, 20 mmol/L for 48 h, six wells in each group. Then the medium was replaced by serum-free F12 medium containing 1.5 mmol/L uric acid for 30 min. The contents of uric acid in the medium were detected by uric acid kit (Nanjing Jiancheng Bioengineering Institute, China). The value of uric acid uptake was calculated by the difference of uric acid content in the medium before and after absorption.

2.4 Cell Treatment

The HK-2 cells were cultured in 6-well polystyrene plates with approximately 3×10^5 cells per well and grouped as follows: control group (C); model group (M), in which 1.5 mmol/L uric acid was added to the medium; taurine group (MT), in which 1.5 mmol/L uric acid and 15 mmol/L taurine were added to the medium; and taurine control group (T), in which 15 mmol/L taurine was added to the medium. The mRNA and protein levels of URAT1 and GLUT9 were determined using real-time PCR and western blot analysis, respectively. Triplicate wells per group were cultured for 48 h and the experiments were repeated for three times.

2.5 Real-Time PCR

The total cellular RNA was isolated from the HK-2 cells, and reverse transcriptions were performed with PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Primer pairs for the selected genes were designed with Primer Premier 6.0 software using the human mRNA sequence as published, and the GAPDH gene was selected as a housekeeping gene to normalize variations in cDNA content. For organic anion/urate transporter (URAT1, Slc22a12), the cDNA was amplified using the primer (forward: 5′-CGTGTACTGCCTGTTCCGCT-3′; reverse: 5′-CCACTCCA TCAGGAGAGTGCC-3′); For GLUT9 (SLC2A9), the primer pairs (forward: 5′-AGAGGAGGTCCTGGCTGAGA-3′; reverse:

5′-GTAGCAGG CCATGGTGACAA-3′) were used; For GAPDH, the primer pairs (forward: 5′-AGAAGGCTGGGGCTCATTTG-3′; reverse: 5′-AGGGGCCA TCCA GTCTTC-3′) were used.

2.6 Western Blot Analysis

The total protein of HK-2 cells were extracted with total protein extraction kit according to the protocol of the manufacturer (Applygen, China), and the concentration was determined by Bradford protein quantitation kit (Applygen, China). Equal amounts of protein samples were loaded on SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) by wet transfer method (150 mA, 60 min). After blocking with 5% (W/V) non-fat milk in TBS (Applygen, China) for 2 h at room temperature, the membranes were incubated with primary antibodies directed against SLC22A12 monoclonal antibody (Abnova, 1:1000), Anti-GLUT9 antibody (Abcam, 1:1000) and β-actin monoclonal antibody (Abcam, 1:5000) overnight at 4 °C. After three washes with TBST (0.1% Tween-20), the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) were incubated for 2 h at room temperature. Specific signals were visualized by ECL Western Blotting Substrate. The protein bands were quantified by Image Quant 5.0 software and normalized to individual β-actin expression levels.

2.7 Statistical Analysis

Data were presented as the mean ± SD and significant differences were analyzed by one-way ANOVA and Duncan's multiple range tests using SPSS 16.0 statistical analysis software. P values less than 0.05 were considered statistically significant.

3 Results

3.1 Taurine Decreased the Inhibition Rate of Cell Proliferation in HK-2 Cells Treated with 1.5 mmol/L Uric Acid

HK-2 cells were administered with uric acid (1.5 mmol/L) and taurine in different concentration (0, 1, 5, 10, 15, 20, 30) mmol/L, and the inhibition rates of cell proliferation were detected by MTS assay. As shown in Fig. 1, the inhibition rate of cell proliferation caused by 1.5 mmol/L uric acid was about 50%, and the inhibition rate decreased in a dose-dependent manner when taurine concentration was lower than 15 mmol/L. However, when the concentration of taurine increased to 20 mmol/L

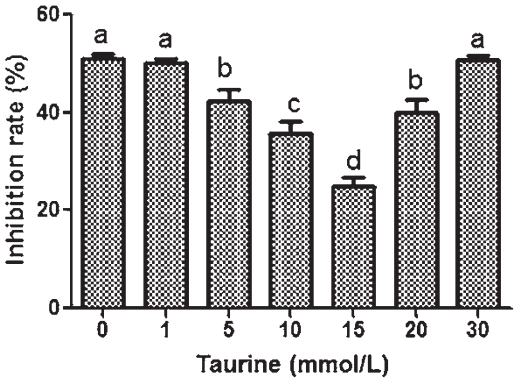

Fig. 1 Effects of taurine on the inhibition rate of cell proliferation in HK-2 cells treated with 1.5 mmol/L uric acid. Data were presented as the mean ± SD. Values with different superscript letters were significantly different ($P < 0.05$)

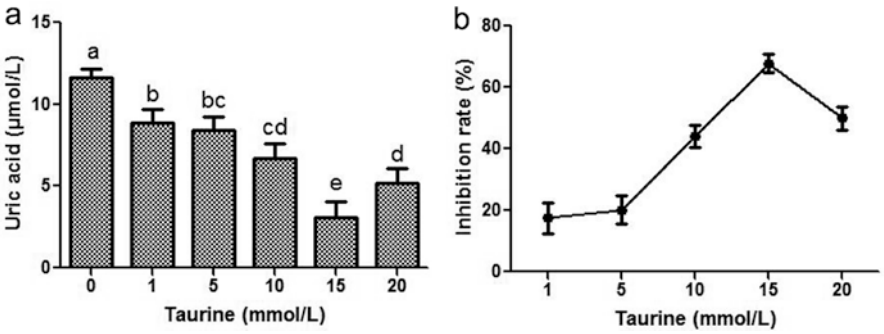

Fig. 2 (**a**) Effects of taurine on uric acid uptake in HK-2 cells treated with 1.5 mmol/L uric acid (**b**) Effects of taurine on the inhibition rate of uric acid uptake. Data were presented as the mean ± SD. Values with different superscript letters were significantly different ($P < 0.05$)

and 30 mmol/L, the cell proliferation inhibition rate showed an upward trend. Thus, the optimal taurine concentration against cell injury caused by 1.5 mmol/L uric acid is 15 mmol/L.

3.2 Taurine Inhibited Uric Acid Uptake in HK-2 Cells Treated with 1.5 mmol/L Uric Acid

As shown in Fig. 2a, with the increase of taurine concentration, uric acid uptake by HK-2 cells was reduced in a dose-dependent manner. Among them, 15 mmol/L of taurine had the greatest inhibition of uric acid absorption ($P < 0.05$), and the inhibition rate was 67.86% (Fig. 2b). However, 20 mmol/L of taurine showed less inhibitive effects on uric acid uptake compared with 15 mmol/L of taurine.

3.3 *Taurine Inhibited URAT1 and GLUT9 Expressions in HK-2 Cells*

As shown in Fig. 3, the mRNA/protein expression levels of URAT1 and GLUT9 in group M were significantly increased compared with group C, whereas in MT group, in which 15 mmol/L of taurine was administered, the expression levels were significantly lower than that in group M, and showed no significant difference from the group C. In addition, the expressions of the two transporters in group T were both significantly lower than that in group C. It indicated that under both normal and high uric acid condition, taurine could down-regulate the expressions of URAT1 and GLUT9 in HK-2 cells.

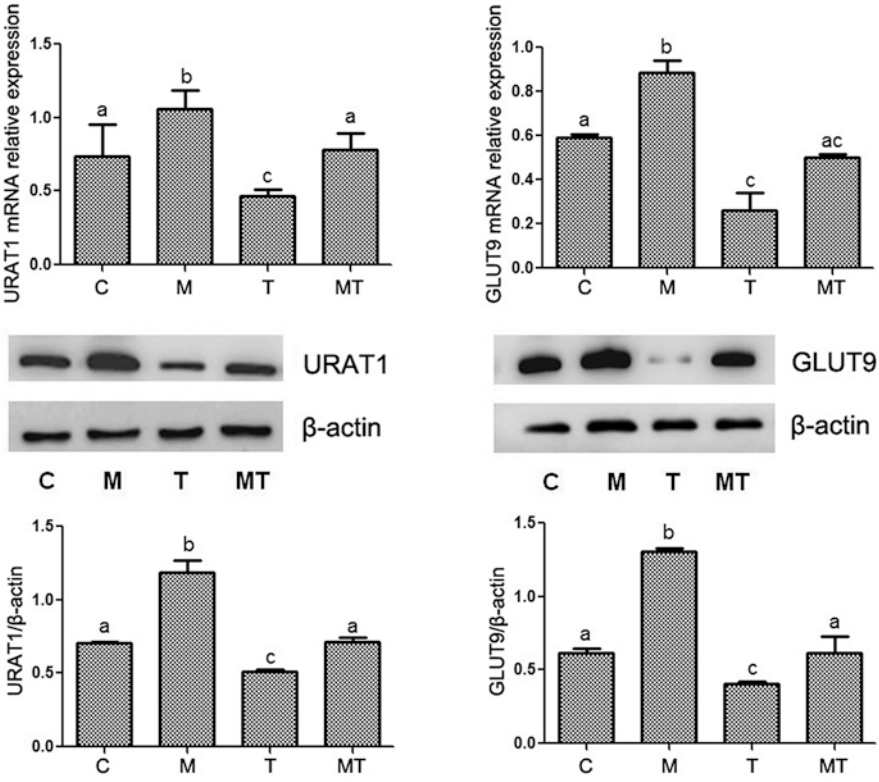

Fig. 3 Effects of taurine on URAT1 and GLUT9 mRNA and protein expressions in HK-2 cells. *C* control group, *M* model group, 1.5 mmol/L uric acid was added to the medium, *MT* taurine group, 1.5 mmol/L uric acid and 15 mmol/L taurine were added to the medium, *T* taurine control group, 15 mmol/L taurine was added to the medium. The mRNA and protein levels of URAT1 and GLUT9 were determined using real-time PCR and western blot analysis, respectively. Triplicate wells per group were cultured for 48 h and the experiments were repeated for three times. Data were presented as the mean ± SD. Values with different superscript letters were significantly different ($P < 0.05$)

4 Discussion

The kidney plays a dominant role in maintaining plasma urate levels through the excretion process, which eliminates 70% of the daily urate production. Too much uric acid in the body, is largely due to excessive reabsorption of uric acid. Urate hardly permeates the plasma membrane of cells in the absence of urate transporters. URAT1 is efficient for urate reabsorption, which contains 553 amino acids and 12 transmembrane domains, specifically localizes at the apical membrane of proximal tubular epithelial cells. Mutations in URAT1 might cause hypouricemia in humans (Enomoto et al. 2002). GLUT9, on the basolateral side of renal proximal tubular epithelial cells, is a voltage-dependent transport system for urate absorption (Anzai et al. 2008). Human GLUT9 loss-of-function mutations were identified in familial hypouricemia, indicating that it is a major determinant of serum uric acid level (Ruiz et al. 2018). Thus, both URAT1 and GLUT9 play critical roles in urate reabsorption. The actual model for urate reabsorption suggests that urate in the lumen is taken up via URAT1 and intracellular urate exits from the cell to the interstitial space via GLUT9 (Nakanishi et al. 2013). In our present study, taurine protected HK-2 cells from uric acid injury in a dose-dependent manner, and the uric acid uptake was significantly inhibited after taurine administration. The results also suggest that under both normal and high uric acid condition, taurine could down-regulate the expressions of URAT1 and GLUT9 in HK-2 cells. URAT1 interchanges uric acid with Cl^- or organic anions in the epithelial cells. Some agents such as probenecid promote the excretion of uric acid by combining with URAT1 (Xu et al. 2017). As a member of GLUT family, GLUT9 has a high capacity for transporting urate in humans and increases the speed of uric acid reabsorption by glucose transport (Caulfield et al. 2008). It is worth mentioning that the active uphill transport of taurine occurs via a sodium and Cl^- dependent taurine transporter (TAUT), which is also interact with glucose (Chesney et al. 2010). Thus, we speculated that taurine might play an important role in uric acid transport by the regulation of urate transporters, the actual mechanism still need to be further studied.

5 Conclusion

Taurine protected HK-2 cells from uric acid injury and inhibited uric acid uptake in a dose-dependent manner. Under both normal and high uric acid condition, taurine could down-regulate the expressions of URAT1 and GLUT9 in HK-2 cells.

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Role of Taurine in Testicular Function in the Fragile x Mouse

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Abstract Fragile X syndrome is an X-linked dominant disorder and the most common cause of inherited mental retardation. It is caused by trinucleotide repeat expansion in the fragile X mental retardation 1 gene (FMR1) at the Xq27.3. The expansion blocks expression of the gene product, Fragile X Mental Retardation Protein (FMRP). The syndrome includes mild to moderate mental retardation and behavioral manifestations such as tactile defensiveness, gaze avoidance, repetitive motor mannerisms, perseverative (repetitive) speech, hyperarousal and it frequently includes seizures. This behavioral phenotype overlaps significantly with autism spectrum disorder. The knockout mice lack normal Fmr1 protein and show macroorchidism, learning deficits, and hyperactivity. Consequently, this knockout mouse may serve as a valuable tool in the elucidation of the physiological role of FMR1 and the mechanisms involved in macroorchidism, abnormal behavior, abnormalities comparable to those of human fragile X patients. In this study we evaluated the effects of taurine on the testicular physiology to better understand the cellular mechanisms underlying macro-orchidism. We found that there was a significant decrease in the number of Leydig cells in the testis of fragile X mouse. Furthermore, the expression of somatostatin was drastically decreased and differential expression pattern of CDK5 in fragile X mouse testis. In the control testis, CDK is expressed in primary and secondary spermatids whereas in the Fmr1 ko mice CDK 5 is expressed mainly in spermatogonia. Taurine supplementation led to an increase in CDK5 expression in both controls and Ko mice. CDKs (Cyclin-dependent kinases) are a group of serine/threonine protein kinases activated by binding to a regulatory subunit cyclin. Over 20 functionally diverse proteins involved in cytoskeleton dynamics, cell adhesion, transport, and membrane trafficking act as CDK5 substrates elucidating the molecular mechanisms of CDK5 function. CDK5 phosphorylates a

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diverse list of substrates, implicating it in the regulation of a range of cellular processes. CDK5 is expressed in Leydig cells, Sertoli cells, spermatogonia and peritubular cells indicating a role in spermatogenesis. In this study we examined the expression levels of CDK5 and how it is affected by taurine supplementation in the testes and found that taurine plays an important role in testicular physiology and corrected some of the pathophysiology observed in the fragile x mouse testis.

Keywords Taurine · Fragile X · Autism · Testes · CDK5 · Leydig cells · Somatostatin · P450scc · Steroidogenesis

Abbreviations

CDK5	cyclin-dependent kinase
TAU	taurine
P450scc	P450 side chain cleavage
Fmr1 KO	fragile x knockout mice

1 Introduction

The fragile X syndrome includes hyperarousal, hypersensitivity to sensory stimuli and an increased prevalence of seizures (Hagerman 2002). The mouse model for this disorder (Bakker et al. 1994) has increased seizure susceptibility (Musumeci et al. 2000; Chen and Toth 2001; Yan et al. 2004) and this may be a direct parallel to elements of the syndrome that suggest reduced inhibition/increased excitability. Our investigations of the molecular basis of increased seizure susceptibility in the fragile X mouse indicated a reduction in $GABA_A$ receptor expression (El Idrissi et al. 2005). Since these receptors play a major role in inhibition, their reduction helps explain the increased seizure susceptibility of this mouse model for fragile X and suggest that the GABAergic system may be affected in the fragile X syndrome.

We also found increased expression of the enzyme responsible for the synthesis of GABA, the neurotransmitter agonist for $GABA_A$ receptors. This increase is likely to be a response of the brain to reduced inhibition – a response that has been observed in other models of elevated excitability (Riback et al. 1993). The excitability of neuronal circuits is kept within a normal range through feed-forward and -backward inhibition, mediated by inhibitory interneurons. These neurons continuously adjust their inhibitory output to match the level of excitatory input. Thus, when there is reduced inhibition of postsynaptic neurons, feedback from these neurons causes the presynaptic neurons to increase their inhibitory output. In the example of fragile X mouse brain, reduced $GABA_A$ receptor expression on postsynaptic membranes would induce an increase in GAD expression, thus increasing the bioavailability of GABA in presynaptic terminals. Therefore, increased GAD may represent a secondary response to the direct effects of Fmrp depletion.

In our previous studies (El Idrissi 2006; El Idrissi et al. 2003), we have shown that mice chronically supplemented with taurine in their drinking water showed biochemical changes in the GABAergic system similar to those observed in fragile x mouse, including reduced $GABA_A$ receptor, increased GAD expression and a lower threshold for seizure induction.

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid. It is one of the most abundant free amino acids in many excitable tissues, including the brain, skeletal and cardiac muscles. Physiological actions of taurine are widespread and include bile acid conjugation, detoxification, membrane stabilization, osmoregulation, neurotransmission and modulation of cellular calcium levels (Foos and Wu 2002; Lombardini 1985; Saransaari and Oja 2000; Schaffer et al. 2000; Solis et al. 1988). Furthermore, taurine plays an important role in modulating glutamate and GABA neurotransmission (El Idrissi and Trenkner 1999, 2004; Militante and Lombardini 1998). We have previously shown that taurine prevents excitotoxicity in vitro primarily through modulation of intracellular calcium homeostasis (El Idrissi and Trenkner 1999). In neurons, calcium plays a key role in mediating glutamate excitotoxicity.

Outside of the central nervous system, taurine also is essential during developmental processes. Taurine is added to milk formula and in solution for parenteral nutrition of premature babies to prevent retinal degeneration and cholestasis (Huxtable 1992; Lourenço and Camilo 2002). Taurine is found at high concentrations in pancreatic islets (Huxtable 1992). Taurine is able to prevent pancreatic alterations induced by gestational malnutrition especially low-protein diet (Boujendar et al. 2002; Cherif et al. 1996; Dahri et al. 1991; Merezak et al. 2001). In addition, taurine administration during gestation delays the mean onset time of diabetes in non-obese diabetic (NOD) mice (Arany et al. 2004). Taurine supplementation to dams fed a normal diet produces weak glucose intolerance and decreases islet sensitivity to cytokines in offspring (Merezak et al. 2001). Moreover, taurine participates in glucose metabolism in adults (Franconi et al. 2006; Hansen 2001).

Further, we sought to determine the role of taurine in testicular function could affect the endocrine function of the testis in both controls and fragile x mouse.

2 Materials and Methods

2.1 Animals

All mice used in this study were 2-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 0.05%, and this solution was made available to the mice in place of drinking water for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Immunohistochemistry

Frozen sections were made as previously described and placed onto gelatin-subbed slides. Non-specific binding sites were blocked using 4% bovine serum albumin (BSA), 2% normal goat serum (NGS), and 0.05% Triton X-100 in 0.01 M phosphate-buffered saline (PBS; pH 7.2). Following the blocking step, the slides were rinsed in an antibody dilution cocktail (ABD) consisting of 2% BSA and 1% NGS in 0.01 M PBS. Primary antibodies (Thermo Fisher) employed were directed against the indicated antigens diluted 1:500 in ABD. The primary antibodies were incubated overnight at 4 °C and then unbound antibodies rinsed with ABD. Secondary antibodies were all raised in goat and directed against appropriate primary antibody type. The anti-mouse IgG was conjugated to Alexa Fluor 488 (Invitrogen/Molecular probes). Images were obtained by confocal microscopy (Leica SP2 AOBS). Nuclei were counter-stained with SlowFade with DAPI (Invitrogen). To determine relative changes in protein expression, the gain and offset was identical for all comparisons. The intensity ratios of immunoreactivity was determined by importing the data from the Leica confocal software into Imaris X64 (Bitplane). For each Z stack, the threshold values for insulin receptor immunoreactivity were set for the untreated tissues. When the Z stacks for the taurine-treated tissues were imported, the Z stack were treated the same as the control. Coupling these manipulations with the consistent imaging parameters (same lens, gain and offset for each laser), the data changes are treatment-related. The mean pixel intensity values for each thresholded channel were obtained from the Imaris software and those data imported into InStat statistical software (GraphPad Software Inc.)

2.3 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Fmr1 KO Mice Have Reduced P450scc in the Testis

The biosynthesis of steroids proceeds through the transfer of cholesterol from the outer to the inner mitochondrial membrane through the steroidogenic acute regulatory protein. The cholesterol side chain is cleaved by cytochrome P450 side chain cleavage (P450scc), a rate limiting mitochondrial enzyme originally found in peripheral steroidogenic glands and subsequently in the brain. The cleavage results

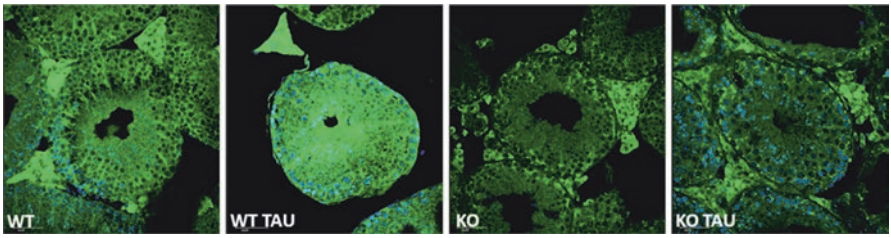

Fig. 1 Effect of taurine supplementation on P450scc immunoreactivity in the testis. Images are z-stacks obtained with a confocal microscope. Testis from taurine-fed mice show a significant increase in immunoreactivity for P450scc while KO mice showed reduction in immunoreactivity for P450scc

in cholesterol conversion to pregnanolone or estrogen, depending on the activation of other enzymes responsible for their synthesis. To investigate the functional significance of the histological changes occurring in the pancreas and the increased insulin production. We found that Fmr1 KO mice have significant decrease in P450scc expression in the testis compared to controls. Taurine supplementation significantly increased P450scc expression in the testis (Fig. 1).

3.2 Taurine Significantly Increases Somatostatin Expression

We have previously showed that taurine supplementation led to a significant increase in somatostatin expression in the brain and pancreas. Here we report that taurine supplementation resulted in a significant increase in somatostatin expression in the testes of both controls and Fmr1 KO mice, mainly in in terstitial cells of the testis (Fig. 2).

3.3 Increased CDK5 Expression in the Testis of Fmr1 KO Mice

In the control testis, CDK is expressed in primary and secondary spermatids whereas in the Fmr1 ko mice CDK 5 is expressed mainly in spermatogonia. Taurine supplementation led to an increase in CDK5 expression in both controls and Ko mice. CDKs (Cyclin-dependent kinases) are a group of serine/threonine protein kinases activated by binding to a regulatory subunit cyclin. Over 20 functionally diverse proteins involved in cytoskeleton dynamics, cell adhesion, transport, and membrane trafficking act as CDK5 substrates elucidating the molecular mechanisms of CDK5 function. CDK5 phosphorylates a diverse list of substrates, implicating it in the regulation of a range of cellular processes. CDK5 is expressed in Leydig cells, Sertoli cells, spermatogonia and peritubular cells indicating a role in

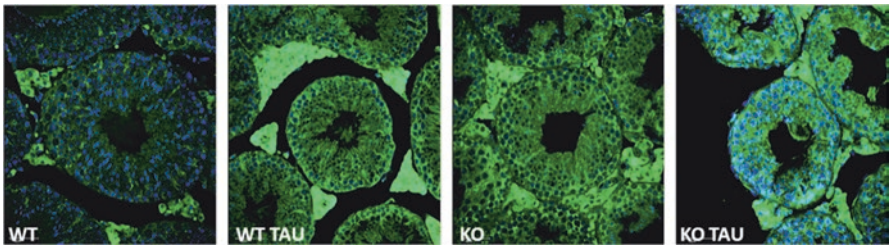

Fig. 2 Effect of taurine supplementation on somatostatin immunoreactivity in the testis. Images are z-stacks obtained with a confocal microscope. Testis from taurine-fed mice show a significant increase in immunoreactivity for somatostatin

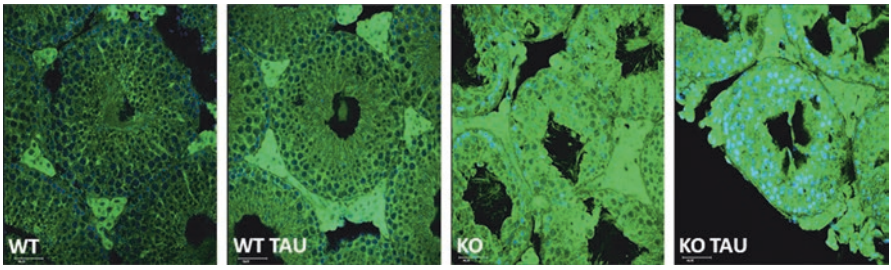

Fig. 3 Effect of taurine supplementation on CDK5 immunoreactivity in the testis. Images are z-stacks obtained with a confocal microscope. Testis from Fmr1 KO mice have increased CDK5 expression and taurine further increased the intensity for immunoreactivity for CDK5

spermatogenesis. Taurine supplementation resulted in a significant increase in CDK5 immunoreactivity in both controls and Fmr1 KO mice (Fig. 3).

4 Discussion

In this study we report changes in the expression of P450scc, somatostatin and CDK5 in the fragile x mouse testis. Reduced P450 expression in the testis of Fmr1 KO mice indicate a reduced biosynthetic capacity for steroid hormones as this enzyme is the rate limiting enzyme for steroids biosynthesis. Taurine supplementation affect did not significantly the expression of this enzyme. On the other hand, taurine supplementation resulted in a significant increase in somatostatin expression in the testes of both controls and Fmr1 KO mice, mainly in in terstitial cells of the testis (Fig. 2). This is consistent with previous observation on the effects of taurine on somatostatin expression in the brain and the pancreas. We also found that the expression of CDK5 was altered in the testis of Fmr1 mice. Fmr1 KO mice testis showed a significant increase in the expression levels of CDK5 and taurine supplementation further increased CDK5 expression in both controls and Fmr1 testis

(Fig. 3). Over 20 functionally diverse proteins involved in cytoskeleton dynamics, cell adhesion, transport, and membrane trafficking act as CDK5 substrates elucidating the molecular mechanisms of CDK5 function. CDK5 phosphorylates a diverse list of substrates, implicating it in the regulation of a range of cellular processes. CDK5 is expressed in Leydig cells, Sertoli cells, spermatogonia and peritubular cells indicating a role in spermatogenesis.

5 Conclusion

Taurine wide spread tissue distribution makes its physiological roles equally broad. Here we report that Fmr1 KO mice have altered expression in the expression of several proteins important for testicular function and taurine supplementations affects expression of these proteins, indicating the role of taurine in testicular function.

Acknowledgement This work was supported by CDN, PSC-CUNY and CSI. The authors declare that they have no conflict of interest. This chapter was modified from the paper published by our group in *Results Probl Cell Differ* (El Idrissi et al. 2012; 54:201–21) in *Neuroscience Letters*, (El Idrissi et al. 2005; 377:141–146) and Adv Exp Med Biol (El Idrissi et al. 2009; 191–198). The related contents are re-used with the permission.

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Effect of Taurine on Cell Function via TXNIP Induction in Caco-2 Cells

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Abstract Taurine (2-aminoethanesulfonic acid), a sulfur-containing β-amino acid, is a free amino acid present in high concentrations in mammalian tissues. Taurine has pivotal roles in anti-oxidation, membrane stabilization, osmoregulation, anti-inflammation, and other process. In a DNA microarray analysis, we previously found that taurine markedly increases the mRNA expression of thioredoxin interacting protein (TXNIP) in Caco-2 cells. In this study, we investigated the effect of these taurine-induced changes in TXNIP on the function of Caco-2 cells. We found that taurine decreases glucose uptake in a dose-dependent manner. The taurine-induced decrease in glucose uptake was completely abolished by transfection with siRNA against TXNIP, suggesting that TXNIP is involved in the taurine-induced down-regulation of glucose uptake. We also revealed that taurine induces AMPK activation and further increases the intracellular ATP content in Caco-2 cells. These results suggest that taurine could regulate the function of Caco-2 cells via TXNIP induction, leading to extend our understanding of the functions of taurine.

Keywords TXNIP · Caco-2 · GLUT · AMPK · ATP

Abbreviations

TXNIP	thioredoxin interacting protein
AMP	adenosine monophosphate
AMPK	adenosine monophosphate kinase
ATP	adenosine triphosphate

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GLUT1	glucose transporter 1
GLUT3	glucose transporter 3
ChoRE	carbohydrate response element
ChREBP	carbohydrate response element binding protein
mTOR	mammalian target of rapamycin

1 Introduction

Taurine (2-aminoethanesulfonic acid), a sulfur-containing β-amino acid, is a free amino acid present in high concentrations in mammalian tissues (Huxtable 1992; Schaffer et al. 2003). Taurine is found to be involved in various biological processes including anti-oxidation, membrane stabilization, osmoregulation, and bile acid conjugation. Taurine also has anti-inflammatory effects based on in vivo and in vitro analyses (Zhao et al. 2008). We have previously observed that the functioning of intestinal taurine transporter (TAUT) is regulated by various factors such as the extracellular concentration (Satsu et al. 1997), hyperosmolarity (Satsu et al. 1999, 2004), lysophosphatidylcholine (Ishizuka et al. 2000, 2002), and inflammatory cytokines including TNF-α and IL-1β (Mochizuki et al. 2002, 2005). Despite extensive studies of the functions of taurine (Schaffer et al. 2003; Murakami 2017), the detailed regulatory mechanism underlying taurine function is unclear, especially at the level of molecule and cell.

A nutrigenomics approach (Masotti et al. 2010; Noe et al. 2004; Murphy et al. 2007) refers to the analyses of the physiological functions of nutrients and food factors based on changing of gene expression profiling. We previously investigated the result of taurine on Caco-2 cells gene expression by using DNA microarrays and found that taurine markedly increases the mRNA expression of thioredoxin interacting protein (TXNIP) (Gondo et al. 2012). We further confirmed that taurine increases TXNIP protein expression, which was induced at the level of transcription. In this study, the effect of taurine on the function of Caco-2 cells via TXNIP induction was investigated.

2 Methods

2.1 Cell Culture

Caco-2 cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 1% non-essential amino acids, 2% glutamine, and an appropriate amount of penicillin-streptomycin (10,000 U/mL and 10 mg/mL). Caco-2 cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air and having temperature of 37 °C.

Table 1 Primers for real-time RT-PCR

Gene		Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
β-actin	Forward	ccagcacaatgaagatcaaga	60	198
	Reverse	agaaagggtgtaacgcaactaa		
TXNIP	Forward	acgcttcttctggaagacca	60	93
	Reverse	aagctcaaagccgaacttgt		
GLUT3 (SLC2A3)	Forward	gccctgaaagtcccagattt	60	115
	Reverse	ttcatctcctggatgtcttgg		
GLUT1 (SLC2A1)	Forward	ggttgtgccatactcatgacc	60	66
	Reverse	cagataggacatccagggtagc		

2.2 Real-Time PCR

Total RNA was isolated from cells to perform real-time PCR by using Isogen (Nippon Gene, Tokyo, Japan), by following manufacturer's instructions. Total RNA (0.5 μg) was reverse-transcribed to cDNA using the Primescript RT Reagent Kit (TAKARA, Shiga, Japan), and the single stranded cDNA was amplified using a SYBR Green Kit (TAKARA). The PCR conditions were; denaturation at 95 °C for 10 s, followed by 35 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s, and extension at 72 °C for 15 s. The PCR primers for β-actin, TXNIP, GLUT1(SLC2A1), and GLUT3 (SLC2A3) are listed in Table 1. The mRNA expression of β-actin did not change significantly at any stage when treated with taurine, demonstrating the use of β-actin as stable housekeeping gene.

2.3 Glucose Uptake Experiment

The Caco-2 cells were washed (twice) with 700 μL of phosphate-buffered saline (PBS) and incubated with 300 μL of glucose-free Hank's balanced salt solution (HBSS) at 37 °C for 10 min. Then, HBSS containing D-[^{3}H] glucose was added and further incubated at 37 °C. After 10 min, the solution was removed and each cell was washed with 0.05% sodium azide containing ice-cold PBS. The monolayer was then solubilized with 0.1% Triton X-100, and the radioactivity in each monolayer was determined using LSC 5100 liquid scintillation analyzer (Aloka, Tokyo, Japan).

2.4 Western Blot Analysis

Caco-2 cells were cultured on 6-well plates with taurine for 48 h and used for western blotting. A whole cell extract was prepared using lysis buffer (50 mM Tris–HCl, pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.1% NP-40, 10% glycerol, 0.1% inhibitor

cocktail) and analyzed by western blot as described previously (Gondo et al. 2012). The protein assay was performed using BIO-RAD Protein Assay Solution (BIO-RAD, Hercules, CA, USA). The primary antibody was the rabbit anti-human AMPKα (Thr172) antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-human AMPKα (Cell Signaling Technology). Goat anti-rabbit IgG antibody linked to horseradish peroxidase (Amersham Biosciences, Little Chalfont, UK) was used as the secondary antibody. Bound antibodies were analyzed using an ECL chemiluminescent substrate (GE Healthcare, Chicago, IL, USA) and the Lumino Image Analyzer (LAS-4000miniPR; Krefeld, Germany).

2.5 Measurement of Intracellular ATP Content

ATP was measured using the ATP Bioluminescence Assay Kit HS II (Roche, Basel, Switzerland) according to the instructions of manufacturer. Caco-2 cells were cultured on 24-well plates and incubated with or without taurine for 48 h. Caco-2 cells were washed with ice-cold PBS, scraped with 500 μL of ice-cold PBS and recovered in Eppendorf tubes. By centrifugation at 1,700 rpm for 5 min 4 °C, PBS was removed and the collected cells were diluted with dilution buffer. Further cell lysis reagent was added, and samples were kept at 22 °C for 5 min. The samples were incubated at 100 °C for 2 min, centrifuged at 10,000 × g for 1 min, and the supernatant was used for ATP measurements.

2.6 Statistical Analysis

Values are expressed as means ± SE. Data were analyzed by the Student's *t*-test, Dunnett's test, or Tukey's test. A *P*-value of less than 0.05 was considered significant.

3 Results

3.1 Effect of Taurine on GLUT1/3 mRNA Expression and Glucose Uptake Activity

To confirm the changes in gene expression in response to taurine, real-time PCR was performed using Caco-2 incubated with 50 mM taurine for 24 h. Consistent with previous findings using DNA microarray approach (Gondo et al. 2012), taurine significantly increased the mRNA expression of TXNIP (Fig. 1A) and decreased GLUT1 (Fig. 1B) and GLUT3 (Fig. 1C).

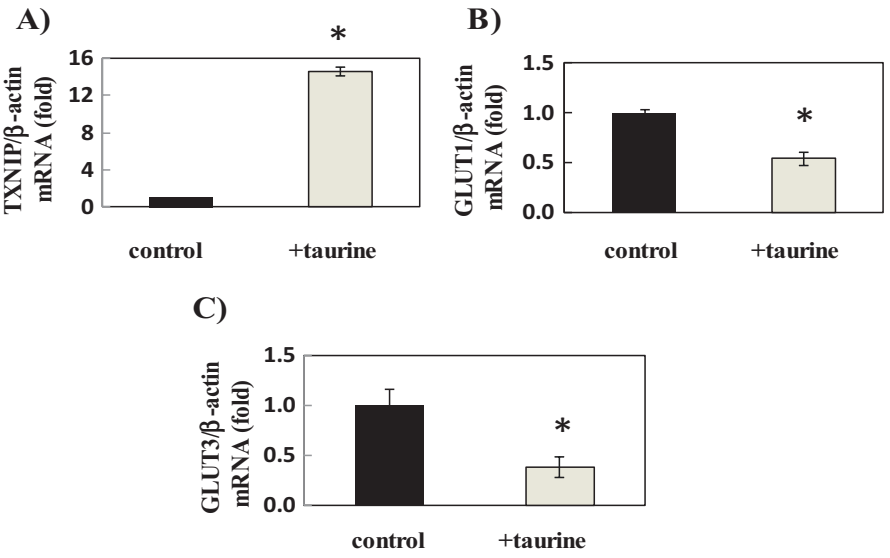

Fig. 1 Effect of taurine on mRNA expression of TXNIP (A), GLUT1 (**B**) and GLUT3 (**C**). Data represents means ± S.E. (n = 3). ∗Significantly different from control ($P < 0.05$), Student's *t*-test

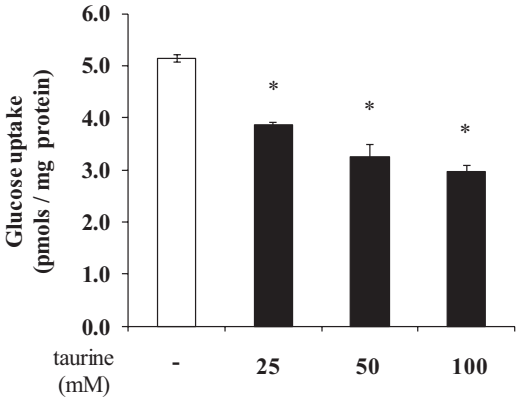

Fig. 2 Effect of taurine on glucose uptake in Caco-2 cells. Caco-2 cells were incubated with 0–100 mM taurine for 48 h. After incubation, glucose uptake was measured as described in Materials and Methods. Data are presented as means ± S.E. (n = 3). ∗Significantly different from control ($P < 0.05$), Dunnett's test

Since GLUT1 and GLUT3 mRNA levels were suppressed by taurine, we further examined whether taurine decreases glucose uptake activity, presumably via GLUT1 and GLUT3, in Caco-2 cells. After Caco-2 cell culturing in medium containing various concentrations of taurine for 48 h, glucose uptake was measured. As shown in Fig. 2, glucose uptake was significantly decreased by taurine in a dose-dependent manner. These results suggest that taurine suppressed glucose uptake in Caco-2 cells, presumably by down-regulation of GLUT1 and GLUT3.

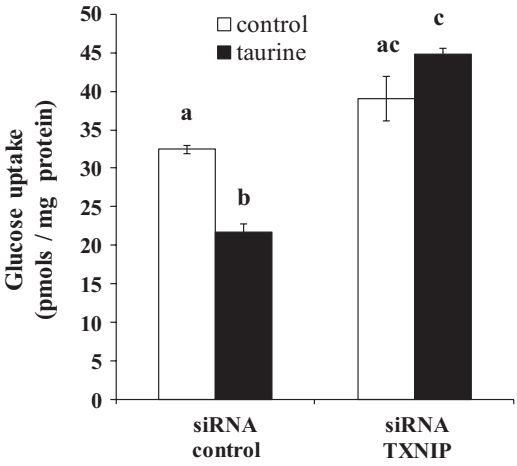

Fig. 3 Role of TXNIP in the taurine-induced down-regulation of glucose uptake in Caco-2 cells. Caco-2 cells transfected with siRNA against TXNIP or control siRNA were incubated with 100 mM taurine. After 48 h, a glucose uptake experiment was performed. Data are presented as means ± S.E. (n = 3). [abc]Values not sharing a common letter are significantly different ($P < 0.05$) based on Tukey's test

3.2 *Role of TXNIP in the Taurine-Induced Down-Regulation of Glucose Uptake in Caco-2 Cells*

It has been reported that the overexpression of TXNIP suppresses glucose uptake (Patwari et al. 2009; Yu et al. 2009). Therefore, we examined the relationship between the induction of TXNIP by taurine and the decrease in glucose uptake by taurine using siRNA against TXNIP. As shown in Fig. 3, taurine decreased glucose uptake by transfection with the control siRNA. However, the taurine-induced decrease in glucose uptake was completely suppressed by transfection with siRNA against TXNIP. These results suggest that taurine decreases glucose uptake via TXNIP induction in Caco-2 cells.

3.3 *Effect of Taurine on AMPK Activity*

AMPK activity is suppressed in the myocardium under starvation conditions in TXNIP-knockout mice (Andres et al. 2011). Accordingly, we examined the effect of taurine on AMPK activity. Caco-2 cells were incubated with 0, 25, 50, and 100 mM taurine for 48 h, and the cell lysate was recovered and used for a western blot analysis, as described in the Materials and Methods. The expression level of phosphorylated (Thr172)-AMPK (pAMPK) was higher than that of inactivated AMPK, with a 1.5-fold induction in response to 100 mM taurine (Fig. 4). This indicates that taurine induced AMPK activation (phosphorylation) in Caco-2 cells.

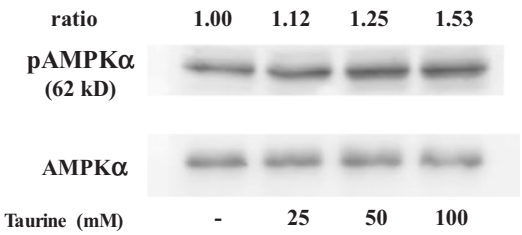

Fig. 4 Effect of taurine on the phosphorylation of AMPKα in Caco-2 cells. Caco-2 cells were cultured in medium containing 0–100 mM taurine for 48 h. The cell lysate was recovered and used for western blotting, as described in the Materials and Methods. The obtained bands were quantified using Image gauge

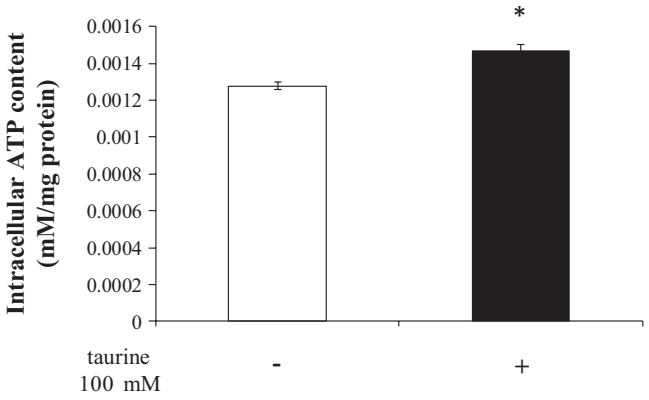

Fig. 5 Effect of taurine on the intracellular ATP content in Caco-2 cells. Caco-2 cells were incubated for 72 h with 100 mM taurine, and the intracellular ATP content was measured. The ATP content was correlated with the protein content. Data are presented as means ± S.E. (n = 3). ∗Significantly different from the control ($P < 0.05$), Student's *t*-test

3.4 Effect of Taurine on the Intracellular ATP Content

AMPK is involved in synthesis and maintenance of ATP, an initial energy source. We measured the intracellular ATP content in Caco-2 cells with medium containing 0 or 100 mM taurine for 72 h. Taurine significantly increased the intracellular ATP content in Caco-2 cells (Fig. 5), suggesting that taurine enhanced energy production.

4 Discussion

In our study, we analyzed the effect of taurine on gene expression, especially energy metabolic-related genes, such as TXNIP, GLUT1, and GLUT3. Further, we examined the energy metabolism related cell functions that were affected by taurine via TXNIP induction.

Glucose uptake activity is suppressed by the overexpression of TXNIP (Patwari et al. 2009; Yu et al. 2009). Taurine increased TXNIP expression in Caco-2 cells (Fig. 1A), suggesting that it suppresses glucose uptake by inducing TXNIP. In support of this, we found that taurine significantly suppressed glucose uptake in a dose-dependent manner (Fig. 2). Further, we analyzed whether TXNIP was involved in taurine-induced downregulation of glucose uptake. The knockdown of TXNIP using siRNA against TXNIP completely abolished the taurine-induced decrease of glucose uptake (Fig. 3), suggesting that taurine decreases glucose uptake via the induction of TXNIP. Based on real-time PCR, taurine decreased the mRNA expression of GLUT1 and GLUT3 (Fig. 1B, C). The downregulation of GLUT1 and GLUT3 may result in the decrease in glucose uptake by taurine. Since TXNIP is located in the nucleus (Patwari et al. 2009; Nishinaka et al. 2004), it might regulate the expression of GLUT1 and GLUT3 mRNAs at the transcriptional level. We will analyze the intracellular localization of TXNIP in Caco-2 cells as well as the role of TXNIP induction by taurine on the suppression of GLUT1 and GLUT3 in future studies.

Glucose uptake is markedly increased by the knockdown of TXNIP in the heart (Yoshioka et al. 2007). Glucose or adenosine-containing molecules suppress glucose uptake by inducing TXNIP, and the carbohydrate response element binding protein (ChREBP)-Mlx complex binds to ChoRE in the promoter region (Yu et al. 2009, 2010). Because the ChREBP-Mlx complex regulates many genes related to glucose metabolism, it has been a focus of many studies of glucose metabolism (Poupeau and Postic 2011; Iizuka and Horikawa 2008). However, in our preliminary experiment, ChoRE was not involved in the induction of TXNIP promoter activity by taurine. Therefore, TXNIP induced the suppression of glucose uptake by a different mechanism.

AMPK activity is suppressed in TXNIP-knockout mice (Andres et al. 2011) and TXNIP suppresses mTOR signaling (Jin et al. 2011). In general, mTOR signaling pathway is suppressed by AMPK (Shaw 2009). Taken together, TXNIP is expected to be involved in AMPK activation. Therefore, we examined whether taurine, which increases TXNIP expression, could induce the activation of AMPK. We observed that AMPK activity increases in a taurine dose-dependent manner (Fig. 4). AMPK is usually activated under energy-insufficient conditions (i.e., high AMP/ATP or ADP/ATP values). AMPK also induces the synthesis or maintenance of ATP (Hardie et al. 2012). In this study, taurine activated AMPK, suggesting that it could promote the synthesis and maintenance of ATP. We found that the intracellular content of ATP increased significantly in response to taurine (Fig. 5). These results indicate that taurine activates AMPK by inducing TXNIP, thereby increasing the intracellular ATP content.

The increase in TXNIP expression by taurine has also been observed in human hepatic HepG2 cells and in murine macrophage-like J774.1 cells (unpublished data). Thus, the induction of TXNIP expression as well as the regulation of cell function via TXNIP induction by taurine may not be specific in Caco-2 cells, but rather be observed in other cell types such as hepatic cells or macrophages although more experiments using various culture cell lines are necessary to evaluate this.

5 Conclusion

We found that taurine resulted in increased expression of TXNIP and it decreased the expression of GLUT1 and GLUT3 in Caco-2 cells. Taurine decreased glucose uptake via TXNIP induction. Furthermore, taurine activated AMPK activity and increased the intracellular ATP content. These results indicate that taurine could regulate the expression of genes, such as TXNIP, leading to the regulation of energy metabolism.

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Taurine Regulation of Peripheral Hemodynamics

Francoise Sidime and Abdeslem El Idrissi

Abstract Taurine plays an important role in the modulation of cardiovascular function by acting not only within the brain but also within peripheral tissues. We found that IV injection of taurine to male rats caused hypotension and tachycardia. A single injection of taurine significantly lowered the systolic, diastolic and mean arterial pressure blood pressure in freely moving long Evans control rats. Previousely, we found that the endothelial cells express high levels of taurine transporters and $GABA_A$ receptors and showed that taurine activates $GABA_A$ receptors. Thus we suggest that the functional implication of $GABA_A$ receptors activation is the relaxation of the arterial muscularis, vasodilation and a decrease in blood pressure. Interestingly however, the effects of acute taurine injection were very different that chronic supplementation of taurine. When rats were supplemented taurine (0.05%, 4 weeks) in their drinking water, taurine has significant hypertensive properties. The increase in blood pressure was observed however only in females, males supplemented with taurine did not show an increase in systolic, diastolic or mean arterial pressure. In both genders however, taurine supplementation caused a significant tachycardia. Thus, we suggest that acute administration of taurine may be beneficial to lowering blood pressure. However, our data indicate that supplementation of taurine to females caused a significant increase in blood pressure. It remains to be seen the effect of taurine supplementation on hypertensive rats.

Keywords Taurine supplementation · Taurine · GABA · Vasoactivity · Blood pressure · Hypertension

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Abbreviations

TAU taurine
GABA γ-Aminobutyric acid

1 Introduction

γ-Aminobutyric acid (GABA), one of the major inhibitory neuro-transmitters in the central nervous system and is also found in many peripheral tissues. GABA has been shown to play an important role in the modulation of cardiovascular function by acting not only within the central nervous system but also within peripheral tissues. GABA has been reported to reduce blood pressure in experimental animals (Takahashi et al. 1955) and humans (Elliott and Hobbiger 1959) following its systemic or central administration, and it has been suggested that the depressor effect induced by systemic administration of GABA is due to the blockade of sympathetic ganglia. The blood–brain barrier is impermeable to GABA, and its concentration in the brain is not changed following i.v. injection (Tsukada et al. 1960). Thus, the antihypertensive effects seen following i.p. or i.v. administration of GABA are due to its actions within the peripheral tissues presumably, blood vessels or autonomic nervous system. It has been reported that GABA can modulate the vascular tone by suppressing the noradrenaline release in the isolated rabbit ear artery and rat kidney (Manzini et al. 1985). The effects produced by GABA in many kinds of peripheral tissues as well as within the central nervous system are mediated by at least two distinct receptor types, $GABA_A$ and $GABA_B$. It has been reported that GABA inhibits sympathetic neurotransmission in the rabbit ear artery through the stimulation of a presynaptic of GABAB receptor subtype (Manzini et al. 1985), and that GABA acts on presynaptic $GABA_B$ receptors to suppress neurotransmitter release (and thereby attenuate renal vasoconstriction) during the activation of the sympathetic nervous supply to the rat kidney (Monasterolo et al. 1996). Baclofen, a selective $GABA_B$ receptor agonist, attenuated the perivascular nerve stimulation-induced increase in perfusion pressure and noradrenaline release to the same extent as did GABA itself (Bowery 1993). Consistent with this, It has been reported that baclofen has hypertensive properties after systemic or intracerebro-ventricular administration in rats (Persson and Henning 1980). Furthermore, these inhibitory effects of GABA were completely antagonized by the selective $GABA_B$ receptor antagonist, saclofen (Bowery 1993), but not by the selective $GABA_A$ receptor antagonist, bicuculline (Curtis 1973). These results strongly suggest that GABA acts on presynaptic $GABA_B$ receptors to inhibit noradrenaline release, and thus the increase in perfusion pressure, induced by perivascular nerve stimulation. Because taurine has been shown to act as an agonist for $GABA_A$ receptors (El Idrissi and Trenkner 2004), we tested the effects of taurine on cardiovascular function, specifically on blood pressure and heart rate. But unlike GABA, taurine crosses the blood-brain barrier. Thus the effects of taurine on cardiovascular function could be mediated either centrally

or peripherally. Based on the results of the present study, we show that taurine injection significantly lowered systemic blood pressure in fully awake rats. Using aortic rings preparation, we further confirmed that taurine acts as a vasodilator. In the aortic preparations taurine-induced vaso-relaxation may be due primarily to the activation of $GABA_A$ receptors expressed on smooth muscle. Interestingly however, the chronic supplementation of taurine to rats resulted in gender-specific increase in blood pressure. This increase in blood pressure was observed only in females, males supplemented with taurine did not show any increase in systolic, diastolic or mean arterial pressure. In both genders however, taurine supplementation caused a significant tachycardia.

Taurine is usually described as a free amino acid and does not participate in protein synthesis. Most animals (but not cats) can synthesize taurine from cysteine in a reaction pathway that involves decarboxylation and multiple oxidations of the sulfhydryl group (Huxtable 1992). However, capacity for endogenous synthesis is limited in humans and the majority of body taurine stores are usually derived from food sources. The neonatal brain contains high levels of taurine (Huxtable 1989, 1992; Sturman 1993). As the brain matures its taurine content declines and reaches stable adult concentrations that are second to those of glutamate, the principal excitatory neurotransmitter in the brain. Taurine levels in the brain significantly increase under stressful conditions (Wu et al. 1998), suggesting that taurine may play a vital role in neuroprotection. A possible mechanism of taurine's neuroprotection lies in its calcium modulatory effects. We have shown that taurine modulates both cytoplasmic and intra-mitochondrial calcium homeostasis (El Idrissi and Trenkner 1999, 2003, 2004). Furthermore, taurine acts as an agonist of $GABA_A$ receptors (Quinn and Harris 1995; Wang et al. 1998; del Olmo et al. 2000; Mellor et al. 2000; El Idrissi et al. 2003; El Idrissi and Trenkner 2004). The effect of taurine on excitable tissues has been well studied with the exception of smooth muscle cells where not much attention has been devoted. Ristori and Verdetti have shown that that perfusion of aortic rings from rats with taurine perfusion (1–10 mM) reduced basal tone and had a relaxant effect on rings preconstricted with KCl or norepinephrine (Ristori and Verdetti 1991). This effect was not mediated by endothelium or by muscarinic or adrenoreceptors, and thus probably represented a direct effect of taurine on vascular smooth muscle cells. Although the mechanism of vasorelaxation mediated by taurine were not elucidated, taurine may be minimizing $[Ca^{2+}]_i$ by enhancing the activity of calcium transporting enzymes. Consistent with this, taurine has been shown to stimulate Ca-ATPase and Na/Ca-antiport in cardiac sarcolemma. In cardiac myocytes, taurine inhibits the rise in $[Ca^{2+}]_i$ induced by beta adrenergic receptor stimulation (Failli et al. 1992). Clearly, more work is required to define the impact of taurine on calcium transport mechanisms in vascular smooth muscle; however, the net effect of these actions appears to be a reduction of $[Ca^{2+}]_i$. The calcium modulatory role of taurine has been well established (El Idrissi and Trenkner 1999, 2003, 2004). In this study, we found that the effect of taurine on blood pressure was dependent on the duration of treatment. Acute taurine injection induced hypotension whereas chronic supplementation proved hypertensive but interestingly, only in females. Several clinical studies indicate that chronic oral administration of taurine reduces elevated blood pressures (Meldrum et al. 1994). Therefore, it seems that the effects

of taurine on blood pressure are not only gender-specific but also depend on the level of blood pressure prior to taurine supplementation. Thus, the findings of this study cast some light on the ability of dietary taurine to regulate blood pressure. The benefit of dietary supplementation may depend on the model examined (Failli et al. 1992; Nara et al. 1978; Nakagawa et al. 1994; Meldrum et al. 1994).

2 Materials and Methods

2.1 Animals

All rats used in this study were 2- to 3-month-old Long Evans. All rats were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All pro-cedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of rats sufficient to provide statistically reliable results was used in these studies.

2.2 Blood Pressure Measurements

For indirect blood pressure and heart rate readings, the rats were placed in a chamber at 37 °C for 10 min, and then transferred to a standard setup with heating pad and acrylic restrainer, tail cuff and pulse sensor (CODA monitor, Kent Scientific, Torrington, CT). The tail cuff was connected to a blood pressure monitor that through an ar-rangement of inlet and outlet valves permitted inflation and deflation of the cuff at a constant rate. The tail cuff pressure was continuously recorded with a solid state pressure sensor (Kent Scientific). The sig-nals from the pulse and pressure sensors were conveniently amplified and then digitized with an analog–digital board directly on the blood pressure monitor. For each indirect BP determination the inflation and deflation readings were always recorded, as well as the compression interval. The indirect measurements were all performed by the same person, who was kept blind about the purpose of the study. The ani-mals quickly became familiar with the procedure and remained calm within the restrainer. In the rare cases when signs of discomfort were present the procedure was interrupted and the animal was disqualified from the study.

2.3 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statis-tically significant when the calculated P value was less than 0.05.

3 Results

3.1 Effects of Taurine on Blood Pressure

To investigate the effects of taurine on the regulation of hemodynamics and peripheral resistance, we supplemented taurine (0.05%) to rats in their drinking water for 4 weeks and monitored their blood pressure. We found that the effects of taurine on blood pressure were gender-specific. While the blood pressure of adult male rats was not affected by taurine supplementation, females on the other hand showed a significant increase in systolic, diastolic and mean arterial pressure (Fig. 1).

3.2 Taurine Effects on Cardiac Function

While the effects of taurine on peripheral resistance were gender-specific with only females being affected, the effect of taurine supplementation on cardiac function was observed in both males and females. In response to 4 weeks of taurine supplementation, rats of both gender showed a drastic increase in heart rate (Fig. 2). The increase in heart rate in response to the increase in vascular resistance in female could be a mechanism to increase tissue perfusion. However, the tachycardia observed in males in the absence of an effect on vascular resistance may suggest a direct effect of taurine on heart physiology. This could be mediated at the myocardiocytes level (e.g. regulation of calcium homeostasis and contractile properties) or through interaction of taurine with the autonomic nervous system innervating the heart.

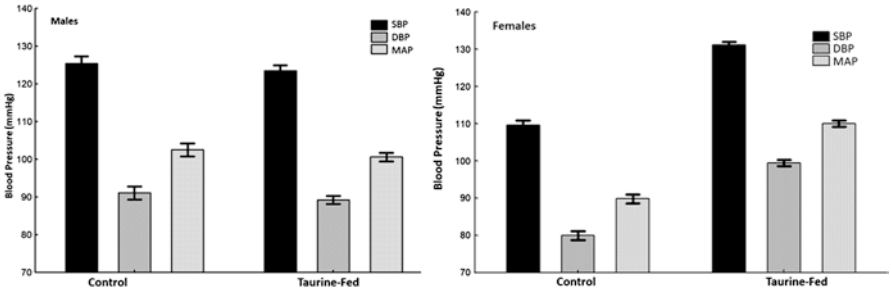

Fig. 1 Blood pressure measurements from male and female rats supplemented with taurine (0.05%) for 4 weeks. In males, taurine induced a slight but not significant decrease in systolic (SBP), diastolic (DBP) and mean arterial pressure (MAP). In females however supplementation with taurine induced a significant ($p < 0.01$) increase in systolic (SBP), diastolic (DBP) and mean arterial pressure (MAP). Rats were 2 months old and 15 rats from each gender were used for these experiments

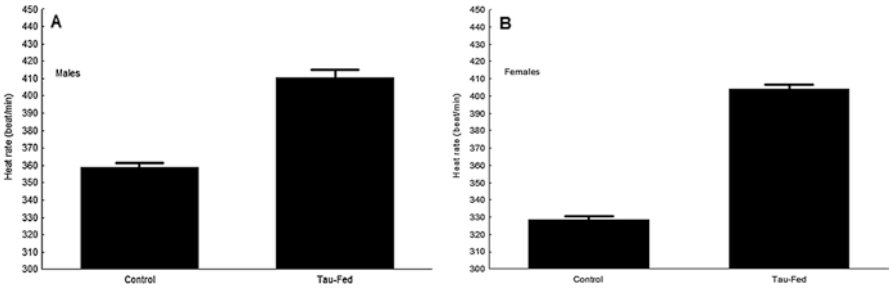

Fig. 2 Heart rate measurements from male female rats supplemented with taurine (0.05%) for 4 weeks. Taurine induced a significant ($p < 0.01$) increase heart rates in both genders. Rats were 2 months old and 15 male and 15 female rats were used for this experiment. These measurements were taken from the same rats used in Fig. 1. Recording of heart rate was simultaneous with blood pressure

4 Discussion

GABA has been shown to play an important role in the modulation of cardiovascular function and hemodynamics. GABA mediates these actions by acting not only within the central nervous system but also within peripheral tissues. Since taurine is an agonist for $GABA_A$ receptors, we sought to determine the vasoactive properties of taurine, presumably through activation of $GABA_A$ receptors.

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid. It is one of the most abundant free amino acids in many excitable tissues, including the brain, skeletal and cardiac muscles. Physiological actions of taurine are widespread and include bile acid conjugation, detoxification, membrane stabilization, osmoregulation, neurotransmission and modulation of cellular calcium levels (Foos and Wu 2002; Lombardini 1985; Saransaari and Oja 2000; Schaffer et al. 2000; Solis et al. 1988). Furthermore, taurine plays an important role in modulating glutamate and GABA neurotransmission (El Idrissi and Trenkner 1999; El Idrissi and Trenkner 2004; Militante and Lombardini 1998). We have previously shown that taurine prevents excitotoxicity in vitro primarily through modulation of intracellular calcium homeostasis (El Idrissi and Trenkner 1999). In neurons, calcium plays a key role in mediating glutamate excitotoxicity.

Outside of the central nervous system, taurine also is essential during developmental processes. Taurine is added to milk formula and in solution for parenteral nutrition of premature babies to prevent retinal degeneration and cholestasis (Huxtable 1992; Lourenco and Camilo 2002). Taurine is found at high concentrations in pancreatic islets (Huxtable 1992). Taurine is able to prevent pancreatic alterations induced by gestational malnutrition especially low-protein diet (Boujendar et al. 2002; Cherif et al. 1996; Dahri et al. 1991; Merezak et al. 2001).

The antihypertensive effects of taurine have been demonstrated in several experimental models (Fujita and Sato 1984; Harada et al. 2000; Nara et al. 1978). Studies in vitro showed that taurine relaxed pre-constricted rabbit ear artery (Franconi et al. 1982), rat aorta (Ristori and Verdetti 1991) and rat mesenteric artery (Li et al. 1996).

Thoracic aortic rings isolated from rats that were chronically given beta-alanine to deplete internal taurine showed enhanced contractile responses to norepinephrine and high potassium, and reduced relaxant responses to sodium nitroprusside and acetylcholine (Abebe and Mozaffari 2003). Thoracic aortic rings isolated from rats that were chronically given taurine showed reduced contractile responses to norepinephrine and high potassium nonspecifically (Abebe and Mozaffari 2000). These experiments suggest that taurine plays an important role in the maintenance and regulation of vascular tone in normal and pathological situations.

Peripheral resistance within the large arteries is predominantly controlled by the level of tonic activity of the sympathetic nervous system and the level of adrenergic receptors activation. Thus one could suggest an antagonistic system to the sympathetic innervation of the vasculature. While the sympathetic nervous system causes vasoconstriction proportional to the level of activation of adrenergic receptors, the GABAergic system opposes that by mediating vasodilation acting on the peripheral vasculature. The GABAergic system mediates its vasoactive properties through activation of $GABA_A$ receptors. $GABA_A$ receptors can be activated either with taurine or GABA both of with are found at relatively high levels in the plasma.

The finding that acute taurine had an opposite effect on peripheral resistance than chronic suggest that the chronic supplementation of taurine in drinking water may cause alterations to the mechanisms responsible for taurine regulation of blood pressure and peripheral resistance. Consistent with this observation, we found that the effects of taurine on the GABAergic system in the brain are dependent on the duration of treatment.

We have previously shown that taurine-fed mice have reduced expression of $GABA_A$ receptors in the hippocampus (El Idrissi and Trenkner 2004). We suggested that a down-regulation of $GABA_A$ receptors expression was due to the sustained interaction of taurine with $GABA_A$ receptors which causes a change in subunit composition of the $GABA_A$ receptors and concomitant decrease in the efficacy of the inhibitory system (El Idrissi and Trenkner 2004). Similar observations were noted in peripheral tissues mainly the pancreas. Therefore, we suggest that a potential decrease in $GABA_A$ receptor expression in response to chronic treatment with taurine would result in a reduced efficiency of vasodilative properties of GABA on peripheral resistance. This would lead to hypertensive effect when taurine is chronically supplemented to rats. However, the hypertensive properties of taurine were gender specific. Only females showed a significant decrease in blood pressure when chronically fed taurine. The gender specificity of taurine on peripheral resistance is intriguing are requires further investigation of the mechanism of action. We suggest that $GABA_A$ could mediate this gender specificity. Steroid hormones have been shown to act as allosteric modulators of $GABA_A$ receptors. Thus, the gender specific hormonal phenotype could underlie the selective modulation of $GABA_A$ receptors conductance by taurine. This however remains to be elucidated. Alternatively, taurine may mediates its vasoactive properties, in addition to activating $GABA_A$ receptors, through other known vasoactive substances. These include vasorelaxant prostaglandins and/or nitric oxide, the opening of K^+ channels and facilitating K^+ efflux, and/or reduced Ca^{2+} availability or other mechanism(s) may be involved in the vasorelaxant effects of a taurine (Félétou and Vanhoutte 2006).

5 Conclusion

In summary, this study shows that taurine could have both hypo- and hyper-tenssive properties. If chronically administered, taurine induces hypertension in female and tachycardia in both female and male rats. Taurine therefore, could act as a vasorelaxant when acutely injected. Furthermore, this study shows that GABA plays an important role in the regulation of cardiovascular function both centrally and peripherally. Drugs that target $GABA_A$ in the CNS would affect peripheral resistance in addition to the intended central effects.

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Part II
Taurine and Nutrition

Antioxidant and Anti-Stress Effects of Taurine Against Electric Foot-Shock-Induced Acute Stress in Rats

Dong-Sung Lee, Hee Geun Jo, Min Ji Kim, Hwan Lee, and Sun Hee Cheong

Abstract In the present study, we evaluated the antioxidant and anti-stress activities of taurine in electric foot-shock stress model rats. Taurine supplementation markedly increased the hepatic glutathione (GSH) levels, compared to the levels in the stress group. In addition, activities of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were improved in the taurine-treated group. Plasma cortisol and dehydroepiandrosterone-sulfate (DHEA-S) levels were significantly reduced in the taurine-supplemented group compared to those in the stress group. In contrast, the levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were markedly increased in the taurine or betaine-treated group compared to those in the stress group. It may be concluded that taurine produces beneficial effects in the form of antioxidant status and biochemical alterations in foot-shock-induced acute stress in rats.

Keywords Antioxidant · Anti-stress · Electric foot-shock · Taurine

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
AAPH, 2	2-azobis (2-amidinopropane) hydrochloride
CAT	Catalase
DHEA-S	Dehydroepiandrosterone-sulfate
DMPO, 5	5-dimethyl-1-pyrroline N-oxide
DPPH, 1	1-diphenyl-2-picrylhydrazyl

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GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferase
SOD	Superoxide dismutase
Tau	Taurine

1 Introduction

Stress is a common phenomenon which is experienced by every individual. It leads to changes in the immune system, the sympathetic nervous system, and the neuroendocrine system, which could cause disturbance in host defense system (Goldstein and McEwen 2002). Stress responses are often mediated by the activation of the hypothalamic-pituitary-adrenal-axis, leading to secretion of glucocorticoid hormone from the adrenal gland. In general, glucocorticoids interact with their receptors in the brain and peripheral tissues to control various stress responses (Smith and Vale 2006). Very few studies have investigated the release of the adrenal androgen dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEA-S), which is a more stable sulfate ester form of DHEA. DHEA-S is a crucial and most abundantly secreted steroid hormone by the adrenal gland in the human body (Orentreich et al. 1984; Villette et al. 1990). The concentrations of cortisol and DHEA-S have been observed to be altered in a variety of chronic diseases, such as diabetes and rheumatoid arthritis as well as chronic stress (Yamauchi et al. 1996; Masi et al. 2006). Under stressful conditions, energy requirement is increased in the body, leading to an increase in the reactive oxygen species (ROS), due to the oxidative stress. ROS can cause several tissue damages by interacting with proteins, lipids, and DNA, resulting in a variety of pathological conditions (Joshi et al. 2012). Moreover, oxidative stress is implicated in the pathogenesis of neurologic diseases as well as stress responses (Colaianna et al. 2013). Therefore, it is a necessary to develop beneficial and effective anti-stress agents to prevent the stress-mediated diseases. Taurine is well known a sulfur-containing semi-essential amino acid and it is endogenously synthesized from diet, especially meat and fish (Maia et al. 2014). It has many therapeutic benefits, such as antioxidant and anti-inflammatory (Merezak et al. 2001; Wu et al. 2005), osmoregulatory (Pasentes-morales and Schousboe 1997), anti-obesity (Xiao et al. 2008), hypotension (Liu et al. 2001), hypocholesterolemic (Zhang et al. 2004), and hypoglycemic effects (Winiarska et al. 2009). However, in vivo studies and the mechanisms involved in its pharmacological activities against stress are still not elucidated. Therefore, the present study was aimed to investigate the anti-stress and antioxidant potential of taurine in in vitro and in a foot-shock-induced stress animal model.

2 Methods

2.1 Reagents

Taurine, betaine hydrochloride, 2, 2-azobis (2-amidinopropane) hydrochloride (AAPH), 5, 5-dimethyl-1-pyrroline N-oxide (DMPO), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and (4-pyridyl-1-oxide)-N-tert-butylnitrone were obtained from Sigma Chemical Co. (St Louis, MO, USA). Total GSH and CAT kits were obtained from Biovision (Milpitas, CA, USA). All other reagents were of the highest grade available commercially.

2.2 Radical Scavenging Activities

DPPH radical scavenging activity was measured using the method previously described by Nanjo et al. (1996). Sixty microliters of taurine at different concentrations were vigorously mixed with equal amounts of DPPH (60 μM) in methanol for 10 s. Then, the mixture was moved into a 100 μL Teflon capillary tube. Measurement conditions of DPPH radical scavenging activity were as follows: modulation frequency, 100 kHz; microwave power, 5 mW; central field, 3475 G; modulation amplitude, 2 G; gain, 6.3 × 105; and temperature, 298 K. Alkyl radicals were produced by AAPH. The phosphate-buffered saline (pH 7.4) reagent containing 10 mM 4-POBN, 10 mM AAPH, and the different concentrations of taurine were incubated at 37 °C for 30 min and transferred to 100 μL quartz capillary tube. The DPPH and alkyl radical scavenging activity were measured using an ESR spectrometer (JEOL Ltd., Tokyo, Japan). Measurement conditions of alkyl radical scavenging activity were as follows: modulation frequency, 100 kHz; microwave power, 1 mW; central field, 3475 G; modulation amplitude, 2 G; gain, 6.3 × 105; and temperature, 298 K.

2.3 Experimental Design

Male Sprague-Dawley rats (n = 24) (Dae Han Bio Link CO. LTD., Korea) weighing about 100 g were maintained at 24 ± 1 °C temperature, 60 ± 10% humidity, and 12 h light/dark cycle, and fed ad libitum with water and pelleted commercial diet (Samyang Co., Korea). After 1 week of acclimation, the rats were randomly divided into four groups: no electric foot-shock with saline treatment (control, n = 6), electric foot-shocked rats with saline treatment (stress, n = 6), electric foot-shocked rats with taurine treatment (ST-Tau, n = 6) and electric foot-shock with betaine

treatment (ST-B, n = 6) as a positive control. Taurine (15 mg/kg, in saline) and betaine (10 mg/kg, in saline) were orally administered with a daily dose of 15 mL/kg between 9:00 and 10:00 am for 14 days. Rats were moved to a separate quiet room, and then treated with electric foot shocks through the grid floor of a Plexiglas cage (30×30×30 cm) under the approximately 400 lx of light intensity between 12:00 and 13:00 h. Electric foot-shocks (100 V, 3 mA) were stimulated per minute for 5 min, i.e. each rat was administered 10 shocks. Each shock was administered for 1 s and was made up of 0.01 s shocks separated by 0.02 s-long breaks. The control group was placed into a similar cage for same time without electric foot shocks. Weekly measurement of body weight was done. All animals were treated in accordance with the Guidelines for Care and Use of Laboratory Animals of Chonnam National University, Yeosu, Republic of Korea.

2.4 Sampling Procedures

One hour after the last electric foot-shock treatment, blood was collected from the heart puncture into heparin tubes to separate plasma samples and then centrifuged at 2000 rpm for 5 min at 4 °C. Organs including the liver, heart, kidneys, and whole brain were quickly removed, washed with saline buffer, and then immediately stored at −80 °C for biochemical analysis.

2.5 Determination of Hepatic Antioxidant Enzyme Activities

To obtain a 1:9 (w/v) whole homogenate, the liver tissue was homogenized in a glass-Teflon homogenizer with 50 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 25,000 rpm for 20 min at 4 °C to remove any cell debris, and then the supernatant was used to determine the antioxidant enzyme activities. Total GSH level was determined using a commercially available kit supplied by Biovision (Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, 0.05 mL supernatant was mixed with 100 mM phosphate buffer at pH 7.4 containing 5 mM NADPH, 5 mM EDTA, and 10 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB). After 3 min of equilibration at 25 °C, the solution was mixed two units of glutathione reductase (Type III from Baker's yeast, Sigma Chemical Co. St. Louis, MO) and the total amount of GSH was measured at 412 nm using a UV/VIS spectrophotometer. GST activity was measured according to the method previously described by Habig et al. (1974). The dinitrophenyl thioether formation by GST was monitored for 3 min at 37 °C using a spectrophotometer at 340 nm. GR activity was measured by the method previously described by Pinto et al. (1984). Briefly, the supernatant was mixed with 5 mM NADPH in 0.1 M phosphate/0.5 mM EDTA buffer (pH 7.0) and 1 M glutathione disulfide, and then the NADP+ formation was monitored at 340 nm. To determine GPx activity, the supernatant was mixed with

100 mM GSH, 1 mM EDTA, 5 mM NADPH, and 1 unit of GR (0.1 M phosphate buffer, pH 7.0), then incubated for 3 min. The solution was mixed with 10 mM cumene hydroperoxide, then the oxidation of NADPH into NADP+ was monitored at 340 nm. The enzyme activities of SOD and CAT were estimated using commercial kits supplied by Biovision (Milpitas, CA, USA) according to the manufacturer's instructions. SOD and CAT activities were measured at 450 nm and 570 nm, respectively, and then, expressed as SOD activity (inhibition rate %) and as an international unit/mg protein, respectively.

2.6 Biochemical Analyses

Plasma cortisol and DHEA-S levels were measured by a double-antibody radioimmunoassay using a commercially available kit (Biochem Immunosystem) and a commercially available radioimmunoassay kit (Radim S.p.A.), respectively.

2.7 Monoamine Neurotransmitter Concentrations in Rat Brain

The concentrations of monoamine neurotransmitters including 5-HT and 5-HIAA were determined by HPLC coupled with a scanning fluorescence detector. Whole brain tissues were homogenized in ice-cold 0.4 M perchloric acid (6.6 μL/mg) containing 5 mM sodium bisulfate and 0.04 mM EDTA, and then centrifuged at 14,000 rpm for 30 min at 4 °C. The HPLC procedure was conducted by the method described by Byers et al. (2006) using an Agilent HC-C18 (250×4.6 mm, 5 μm; Agilent, USA) analytical column. The mobile phase (80% aqueous solution and 20% methanol), contained 0.2 mM EDTA disodium salt, 40 mM sodium acetate, 30 mM citric acid, and 0.5 mM octanesulfonic acid sodium salt, at pH 3.8 with a 1.0 mL/min flow rate. The concentrations were detected using a Waters 474 scanning fluorescence detector (Waters, USA) at 280 nm of excitation and 330 nm of emission wavelengths, respectively, and then compared the peak area of samples with the reference using specific HPLC software (Chromatography Station for Windows).

2.8 Statistical Analysis

All data were presented as mean ± SEM, and statistical analyses were performed using Statistical Analysis System version 8.0 (SAS Institute, Cary, NC, U.S.A). Multi-group comparisons were carried out by one-way analysis of variance followed by Tukey-Kramer multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Free Radical Scavenging Activities of Taurine

In the present study, we determined the DPPH and alkyl radical scavenging activities of taurine using an ESR spectrometer. It is well known that DPPH is a stable radical. Therefore, it is often used to assess the antioxidant properties of several compounds. Figure 1 shows the effect of taurine on DPPH radical scavenging activity. It was observed that IC50 value, the concentration inhibiting 50% of free radical production efficacy of taurine against DPPH radical was 0.058 mg/mL. Moreover, the effect of taurine on alkyl radical scavenging activity is shown in Fig. 1b. Taurine exhibited significant scavenging activity (0.086 mg/mL) against alkyl radical. These results indicated that taurine effectively scavenged various reactive radicals in vitro.

3.2 Effects of Taurine on Changes in Body Weight and Weights of Organs in Electric Foot-Shock Stress Model Rats

The effects of taurine on the alterations in the body weight in rats are presented in Table 1. The body weight in the electric foot-shock stress administered rats was significantly increased than the ST-Tau group with no changes in food intakes. In addition, it tended to increase in the stress group compared to that in control and ST-B groups, although there was no significant difference among the groups. On the other hand, weights of organs, including liver, kidney, heart, and brain were not affected by taurine or betaine treatment, which indicated that taurine has no adverse effects in in vivo.

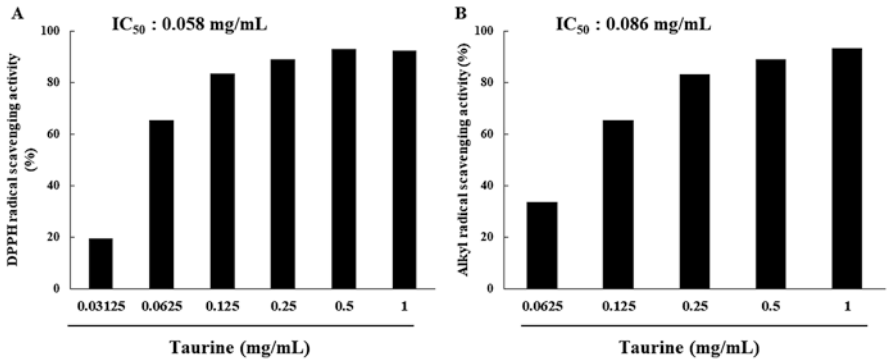

Fig. 1 Free radical scavenging activities of taurine. IC_{50} values (expressed in mg of taurine per mL organic solvent) that exhibited its (**a**) DPPH radical scavenging activity, and (**b**) alkyl radical scavenging activity

Table 1 Changes in body weights and organ weights in taurine-administered rats

	Initial body weight (g)	Body weight gain (g)	Liver (g)	Kidney (g)	Heart (g)	Brain (g)
Control	99.1 ± 10.8^{NS}	49.9 ± 5.6^{ab}	4.85 ± 0.71^{NS}	1.30 ± 0.07^{NS}	0.63 ± 0.06^{NS}	1.54 ± 0.29^{NS}
Stress	100.8 ± 11.3	57.1 ± 4.8^{a}	5.11 ± 1.13	1.33 ± 0.12	0.65 ± 0.04	1.56 ± 0.31
ST-Tau[1]	97.5 ± 8.1	44.3 ± 7.1^{b}	4.98 ± 0.41	1.29 ± 0.09	0.63 ± 0.02	1.59 ± 0.18
ST-B[2]	99.4 ± 6.2	51.6 ± 0.9^{ab}	4.74 ± 0.26	1.28 ± 0.10	0.62 ± 0.05	1.61 ± 0.12

Data are presented as the mean ± SEM values (n = 6 per group)
[1]ST-Tau: electric foot-shocked rats with taurine treatment
[2]ST-B: electric foot-shocked rats with betaine treatment
^{NS}Not significantly different between the groups
$^{a, b}$Mean values with different superscript letters in the same column are significantly different ($p < 0.05$)

Table 2 Hepatic antioxidant enzyme activities in taurine-administered rats

Biomarkers	GSH (nmol GSH/min/mg protein)	SOD (%)	GST (μmol GST/min/mg protein)	GPx (nmol NADPH/min/mg protein)	CAT (μmol H_2O_2/min/mg protein)
Control	21.18 ± 1.82^{b}	109.56 ± 3.28^{NS}	26.88 ± 1.68^{a}	1.18 ± 0.01^{a}	0.116 ± 0.015^{a}
Stress	15.48 ± 0.96^{c}	108.48 ± 6.89	15.60 ± 0.64^{b}	0.69 ± 0.07^{c}	0.044 ± 0.007^{b}
ST-Tau[1]	21.96 ± 3.24^{a}	110.28 ± 9.29	24.52 ± 4.32^{a}	1.36 ± 0.13^{a}	0.095 ± 0.018^{a}
ST-B[2]	25.80 ± 2.04^{a}	114.12 ± 7.38	25.44 ± 2.28^{a}	0.83 ± 0.06^{b}	0.069 ± 0.011^{ab}

Data are mean ± SEM values (n = 6 per group)
[1]ST-Tau: electric foot-shocked rats with taurine treatment
[2]ST-B: electric foot-shocked rats with betaine treatment
^{NS}Not significantly different between the groups
$^{a, b, c}$Mean values with different superscript letters in the same column are significantly different ($p < 0.05$)

3.3 Hepatic GSH Levels and Antioxidant Enzyme Activities

The effects of taurine on the hepatic GSH levels and antioxidant enzyme activities in electric foot-shocked rats are shown in Table 2. The GSH level was significantly higher in the taurine and betaine treatment groups than that in the stress group. On the other hand, SOD enzyme activity was not significantly different among the groups. GST enzyme activity, which is well known to play a major role against various radical damages, was significantly increased by 1.57-fold in the ST-Tau group, and 1.63-fold in the ST-B group, compared with that in the stress group. In addition, the activities of GPx and CAT were markedly higher in the ST-Tau or ST-B groups compared to those in the stress group. These findings suggested that taurine may promote antioxidant enzyme activities.

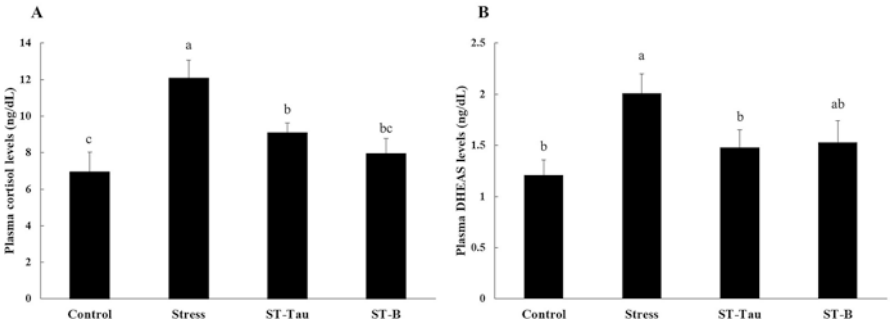

Fig. 2 Plasma stress-related hormone levels, (**a**) cortisol (**b**) dehydroepiandrosterone-sulfate (DHEA-S) in taurine administered rats. ST-Tau: electric foot-shocked rats with taurine treatment, ST-B: electric foot-shocked rats with betaine treatment. Data are shown as mean ± SEM. [a, b, c] Means with different superscript among the groups are different ($p < 0.05$)

3.4 Effects of Taurine on Stress-Related Hormone Levels

The effects of taurine on the plasma concentration of stress-related hormones are shown in Fig. 2. The concentration of cortisol in plasma was dramatically higher in the electric foot-shock induced stress group compared to that in the control group. On the other hand, the plasma cortisol level was significantly reduced in the taurine or betaine-administered group (Fig. 2a). Similarly, the plasma DHEA-S level in the electric foot-shocked rats was markedly higher than that in the control group. In contrast, treatment of taurine as well as betaine markedly suppressed the increase in the plasma DHEA-S level (Fig. 2b).

3.5 Effects of Taurine on the Monoamine Neurotransmitter Levels in Rat Brain

Figure 3 shows the effects of taurine on the concentrations of monoamine neurotransmitter, such as 5-HT and 5-HIAA, in rat brain. The 5-HT concentration was markedly decreased in the stress group (Fig. 3a). In contrast, taurine and betaine effectively recovered the reduction in 5-HT level induced by stress in rat brain. Similar to the 5-HT level, the concentration of 5-HIAA was significantly reduced in the electric foot-shock induced stress group (Fig. 3b). However, the concentration of 5-HIAA was markedly higher in the taurine or betaine-administered group than that in the stress group, although there were no significant differences among the groups.

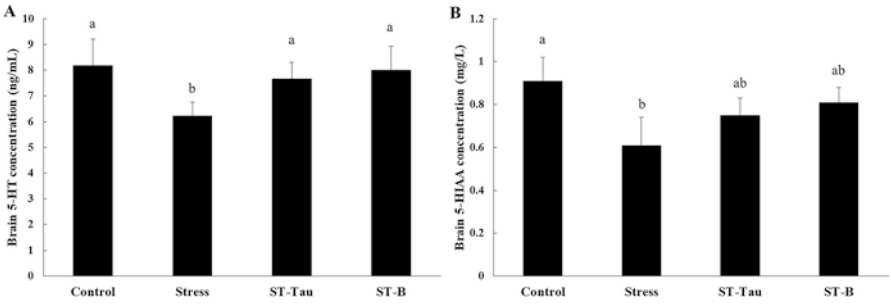

Fig. 3 The concentrations of brain monoamine neurotransmitter (**a**) 5-HT and (**b**) 5-HIAA in taurine administered rats. ST-Tau: electric foot-shocked rats with taurine treatment, ST-B: electric foot-shocked rats with betaine treatment. Data are shown as mean ± SEM. [a, b, c] Means with different superscript among the groups are different ($p < 0.05$)

4 Discussion

Among the sulfur-containing β-amino acids, taurine is the most abundant in several mammalian tissues. Numerous evidences have demonstrated that taurine serves a variety of important roles in physiological processes, including inhibition of pro-inflammatory cytokines (Kontny et al. 2012), cell membrane stabilization (Pasantes-Morales et al. 1985), regulation of calcium concentration (Huxtable 1992), and protection of endothelial cells (Maia et al. 2014). However, very few studies investigating the anti-stress effects of taurine have been reported. In the present study, therefore, we investigated the anti-stress and antioxidant potentials of taurine in in vitro as well as in foot-shock stress animal model. On the other hand, it has been reported that betaine is a nontoxic and chemically stable natural substance and is beneficial in several human diseases, including cancer, obesity, diabetes, and Alzheimer's disease (Zhao et al. 2018). Therefore, we also used betaine as a positive control.

In the present study, taurine exhibited potent scavenger activities against DPPH and alkyl radicals in in vitro. Moreover, we confirmed that taurine markedly improved the GSH levels as well as activities of several hepatic antioxidant enzyme including GST, GPx, and CAT in the rats suffering from stress induced by electric foot-shock. Similar results were found by several other researchers. In a previous study, it was demonstrated that restraint stress caused the depletion of GSH, and activities of antioxidant enzymes, such as SOD and CAT, in mouse brain (Nagasirisha and Mohamed Saleem 2014). Jia et al. (2016) reported that taurine treatment reduced the elevation in mitochondrial ROS levels, recovered the ATP levels, and the activities of SOD2 and CAT induced by prenatal stress in the hippocampus. Tu et al. (2017) also demonstrated that taurine treatment suppressed an aggregation of advanced glycation end-products (AGEs) but increased antioxidant capacity of brain tissue in D-galactose induced age-related disease model of mice. These results demonstrated that taurine exhibited antioxidant activities by acting as an efficient free radical scavenger in vivo as well as in vitro.

The antioxidant system and brain monoamine react with the HPA-axis. They are also involved in mediating several stress responses against psychological and physical stresses (Zafir and Banu 2009). Therefore, it is crucial to study the anti-stress properties of a variety of biological constituents with respect to the alterations in the levels of brain monoamines in cellular redox state. Cortisol, the crucial stress hormone, and DHEA are released by activated HPA-axis under physical as well as mental stress conditions (Lundberg 2005). In neuropsychiatric conditions, it has been reported that levels of DHEA and its sulfate ester, DHEA-S, are altered (Chatzittofis et al. 2013). In our in vivo study, we observed that taurine markedly reduced the levels of plasma stress-related hormones, such as cortisol and DHEA-S. In a previous study, anti-stress properties of DHEA in rats were reported and these effects may be adequate in antagonizing cortisol, leading to modified emotional stress for humans (Cruess et al. 1999). It has been reported that chronic social stress decreases the levels of 5-HT and its metabolite in the rat brain (Berton et al. 1998). In the present study, we observed that stress induced by foot-shocks significantly diminished the levels of monoamine neurotransmitter, including 5-HT and 5-HIAA, in rat brain. In contrast, the concentrations of both 5-HT and 5-HIAA were markedly increased by treatment with taurine or betaine. Similar to our findings, it has already been shown that the forced swimming test-induced stress markedly increased the serum cortisol levels, but decreased the 5-HT and 5-HIAA concentrations in the brain tissue in mice. The ethanolic extracts of *Curcuma longa*, however, reversed the concentrations of monoamine neurotransmitter mediated by swim stress (Xia et al. 2007). Chatzittofis et al. (2013) reported that suicide attempters showed higher plasma cortisol and DHEA-S levels but lower 5-HIAA levels compared to healthy volunteers. Ahmad et al. (2012) also reported that the depleted 5-HT and 5-HIAA levels in the frontal cortex as well as GSH content, antioxidant enzyme activities, and the extent of lipid peroxidation were normalized by the administration of biological compounds isolated from *Ocimum sanctum* in the restraint-induced stress model rats. The perturbed central monoamine responses might have amplified the oxidative damages during chronic stress condition (Ahmad et al. 2012). In our study, the anti-stress effects and differential alterations arising during the antioxidant action of taurine may emerge as an efficient scavenging activity against a variety of free radicals and regulating stress-related hormone as well as monoamine neurotransmitter. Therefore, it is expected that taurine can be used to develop beneficial substitutes for the food or pharmaceutical industries owing to its stress-alleviating and antioxidant properties.

5 Conclusion

Our results showed that taurine exhibited potent antioxidant and anti-stress activities in vitro and in vivo. However, further pre-clinical and clinical research seems warranted to investigate the anti-stress activity of taurine in terms of the underlying mechanism, as well as its therapeutic roles.

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Taurine Promotes In-vitro Follicle Development, Oocyte Maturation, Fertilization and Cleavage of rats

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Abstract It is well known that a large quantity of taurine is present in mammalian ovaries. Taurine reportedly promotes the secretion of female reproductive hormones by stimulating hypothalamus-pituitary-gonadal axis function. Therefore, we speculated that taurine may have beneficial effects on follicle growth, oocyte maturation, fertilization and cleavage. Here, we cultured rat follicles, immature oocytes and sperms in vitro and treated with taurine to observe the changes in follicle diameter, estradiol concentration as well as the rate of oocytes maturation, fertilization and cleavage using an inverted microscope. The results showed that taurine can elevate ovarian follicles growth and oocyte maturation, fertilization, and cleavage rates in vitro, which may be attributed to its osmoregulation and stimulation on the estradiol secretion. Our results provide important insights into taurine application in female production, although the underlying mechanism need to be further addressed.

Keywords Taurine · Follicle development · Oocytes maturation · Fertilization · Cleavage · Rat

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1 Introduction

Taurine, a sulfur-containing amino acid, one of the most abundantly free amino acids in many tissues and cells including hypothalamus, hypophysis and reproductive organs et al., is a major constituent in healthy bodies (Tappaz 2004; Pow et al. 2002). It has been found that person with 70 kg weight has approximately 70 g taurine in serum (Huxtable 1992), and it was a major amino acid in the female uterus and tubal secreted possibly by oocyte (Lobo et al. 2001). Taurine has been proved to be associated with many aspects of general health, such as osmoregulation, antioxidation, neuroregulation, bile formation, calcium modulation and biological membrane stabilization (Schuller-Levis and Park 2010). Dietary taurine deficiency has been demonstrated to result in female reproductive dysfunction (frequently abnormal estrus, stillbirth, fetus absorption and abortion) (Sturman et al. 1985), surviving fetus also exhibited a number of abnormalities such as birth defects, live low-birth-weight, decline in weight and survival rate of weaning offspring and psychological problem (Sturman et al. 1985). It also was reported that taurine was stored in maternal tissues in early pregnancy stages for the needs of the fetus and suckling infants (Naismith et al. 1987). In female rats, taurine immunoreactivity is specifically localized in the ovarian granulosa cells, oocytes and luteal cells, epithelial cells of oviduct and uterus (Lobo et al. 2001). Moreover, taurine transporter mRNA has been found in the ovary (Jhiang et al. 1993), and taurine could promote oocyte maturation (Silva et al. 2009). Our recently study verified that taurine could increase reproductive hormones secretion by the hypothalamus-pituitary-gonadal axis in female mammals (Teng et al. 2015). In addition, Taurine and its' precursors has been proved to support embryonic growth after in vitro fertilization (Barnett and Bavister 1992), as well as the development of bovine embryos in vitro (Guyaderjoly et al. 1998). In brief, previous researches reminder us taurine may have crucial effects on ovarian function and female reproduction, but until now, the exact effects of taurine on follicle growth and oocyte maturation is still unclear.

In the present study, rat follicles, immature oocytes, and sperms were cultured in vitro, and subjected different concentrations of taurine to observe the changes in follicle diameter as well as the process of oocyte maturation, fertilization, and cleavage using an inverted microscope.

2 Methods

2.1 Animals

Sprague-Dawley rats were purchased from Liaoning Changsheng biotechnology company, China. All the experimental protocols were approved by Shenyang Agricultural University Ethical Committee and were followed the Declaration of Helsinki.

2.2 Follicular Development and Detection

Twenty-day-old female rats were sacrificed, and the ovarian tissue was cut into small blocks after exposure to 0.5% collagenase. The follicles were separated by tissue digestion for 30 min at 37 °C. Taurine was then added at varying concentrations of 0, 10, 50, 100, and 200 μM, and follicles were cultured at 37 °C in a 5% CO2 incubator. Follicular growth was observed on the 2nd and 4th day of culture, and the diameter of the follicles was measured using phase contrast microscopy. The supernatant of 4th day was collected for estradiol analysis according to the procedure of the supplier (Dingguo Biological Technology Co., Ltd., China).

2.3 Oocytes Maturation Detection

Thirty female rats aged 8–12 weeks were sacrificed, and their ovaries were removed. Immature oocytes were released from the needle-punctured follicles, and were placed in the 10% SSS Quinn's 1023 culture medium. Taurine was then added at varying concentrations of 0, 10, 50, 100, and 200 μM, and the oocytes were cultured at 37 °C in a 5% CO_2 incubator for 24 h. Under the inverted microscope, the numbers and morphological characters of oocytes were observed. When distinguishing characteristic chromosome were observed in the oocytes, the oocytes were identified as metaphase I (MI). While oocytes germinal vesicle breakdown and the released first polar were observed, the oocytes were identified as metaphase II (MII), which suggest that oocytes obtain the fertilization ability. Then the rate of MI and MII was calculated respectively. MI rate of oocytes was calculated by the numbers of MI oocytes/total numbers of oocytes ×100%, MII rate of oocytes was the numbers of MII oocytes/total numbers of oocytes×100%.

2.4 Fertilization and Cleavage Detection

Fifty male rats aged 10–12 weeks were sacrificed, and their epididymis and adjacent spermaduct were cut into sections. These sections were then placed in 10% SSS Quinn's culture medium; semen samples were obtained by gently squeezing the spermaduct, and sperms were cultured at 37 °C in 5% CO_2 incubator for 1 h. Sperm density was adjusted to $2–5 \times 10^5$/ml. The MII oocytes were added to 25 μl semen and cultured at 37 °C in a 5% CO_2 incubator. Oocytes fertilization was observed for 4 h until the emergence of the second polar body, indicating fertility. The fertilized egg was placed in 10% Quinn's 1026 culture medium for cleavage observation. The rate of fertilization and cleavage were subsequently calculated. Fertility rate was the numbers of fertility oocytes/numbers of MII oocytes×100%, cleavage rate was numbers of cleavage zygotes/total numbers of zygotes×100%.

2.5 Statistical Analysis

Data were presented as the mean ± SE values and significant differences were determined using Duncan's multiple range and X^2 test by employing SPSS 16.0 statistical analysis software. P values of <0.05 were considered to be statistically significant.

3 Results

3.1 Taurine Increases the Follicular Diameter

As shown in Table 1, compared to 0 μg/mL taurine group (control group), although 10 and 200 μg/mL taurine supplementation for 2 or 4 days increased ovarian follicular diameter, this effect was not statistically significant. The diameter of isolated follicles was significantly increased when exposed to 50 and 100 μg/mL taurine for 2–4 days ($p < 0.05$). The experimental results suggested that taurine can promote ovarian follicular development. Table 1 also illustrated that taurine (50 and 100 μg/mL) could obviously stimulate estradiol secretion in cultured rat ovarian follicles ($p < 0.05$).

3.2 Taurine Elevates the Rate of MII Oocytes

Oocytes get the ability of fertilization when oocytes go through meiosis and arrive in metaphase II. We determined the effect of taurine on the rate of MI and MII oocytes. As Table 2 illustrates, compared with control group, there are no statistic difference in the rate of MI oocytes ($P > 0.05$) by taurine treatment. The rate of MII oocytes showed no obvious changes by 10 and 200 μM taurine supplementation ($P > 0.05$), while 50 and 10 μM taurine treatment significantly increase the rate of MII oocytes ($P < 0.05$).

Table 1 Effects of taurine on the diameter of ovarian follicles (n = 6)

Taurine level (μg/mL)	0d follicular diameter (μm)	2d follicular diameter (μm)	4d follicular diameter (μm)	Estradiol level (ng/L)
0	137.2 ± 5.6	144.3 ± 7.4	160.4 ± 7.8	19.78 ± 2.37
10	137.6 ± 6.2	150.5 ± 8.6	174.6 ± 9.3	22.34 ± 3.16
50	139.3 ± 7.6	165.4 ± 8.2[a]	201.2 ± 8.9[a]	30.46 ± 4.32[a]
100	138.5 ± 6.4	181.5 ± 7.6[a]	205.3 ± 9.2[a]	36.78 ± 4.56[a]
200	135.5 ± 4.9	151.6 ± 9.2	171.5 ± 6.6	20.47 ± 3.68

[a]Significantly different from the control group (0 taurine treatment) ($p < 0.05$)

Table 2 Effects of taurine on the rat of MI and MII oocytes

Taurine level (μg/mL)	Numbers of follicles	Numbers of MI oocytes	Rate of MI(%)	Numbers of MII oocytes	Rate of MII (%)
0	80	41	51.25	31	38.75
10	75	36	48.00	29	38.67
50	72	36	50.00	33	45.83[a]
100	70	38	52.86	34	48.57[a]
200	76	37	48.68	30	39.47

[a]Significantly different from the control group (0 taurine treatment) ($p < 0.05$)

Table 3 Effects of taurine on the fertilization and cleavage rate of oocytes

Taurine level (μg/mL)	Numbers of oocytes	Numbers of fertilization oocytes	Rate of fertilization (%)	Numbers of cleavage oocytes	Rate of cleavage (%)
0	80	8	10.00	3	37.5
10	75	9	12.00	4	44.44
50	72	10	13.89	4	40.00
100	70	13	18.57[a]	7	53.85[a]
200	76	9	11.84	4	44.44

[a]Significantly different from the control group (0 taurine treatment) ($p < 0.05$)

3.3 Taurine Promotes the Fertilization and Cleavage Rat of Oocytes

Table 3 shows the effect of taurine on the rate of oocytes fertilization and cleavage. When taurine supplementation reached 100 μM, oocytes fertilization rate reached 18.57% as opposed to the rate of 10% in the control group, this difference was statistically significant ($P < 0.05$). While taurine supplementation of 10 μM, 50 μM, and 200 μM had no significant effect on the fertilization rate of oocytes ($P > 0.05$). In addition, although taurine treatment of 10 μM, 50 μM, and 200 μM had no significant effect on cleavage rate of oocytes ($P > 0.05$), 100 μM taurine treatment obviously increased the cleavage rate of oocytes (reached 53.85%, while control group was 37.5%) ($P < 0.05$).

4 Discussion

Ovarian follicle is the basic structural and functional unit of mammalian ovary. The proper development of ovarian follicle is the base of female reproduction, the follicular diameter is the popular index to measuring the development level of the follicle. Follicular dysplasia will cause the formation of immature eggs, and which result in female sterility. The results of our present study showed that taurine obviously promoted an increase in follicle diameter at 100 μM in the 2nd and 4th day

in vitro, suggesting taurine can stimulate follicular development directly. It has been reported that taurine may work as an osmolyte in mouse and human oocytes as well as embryos, and protect the development of follicles and embryos (Dumoulin et al. 1997). It was well known that many factors are involved in follicular development regulation, especially hypothalamus-pituitary-ovary axis hormones (follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol et al.). Our previous study found that taurine could stimulate FSH, LH and estradiol secretion in rats by the hypothalamus-pituitary axis (Teng et al. 2015). These results indicated that taurine promotes ovarian follicular development in vitro by its direct and indirect effects, direct effect may be attributed to its' osmoregulation, and indirect effect may be due to its' stimulation on estradiol secretion.

Oocyte maturation is the decisive event for oocyte fertilization process and fertility. In rats, oocyte becomes maturation by going through two meiosis. When the first polar body was discharged from the oocyte, oocyte goes into the second meiosis division and stops in metaphase II, which means that the oocyte has the ability of fertility. The MII rate of oocytes was used as a marker for checking the development of oocytes in vitro. The results of the current study revealed that taurine treatment had no significant effect regarding the promotion of oocytes from the germinal vesical-stage to the MI stage, but significant effect was found at taurine supplementation of 50–100 μM on the MII rate of oocytes, suggesting taurine can promote oocyte maturation in vitro. Many factors have been demonstrated involving the development of oocytes, such as female gonadal hormones (FSH, LH, and estradiol) and its' receptors, follicular size, follicular fluid et al.. We speculated that taurine stimulates oocytes maturation by multi ways, and the exact mechanism need be explored in the future. Our finding is consistent with a previous study which reported that taurine promoted the embryonic development of bovine oocytes (Silva et al. 2009), although the animals of experiment were different.

When MIIoocytes meet with sperm, oocytes were activated to restart the second meiosis, and discharge the second polar body, then become fertilized eggs and begin cleavage. Oocyte fertilization and cleavage are the most crucial base of embryonic normal development. In this study, we found that taurine treatment could significantly increase the fertilization and cleavage rate of oocytes in vitro, while other taurine groups have no statistic difference. A study by Dumoulin and his partners in 1992 has reported that taurine treatment have no significant effects on in vitro fertilization of mouse oocytes (Dumoulin et al. 1992). This differences with our findings may be due to the difference of culture medium (Tyrode's medium and SSS Quinn's 1023 medium), and the measure time. But a paper reported that taurine could recover the ovarian activity and fertility in cows under field conditions (Rao et al. 1993), the results is in line with our findings. In addition, a lot of papers have identified that taurine could increase sperm fertilizing ability (Fraser 1986). It has been revealed that taurine and its precursor (hypotaurine) could promote the development of the embryo before imbed (Guyaderjoly et al. 1998; Silva et al. 2009). Collectively, our results, combined with previous studies, taurine has beneficial effects on the oocyte fertilization and cleavage in vitro.

5 Conclusion

Taurine can promote ovarian follicles growth and oocyte maturation, fertilization, and cleavage rates *in vitro*, however, its exact mechanism needs further investigations.

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Consumption Patterns and Importance-Performance Analysis of Home Meal Replacements by Level of Taurine-Related Nutritional Knowledge in Korean Adults

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Abstract The purpose of this study was to investigate consumption patterns and perform Importance-Performance Analysis (IPA) of selective attributes of Home Meal Replacement (HMR) products according to taurine-related nutritional knowledge levels in Koreans aged 40–64 years as a basis for developing additional HMR products. The study included 793 adults (297 males and 496 females) who had experience in consuming HMR products and who lived in Seoul and its metropolitan areas, Korea. Data were collected using self-administered questionnaires. Statistical analysis was performed by using the SPSS 18.0 program. The subjects were classified into a high-level group (HG, 467 adults) and low-level group (LG, 326 adults) based on their taurine-related nutritional knowledge scores. Analysis of HMR consumption patterns showed that the frequency of HMR consumption in the HG was one to two times a month in 41.1% of the subjects and once every 3–4 months in 22.7% of the subjects, whereas, in the LG, it was one to two times a month in 39.3% of the subjects and four to six times a month in 24.5% of the subjects. With regarding to the reasons for purchasing HMR products, there was no significant difference between HG and LG ($p = 0.089$). The IPA analysis of HMR selective attributes included factor analysis of 14 selective attributes that were divided into three factors: 'convenience and taste', 'reliability and health', and 'brand and awareness'. The average importance scores of the first ($p < 0.01$), second ($p < 0.001$), and third ($p < 0.01$) factors in the HG were significantly higher than those in the LG. In addition, the average satisfaction with the first factor ($p < 0.01$) in the HG was significantly higher than that in the LG. Based on the IPA results, the selective attributes with low satisfaction and high importance were price, origin, food additives, and nutrient content in both the HG and LG. In the second IPA

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quadrant was safety, but only in the LG. Multiple regression analysis revealed that the importance of the reliability and health factor and the satisfaction with the convenience and taste factor were positively influenced by the subject's taurine-related nutritional knowledge score. These results suggest that reliability and safety of HMR products need to be improved to meet the expectations of Korean consumers aged 40 years and older with a high level of taurine-related nutritional knowledge. Therefore, there is a need to produce HMR products that use safe and reliable food ingredients.

Keywords Consumption pattern · Home meal replacement · Importance-performance analysis · Korean adults · Taurine-related nutritional knowledge

Abbreviations

HG	High-level group
HMR	Home meal replacement
IPA	Importance-performance analysis
LG	Low-level group
SD	Standard deviation

1 Introduction

A home meal replacement (HMR) is a food product that can be substituted for a home meal without being cooked at home and that can be cooked easily when presented as a food product which is completely cooked or requires heating. The size of the global HMR market was expected to reach $157.3 billion in 2016 and $189.1 billion in 2021, an increase of approximately 20.2% over those 5 years (FOOD BANK). The size of the HMR market in Korea increased to 2254.2 billion won in 2016, 34.8% from that in 2015, and it will soon surpass a market size of 3 trillion won (Korea Agro-Fisheries & Food Trade Corporation 2017). The main growth factors in the HMR market in Korea are the increase in single or two-person households and the changes in the economic activity participation of women. Along with such changes in sociocultural phenomena, the rapid growth of the HMR market has led to the development of premium HMR products that have improved taste, quality, and functionality in order to satisfy consumers' needs (Park et al. 2016). An individual's level of nutrition knowledge affects both the selection and consumption of food items (Chang and Kim 2001). Therefore, there is a need to understand consumers' needs when developing HMR products.

Taurine has been reported as an effective anti-obesity factor, an antioxidation factor, and as effective in both fatigue recovery and cognitive function improvement (Stapleton et al. 1997). Taurine is not used for protein synthesis, but it is a conditionally essential amino acid in certain aspects of mammalian development (Lourenco and Camilo 2002). The main source of taurine is animal products such as fish, shellfish, and meat, while taurine is a rare constituent in plant-based foods. However, low levels of taurine intake, such as that of vegans and vegetarians, can lead to cardiomyopathy, retinal degeneration, and growth retardation (Birdsall 1998).

The purpose of this study was to investigate the consumption patterns and perform importance-performance analysis (IPA) of selective attributes of HMR according to the level of taurine-related nutritional knowledge in Korean adults 40–64 years old. Through the IPA analysis, we sought to determine what aspects need to be improved by importance and satisfaction among several selective attributes of HM products and to suggest a direction for customized HMR product development that will reflect consumer needs.

2 Methods

2.1 Subjects

This study included 793 adults (40–64 years old, 297 male and 496 female) who had experience in consuming HMR products and who lived in Seoul and its metropolitan areas, Korea. Subject surveys were conducted anonymously via self-administered questionnaires.

The subjects were classified into two groups according to their taurine-related nutritional score: a 467-member high-level group (HG, average score 6.4 points) and a 326-member low-level group (LG, average score 2.6 points). Taurine-related nutritional knowledge was assessed based on responses to ten questions regarding the effects of taurine and the major taurine source foods. Each item was answered with “Yes”, “No”, or “Do not know” with responses scored as 1 point for a correct answer and 0 points for wrong and ‘do not know’ answers. The total score for those ten questions ranged from 0 to 10 points. The Cronbach’s coefficient associated with taurine-related nutritional knowledge was 0.684. The study was approved by the institutional review board of Inha University, Korea (171120-9A).

2.2 Questionnaire

The questionnaire was comprised of three sections: general characteristics, HMR consumption patterns, and importance of and satisfaction with HMR selective attributes which were based on previous studies (Chung et al. 2007; Park et al. 2016).

Data collected on general characteristics of the subjects included gender, age, marital status, education level, and participation in food purchase.

The HMR consumption patterns were assessed by questions related to consumption frequency, purchase time, consumption time, and reason for purchase.

For IPA analysis, the importance of and satisfaction with HMR selective attributes were assessed by using 17 questions that used a Likert 5-point scale to score the responses. On that scale, 1 point indicated "strongly unimportant or unsatisfactory", 2 points represented "unimportant or unsatisfactory", 3 points indicated a "so-so" response, 4 points indicated "important or unsatisfactory", and 5 points indicated "strongly important or satisfactory", respectively.

2.3 Statistic Analysis

Data were analyzed by using SPSS 20.0 (SPSS Inc., IBM Corp., NY, USA). Values are expressed as frequency and percentage, or as mean and standard deviation. Statistical significance was determined by using the Student t-test or chi-square test for data differences between HG and LG. IPA was conducted to analyze the importance of and satisfaction with selective attributes of HMR according to the level of taurine-related nutritional knowledge. Differences were considered statistically significant when the calculated p-value was less than 0.05.

3 Results

3.1 General Characteristics

General characteristics of the subjects are summarized in Table 1. The proportions of male and female subjects were 32.3% and 67.7% in the HG, and 44.8% and 55.2% in the LG, respectively. The average age of the HG was 47.8 years while that of the LG was 48.0 years. With regard to participation in food purchasing, direct and occasional participation was present in 69.6% and 25.7% in the HG and in 61.3% and 27.9% in the LG, respectively.

3.2 HMR Consumption Patterns

Results of the analysis of HMR consumption patterns are shown in Table 2, and there were no significant differences detected between the HG and LG. The frequency of HMR consumption was one to two times a month in 41.1% of the subjects and once every 3–4 months in 22.7% of the subjects in the HG, while that in

Table 1 General characteristics of the subjects

Variables		HG (n = 467)	LG (n = 326)
Gender	Male	151 (32.3)	146 (44.8)
	Female	316 (67.7)	180 (55.2)
Age (years)		47.8 ± 6.9	48.0 ± 7.5
Marriage	Married	418 (89.5)	285 (87.4)
	Single	49 (10.5)	41 (12.6)
Education level	Middle school	13 (2.8)	8 (2.5)
	High school	138 (29.6)	132 (40.5)
	College	316 (67.7)	186 (57.1)
Food purchase	Direct participation	325 (69.6)	200 (61.3)
	Occasional participation	120 (25.7)	91 (27.9)
	Almost never	22 (4.7)	35 (10.7)

HG High-level group, *LG* Low-level group
n (%), Mean ± SD

Table 2 Consumption patterns of home meal replacement of the subjects

Variables	HG (n = 467)	LG (n = 326)	χ^2
Frequency of consumption			
1 time/3–4 months	106 (22.7)	77 (23.6)	5.077
1–2 times/month	192 (41.1)	128 (39.3)	
4–6 times/month	101 (21.6)	80 (24.5)	
2–3 times/week	49 (10.5)	36 (11.0)	
More than 4 times a week	19 (4.1)	5 (1.5)	
Time of purchase			
Weekday	48 (10.3)	34 (10.4)	1.427
Weekend	207 (44.3)	131 (40.2)	
Any time	212 (45.4)	161 (49.4)	
Time to consume			
Before meals	3 (0.6)	3 (0.9)	1.403
Breakfast	90 (19.3)	59 (18.1)	
Lunch	88 (18.8)	63 (19.3)	
Dinner	130 (27.8)	86 (26.4)	
Snack	96 (20.6)	65 (19.9)	
Midnight snack	60 (12.8)	50 (15.3)	
Reason for purchase			
Saving time	241 (51.8)	141 (43.4)	9.560
Nutrition and taste	16 (3.4)	9 (2.8)	
Affordable price	37 (8.0)	29 (8.9)	
Easy cooking	82 (17.6)	78 (24.0)	
Bothering and eating alone	75 (16.1)	51 (15.7)	
Etc. (habitually, convenient)	14 (3.0)	17 (5.2)	

n (%), Mean ± SD

the LG was one to two times a month by 39.3% and four to six times a month by 24.5% of the LG. The commonest purchase times were 'any time' (45.4% of HG and 49.4% of LG) and at the 'weekend' (44.3% of HG and 40.2% of LG). With regard to the reason for purchasing HMR products, there was a trend toward a significant difference between the HG ('saving time' by 51.8%, 'easy cooking' by 17.6%, and 'nutrition and taste' by 3.4%) and LG ('saving time' by 43.4%, 'easy cooking' by 24.0%, and 'nutrition and taste' by 2.8%) ($p = 0.089$).

3.3 Analysis of Importance of and Satisfaction with HMR Selective Attributes

As shown in Table 3, 14 selective attributes were classified into three factors: "convenience and taste", "reliability and health", and "brand and awareness" by factor analysis. As for total average scores of HMR selective attributes by level of taurine-related nutritional knowledge, the importance of and satisfaction with factor 1 ($p < 0.01$), the importance of factor 2 ($p < 0.001$), and the importance of factor 3 ($p < 0.01$) in the HG were significantly higher compared to LG. Among the 14 selective attributes, the average scores for the importance of price, origin, food additives, nutrient content ($p < 0.01$), taste, safety, chewing ($p < 0.001$), cooking time, and manufacturing company ($p < 0.05$) in the HG were significantly higher compared to the LG. Whereas, among the 14 selective attributes, the average scores for satisfaction with taste, volume ($p < 0.05$), and cooking time ($p < 0.001$) in the HG were significantly higher compared to the LG.

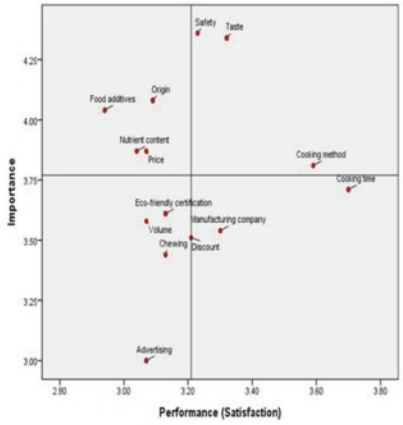
3.4 Importance-Performance Analysis of HMR Selective Attributes by Level of Taurine-Related Nutritional Knowledge

The results of the IPA analysis of HMR selective attributes according to level of taurine-related nutritional knowledge of the subjects are shown in Fig. 1 for the HG and in Fig. 2 for the LG. Among the selective attributes in both HG and LG, taste and cooking method were positioned in IPA quadrant I, which indicates they have both high importance and high satisfaction. Price, origin, food additives, and nutrient content were placed in IPA quadrant II, which had high importance but relatively low satisfaction; thus, there is need for these selective attributes to be focused on and improved. Volume, eco-friendly certification, advertising, and chewing all belonged to IPA quadrant III which indicates both importance and satisfaction were lower than those of other items. Finally, cooking time and manufacturing company belonged to IPA quadrant IV, which indicates low importance but high satisfaction. Interestingly, attributes of the safety and discount were positioned in quadrants I and III in the HG and quadrants II and IV in the LG, respectively.

Table 3 Analysis of differences in the importance of and satisfaction with HMR selective attributes by level of taurine-related nutritional knowledge of the subjects

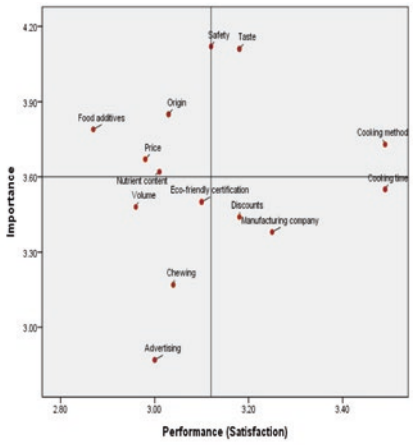
Variables	Importance			Satisfaction		
	HG	LG	t	HG	LG	t
Factor 1. Convenience and taste						
Price	3.8±0.9	3.7±1.0	3.067**	3.1±0.8	3.0±0.7	1.653
Taste	4.3±0.8	3.8±0.9	3.632***	3.3±0.9	3.2±0.8	2.292*
Cooking method	3.8±0.9	3.8±0.9	1.226	3.6±0.8	3.5±0.8	1.862
Cooking time	3.7±0.9	3.6±0.9	2.460*	3.7±0.8	3.5±0.8	3.653***
Volume	3.6±0.9	3.8±0.9	1.484	3.1±0.8	3.0±0.8	2.027*
Total	3.9±0.6	3.8±0.9	3.286**	3.4±0.6	3.2±0.6	3.22**
Factor 2. Reliability and health						
Safety	4.4±0.9	4.1±1.0	3.752***	3.2±0.9	3.1±0.8	1.774
Origin	4.1±0.9	3.9±1.1	3.328**	3.1±0.9	3.0±0.8	0.913
Food additives	4.0±1.0	3.8±0.9	3.421**	2.9±0.9	2.9±0.8	1.179
Nutrient content	3.9±0.9	3.6±1.1	3.489**	3.0±0.8	3.0±0.7	0.576
Eco-friendly certification	3.6±1.0	3.5±1.1	1.513	3.1±0.8	3.1±0.8	0.435
Total	4.0±0.7	3.8±0.9	3.750***	3.1±0.7	3.0±0.6	1.237
Factor 3. Brand and awareness						
Advertising	3.0±1.0	2.9±0.9	1.951	3.1±0.7	3.0±0.6	1.524
Discounts	3.5±1.0	3.4±1.0	1.086	3.2±0.8	3.2±0.8	0.566
Manufacturing company	3.5±0.9	3.4±1.0	2.252*	3.3±0.7	3.3±0.7	0.932
Chewing	3.4±1.0	3.2±1.0	3.685***	3.1±0.7	3.0±0.7	1.694
Total	3.4±0.7	3.2±0.7	3.072**	3.2±0.5	3.1±0.5	1.601

Mean ± SD for a Likert 5-point scale: 1 point (strongly unimportant) to 5 points (strongly important)
Mean ± SD for a Likert 5-point scale: 1 point (strongly unsatisfactory) to 5 points (strongly satisfactory)
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student t-test



Variables	Attributes of HG
I (Keep up the good work)	Taste Cooking method **Safety**
II (Concentrate here)	Price Origin Food additives Nutrient content
III (Low priority)	Volume **Discount** Eco-friendly certification Advertising Chewing
IV (Possible overkill)	Cooking time Manufacturing company

Fig. 1 Importance-performance analysis chart for the HMR selective attributes in the HG by level of taurine-related nutritional knowledge of the subjects



Variables	Attribute of LG
I (Keep up the good work)	Taste Cooking method
II (Concentrate here)	Price **Safety** Origin Food additives Nutrient content
III (Low priority)	Volume Eco-friendly company Advertising Chewing
IV (Possible overkill)	Cooking time **Discount** Manufacturing company

Fig. 2 Importance-performance analysis chart for the HMR selective attributes in the LG by level of taurine-related nutritional knowledge of the subjects

Table 4 Multiple regression results for importance of and satisfaction with HMR selective attributes and level of taurine-related nutritional knowledge

Variables		B	β	t
	(constant)	1.660		2.644**
Importance	Convenience and taste	0.081	0.024	0.551
	Reliability and health	0.287	0.105	2.416*
	Brand and awareness	0.170	0.053	1.175
Satisfaction	Convenience and taste	0.407	0.104	2.351*
	Reliability and health	0.017	0.005	0.111
	Brand and awareness	−0.070	−0.016	−0.354

*$p < 0.05$, **$p < 0.01$

3.5 *Effect of Taurine-Related Nutritional Knowledge on the Importance and Satisfaction of HMR Selective Attributes*

Based on the results of multiple regression analysis (Table 4), the importance of the reliability and health factor and the satisfaction with the convenience and taste factor were positively influenced by taurine-related nutritional knowledge scores of the subjects.

4 Conclusion

These results suggest that the reliability and safety of HMR products should be improved to meet the HMR expectations of Korean consumers aged 40 years and older with a high level of taurine-related nutritional knowledge. As the difference of HMR selective attributes appears according to the level of taurine-related nutritional knowledge, it is necessary to develop HMR products especially considering safety and discount.

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Demands for Development of Taurine-Containing Home Meal Replacement for Prevention of Dementia According to Age

So Hyun Park, Mi Ae Bae, So Hee Han, Young Deok Seo, Sung Hoon Kim, and Kyung Ja Chang

Abstract It has been reported that taurine intake in the past may have a positive effect on present cognitive function in the elderly. The purpose of this cross-sectional study was to investigate the need to develop an elderly-friendly home meal replacement (EF-HMR) containing taurine for the prevention of dementia in Korean adults aged 40–84 years. Study subjects included 481 adults 40–49 years group, 319 adults 50–64 years group, and 181 elderly group (65–84 years old) residing in Seoul and its metropolitan area, Korea. Data were collected from adults aged 40–64 years by self-administered questionnaires and from elderly through face-to-face interviews. Statistical analysis was performed using SPSS 18.0. The level of need for EF-HMR was significantly higher in 40–49 years and 50–64 years groups compared to the elderly group ($p < 0.001$). With regard to the preferred EF-HMR taste, the needs for less salty ($p < 0.01$) and less sweet ($p < 0.001$) foods were significantly higher in 40–49 years group compared to the elderly group. The main factors of consideration in the development of EF-HMR were nutrition, taste, and freshness. The level of need for EF-HMR containing taurine was significantly lower in those 40–49 years and 50–64 years groups compared to the elderly group ($p < 0.01$). If an EF-HMR containing taurine for prevention of dementia was developed, willingness to buy

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such a food was significantly higher in the elderly group than in those 40–49 years and 50–64 years groups ($p < 0.01$). Regarding cooked EF-HMR containing taurine, semi-prepared and ready-made meals were preferred in 71.1% and 25.4% in 40–49 years group, 69.6% and 22.6% in 50–64 years group, and 47.0% and 33.7% in the elderly group, respectively ($p < 0.001$). EF-HMR containing taurine sales unit sizes containing 3 servings or 1 serving was preferred by 37.2% and 26.3% of all subjects. The preferred places to purchase EF-HMR (in descending order of super-supermarket (mart), supermarket, and convenience store) were the same among the age groups. Therefore, it is necessary to understand the diversity present in the needs and purchasing characteristics of adults involved purchasing EF-HMR containing taurine, and there is a need to develop age-specific customized products for the elderly and for Korean adults 40–64 years old.

Keywords Dementia prevention · Demands · Development · Taurine-containing home meal replacement · Korean elderly

Abbreviations

EF-HMR	Elderly-friendly home meal replacement
HMR	Home meal replacement
SD	Standard deviation

1 Introduction

As life expectancy in Korea increased due to improvements in living standards, nutritional status, and healthcare services, Korea became an 'aged society' in September 2017 (Lee et al. 2016; Statistics Korea 2017). With the increase in the Korean elderly population, it has resulted in a social phenomenon in which there is an increase in single-person-elderly households and getting worse in the nutritional status of the elderly (Jung 2015). In 2006, the government of Korea enacted the 'elderly-friendly industrial promotion law' to increase interest in health and nutrition for the elderly. However, compared to the markets in other parts of the world, the Korean market for elderly-friendly food is underdeveloped (Lee 2015; OHMYNEWS 2018). Thus, the development of elderly-friendly foods is direly needed in Korea.

A home meal replacement (HMR) is a meal that has been produced and sold away from the home for "at home" consumption. HMR may be classified into four convenience classes systems according to its cooking status; ready to eat, ready to heat, ready to cook, and ready to prepare (Costa et al. 2001). The HMR market in Korea is growing rapidly and increased by 37.6% over the period 2015–2016 and amounted to 2.3 trillion won in 2016 (Korea agro-fisheries & food trade corporation 2017). HMR has steadily improved through the development of diverse, functional, and convenient meals.

Taurine has been recognized for its physiological benefits and effects (fatigue recovery, life stress improvement, anti-obesity, etc.) by previous researchers (Lee et al. 2003; Sung and Chang 2009). Moreover, taurine is used as a functional ingredient in foods such as milk, soy milk, beverages, and energy drink. Recently, it was reported that taurine has been effective in the treatment of Alzheimer's disease in a mouse model (Kim et al. 2014). In addition, dietary taurine intake in the past may have a positive effect on present cognitive function in the elderly (Bae et al. 2017).

The purpose of this study was to investigate the need to develop an elderly-friendly home meal replacement (EF-HMR) containing taurine to assist in the prevention of dementia in Korean adults. The study included subjects aged between 40 and 84 years.

2 Methods

2.1 Subjects

Subjects of this study included 990 participants who had experience purchasing HMR. All participants were between 40 and 84 years old and resided in Seoul and its metropolitan areas, Korea. Included in the final analysis were 981 participant questionnaires as 9 of the selected participants provided incomplete data. The subjects were divided into three groups based on age (The Korean Nutrition Society 2015); 481 adults 40–49 years old, 319 adults 50–64 years old, and 181 elderly 65–84 years old. In the case of 40–64 years old subjects, data were collected via self-administered questionnaires, whereas data from the elderly were collected via face-to-face interviews. This study was approved by the institutional review board of Inha University, Korea (171120-9A).

2.2 Questionnaires

The questionnaire was based on results in previous studies (Park et al. 2016; Yang 2018). A preliminary survey was conducted on ten persons in each age group and the results were used to remove duplicate content and increase readability. The final questionnaire was comprised of three major sections: general subject characteristics, EF-HMR, and EF-HMR containing taurine.

General characteristics included gender, age, number of family living together, and living arrangement.

Demands for the development of EF-HMR were investigated by using four items: degree of need, purchase motivation, expected characteristics, and development factors. The level of need and the expected characteristics for development of EF-HMR were measured by using a 5-point Likert scale. With regard to factors to be considered in the development of EF-HMR, the subjects selected one of the most important factors in the development of EF-HMR.

Demands for the development of EF-HMR containing taurine for the prevention of dementia were investigated by using five items: the need for development, willingness to buy, desired cooked status, sales package unit size, and purchase place. The degree of need and the willingness to buy for EF-HMR containing taurine were measured by using a 5-point Likert scale.

2.3 Statistic Analysis

Statistical analysis was performed by using the SPSS 18.0 program. Statistical significance of differences among three groups was determined by applying the chi-square test and the one-way ANOVA followed by post-hoc analysis with Scheffe's test. Each value was expressed as a frequency and percentage or as a mean ± standard error (SE). Statistical significance was set at p less than 0.05.

3 Results

3.1 General Characteristics

As shown in Table 1, the number of family members living together averaged 2.5 members in the 40–49 years and 50–64 years groups, but only averaged 1.4 members in the elderly group. In particular, more than approximately 66.0% of the 40–49 years and 50–64 years groups members were living with a spouse and child, but among the elderly 34.8% were alone at home and 26.5% lived with their spouse

Table 1 General characteristics of the subjects by age

Variables		40–49 years (n = 481)	50–64 years (n = 319)	65–84 years (n = 181)	Total (n = 981)
Gender	Male	195 (40.5)	104 (32.6)	474 (26.0)	346 (35.3)
	Female	286 (59.5)	215 (67.4)	134 (74.0)	635 (64.7)
Age (years)		42.9 ± 3.1	55.6 ± 4.0	72.4 ± 6.0	52.5 ± 11.8
Number of family living together		2.5 ± 1.2	2.5 ± 1.1	1.4 ± 1.3	2.3 ± 1.3
Living arrangement					
Alone at home		44 (9.1)	12 (3.8)	48 (26.5)	104 (10.6)
With spouse only		27 (5.6)	51 (16.0)	63 (34.8)	141 (14.4)
With spouse and children		325 (67.6)	211 (66.1)	31 (17.1)	567 (57.8)
With children only		0 (0.0)	0 (0.0)	32 (17.7)	32 (3.3)
Other (with friend, with relatives, etc.)		85 (17.7)	45 (14.1)	7 (3.9)	137 (14.0)

n (%), Mean ± SD

only. These results are similar to those reported previously in Korea (proportion of single-person-elderly households was 33.5% of total elderly households in 2016; Statistics Korea 2017).

3.2 Demands for Development of EF-HMR

Demands for the development of EF-HMR are summarized in Table 2. The levels of need for EF-HMR were significantly high in the 40–49 years and 50–64 years groups compared to that in the elderly group ($p < 0.001$). Purchase motivation was significantly different among the groups: respectively, 37.2% and 1.5% in the 40–49 years group, 24.8% and 2.5% in the 50–64 years group, and 28.2% and 9.9% in the elderly group ($p < 0.001$). The differences in purchasing motivation for EF-HMR are probably related to physical changes such as bad teeth and decrepitude in the elderly. However, one reason why is the low level of need for EF-HMR in the elderly may be because meal rejection can be more than 50% for HMR (Park et al. 2012).

Regarding the preferred taste characteristics associated with the development of EF-HMR, the needs for less salty ($p < 0.01$) and less sweet ($p < 0.001$) tastes in the

Table 2 Demands and expected characteristics for development of EF-HMR by age

Variables	40–49 years (n = 481)	50–64 years (n = 319)	65–84 years (n = 181)	F/χ^2
Level of need for development	3.9 ± 0.7[a]	3.8[a] ± 0.7	3.6[b] ± 1.0	7.745***
Purchase motivation of elderly-friendly HMR				
Delicious meal	6 (1.2)[b]	11 (3.4)	12 (6.6)	68.554***
Buying cheaper price	22 (4.6)	38 (11.9)	13 (7.2)	
Promotion of health	171 (35.6)	116 (36.4)	56 (30.9)	
Decrepitude	179 (37.2)	79 (24.8)	51 (28.2)	
Dislike to cook	96 (20.0)	67 (21.0)	31 (17.1)	
Having bad teeth	7 (1.5)	8 (2.5)	18 (9.9)	
Expected characteristics for elderly-friendly HMR				
Low calorie	3.8 ± 0.8	3.9 ± 0.9	3.8 ± 1.0	1.074
Less saltiness	4.4 ± 0.7[a]	4.3 ± 0.8	4.2 ± 0.9[b]	6.577**
Less sweetness	4.3 ± 0.7[a]	4.2 ± 0.8	3.9 ± 1.0[b]	11.473***
Premium grade	3.7 ± 0.9[a]	3.6 ± 0.8[a]	3.2 ± 1.1[b]	16.577***
Nutritionally enhancement	4.5 ± 0.7[a]	4.2 ± 0.8[b]	4.3 ± 0.9[b]	13.805***
Easy-to-carry	3.8 ± 0.9[a]	3.7 ± 0.9[a]	3.4 ± 1.0[b]	13.233***
Dementia prevention	4.3 ± 0.8	4.3 ± 0.8	4.4 ± 0.9	1.782

[a]Score; 1=Not needed at all, 2=Not that needed, 3=Neither necessary nor unnecessary, 4=Needed, 5=Definitely needed, [a,b] Different letters indicate a significant difference at 5% significance level by Scheffe's multiple range test. **p<0.01, ***p<0.001

[b]n(%); p-value was analyzed by chi-square test. ***p<0.001

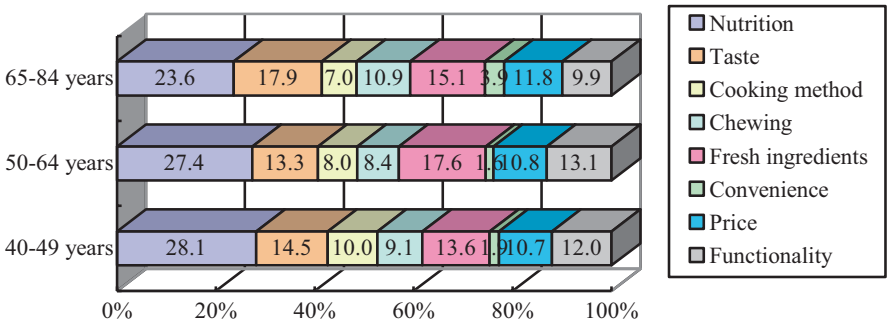

Fig. 1 Factors considered in the development of EF-HMR

40–49 years group were significantly higher than that in the elderly group. Scores for premium grade and easy-to-carry EF-HMR in the 40–49 years and 50–64 years groups were significantly higher than that in the elderly group ($p < 0.001$). Moreover, the needs for nutritional enhancement in the 50–64 years and 65–84 years groups were significantly higher than that in the 40–49 years group ($p < 0.001$).

Among the three groups, the highest level of demand was for less salty food. In Kwak's study, it was shown that less saltiness and less sweetness were preferred by those aged 65 years and over (Kwak et al. 2013).

Regarding the factors to be considered in the development of EF-HMR, nutrition, taste, freshness, and functionality, in that order, were preferred in the 40–49 years group, nutrition, freshness, taste, and functionality, in that order, in the 50–64 years group, and nutrition, taste, freshness, and price, in that order, in the 65–84 years group (Fig. 1).

3.3 Demands for Development of EF-HMR Containing Taurine

Demands for the development of an EF-HMR containing taurine for the prevention of dementia are summarized in Table 3. The level of need for the development of ($p < 0.01$) and the willingness to buy ($p < 0.001$) EF-HMR containing taurine were significantly higher in the elderly group compared to 40–49 years and 50–64 years groups.

As for desired cooked status of the EF-HMR containing taurine, there were significant differences in the preferences for semi-prepared meals and ready-made meals among the groups: respectively, 71.1% and 25.4% in the 40–49 years group, 69.6% and 22.6% in the 50–64 years group, and 47.0% and 33.7% in the elderly group ($p < 0.001$).

There was no significant difference among the three groups in the desired sales package unit; in descending order of preference, a 3 serving unit, a 1 serving unit, and a 5 serving unit. The results of multiple responses to questions regarding the desired purchase place revealed the preferred locations were mart (super-supermarket), supermarket, and convenience stores, in that order, in all three groups,

Table 3 Demands for development of EF-HMR containing taurine by age

Variables	40–49 years (n = 481)	50–64 years (n = 319)	65–84 years (n = 181)	F/χ^2
Level of need for development	$4.0^{a} \pm 0.7$	$3.9^{a} \pm 0.7$	$4.2^{b} \pm 0.7$	7.402**
Willingness to buy	$3.4^{a} \pm 0.8$	$3.5^{a} \pm 0.8$	$3.7^{b} \pm 0.9$	8.214***
Desired cooked status				
Ready-made meals	122 (25.4)	72 (22.6)	61 (33.7)	75.032***
Semi-prepared meals	342 (71.1)	222 (69.6)	85 (47.0)	
Washed food	16 (3.3)	22 (6.9)	22 (12.2)	
Raw material food	1 (0.2)	3 (0.9)	13 (7.2)	
Desired sales package unit				
1 serving unit	129 (26.8)	88 (27.6)	41 (22.7)	10.745
3 servings unit	181 (37.6)	109 (34.2)	75 (41.4)	
5 servings unit	103 (21.4)	69 (21.6)	41 (22.7)	
10 servings unit	57 (11.9)	35 (11.0)	15 (8.3)	
More than 10 serving unit	11 (2.3)	18.0 (5.6)	9 (5.0)	
Desired purchase place (multiple responses)				
Hospital	41 (8.5)	20 (6.3)	14 (7.7)	–
Pharmacy	76 (15.8)	51 (16.0)	24 (13.3)	
Mart (super-supermarket)	351 (73.0)	215 (67.6)	115 (63.5)	
Convenience stores	210 (43.7)	112 (35.2)	45 (24.9)	
Supermarkets	260 (54.1)	166 (52.2)	98 (54.1)	
Online shopping	171 (35.6)	77 (24.2)	14 (7.7)	
Nursing home	93 (19.3)	32 (10.1)	13 (7.2)	
Grocery store	32 (6.7)	21 (6.6)	10 (5.5)	
Department store	29 (6.0)	17 (5.3)	10 (5.5)	

Mean ± SD, [a, b]: different letters indicate a significant difference at the 5% significance level by Scheffe's multiple range test; $^{**}p < 0.01$ and $^{***}p < 0.001$
n(%), p values was analyzed by chi-square test; $^{***}p < 0.001$.

followed by online shopping in the 40–49 years and 50–64 years groups, and by pharmacy in the elderly group.

4 Conclusions

In summary, the levels of need for EF-HMR and EF-HMR containing taurine were the highest in subjects 40–49 years old and 65–85 years old, respectively. The main factors to be considered in the development of EF-HMR were nutrition, taste, and freshness. In addition, the most desired cooking status, sales package unit size, and purchase place for EF-HMR containing taurine were semi-prepared meals, a 3 servings unit, and a super-supermarket, respectively.

These results show that there are different levels of needs for development of EF-HMR containing taurine for prevention of dementia among Korean adults aged 40–84 years old. Thus, there is a need to understand the diversity of needs and purchasing characteristics for development of EF-HMR containing taurine in Korean adults of various ages. Therefore, it is necessary to develop age-specific customized products in Korea to assist in the prevention of dementia.

Acknowledgments This project was conducted by the generous financial support of the Youlchon Foundation (Nongshim Corporation and its affiliated companies) in Korea.

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Dietary Taurine Intake and Its Food Sources in Korean Young Adults Using 2015 Korea National Health and Nutrition Examination Survey Data

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Abstract This study aimed to estimate the dietary taurine intake and its food sources in Korean's aged between 19 and 29 years. The study included 619 participants (292 males and 327 females) who had provided data via a 24-h recall method to the 2015 Korea National Health and Nutrition Examination Survey (KNHANES). Dietary taurine intake and the sources of dietary taurine were estimated by using CAN-Pro 4.0 software. Statistical analysis was performed by using SPSS 20.0. Average height and weight of the subjects was 174 cm and 73.5 kg in males and 161 cm and 56.4 kg in females, respectively. The prevalence of obesity in males was significantly higher compared to females ($p < 0.001$). Overall, the nutrient intakes of subjects were higher than the dietary reference intakes for Korean's (KDRIs). In particular, phosphorus and sodium intakes of males and females were higher, whereas, potassium and calcium intakes of males and females were lower than the KDRIs. The average intake of taurine 327.3 mg by males was significantly higher compared to 245.1 mg by females ($p < 0.05$). With regard to dietary taurine intake from the main food groups, meat ($p < 0.001$), vegetable ($p < 0.001$), beverages and alcohol ($p < 0.05$), and cereal ($p < 0.001$), in males was significantly higher compared to females. This study showed that 19–29 years old Korean young adults had a high intake of taurine due to high intake of protein. However, since high intake of meat can lead to chronic disease, it is necessary to provide nutrition education to increase the intake of fishes and shellfishes as a taurine source food.

Keywords Dietary taurine intake · Food source · KNHANES (Korean National Health and Nutrition Examination Survey) · Korean young adults

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Abbreviations

CAN-Pro	Computer-aided nutrition program
KDRIs	Dietary reference intakes for Koreans
KNHANES	Korea National Health and Nutrition Examination Survey
SE	Standard error

1 Introduction

Taurine is an amino acid found in animal proteins including those in meat, fish, and shellfish (Lee 2013). Taurine has been shown to have effects on the restoration of fatigue (Lee et al. 2003), prevention of obesity (Zhang et al. 2004), and reduction of LDL-cholesterol in serum (Xu et al. 2008). Taurine has no reported side effects, and it is discharged into the urine when consumed in excess (Yoon et al. 2015). Many countries have developed food products that contain taurine (such as Lipovitan in Japan, Redbull in Austria, etc.). Korea is actively developing food products that contain taurine (Korea IT Times; Newsis; Yonhapnews). Recently, it has been reported that consumption of energy drinks and animal protein foods with a high taurine content is increasing in young adults (Lee et al. 2013; Park et al. 2016).

The Korea National Health and Nutrition Examination Survey (KNHANES), conducted by the Korea Centers for Disease Control and Prevention since 1998, is an ongoing nationwide survey of selected Korean participants of more than 1 year of age that collects data on the health and nutritional status of Koreans (Korea Centers for Disease Control and Prevention 2015). However, taurine-specific data collection is not included in the food intake survey of the KNHANES database. In addition, reports on studies into the dietary taurine intake and food sources of dietary taurine in a representative population of young adults are rare. Therefore, the purpose of this study was to analyze the intake and food sources of dietary taurine for young adult Koreans (19–29 years old) by using the data collected by KNHANES.

2 Methods

2.1 Subjects

The subjects of this study were 950 participants aged 19–29 years obtained from data of the 2015 KNHANES. Of the 950 participants, 331 participants were excluded because they failed to provide complete dietary intake data (304 participants) or reported an implausible energy intake level (<500 or >5,000 kcal/day, 27 participants). The final analysis included data for 619 participants (292 males and 327 females).

2.2 Dietary Nutrient Intakes

The 2015 KNHANES used a 24-h dietary recall method, with data collection conducted via face-to-face interviews with a trained staff (KNHANES 2015). Dietary taurine and nutrient intakes were estimated by using computer-aided nutrition program (CAN-Pro) version 4.0 (a Korean nutrient analysis program) to examine raw data for food intake in the 2015 KNHANES database.

The total dietary intake of taurine according to each food group was summed to determine the total dietary taurine content consumed by the subjects.

2.3 Construction of Database for Dietary Taurine and Nutrient Intakes Assessment

To evaluate dietary taurine intake, the taurine content of 315 foods in 17 food groups was entered into the CAN-Pro 4.0 database (National Institute of Fisheries Science 2016; Park et al. 1998).

To evaluate nutrient intakes, 2016 foods and their respective codes included in the 2015 KNHANES were converted to food codes for the 17 food groups in the CAN-Pro database. As shown in Table 1, of the 2016 foods included in the study, 1328 foods were converted through a direct match with those in the CAN-Pro database (The Korean Nutrition Society), 404 foods were matched by using a substitute code in the CAN-Pro 4.0 database, and 284 foods were matched after substitution that used codes from other data sources (Fatsecret n.d.; Health Encyclopedia n.d.).

2.4 Statistical Analysis

Differences of statistical significance were determined by performing Student t-tests or chi-square test as provided in the SPSS 20.0 program. Values are expressed as means ± standard error (SE) or frequencies and percentages. Differences in the

Table 1 Types of food matching of 2015 KNHANES foods to CAN-Pro 4.0

Variables	Description of matching	Number of foods (% of 2016 foods)
Taurine-containing foods	Data sources entered in CAN-Pro 4.0	315 (6.4)
All foods	Matching 2015 KNHANES data directly to CAN-Pro database	1328 (65.9)
	Substituted with CAN-Pro 4.0 data	404 (20.0)
	Substituted with data from other sources	284 (14.1)
	Total	2016 (100.0)

average dietary total taurine intake and tau-rine intake by food group between males and females were analyzed by using the Student t-test. Differences were considered statistically significant when the calculated p-value was less than 0.05.

3 Results and Discussion

3.1 Anthropometric Data

Anthropometric data of the subjects are summarized in Table 2. Average height and weight of the subjects were 174 cm and 73.5 kg in males and 161 cm and 56.4 kg in females, respectively. Subjects were classified as underweight, normal, or obese by applying body mass index (BMI) standards. There was a significant difference in the distribution of subjects by weight group between males and females ($p < 0.001$). Most male and female subjects were in the normal weight group, followed in descending order by the obese and underweight groups. In the underweight group, there was a greater proportion of 13.3% by females than 5.0% by males, while in the obese group, there was a higher proportion of 33.1% by males than 15.3% by females.

The average BMI of Korean males was reported to be 23 kg/m^2 in 2010 (Survey of Korean Human Body Dimensions 2010). In this study of 2015 data, the average BMI of the males was 24.0 kg/m^2, indicating that, over that period, the BMI of Korean males had increased. The average BMI of Korean females also slightly increased from 20.8 kg/m^2 in 2010 (Survey of Korean Human Body Dimensions 2010) to 22.7 kg/m^2 in the 2015 data included in the present study.

3.2 Dietary Nutrient Intakes

As shown in Table 3, protein, which is a major food source of taurine, intake was 91.1 g for males and 71.5 g for females. The overall nutrient intakes and dietary habits of the subjects were assessed. In the subjects, dietary phosphorus and sodium

Table 2 Anthropometric data of the subjects by gender

Variables	Male (n = 260)	Female (n = 300)	χ^2-value
Height (cm)	174.4 ± 0.4	161.5 ± 0.3	–
Body weight (kg)	73.5 ± 0.9	56.4 ± 0.6	
Body mass index (n = 560)[a]	24.0 ± 0.3	21.6 ± 0.2	
Underweight (<18.5)	13 (5.0)[b]	40 (13.3)	30.666***
Normal (18.5–24.9)	161 (61.9)	214 (71.3)	
Obese (≥25)	86 (33.1)	46 (15.3)	

p-value was analyzed by chi-square test, ***$p < 0.001$
[a]Data missing for 59 subjects
[b]n (%)

Table 3 Nutrient intakes and % dietary reference intakes for Korean (KDRIs) of the subjects by gender

	Male (n = 292)		Female (n = 327)	
Variables	Mean ± SE	% of KDRI[a]	Mean ± SE	% of KDRI
Energy (kcal)	2666.8 ± 74.6	102.6	1922.1 ± 48.5	91.5
Protein (g)	91.1 ± 2.7	182.2	71.5 ± 2.1	158.9
Fat (g)	76.2 ± 3.4	–	57.0 ± 2.3	–
Carbohydrate (g)	359.1 ± 8.9	–	271.0 ± 6.2	–
Ca (mg)	549.7 ± 20.4	84.6	429.4 ± 14.4	81.0
P (mg)	1388.0 ± 38.7	239.3	1022.9 ± 26.3	176.4
Fe (mg)	16.0 ± 0.6	200.0	11.9 ± 0.5	108.2
Na (mg)	5254.3 ± 173.6	350.3	3696.7 ± 125.8	246.4
K (mg)	3289.5 ± 95.6	94.0	2569.3 ± 74.7	73.4
Vitamin A (RE)	882.1 ± 47.6	154.8	683.3 ± 48.6	148.5
Vitamin B_1 (mg)	1.9 ± 0.1	190.0	1.0 ± 0.1	155.6
Vitamin B_2 (mg)	1.7 ± 0.1	130.8	1.3 ± 0.1	130.0
Niacin (mg)	22.7 ± 0.8	189.2	15.8 ± 0.5	143.6
Vitamin C (mg)	124.4 ± 10.4	165.9	102.6 ± 10.3	136.8

[a]Nutrient intake percentages of dietary reference intakes for Koreans, % Estimated Energy Requirement (EER) for energy, % Recommended Intake (RI) for protein, calcium, phosphorus, iron, Vitamin A, Vitamin B1, Vitamin B2, Niacin, Vitamin C, and % Adequate Intake (AI) for sodium and potassium

intakes were markedly higher than the KDRI values (239.3% and 350.3% of the KDRIs in the male group and 176.4% and 246.4% of the KDRIs in the female group). In female subjects, energy intake was lower than the KDRI but it was slightly higher than the KDRI in the male subjects. Potassium and calcium intakes in males and females were lower than the KDRIs.

Similarly, Shin's study (Shin 2016) of Korean college students, the intakes of sodium in male and female college students were notably higher than the KDRI (300.7% of KDRI for males and 257.3% for females). Kim and Kim (2015) reported that the calcium intakes of male and female Korea college students were lower than the KDRI (58.0% of KDRI for males and 58.6% for females).

3.3 Average Total Dietary Taurine Intake and Main Food Groups

Average dietary taurine intake and main food groups by gender are shown in Table 4. Dietary taurine intake in male group (327.3 mg) was significantly higher compared to the female group (245.1 mg) ($p < 0.05$). Dietary taurine intake of all the subjects was 284.7 mg. In previous studies, dietary taurine intake of Japanese aged 20–59 years in 2000 was 225.5 mg/day in males and 162.6 mg/day in females

Table 4 Average dietary taurine intake and main food groups by gender

Variables	Male	Female	Total	t-value
Dietary taurine intake (mg/day)	327.3 ± 30.0 (100)	245.1 ± 22.0 (100)	284.7 ± 18.5 (100)	2.207*a
Main food groups				
Fishes and shellfishes	170.3 ± 25.1 (52.0)	165.1 ± 21.6 (67.3)	167.6 ± 16.4 (58.9)	0.158
Meats	85.2 ± 8.9 (26.0)	53.7 ± 5.3 (21.9)	69.1 ± 5.6 (24.3)	2.807***
Vegetables	24.5 ± 1.6 (7.5)	14.1 ± 1.1 (5.7)	19.1 ± 1.0 (6.7)	5.506***
Beverages and alcohols	30.4 ± 13.1 (9.3)	3.7 ± 3.1 (1.5)	16.7 ± 6.6 (5.9)	1.988*
Cereals	5.2 ± 0.2 (1.6)	3.7 ± 0.2 (1.5)	4.5 ± 0.2 (1.6)	4.338***
Fruits	0.5 ± 0.2 (0.1)	1.3 ± 0.5 (0.5)	0.9 ± 0.3 (0.3)	−1.484
Eggs	0.6 ± 0.1 (0.2)	0.7 ± 0.1 (0.3)	0.7 ± 0.0 (0.2)	−1.658
Others (snacks, etc.)	0.0 ± 0.0 (0.0)	0.6 ± 0.6 (0.2)	0.3 ± 0.3 (0.1)	−1.149

Mean ± SE (% of total), P values were analyzed by Student t-test (*$p < 0.05$, ***$p < 0.001$)

(Kibayashi et al. 2000) and dietary taurine intake of Korean aged 16–59 years in 2001 was 216 mg/day in males and 181 mg/day in females (Park et al. 2001).

With regard to the food groups contributing to dietary taurine intake, meats, vegetables, cereal ($p < 0.001$), and beverages and alcohol ($p < 0.05$) in male group (85.2, 24.5, 30.4, and 5.2 mg/day, respectively) were significantly higher compared to female group than in the female group (53.7, 14.1, 3.7, and 3.7 mg/day, respectively). As for the proportion of total food groups contributing to dietary taurine intake, in the male group, fish and shellfish provided 52.0% of the intake, meats 26.0%, beverages and alcohol 9.4%, and vegetables 7.5%. In the female group, fish and shellfish provided 67.3% of the intake, meats 21.9%, and vegetables 5.7%.

3.4 Food Sources Contributing to Dietary Taurine Intake

The five food sources contributing the greatest amount to a dietary taurine intake of the subjects are shown in Table 5. In the male group, five food sources contributing to dietary taurine intake were: raw chicken meat 43.6 mg/day (15.0%), raw squid 28.3 mg/day (8.8%), Bacchus 25.4 mg/day (7.9%), whip-arm octopus 24.0 mg/day (7.4%), and kimchi and cabbage 21.8 mg/day (6.8%). In the female group, five food sources contributing to dietary taurine intake were: whip-arm octopus 28.2 mg/day (11.4%), raw chicken meat 27.9 mg/day (11.2%), raw squid 26.9 mg/day (10.8%), raw abalone 20.6 mg/day (8.3%), and granulated ark shell, raw 13.4 mg/day (5.4%). In the female group, all five food items contributing to taurine were classified as ‘natural’ foods. However, among the male group, the amount of taurine intake from Bacchus (a ‘non-natural’ energy drink) was the third greatest contributor with an average of 25.4 mg/day.

Table 5 Five food sources contributing to dietary taurine intake in high order by gender

Male			Female		
Food sources	Intake (mg/day)	% of total	Food sources	Intake (mg/day)	% of total
Raw chicken meat	43.6a	15.0	Whip-arm octopus	28.2	11.4
Raw squid	28.3	8.8	Raw chicken meat	27.9	11.2
Bacchus	25.4	7.9	Raw squid	26.9	10.8
Whip-arm octopus	24.0	7.4	Raw abalone	20.6	8.3
Kimchi, cabbage	21.8	6.8	Granulated ark shell, raw	13.4	5.4

4 Conclusion

This study showed that Korean aged 19–29 years had a relatively high intake of taurine, most of which is obtained from fish, shellfish, and meats. Interestingly, in males, the intake of taurine through energy drinks was high. However, since high intake of meat can lead to chronic disease, it is necessary to provide nutrition education to increase the intake of fishes and shellfishes as a taurine source food. This study is the first study to compare taurine intake by gender in Korean aged 19–29 years. This study is expected to be used as basic data for future studies evaluating the intake of taurine.

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Effects of Dietary Taurine Supplementation on Blood and Urine Taurine Concentrations in the Elderly Women with Dementia

Ranran Gao, Mi Ae Bae, So Hee Han, Kyung Ja Chang, and Sung Hoon Kim

Abstract The purpose of this research is to investigate the effects of dietary taurine supplementation on blood and urine taurine concentrations of the elderly women with dementia. Subjects were 31 female elderly with dementia hospitalized in a geriatric hospital. They were divided randomly into control group and dietary taurine supplemented group. Basically, same meals were served to both groups. Scorched rice water without taurine were served to control group. Scorched rice water containing 3 g of taurine were reserved to taurine group with lunch similarly. Food ingredients containing high concentration of taurine were eliminated from the meal menu. Blood and urine samples were obtained from each subject at the beginning of study, after 2 week and 4 weeks in the morning fasting state. Taurine concentrations in serum and urine were measured as taurine-fluorescamine derivatives using high performance liquid chromatography (HPLC). Data were analyzed using SPSS 20.0. The average taurine concentrations in serum and urine of subjects were 89.2 ± 9.5 μM and 876.7 ± 97.1 μM at the beginning. After 4 weeks, the taurine concentrations in serum and urine of dietary taurine supplemented group were 218.0 ± 15.6 μM and 6502.6 ± 380.6 μM, which were significantly higher compared to control group. Dietary taurine supplemented group showed positive changes in the score on language and execute performance. So taurine supplementation can provide beneficial effects to the elderly and the elderly with dementia.

Keywords Dietary taurine supplementation · Blood taurine concentration · Urine taurine concentration · Elderly · Dementia

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1 Introduction

Taurine is the most abundant non-protein amino acid in human. Major sources of taurine are hepatic biosynthesis and dietary intake of seafood, eggs, meat, and milk. Taurine contributes several beneficial physiological functions in mammalian cells, such as anti-inflammation, ion transport modulation, balance of oxidative stress (Schaffer et al. 2014). Based on animal and human taurine supplementation studies, taurine also showed some advantages such as reducing excise muscle fatigue (Yatabe et al. 2009), improving protection in the brain and liver of hyperammonemia animal (Jamshidzadeh et al. 2017). Recently, several researches of brains function related were reported. Del indicated that taurine activates GABAA receptor located on the cell body layer in rat brain (Olmo et al. 2000). In 2004, Monti reported that brain becomes less efficient in producing and responding to GABA due to aging (Monti et al. 2004). Idrissi studied that taurine supplementation in aged mice showed a significant improvement in memory formation and retention based on experiments (Idrissi 2008). In 2017, with the aim of investigating the differences of taurine metabolism between normal elderly and the elderly with dementia, and it is proved that elderly with dementia shows significant higher urinary excretion of taurine based on the test of measuring the taurine concentrations in serum and urine (Gao et al. 2017).

Until now, most of the taurine studies were based on the animals, cells and healthy subjects. There is lack of the research about human with dementia. In this retrospective study, the changes of taurine concentrations in serum and urine after taurine supplementation were assessed and the correlation among MMSE-score, serum taurine concentration and urine taurine concentration was explored too.

2 Methods

2.1 Subjects

Thirty-one elderly women with dementia who live in geriatric hospital were divided randomly into control group (CG, n = 15) and dietary taurine supplementation group (TG, n = 16). All subjects' caregivers had given their cognitive informed consent. The study protocol was approved by the IRB (Institutional Review Board) of Inha University (161010-8ARC).

2.2 Dietary Taurine Supplymentation

Subjects took the same meal which was planned by nutritionists. The food which contains high taurine concentration were removed from the menu during the period of taurine being provided. Besides, one cup of scorched rice water was provided with every lunch for these subjects. There were 5 g glutinous rice powder and 180 mL water in one cup of scorched rice water. Rice powder was put into boiling water, which was heated for 15 min. When the temperature of scorched rice water was up to 60 °C, taurine 3 g were put in and stirred until it was dissolved (Chen et al. 2006). Calories of 180 mL scorched water was 19.4 kcal.

Control group just took scorched rice water. However, taurine group took scorched rice water with 3 g of taurine. European safety recommendation suggested that 6 g per day of taurine supplementation is safe. Taurine has been shown several benefits on brain, and no animal or human study data has indicated any toxic effects (Parvez et al. 2008).

2.3 Data Collection

Serum, urine samples and BMI of subjects were tested. Due to the healthy state of the elderly, BMI was measured under the condition of subjects lying down. During dietary taurine supplementation, the health conditions of these subjects were monitored every day. Their blood and urine samples (after 12 h of overnight fasting) were collected with the agreement of elderly by themselves or their family caregivers. Then, urine and serum samples were stored at −20 °C before taurine concentration measurement. In order to know the change of taurine concentration in serum and urine, the serum and urine were tested after 2 weeks of dietary intake.

2.4 Chemicals

Taurine (99%) was from Dong-A Pharmaceutical Company. Super-purity acetonitrile (HPLC grade), boric acid, and fluorescamine (99%) were from Sigma-Aldrich. Isopropyl alcohol (HPLC grade), methanol, and tetrahydrofuran were from J.T.Baker.

2.5 Sample Preparation

One hundred microliters of serum or urine samples were mixed with 150 μL of super-purity acetonitrile and then centrifuged at 3000 rpm for 20 min to obtain supernatants. Borate buffer (50 μL), which was prepared from aqueous disodium tetraborate solution (100 mM) adjusted to pH 9.2 with 10 mM boric acid, were added to the supernatants to give approximately pH 9.2 solution. Then 50 μL of fluorescamine reagent in acetonitrile (5 mM) was added to each solution, immediately vortex mixed (McMahon et al. 1996). The resulting sample solutions (20 μL) were analyzed on the HPLC system within 45 min.

2.6 Determination of Taurine Concentration

The taurine concentrations were measured using HPLC system (Agilent Technologies 1200 series HPLC) and Waters C18 reverse-phase column (250 × 4.6 mm i.d.) at 20 °C. The mobile phase was composed of tetrahydrofuran-isopropyl alcohol-acetonitrile-phosphate buffer (pH 3.5) (7:1:23:69 v/v/v). The flow rate was 1 mL/min. UV/VIS detection was carried out at 382 nm, which is the maximum absorbance wavelength for the taurine-fluorescamine derivative.

2.7 Statistic Analysis

The significance was determined by student t-test. Each value was expressed as mean ± SE (standard error). Difference was considered statistically significant when the calculated P value was less than 0.05.

3 Results and DISSCUSSION

3.1 General Characteristics and Anthropometric Data

The purpose of this study was to investigate the effects of dietary taurine supplementation on blood and urine taurine concentrations of the elderly women with dementia. Subjects were supplemented with 3 g taurine in 180 mL scorched rice water to taurine supplement group (TG) for 4 weeks. The average age of the elderly was 80.8 ± 2.1. The subjects had more than one disease such as hypertension and diabetes, as well as dementia. The body weight and BMI of these subjects showed no difference after 4 weeks of dietary taurine supplementation (Table 1).

Table 1 General characteristics and anthropometric data

Variables			Total (N = 31)	CG (N = 15)	TG (N = 16)	*P*-value
General characteristics	Age (year)		81.5 ± 1.2	82.3 ± 1.2	80.8 ± 2.1	0.874
	Care period (month)		26.6 ± 3.4	27.1 ± 5.7	26.1 ± 4.2	0.984
	Number of diseases (except dementia)		1.7 ± 0.2	1.7 ± 0.3	1.7 ± 0.3	0.885
	Types of dementia	Alzheimer	16(51.6)[a]	8(53.3)	8(50.0)	1.000
		Other	15(48.4)	7(46.7)	8(50.0)	
Anthropometric data	Height (cm)		151.8 ± 0.8	151.0 ± 1.1	152.6 ± 1.1	0.302
	Weight (kg)		52.4 ± 1.9	50.3 ± 2.8	54.4 ± 2.6	0.429
	BMI (kg/m^2)[b]		22.7 ± 0.8	22.0 ± 1.2	23.3 ± .0	0.452

[a]Data were expressed as N (%)
[b]BMI (body mass index) was calculated as body weight (kg) divided by height squared (m^2): BMI < 18.5 (underweight), 18.5 ≤ BMI < 23(normal), BMI ≥ 23(overweight)

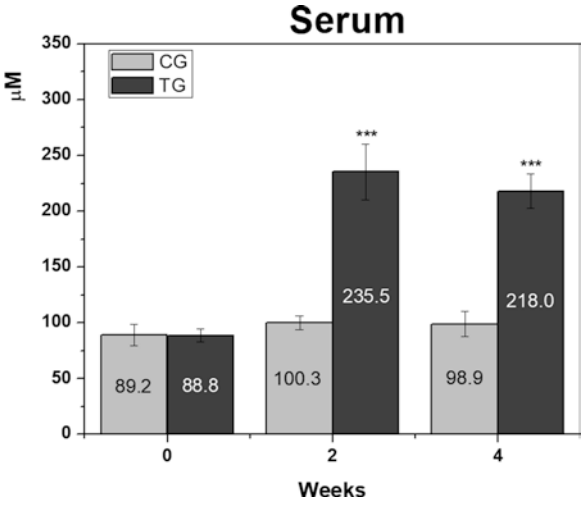

Fig. 1 Changes of taurine concentrations in serum

3.2 Changes of Taurine Concentrations

In order to measure the change of taurine concentrations in serum and urine of these subjects after dietary taurine supplementation, it was measured before and after dietary taurine supplementation 2 and 4 week. The serum taurine concentrations are shown in Fig. 1. There was significant increase in serum taurine concentration after dietary supplementation. Before dietary taurine supplementation the serum average taurine concentrations in CG and TG were 89.2 ± 9.5 μM and 88.8 ± 6.0 μM. The results were similar to those previously reported (Tcherkas et al. 2001; Spelbrink et al. 2016). But the taurine concentrations of serum in this research were higher than our previous study with the mean value was 56.7 μM. Based on Spelbrink's research, the range of taurine concentration in serum was 9–496 μM (Spelbrink

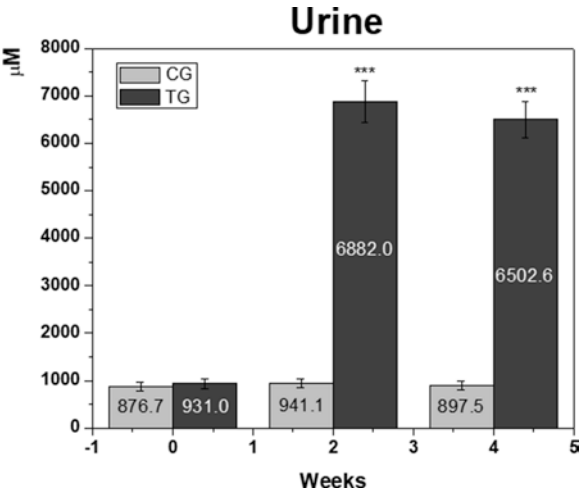

Fig. 2 Changes of taurine concentrations in urine

et al. 2016). The serum result of this research was in this range. Figure 2 showed the change of taurine concentration in urine. There was significant increase in urine concentration after dietary taurine supplementation. Before taurine supplementation, the taurine concentration was about 876 μM, Healthy people have a wide range in urine taurine concentration which varies from 8 to 1500 μM (Qu et al. 1999; Mou et al. 2002).

3.3 Correlation among MMSE–Score, Serum Taurine Concentration and Urine Taurine Concentration

Table 2 showed the correlation among MMSE-score, serum taurine concentration and urine taurine concentration in two groups, The MMSE-DS (Mini Mental State Examination for Dementia Screening) results was from Bae's unpublished data about taurine taurine supplementation and cognitive function (Bae et al. 2018). In control group, there was no correlation among MMSE-Score and taurine concentration of serum and urine. But in taurine supplementation group, there were negative correlations between taurine excretion in urine and language and execute score. There have some studies about the correlations of MMSE score and education level, depression, or some diseases in the elderly person. (Kose et al. 2005; Andel et al. 2006).

Table 2 Correlation among MMSE-score, serum taurine concentration and urine taurine concentration

	Concentration of taurine	Total MMSE score	Language	Time orientation	Place orientation	Memory	Attention	Ability to Execute	Judgement
CG	Serum	0.502	0.449	0.079	0.527	0.159	0.445	0.034	0.554
	Urine	0.319	0.217	0.438	0.559	−0.160	0.354	−0.150	0.564
TG	Serum	−0.137	0.469	0.038	−0.311	−0.395	−0.227	0.612*	−0.003
	Urine	−0.511	−0.628*	−0.242	0.247	−0.173	−0.067	−0.579*	−0.393

4 Conclusion

After 4 weeks dietary taurine supplementation, the taurine concentrations of serum and urine in taurine group were significantly increased compared to control group. There was significantly negative correlation among MMSE-score, score of language and execute and taurine concentrations of serum and urine. It is needed to utilize dietary taurine supplementation for the treatment and prevention of dementia.

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Effects of Taurine on Eusociality of Ants

Ha Won Kim and Dong Hee Lee

Abstract The effects of taurine have been characterized primarily in mammals, and insects are not generally used to study taurine. In this study, ants were used to examine the effect of taurine on eusociality. Ants are the principal models for studying eusociality and superorganisms. Japanese carpenter ants (Camponotus japonicus) were fed a taurine-supplemented diet and tested using ant eusocial indexes. Ant farm structures were constructed using transparent PET bottles containing autoclaved soil. Three categories of vital index were used to study the effect of taurine on group activity: creation of formicaries (residence chambers), cooperative defense efforts, and population density (or group size and composition). Control, low-, and high-taurine diets were prepared using three different levels of taurine in sucrose powder: 0, 5, and 20% (g/g), respectively. The cooperative defense efforts against exogenous queen ants were recorded daily. The high-taurine group took less time to complete their defense formation than the other groups. At least 16% more formicaries (chambers) were observed in the taurine-fed groups than in the control. There were evident differences between control and taurine-fed groups in the total numbers of ants and eggs. The taurine-fed group sustained higher total numbers of ants, excluding the queen. Taurine-fed groups showed a significant increase both in the number of workers and eggs. When fed with taurine, ants responded positively on the eusocial vitality indexes. These results show that taurine exerts a positive effect on the eusociality of ants at the level of the superorganism.

Keywords Ants · Eusociality · Eusocial vital index · Superorganism · Taurine

Abbreviations

CDI	Cooperative Defense Index
PET	polyethylene terephthalate

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1 Introduction

Taurine, 2-aminoethanesulfonic acid, comprises sulfonic acid and amino functions that branch from each carbon backbone. In mammals, taurine protects the brain from various impairments, lessens withdrawal syndromes, and helps to augment visual function. Taurine is a vital nutritional constituent, especially during the infant stage (Whittle et al. 2007). Taurine plays an important role in muscle maturation during the developmental period, and is indispensable for cardiovascular function, development and function of skeletal muscle, the retina, and central nervous system (Schaffer et al. 2000). Taurine has many essential biological roles in antioxidation, osmoregulation, and membrane stabilization, and modulation of calcium signaling (Solis et al. 1988).

To date, the effects of taurine have been studied primarily in mammals. There is no report of its effects in insects. Taurine is widely found in animal organs and accounts for 0.1% of total body weight of humans (Huxtable 1992). Taurine is also abundant in ants (Hymenoptera: Formicidae) and can account for up to 6.5 mg/g of dry matter (McCusker et al. 2014). This is surprising, since taurine content does not exceed 1.0 mg/ml in most insects (Spitze et al. 2003). The taurine content in ants even exceeds that of many oceanic fishes and mollusks (Table 1). Despite its extremely high content in ants, the actual function of taurine in ants is unknown.

Eusociality refers to a highly advanced social structure between group members (Crespi and Yanega 1995). Eusociality evolved after recurrent modifications of social behaviors in insects and other hexapods more than three million years ago (Wilson and Nowak 2014; Wilson and Holldobler 2005). Most ants show a form of eusociality, but it is rare in other Hymenoptera, such as bees and wasps, and vertebrates, such as mammals (Linksvayer and Wade 2005; Nowak et al. 2010). One of the characteristic features of eusociality is a division of labor between reproductive and non-reproductive members (Purcell et al. 2014). A eusocial colony has a distinctive hierarchy, maintained under a strict caste system (de Wilde and Beetsma 1982). Queens and reproductive males are the sole reproducers, while most offspring assume the roles of workers or soldiers who gather food, protect the colony, or raise the offspring produced by the queen (Rehan and Toth 2015). Soldiers and workers are interdependent on the overall growth, solidarity, and proliferation of the colony for their survival. Eusocial ants defend their colonies by subduing invaders, and alert nest mates to rich food sources. Eusocial ants have a high level of interaction

Table 1 Taurine contents in selected organisms

Kingdom	Species	Source of sample	Dried Matter (mg/g)
Planta	Purple kale (*Brassica oleracea*)	Leaf	0.02[a]
Fungi	Yeast (*Saccharomyces cerevisiae*)	Whole	0.00[a]
Animalia	Cow (*Bos taurus*)	Heart	3.64[b]
	Ant (*Pogonomyrmex occidentalis*)	Whole	6.42[a]
	Tuna (*Thunnus thynnus*)	Red meat	8.69[b]

[a]McCusker et al. (2014); [b]Spitze et al. (2003)

among peers and are highly collaborative with other members to ensure the success of their community (Wilson and Nowak 2014; Crozier and Pamilo 1996). However, individual ants within a eusocial community may also experience a high level of pressure and stress, which might be relieved by taurine.

This study used Japanese carpenter ants (Camponotus japonicas) to examine the effect of taurine on eusociality. Ants were fed with taurine-supplemented diets and tested according to eusocial indexes. The aim was to analyze the potential function of taurine in eusociality. The effect of taurine as a eusocial enhancer in ants was measured using three indexes: residence number, cooperative defense index, and population density. The three indexes are summarized in a composite diagram.

2 Methods

2.1 Maintenance of Ant Farms

Stocks of carpenter ants (Camponotus japonicas) were purchased from Biobiba Ants (Daegu, Korea) and maintained in transparent PET bottles containing autoclaved soil. Ant farm structures were created by inserting a 500-ml PET bottle into a 2000-ml bottle. The larger bottle was cut open to accommodate the smaller one then sealed using transparent tape and the space between the two bottles was filled with autoclaved soil.

Ventilation holes were made by piercing the outer (2000 ml) bottle with a probe smaller than ant body size. Water was supplied through the ventilation holes using a squeezable bottle twice a week. The ant farms were wrapped in aluminum foil and kept in the dark. Ants were regularly fed with sucrose. Three groups of ants were fed a taurine-free or taurine-supplemented diet. Control, low-, and high-taurine diets were created using taurine and sucrose at 0, 5, and 20% (g/g), respectively. The effects of taurine were tested using a combination of ant eusocial indexes. Three indexes of social strength were used to study the effect of taurine on group activity: number of formicaries (chambers) formed, cooperative defense efforts, and population density (or group size and composition).

2.2 Observation of Newly Formed Formicaries

Ant colonies were initiated by implanting 1 queen and 10 workers into the ant farms. The farms were maintained for 30 days and fed with sucrose or a mixture of sucrose and taurine in the concentrations described above. The number of formicaries was recorded daily and newly formed formicaries were circled with a marking pen. The cumulative number of newly made formicaries was calculated every 3 days.

2.3 Assessment of Cooperative Defense Efforts

The level of cooperative defense effort was assessed by recording the time taken to complete the defense formation against a foreign queen. To initiate a defensive response, a foreign queen was carefully added to an established ant farm using a pair of forceps. When soldiers or workers responded to the extraneous queen, the time taken for four workers to pull each leg of the foreign queen was measured. The time taken in hours was converted into reciprocals to obtain the cooperative defense index (CDI). The CDI was calculated as follows: CDI = 1/(hours taken until completion of defense formation).

2.4 Comparison of Population Density

After 30 days, the number of ants and eggs in each group, and the composition of each group were measured. To count the number of ants in each class, the whole ant farm was emptied into a container and the soil was carefully removed. The number of ants was compared between taurine-free and taurine-supplemented groups.

2.5 Composite Strength Index

Composite diagrams for the three indexes of eusocial strength were prepared using a three-dimensional chart by integrating the social data from the three experiments: number of formicaries formed, CDI, and total numbers of ants and eggs. The performance of each group was categorically quantitated using a control performance of 100.

3 Results

Three eusocial strength indexes were measured to examine the effect of taurine on group activity: number of formicaries formed, CDI, and population density (group size and composition).

3.1 Taurine Promoted Construction of Formicaries

Ants began to assemble formicaries 3 days after they were introduced into the ant farms. The number of formicaries in the three different taurine concentrations in sucrose was counted every 3 days. Figure 1 shows the cumulative number of

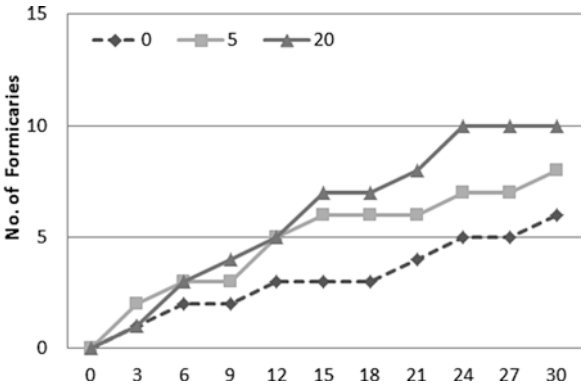


Fig. 1 Formicary Construction Comparison. After 3 days with three different taurine diets: control, low and high [0, 5, and 20% (g/g)], respectively, construction of formicary chambers was monitored. The number of formicary was counted every 3 days up to 30 days since the deployment of ants into the chamber culture bottles. The numbers represent those which were observed on the day of counting. The *x-axis* refers to days after transplantation and *y-axis*, # of formicaries

formicaries up to 30 days post-implantation of individuals into the ant farms. The number of formicaries increased as the taurine content in the ant diet increased. This indicated that taurine prompted ants to build more formicaries and/or that more dynamic activity, stimulated by taurine, resulted in a higher number of formicaries.

3.2 Cooperative Defense Index (CDI) Was Enhanced under Treatment with Taurine

Defensive responses were observed after a foreign queen was introduced. The defensive response began as early as 10 minutes after the implantation of a foreign queen. The cooperative defense index (CDI) was calculated using the reciprocal of the time taken to complete the defensive response, which was several ants holding each leg of the foreign queen. We compared the time taken for the defensive formation between taurine-free and taurine-supplemented groups. There was a significant difference in the time taken to complete the defense formation between the two groups. Taurine-supplemented groups exhibited more rapid response at all taurine concentrations than the control. Accordingly, the taurine-supplemented groups had a higher CDI (Fig. 2). That is, taurine-supplemented groups showed higher levels of readiness against the introduced foreign queen ant. Considering that successful defensive efforts are vital to maintaining a community, taurine may play a significant role in the protection of a eusocial community.

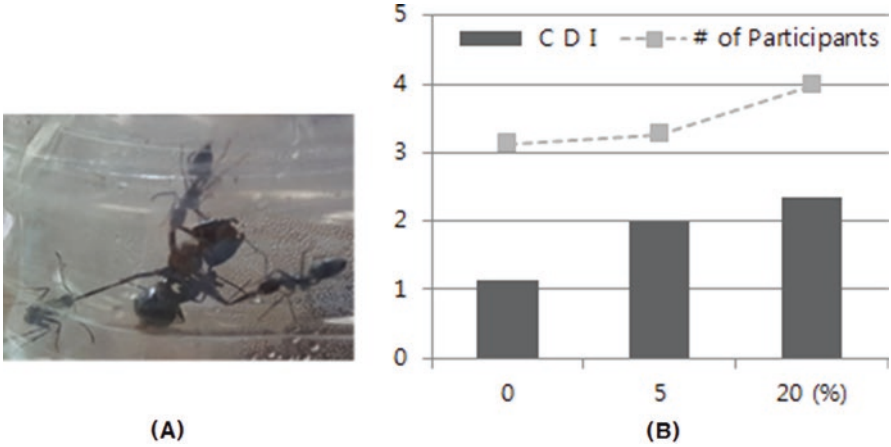
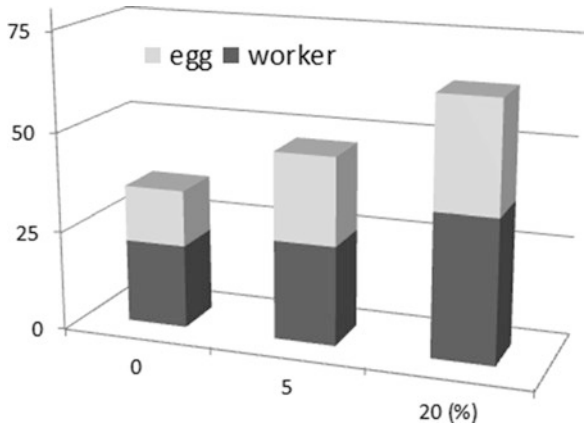


Fig. 2 Cooperative defense (CD) effort. Defensive response was observed immediately after the introduction of a foreign queen. The level of cooperative defense effort was quantitated based on the intensity of readiness against the introduced foreign queen ant. (**a**) Typical image of CD (**b**) CDI and number of participants in the CD at the three different taurine concentrations. CDI = 1/[F] (*F* hours taken for completion of the defense form against a foreign queen)

Fig. 3 Population size and composition. Ant farms were maintained for 30 days by feeding taurine-free or taurine-supplemented diet. At the termination of the experiment, the ant group was evaluated according to the total number and colony composition of worker and egg except for the queen



3.3 Taurine Increased the Size of Ant Community

Ant colonies were initiated using 1 queen and 10 workers and sustained for 30 days. After 30 days, population density and composition was compared among the three taurine-fed groups. There was a significant difference between the groups. Figure 3 shows the population size and composition of each group after 30 days. The high-taurine group had more workers and eggs than did the taurine-free group. There was at least a 40% difference between the taurine-free and the low-taurine group. There was also an obvious difference between high- and low-taurine groups, suggesting the effect was dependent on the concentration of taurine. There was a significant

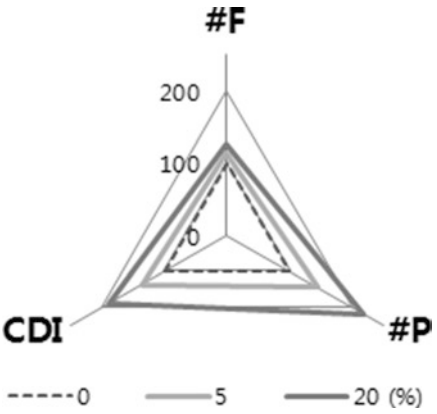


Fig. 4 Integration of the three eusocial indexes. Three indexes in this study were standardized to assess a specific effect of the taurine on the eusociality. *#F* # of formicaries, *#P* population size encompassing workers and eggs, *CDI* cooperative defense index

difference in egg number between taurine-free and taurine-supplemented groups. There was no significant difference between high- and low-taurine groups in the percentage of eggs.

3.4 Composite Eusocial Pattern Indicates the Size of Ant Community Is the most Affected by Taurine

The effects of taurine on eusocial indexes were evaluated using three-dimensional analysis. Three categories of eusocial performance were combined and expressed as a three-dimensional pattern of eusocial strength. The performance of each group was categorically quantitated using a performance control of 100. The most affected aspect was the population size and composition (Fig. 4). The high-taurine group showed the highest numbers of ants and eggs.

4 Discussion

Taurine consistently enabled ant members to enhance their eusocial performance. In the taurine-supplemented groups, ants built more formicaries in a dose-dependent manner. Taurine also enabled ants to respond quickly to a foreign queen and reduce the time taken to complete cooperative defense formations. Taurine augmented the fecundity of the queen ant and the care of offspring by workers. Undoubtedly the positive effects of taurine on all three indexes of group activity are important in sustaining a eusocial community.

Ants have an unusual amount of taurine compared to that in other insects. Ants are typically eusocial insects, and are known as true social insects with complex and diverse biological traits (Thorne and Traniello 2003). The taurine content in ants is

comparable to that in the heart of cow (Bos taurus) and the muscle of tuna (Spitze et al. 2003). The basis for the unusually high taurine content in ants is unknown. The link between taurine and major features of ant sociobiology and physiology has not yet been elucidated; however, taurine may play a major functional role in ants that are members of a eusocial community.

Our results for population size and composition were unexpected. Since the queen is the only reproductive member of the community, her fecundity and capacity to care for the offspring reflect the overall viability of the ant community. The increased number of ants is a strong indicator of the total capacity for reproduction. Taurine may have exerted positive effects both on the queen and workers in producing and caring for offspring despite limited resources in the ant farms.

Variation in taurine content between insect species can explain the prevalence of eusociality in ants, which have very low taurine content. Taurine might have supported specific behaviors during the evolution of eusociality in ants. For example, taurine may have reduced stress from mental and physical exertion in obedience to the queen. In addition, ants, especially workers, perform their assigned roles incessantly. This would be impossible without taurine, which is a powerful antioxidant. Our results imply that ants may utilize taurine to reduce stress from being a member of a eusocial community. Ants were highly responsive to taurine supplements under reduced pressure. Ants with an additional supply of taurine built more formicaries. Also, ant fecundity and population size increased in response to taurine in a dose-dependent manner. Worker ants responded to the invasion of a foreign queen spent in a shorter amount of time when their food was supplemented with taurine.

Eusociality is a highly sophisticated system and a relatively recent development in this group of insects (Hunt 2012; Ratnieks and Helantera 2009). The evolution of eusociality involves an increase in complexity (Wilson and Holldobler 2005). The continual interaction between biological organization and natural selection results in a unique system requiring compulsory collaboration between individuals (Billen 1992). Although the eusocial system is a very efficient system for the survival of a group, it can be very stressful for individual members who must sacrifice their individual reproduction, and commit to the needs of the colony (Robinson 1992). Most members of an ant colony must provide continual labor without any direct individual advantage (Norman and Hughes 2016). Under these circumstances, the unusual amount of taurine in Formicidae may serve to relieve the stress in members of eusocial communities.

To our knowledge, this is the first report of the potential effect of taurine on eusocial organisms characterized using quantitative indexes of eusociality. The results strongly indicate that taurine plays an important role in maintaining ant communities as a eusocial superorganism. Taurine is one the essential biomolecules that sustains eusociality in insects and other higher organisms. Future study is necessary to determine whether taurine helps eusocial organisms increase their eusocial strength.

5 Conclusion

Taurine consistently augmented the level of eusociality in groups of ants fed diets supplemented with taurine. Ants built formicaries in response to taurine in a dose-dependent manner. Taurine also enabled ants to reduce the time required for cooperative defense. Taurine boosted the productivity of queen ants and the care of offspring by workers. This study increases our understanding of eusociality and the contribution of taurine to maintaining eusociality.

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Food Preference of the Elderly for the Development of Taurine-Containing Elderly-Friendly Foods

Mi Ae Bae, So Hyun Park, So Hee Han, Kyung Ja Chang, and Sung Hoon Kim

Abstract The purpose of this study was to investigate food preference of the elderly for the development of taurine-containing elderly-friendly foods (TEF). The subjects in this study were 278 elderly people who were over 65 years old. All data were collected by face to face interview. Statistical analysis was performed by using SPSS 20.0 for Windows. The 'low-salt diet', 'nutritional enriched diet', and 'swallow able diet' were diets that all subjects preferred. All subjects preferred a diet to be sold as three items in separate packaging, as a semi-cooked type. The food types and cooking methods that showed high preference and had a high intention to purchase were cooked rice, porridges, soups and stews, meat side dishes, fish side dishes, kimchi, and vegetable dishes and steaming Among the taurine-containing foods, whip-arm octopus, manila clam, dried anchovy, flatfish, pollack, laver, green laver, sea tangle, seaweed, cod, croaker, and cutlassfish were the preferred foods of most subjects. Elderly females preferred significantly more squid, octopus, eel, mudfish, and sea cucumber than that of elderly males ($p < 0.05$). Elderly males preferred and consumed significantly more taurine-supplement than did elderly females ($p < 0.05$). These results will be used as baseline data for development of a customized TEF for Korean elderly.

Keywords Food preference · Elderly-Friendly · Food development · Taurine · Elderly

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Abbreviations

TEF Taurine-Containing Elderly-Friendly Foods
IPA Importance and Performance Analysis
SE Standard error

1 Introduction

Korea had become an aged society and the elderly population in Korea is rapidly increasing. Furthermore, Korea is expected to become a super-aged society, in which two out of ten people are elderly in 2026 (Korean Statistical Information Service 2016).

The elderly suffer from aging, their teeth are worsened, their chewing ability is lowered, their appetite is reduced, and the secretion of saliva and digestive fluid is reduced, making it difficult to digest (Park and Lee 2006). Foods for the elderly needs to be developed in an easy to eat form considering these changes. However, there is a lack of dietary support that is directly related to health problems.

Taurine is known to play a variety of positive roles in the human body, especially, cognitive function development (Kim et al. 2014). In addition, research has shown that the intake of foods containing a high level of taurine, such as fish and shellfish, has a positive effect on current cognitive function (Bae et al. 2017a). Prevention from dementia along with its management is very important. Thus, it is necessary to actively recommend the intake of foods containing a high level of taurine, which is effective in preventing dementia.

Home meal replacement that is customized to consumer needs and sociocultural environments are being actively developed, but the development of elderly-friendly foods is insufficient. The number of elderly who are alone due to marriage of their children and/or the death of a spouse is increasing, and they are in need of elderly-friendly foods because they can find it difficult to pre-pare food alone. However, Korea is still in its early stages of developing such foods due to a lack of awareness about elderly-friendly foods and the associated industry (Shin et al. 2016).

Therefore, in this study, we investigated some basic data that can reflect the preferences of the elderly as they relate to the future development of TEF.

2 Methods

2.1 Subjects

The subjects of this study were 278 the elderly (men 76, women 202) and aged over 65 years who were attending welfare facilities for the elderly and senior community centers in Incheon, Korea. The subjects voluntarily participated in the study after listening to a detailed explanation of the study by the researcher.

2.2 Data Collection

The data included in this study were collected between January and March 2018 by the researcher. Data collection was accomplished during the conduct of 1:1 face-to-face interview by using a questionnaire.

The general characteristics were examined by assessing the 'Yes' and 'No' responses to four items and five items, respectively. The factors included gender, age, family members, and the person who prepares the meal, participation in food purchase, monthly food purchasing cost, and chewing ability.

Interest in dementia was evaluated by scoring responses on a 5 – point scale. Also, the knowledge level of dementia and taurine were assessed by asking 16 and 10 questions, respectively, and the responses were evaluated by assigning 1 point for the correct answer, and 0 points for the wrong answer. High scores indicated that the subject had a high knowledge of dementia or taurine.

The necessity and intention to purchase by food type and recipe of TEF were examined by applying a 5 – point scale (1 point: never unnecessary, 5 points: very necessary). In this part, the researcher initially provided a detailed explanation of the topic to those subjects who did not know about taurine and then asked the questions.

Finally, the preference for and intake frequency of foods containing a lot of taurine were evaluated scoring by 'Like' or 'Not like' and by using a 5 – point scale, respectively (Bae et al. 2017a).

2.3 Statistical Analysis

The statistical analysis of all data was conducted by using the SPSS 20.0 program. The values were expressed as percentages or means ± standard errors (SE). Statistical significance was decided by applying the Student's t-test, and the significance of differences was assessed at $p < 0.05$.

3 Results and Discussion

3.1 General Characteristics of the Subjects

The general characteristics of the study subjects are summarized in Table 1. Among the subjects, the numbers of male and female elderly were 76 (27.3%) and 202 (72.7%), respectively. With regard to age, the proportion of subjects 65–69 years old in the total was 50.4%, slightly more than half.

The dietary intake and nutritional status of the elderly vary depending on whether the person eats with others or alone, whether the person buys the food, and whether

Table 1 General characteristics of the subjects

Variable	Classification	Total (N = 278)	Male (N = 76)	Female (N = 202)
Gender	Male	76(27.3)		
	Female	202(72.7)		
Age	65–69	140(50.4)	47(61.8)	93(46.0)
	70–79	84(30.2)	21(27.6)	63(31.2)
	80≤	54(19.4)	8(10.5)	46(22.8)
Family members	Alone	59(21.2)	5(6.6)	54(26.7)
	With spouse	81(29.1)	33(43.4)	48(23.8)
	With children	42(15.1)	7(9.2)	35(17.3)
	With spouse and children	80(28.8)	28(36.8)	52(25.7)
	Other	16(5.7)	3(3.9)	13(6.5)
Person who prepares a meal	Myself	183(65.8)	10(13.2)	173(85.6)***
	Spouse	67(24.1)	64(84.2)	3(1.5)
	Children	23(8.2)	1(1.3)	22(10.9)
	Other	5(1.8)	(1.3)	4(2.0)
Participation in food purchase	Always	171(61.5)	8(10.5)	163(80.7)***
	Sometimes	74(26.6)	54(71.1)	20(9.9)
	Rarely	33(11.9)	14(18.4)	19(9.4)
Food purchasing cost (per month)	Less than 100,000 won	27(9.7)	2(2.6)	25(12.4)
	100,000~⟨300,000won	58(20.9)	13(17.1)	45(22.3)
	300,000~⟨500,000won	87(31.3)	24(31.6)	63(31.2)
	500,000~⟨700,000won	45(16.2)	16(21.1)	29(14.4)
	700,000~⟨1,000,000won	35(12.6)	13(17.1)	22(10.9)
	More than 1,000,000 won	26(9.4)	8(10.5)	18(8.9)
Chewing ability	Very good	138(49.6)	36(47.4)	102(50.5)
	Good	106(38.1)	31(40.8)	75(37.1)
	Normal	24(8.6)	8(10.5)	16(7.9)
	Bad	8(2.9)	1(1.3)	7(3.5)
	Very bad	2(0.7)	0(0.0)	2(1.0)

N(%), ***$p < 0.001$ by Chi-squared test

the person cooks (Hong and Choi 1996; Lee and Chang 1999; Son et al. 1996). In the case of family members, most (73.0%) subjects lived with more than one family member with, only 21.2% living alone. Elderly males lived with their spouse (43.4%), with their spouse and children (36.8%), or alone (6.6%). In contrast, 26.7% of elderly females lived alone; there was a significant difference in eating with others or alone between the genders ($p < 0.001$).

More than half (65.8%) of all subjects said that they prepared their own meals. While elderly males were mostly provided with a meal prepared by their spouse (84.2%), elderly females typically prepared their own meals (85.6%); there was a significant difference in meal preparation between genders ($p < 0.001$).

Table 2 Interest in and knowledge about dementia and taurine among the subjects

Variable	Total (N = 278)	Male (N = 76)	Female (N = 202)
Interest in dementia	4.0 ± 0.1	3.7 ± 0.1	4.1 ± 0.1∗
Knowledge about dementia	9.6 ± 0.2	9.5 ± 0.3	9.7 ± 0.2
Knowledge about taurine	4.0 ± 0.2	3.8 ± 0.3	4.1 ± 0.2

Mean ± SE, ∗$p < 0.05$ by Student's t-test

With regard to food purchase, more than half (61.5%) of all subjects always participated in the purchase of food. However, there was a significant difference depending on gender ($p < 0.001$) with 71.1% of elderly males sometimes participating in food purchase, while 80.7% of elderly females always participated in food purchase.

The average monthly food purchasing cost for all subjects was the highest (31.3%) in 300,000 to 500,000won category more than half (87.7%) of all subjects responded that they were very good (49.6%) or good (38.1%) at chewing ability.

3.2 Interest in and Knowledge about Dementia and Taurine

Interest in dementia was high at 4 out of 5 points, and especially high among elderly females (4.1 points) compared to that for elderly males (3.7 points) ($p < 0.05$). On the other hand, the average level of knowledge about dementia was 9.6 out of 16 points, and it was not significantly different between the genders. In addition, the average level of knowledge related to taurine was 4 out of 10 points. The higher the knowledge related to dementia, the more positive the attitude toward preventing dementia (Park et al. 2018). Thus, it was thought that understanding of dementia and taurine is effective in preventing dementia. It was expected to have a direct effect on the preference for and purchase of TEF. To that end, increased education and public awareness will be needed (Table 2).

3.3 Preferences for Development of TEF

As shown in Table 3, the 'low-salt diet' (4.2 points), 'nutritional enriched diet' (4.3 points), and 'swallow able diet' (4.2 points) were diets that all the subjects highly preferred. In particular, the 'swallow able diet' was significantly more preferred among elderly females (4.3 points) than in elderly males (3.9 points) ($p < 0.01$). In previous study, taste, nutrition, hygiene, and swallow able food were preferred when developing elderly-friendly foods (Shin et al. 2016). In particular, protein foods which are a source of taurine are expected to need further development in diets which are easy to be consumed by the elderly.

Table 3 Preferences for development of TEF among subjects

Variable	Classification	Total (N = 278)	Male (N = 76)	Female (N = 202)
Expected diet type	Low-salt diet	4.2 ± 0.1[a]	4.2 ± 0.1	4.3 ± 0.1
	Low-sweet diet	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
	Nutritional enriched diet	4.3 ± 0.0	4.1 ± 0.1	4.3 ± 0.1
	Swallowable diet	4.2 ± 0.1	3.9 ± 0.1	4.3 ± 0.1**
Expected packaging unit	One time	72(25.9)[b]	14(18.4)	58(28.7)
	Three times	111(39.9)	35(46.1)	76(22.8)
	Five times	60(21.6)	14(18.4)	46(22.8)
	Ten times	24(8.6)	10(13.2)	14(6.9)
	More than	11(4.0)	3(3.9)	8(4.0)
Expected cooking type	Cooked type	86(30.9)	28(36.8)	58(28.7)
	Semi-cooked type	148(53.2)	37(48.7)	111(55.0)
	Washed food type	29(10.4)	8(10.5)	21(10.4)
	Raw material food type	13(4.7)	3(3.9)	10(5.0)
	Etc.	2(0.7)	0(0.0)	2(1.0)

[a]Mean ± SE, **p < 0.01 by Student's t-test
[b]N (%)

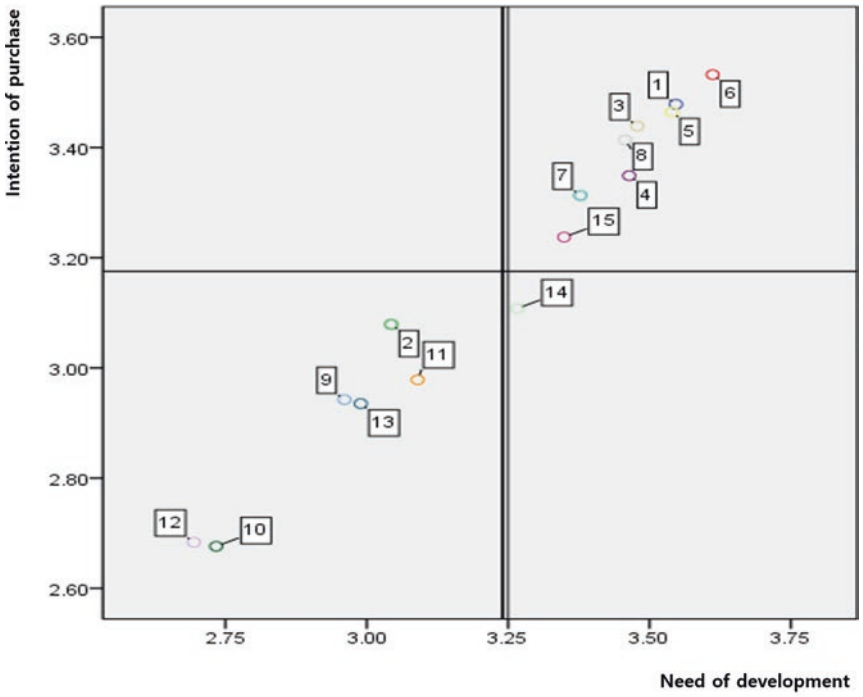
All subjects preferred that a TEF be sold as three items in a packaging unit, as a semi-cooked type. In previous studies, there was a preference for food in a semi-cooked state that can be eaten immediately after purchase (An and Jang 2018; Park et al. 2012).

3.4 Development Needs and Intention to Purchase for Food Type and Cooking Method of TEF

Based on the results of our importance and performance analysis (IPA), in the relationship between development needs and intentions to purchase there were no food or recipe items in quadrant I, which shows items associated with a high purchase intention and a low need.

The TEF types and cooking methods that showed high scores for both need and intention to purchasing (quadrant II) were cooked rice, porridges, soups and stews, meat side dishes, fish side dishes, kimchi, vegetable dishes, and steaming. In particular, although not shown in Fig. 1, 'steaming cooking method' was significantly more preferred by elderly females than by elderly males ($p < 0.05$).

The items in quadrant III, which indicated low needs and low purchase intention included noodle, snacks, roasting, stir-frying, beverages, and frying. Among them, vegetables and frying methods were considered to be excluded from TEF development because they had low scores. Similar to results in previous study of the elderly,



I : Low need and High intention of purchase

II : High need and High intention of purchase

1 Cooked rice
3 Porridges
4 Soups and stews
5 Meat side dishes
6 Fish side dishes
7 Kimchi
8 Vegetable side dishes
15 Steaming

III : Low need and Low intention of purchase

2 Noodle
9 Snacks
11 Roasting
13 Stir-frying

10 Beverages
12 Frying

IV: High need and Low intention of purchase

14 Boil

Fig. 1 Importance and performance analysis of relationships between the need for development and the intention of purchase for food types and recipes of TEF among study subjects

there was a preference for recipes for soft such as those obtained by steaming rather than cooked with a lot of oil such as that involving frying of food items (Bae et al. 2017b).

Quadrant IV with a low purchase intention and high development need included a single recipe prepared by boiling.

3.5 Preference and Intake Frequency of Taurine–Containing Foods

Table 4 summarized the preferences for several taurine-containing foods, and more than 90% of the subjects preferred long-arm octopus, oyster, abalone, manila clam, crab, dried anchovy, flatfish, pollack, dried laver, green laver, sea tangle, seaweed, cod, yellow croaker, and hairtail. Compared to elderly males, elderly females preferred significantly more sea tangle ($p < 0.05$), while elderly males preferred significantly more taurine supplement (as an energy drink), taurine supplement (in tablet form), octopus, eel, mudfish, and sea cucumber than that preferred by elderly females ($p < 0.05$).

With regard to the frequency of intake of taurine-containing foods, dried anchovy, dried laver, and sea tangle were consumed relatively frequently in all subjects. In particular, elderly males consumed more oyster, taurine supplement, squid, cuttlefish, octopus, pacific saury, skate, eel, sea cucumber, and warty sea squirt than that consumed by elderly females ($p < 0.05$). On the other hand, compared to elderly males, elderly females consumed more dried anchovy and sea tangle ($p < 0.05$).

The previous study of elderly people with dementia and normal elderly people showed a high level of preference for soft and white seafood such as croaker, hairtail, mackerel, oysters, octopus, and shrimp (Bae et al. 2017a).

4 Conclusion

In Korea, the rapidly aging population is expected to result in an expansion in the availability of elderly-friendly foods in markets; thus, it is necessary to develop elderly-friendly foods which are practical and customized to the needs of the elderly. The results of this study should be used as baseline data during the development of TEF suitable for elderly Koreans.

Table 4 Preference and intake frequency of taurine-containing foods among study subjects

Taurine-containing foods		Preference[a]			Intake frequency[b]		
		Total (N = 278)	Male (N = 76)	Female (N = 202)	Total (N = 278)	Male (N = 76)	Female (N = 202)
High taurine-content food (≥500 mg taurine/100 g)	Webfoot octopus	88.5	94.7	86.1	2.3 ± 0.0	2.4 ± 0.1	2.3 ± 0.1
	Long-arm octopus	91.0	94.7	89.6	2.4 ± 0.0	2.5 ± 0.1	2.3 ± 0.1
	Oyster	90.6	92.1	90.1	2.5 ± 0.0	2.7 ± 0.1	2.5 ± 0.1*
	Abalone	90.3	94.7	88.6	2.2 ± 0.0	2.3 ± 0.1	2.2 ± 0.1
	Manila clam	91.7	92.1	91.6	2.6 ± 0.0	2.6 ± 0.1	2.5 ± 0.1
	Mussel	87.4	90.8	86.1	2.3 ± 0.0	2.5 ± 0.1	2.3 ± 0.1
	Whelk	79.9	81.6	79.2	2.1 ± 0.1	2.2 ± 0.1	2.0 ± 0.1
	Crab	90.3	86.8	91.6	2.4 ± 0.0	2.3 ± 0.1	2.5 ± 0.1
	Dried anchovy	94.2	90.8	95.5	3.5 ± 0.1	3.1 ± 0.1	3.7 ± 0.1*
	Dried shrimp	82.4	76.3	84.7	2.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
	Taurine supplement (energy drink)	51.4	63.2	47.0*	1.9 ± 0.1	2.2 ± 0.1	1.7 ± 0.1*
	Taurine supplement (tablet)	39.9	50.0	36.1*	1.3 ± 0.0	1.5 ± 0.1	1.3 ± 0.0*
Medium taurine-content food (100~499.9 mg taurine/100 g)	Squid	80.6	96.1	74.8	2.5 ± 0.0	2.8 ± 0.1	2.4 ± 0.1*
	Cuttlefish	74.8	82.9	71.8	1.8 ± 0.0	2.0 ± 0.1	1.7 ± 0.1*
	Octopus	80.6	89.5	77.2*	2.0 ± 0.0	2.1 ± 0.1	1.9 ± 0.1*
	Mackerel	87.1	92.1	85.1	2.9 ± 0.1	3.0 ± 0.1	2.9 ± 0.1
	Spanish makerel	84.2	86.8	83.2	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
	Pacific saury	83.1	89.5	80.7	2.5 ± 0.1	2.8 ± 0.1	2.4 ± 0.1*
	Atka mackerel	77.7	85.5	74.8	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
	Anglerfish	82.0	84.2	81.2	2.1 ± 0.0	2.3 ± 0.1	2.1 ± 0.1
	Skate	75.5	80.3	73.8	1.9 ± 0.0	2.1 ± 0.1	1.9 ± 0.1*
	Flatfish	91.0	89.5	91.6	2.4 ± 0.0	2.4 ± 0.1	2.4 ± 0.1
	Pollack	93.2	94.7	92.6	2.7 ± 0.0	2.7 ± 0.1	2.6 ± 0.1
	Cockle	89.6	92.1	88.6	2.3 ± 0.0	2.4 ± 0.1	2.3 ± 0.1
	Short-necked clam	84.5	77.6	87.1	2.1 ± 0.0	2.0 ± 0.1	2.2 ± 0.1
	Shrimp	88.8	89.5	88.6	2.5 ± 0.0	2.4 ± 0.1	2.5 ± 0.1
	Dried laver	97.8	97.4	98.0	4.0 ± 0.1	3.9 ± 0.1	4.1 ± 0.1

(continued)

Table 4 (continued)

Taurine-containing foods		Preference[a]			Intake frequency[b]		
		Total (N = 278)	Male (N = 76)	Female (N = 202)	Total (N = 278)	Male (N = 76)	Female (N = 202)
Low taurine-ContentFood (<100 mg taurine/100 g)	Butterfish	82.0	82.9	81.7	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
	Eel	80.9	92.1	76.7**	2.1 ± 0.0	2.3 ± 0.1	2.0 ± 0.1*
	Mudfish	76.6	88.2	72.3**	2.1 ± 0.0	2.3 ± 0.1	2.1 ± 0.1
	Sea cucumber	82.0	90.8	78.7*	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.1*
	Warty Sea squirt	74.1	75.0	73.8	2.1 ± 0.0	2.3 ± 0.1	2.1 ± 0.1*
	Green laver	91.4	90.8	91.6	2.6 ± 0.1	2.5 ± 0.1	2.7 ± 0.1
	Sea tangle	92.8	86.8	95.0*	3.1 ± 0.1	2.9 ± 0.1	3.2 ± 0.1*
	Seaweed	92.8	89.5	94.1	2.9 ± 0.1	2.8 ± 0.1	3.0 ± 0.1
	Cod	91.4	93.4	90.6	2.2 ± 0.0	2.4 ± 0.1	2.2 ± 0.1
	Yellow corvine	94.2	94.7	94.1	2.6 ± 0.0	2.6 ± 0.1	2.7 ± 0.1
	Hairtail	95.0	94.7	95.0	2.6 ± 0.0	2.7 ± 0.1	2.5 ± 0.1
	Agar-agar	65.1	64.5	65.3	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
	Dried squid	71.9	65.8	74.3	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.1

[a]%, $*p < 0.05$, $**p < 0.01$ by Chi-squared test
[b]Mean ± SE, $*p < 0.05$ by Student's t-test

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Laxative Effects of Taurine on Loperamide-Induced Constipation in Rats

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Abstract In the present study, we investigated the laxative effects of taurine in a rat model of loperamide-induced constipation. Rats were divided into six groups of six animals each: normal (NOR), control (CON), loperamide + Dulcolax (5.5 mg/kg, p.o.), and loperamide + various doses of taurine (7.5, 15, and 30 mg/kg, p.o.). Laxative activity was determined based on body weight, feeding characteristics, fecal properties, gastrointestinal transit (GIT) ratio, and the levels of serum gastrointestinal hormones. Taurine supplementation significantly increased the number, wet weight, and water content of fecal pellets in rats with loperamide-induced constipation. GIT ratio and loperamide-induced serum metabolic parameters, such as gastrin (GAS), motilin (MTL), and somatostatin (SS) significantly changed after supplementation with taurine in loperamide-induced constipated rats. We suggest that taurine had a potent effect against loperamide-induced constipation in part by increasing gastrointestinal motility.

Keywords Constipation · Laxative effects · Loperamide · Taurine

Abbreviations

CGRP	calcitonin gene related peptide
GAS	gastrin
GIT	gastrointestinal transit
MTL	motilin
SS	somatostatin

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is a major intracellular free β-amino acid and it is the most abundant in blood cells and mammalian tissues, including the brain, and skeletal and cardiac muscles (Hansen 2001). Taurine is involved in several important biological functions, such as detoxification, osmoregulation, thermoregulation, membrane stabilization, anti-oxidative, and anti-apoptotic properties (Hansen 2001; Çetiner et al. 2005). Furthermore, taurine plays an important protective role against diabetic complications, atherosclerosis, and gastrointestinal damage (Balkan et al. 2002; Çetiner et al. 2005). Constipation is one of the most frequent functional gastrointestinal disease and often caused by inadequate fluid intake, fiber deficiency, metabolic imbalance, and medication (Chang et al. 2010). The main symptoms of chronic constipation include difficulty in defecation, infrequent bowel movements, reduced the quantity of feces, and dry stools (McCallum et al. 2009). Many therapeutic approaches, including plenty of fluids, enema, fiber supplement in the diet, and stimulants, are used to treat constipation (Fukudo et al. 2011). Currently, several chemical drugs, such as bulking agents and secretory or osmotic laxatives, are used to improve constipation. However, their applications are limited because of their high expenditures and several side effects, such as bloating, cramps, and pain (Ford and Suares 2011). Therefore, therapeutic interventions with no side effects are needed to prevent or treat chronic constipation in order to improve the quality of patients' life. On the contrary, loperamide, a synthetic piperidine derivative, has often been used to induce constipation in many studies to identify novel compounds with therapeutic effects against constipation (Wintola et al. 2010; Lee et al. 2012). In several animal models, constipation can effectively be induced by treatment with 1.5–3 mg/kg of loperamide for 3–7 days because of a delay in intestinal luminal transit through inhibition of smooth movement and water secretion in the intestinal wall (Yamada and Onoda 1993; Lee et al. 2012). Therefore, the present study was conducted to investigate the laxative potentials of taurine in loperamide-induced constipated rats.

2 Methods

2.1 Materials

Taurine, carmine, and loperamide hydrochloride was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All reagents used were of analytical grade.

2.2 Animals

Thirty-six male Sprague-Dawley rats (5 weeks old) were purchased from SamTacho (Osan, Korea). The animals were housed at an animal room with controlled temperature (23 ± 2 °C) and humidity (55 ± 10%) under a 12-h light/dark cycle. All rats

were provided with standard irradiated chow diet (Purina Mills, Seoungnam, Korea) and tap water *ad libitum.* All animals were treated in accordance with the Guidelines for Care and Use of Laboratory Animals of Chonnam National University, Yeosu, Republic of Korea.

2.3 Induction of Constipation in the Rats

After 1 week of acclimatization to pelleted commercial diet, the rats were randomly divided into six treatment groups, and provided *ad libitum* access to water and AIN-76A basal diets. Constipation was induced in the animals by daily oral administration of 1 mL loperamide (4 mg/kg in 0.9% sodium chloride) at 09:00 and 18:00 for 14 days, whereas the control rats were administered normal saline only. Taurine was administered at 7.5, 15, or 30 mg/kg, p.o.) daily at 10:00 (1 h after loperamide administration) during the experimental period. The latter group was treated with Dulcolax (5.5 mg/kg, p.o.) as a standard drug. We measured daily food intake and body weight throughout the experiment. Feed efficiency ratio (FER) was calculated throughout the treatment period by dividing the dietary intake amount by body weight gain within the same period.

2.4 Number, Weight, and Water Content of Fecal Pellets

Number and wet weight of fecal pellets were measured every day during the experiment. Water content was determined by drying the fecal pellets at 70 °C for 24 h in an oven and calculating the difference in weight between before and after drying. Water content of fecal pellets was calculated as follows: Fecal water content (%) = [(fecal wet weight – fecal dry weight)/fecal wet weight] × 100.

2.5 Gastrointestinal Transit (GIT) Ratio

GIT ratio was measured according to the method of Nakagura et al. (1996), with minor modifications. Briefly, animals were fasted for 18 h prior to the experiment, but they consumed water *ad libitum*. On the 15th day, the rats were orally administered 1 ml of carmine (3 g suspended in 50 mL of 0.5% carboxymethylcellulose) as a marker. After 30 min, the rats were sacrificed and then the small intestines were quickly removed. The heart, kidneys, liver, and fat pad were rapidly removed and washed in saline buffer, placed in cryovials, weighed, and immediately stored in liquid nitrogen. Blood was collected in appropriate tubes using a syringe via the aorta of rats under ether anesthesia, and then centrifuged at 2000 × *g* for 20 min. Serum was separated and stored at −80 °C until further analyses. The distance over

which carmine had moved and the total length of the small intestine were measured. GIT ratio was expressed as the percentage of the distance traveled by carmine relative to the total length of the small intestine.

2.6 Assessment of Serum GAS, MTL, SS, and CGRP

The concentrations of gastrin (GAS), motilin (MTL), somatostatin (SS), and calcitonin gene related peptide (CGRP) in the serum were estimated by ELISA using commercially available kits.

2.7 Statistical Analysis

All data are presented as the mean ± standard deviation (SD), and statistical analyses were carried out using the Statistical Analysis System version 8.0 (SAS Institute, Cary, NC, U.S.A). Multigroup comparisons were conducted by one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. Values of $P < 0.05$ were considered statistically significant.

3 Results

3.1 Body Weight, Food Intake, and Food Efficiency

In the present study, final body weight and body weight gain after constipation induction were significantly higher in the normal control group than the other groups (Table 1). However, there was no significant difference in body weight and food intake among the experimental groups during the experiment period, although the control group showed a slightly lower food intake than the other groups. Moreover, FER was not significantly different among the experimental groups. These results indicated that taurine supplementation did not alter food intake and FER.

3.2 Effect of Taurine on Organ Weight of Constipated Rats

In the present study, constipated rats were treated with Dulcolax, a standard drug for constipation, or taurine. The weights of the organs, including the liver, kidney, and heart, were not significantly difference between the groups, as shown in Table 2.

Table 1 Effects of taurine on body weight gain, food intake, and food efficiency ratio in loperamide-induced constipated rats

	Groups					
	NOR	CON	LTS	MTS	HTS	DS
Initial body weight (g)	78.2 ± 1.5^{NS}	78.0 ± 3.7	77.3 ± 3.3	76.8 ± 3.3	77.0 ± 3.7	76.7 ± 0.9
Final body weight (g)	215.3 ± 3.5^{a}	193.0 ± 10.3^{b}	184.8 ± 6.4^{b}	187.5 ± 6.3^{b}	176.0 ± 2.9^{b}	183.7 ± 7.6^{b}
Body weight gain (g)	156.6 ± 14.1^{a}	141.6 ± 10.7^{b}	132.5 ± 6.5^{b}	136.8 ± 5.8^{b}	124.3 ± 1.5^{b}	131.3 ± 7.8^{b}
Food intake (g/day)	18.4 ± 1.5^{NS}	15.3 ± 1.6	17.3 ± 1.3	17.0 ± 1.2	18.5 ± 0.9	15.0 ± 0.8
FER[a]	0.55 ± 0.01^{NS}	0.56 ± 0.03	0.55 ± 0.02	0.56 ± 0.02	0.53 ± 0.02	0.55 ± 0.01

NOR normal diet group, *CON* constipation group induced by loperamide (4 mg/kg, *LTS* low-dose taurine (7.5 mg/kg, p.o.) and loperamide-treated group, *MTS* medium-dose taurine (15 mg/kg, p.o.) and loperamide-treated group, *HTS* high-dose taurine (30 mg/kg, p.o.) and loperamide-treated group, *DS* Dulcolax (5.5 mg/kg, p.o.) and loperamide-treated group. Values are means ± SD (n = 6). *NS* not significantly different among groups. [a,b]Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparison test.[a]*FER* food efficiency ratio = body weight gain (g)/food intake (g)

[a]*FER* food efficiency ratio = body weight gain (g)/food intake (g)

Table 2 Effect of taurine supplementation on organ weight of constipated rats

	Groups					
	NOR	CON	LTS	MTS	HTS	DS
Liver	8.50 ± 0.64^{NS}	7.91 ± 1.10	7.23 ± 0.78	6.78 ± 0.39	6.59 ± 0.17	7.45 ± 1.09
Kidney	2.15 ± 0.23^{NS}	2.02 ± 0.13	1.80 ± 0.04	1.80 ± 0.17	1.73 ± 0.11	1.87 ± 0.18
Heart	0.96 ± 0.16^{NS}	0.98 ± 0.06	0.87 ± 0.07	0.88 ± 0.09	0.82 ± 0.08	0.89 ± 0.03

NOR normal diet group, *CON* constipation group induced by loperamide (4 mg/kg, *LTS* low-dose taurine (7.5 mg/kg, p.o.) and loperamide-treated group, *MTS* medium-dose taurine (15 mg/kg, p.o.) and loperamide-treated group, *HTS* high-dose taurine (30 mg/kg, p.o.) and loperamide-treated group, *DS* Dulcolax (5.5 mg/kg, p.o.) and loperamide-treated group. Values are means ± SD (n = 6). *NS* not significantly different among groups

Table 3 Effects of taurine on number, wet weight, and water content of fecal pellets in loperamide-induced constipated rats

	Groups					
	NOR	CON	LTS	MTS	HTS	DS
Number of fecal pellets (count/day)	22.6 ± 5.3^{a}	13.4 ± 2.3^{b}	16.8 ± 3.3^{ab}	16.9 ± 5.2^{ab}	17.5 ± 4.9^{ab}	15.4 ± 4.6^{ab}
Wet weight of fecal pellet (g/day)	1.44 ± 0.06^{a}	0.93 ± 0.06^{b}	1.13 ± 0.07^{b}	1.33 ± 0.05^{ab}	1.50 ± 0.05^{a}	1.23 ± 0.10^{b}
Water content of fecal pellet (%)	21.8 ± 3.9^{a}	14.1 ± 0.6^{b}	19.5 ± 1.3^{ab}	19.3 ± 1.0^{ab}	21.9 ± 0.7^{ab}	19.1 ± 2.6^{ab}

NOR normal diet group, *CON* constipation group induced by loperamide (4 mg/kg, *LTS* low-dose taurine (7.5 mg/kg, p.o.) and loperamide-treated group, *MTS* medium-dose taurine (15 mg/kg, p.o.) and loperamide-treated group, *HTS* high-dose taurine (30 mg/kg, p.o.) and loperamide-treated group, *DS* Dulcolax (5.5 mg/kg, p.o.) and loperamide-treated group. Values are means ± SD (n = 6). ^{a,b}Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparison test

3.3 Effects of Taurine on Fecal Parameters in Loperamide-Induced Constipated Rats

To examine the effects of taurine on the number, wet weight, and water content of fecal pellets, constipation was induced by loperamide in rats, and the rats were then supplemented taurine for 14 days. The number, wet weight, and water content of fecal pellets are shown in Table 3. Number of fecal pellets in the constipation control (CON) group (13.4 count/day) was significantly higher than the normal diet (NOR) group (22.6 count/day), suggesting the establishment of loperamide-induced constipation. After constipation was induced, the number and water content of fecal pellets in the low-dose taurine (LTS), medium-dose taurine (MTS), high-dose taurine (HTS), and Dulcolax (DS) groups tended to increase more than in the CON group. Similarly, wet weight of fecal pellets in the CON group was markedly

decreased more than in the NOR group after induction of constipation. On the contrary, after induction of constipation, wet weight of fecal pellets was significantly increased by supplementation with taurine in a dose-dependent manner. Especially, treatment with HTS was observed to increase the wet weight of fecal pellets more than the by normal range (1.50 g/day in HTS vs. 1.44 g/day in NOR).

3.4 *Effect of Taurine on GIT Ratio in Loperamide-Induced Constipated Rats*

In this study, gastrointestinal motility markedly decreased in the constipated rats, as shown in Table 4. On the contrary, supplementation with taurine or Dulcolax tended to increase transit distance and GIT ratio in constipated rats. Especially, the transit distance and GIT ratio of the constipated rats were normalized following supplementation with the high-dose taurine. These results showed that taurine was a useful material for ameliorating constipation.

3.5 *Effect of Taurine on Colonic Motility Index in Loperamide-Induced Constipated Rats*

As shown in Fig. 1, serum levels of GAS and MTL in the CON group were significantly lower than in the NOR group. On the contrary, serum GAS and MTL levels were markedly higher in taurine-supplemented rats than in the CON group, in a dose-dependent manner. Serum SS level in the CON group was significantly higher than in the NOR, LTS, and MTS groups. Serum CGRP level was markedly higher

Table 4 Gastrointestinal transit ratio following supplementation with taurine in loperamide-induced constipated rats

	Gastrointestinal motility (during 2 h)		
	Total small intestine length (cm)	Transit distance (cm)	Gastrointestinal transit ratio (%)
NOR	118.7 ± 5.4^{NS}	92.4 ± 6.9^{ab}	77.8 ± 3.7^{a}
CON	117.8 ± 4.7	78.6 ± 4.2^{b}	66.7 ± 3.3^{b}
LTS	113.1 ± 6.8	81.0 ± 8.3^{b}	71.5 ± 4.7^{ab}
MTS	122.6 ± 5.2	89.8 ± 6.3^{ab}	73.3 ± 6.7^{ab}
HTS	122.9 ± 9.8	98.0 ± 10.8^{a}	79.7 ± 5.6^{a}
DS	120.7 ± 4.0	85.8 ± 2.4^{ab}	71.2 ± 4.4^{ab}

NOR normal diet group, *CON* constipation group induced by loperamide (4 mg/kg, *LTS* low-dose taurine (7.5 mg/kg, p.o.) and loperamide-treated group, *MTS* medium-dose taurine (15 mg/kg, p.o.) and loperamide-treated group, *HTS* high-dose taurine (30 mg/kg, p.o.) and loperamide-treated group, *DS* Dulcolax (5.5 mg/kg, p.o.) and loperamide-treated group. Values are means ± SD (n = 6). *NS* not significantly different among groups. [a,b]Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparison test

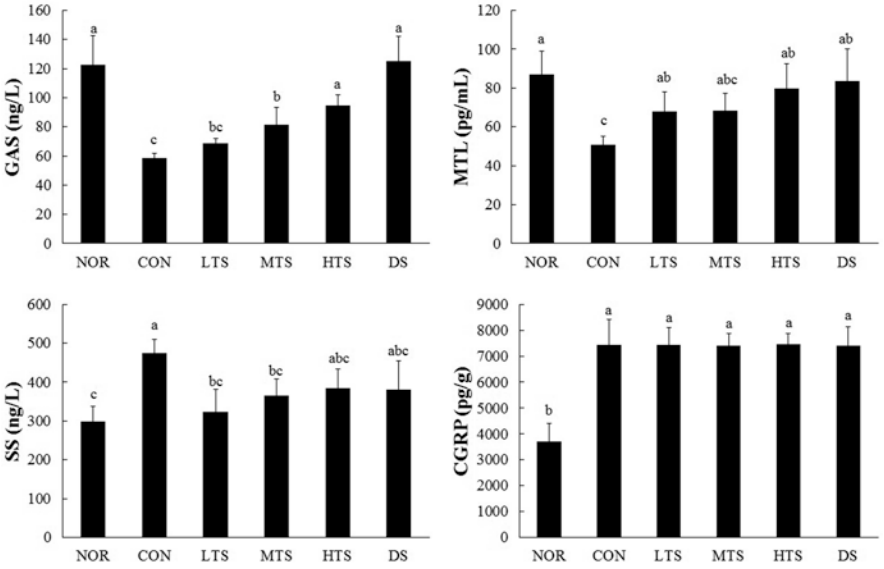

Fig. 1 Colonic motility index function following supplementation with taurine in loperamide-induced constipated rats. *NOR* normal diet group, *CON* constipation group induced by loperamide (4 mg/kg, *LTS* low-dose taurine (7.5 mg/kg, p.o.) and loperamide-treated group, *MTS* medium-dose taurine (15 mg/kg, p.o.) and loperamide-treated group, *HTS* high-dose taurine (30 mg/kg, p.o.) and loperamide-treated group, *DS* Dulcolax (5.5 mg/kg, p.o.) and loperamide-treated group. Values are means ± SD (n = 6). NS: not significantly different among groups. Values not sharing a common letter are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparison test. The concentrations of gastrin (GAS), motilin (MTL), somatostatin (SS), and calcitonin gene related peptide (CGRP) in the serum were estimated by ELISA using commercially available kits

in the CON group than in the NOR group; however, taurine supplementation did not affect serum CGRP level in loperamide-induced constipated rats.

4 Discussion

Constipation is a well-known common gastrointestinal disorder characterized by several symptoms, such as poor bowel movements and difficulty in defecation (Wintola et al. 2010). It is often caused by inadequate fluid and dietary fiber intakes, decreased physical activity, hypothyroidism, and side effects of medical drugs (Leung 2007). In general, loperamide is widely used to induce constipation in animal models. It can delay intestinal luminal transit and induce prolonged duration of stool evacuation (Han et al. 2017). Therefore, in the present study, we studied the therapeutic effects of taurine on loperamide-induced constipated rats. In this study, final body weight and body weight gain of the normal group were significantly higher than those of the other groups after induction of constipation. However, there

was no significant difference in body weight, organ weight, food intake, and FER among the experimental groups during the experimental period. These results indicated that Dulcolax, a standard drug for treatment of constipation, taurine, and loperamide did not alter food intake, FER, and organ weight. Similarly, Yan et al. (2017) reported that food intake is markedly lower in constipated Sprague-Dawley rats than in the non-constipation group; however, loperamide treatment does not alter body weight.

Several studies considered that a significant reduction in fecal excretion is one of the major markers of constipation in loperamide-induced rats. It has been reported that loperamide administration dramatically induces a decrease in stool-related factors, such as the number, wet weight, and water content of fecal pellets (Yang et al. 2008; Kakino et al. 2010). In the present study, the number, wet weight, and water content of fecal pellets in the normal group were significantly different from those in the control group. On the contrary, the number and water content of fecal pellets in the taurine-treated rats tended to increase in a dose-dependent manner to a greater extent than in the loperamide-induced constipated rats. Especially, in the group treated with high-dose taurine, wet weight of fecal pellets was higher than the normal range. These findings suggested that taurine supplementation exerted a preventive effect against constipation induced by loperamide. In a previous study, it was reported that loperamide treatment can cause a decrease in the serum levels of endogenous metabolites, including taurine, glycine, succinate, glycerol, and glutamine (Kim et al. 2014). Zhang et al. (2017) reported that dietary supplementation with 1% L-glutamine ameliorated constipation in sows by regulating endogenous gut microbiota.

In the present study, we used carmine as a marker to measure gastrointestinal transit ratio in loperamide-induced constipated rats. It has been reported that transit through the gastrointestinal tract reflects the overall gastrointestinal motor activity; therefore, measurement of the gastrointestinal transit ratio is useful to diagnose constipation (Wintola et al. 2010). A decrease in the gastrointestinal transit ratio of carmine is indicative of constipation. In the present study, loperamide administration markedly reduced transit distance and gastrointestinal transit ratio in the Sprague-Dawley rats. On the contrary, taurine supplementation, in a dose-dependent manner, significantly increased gastrointestinal motility in loperamide-induced constipated rats. Choe et al. (2012) showed that methionine improves the contractile activity of colon circular smooth muscle in vitro. In contrast, Rodriguez et al. (2013) reported correlations between normal and abnormal postprandial colon motility and alterations in some specific metabolites such as, glycerol, alanine, cytosine, carnosine, asparagine, choline, phosphocholine, thyroxine, and triiodothyronine in children with constipation.

In the present study, we also investigated the effect of taurine on serum gastrointestinal hormones, such as GAS, MTL, SS, and CGRP in loperamide-induced constipated rats. It has been known that GAS and MTL can stimulate the secretion of digestive juice, movement of gastrointestinal contents, and gastrointestinal motility. On the contrary, SS and CGRP can inhibit digestive juice secretion and gastrointestinal emptying (Chen et al. 2015). In this study, the serum levels of GAS and MTL

in the CON group were markedly lower than in the NOR group. However, these levels were significantly increased by taurine in a dose-dependent manner; thus, taurine enhanced colonic motility. Serum SS level in the CON group was significantly higher than in the NOR, LTS, and MTS groups. In contrast, taurine did not affect serum CGRP level in loperamide-induced constipated rats. These results showed that taurine may be useful and effective in ameliorating constipation. However, our study was limited in that it used only one type of animal model of constipation. Therefore, more studies are necessary to clarify the laxative effects of taurine to improve human health.

5 Conclusion

In conclusion, our findings showed that loperamide-induced constipation in rats was alleviated by taurine supplementation. Taurine treatment improved several fecal parameters, such as the number, wet weight, and water content of fecal pellets, gastrointestinal transit, and serum colonic motility index, including gastrin, motilin, and somatostatin. Therefore, our results suggested that taurine alleviated the symptoms of loperamide-induced constipation.

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Positive Changes in Blood Lipid Profiles, Nutrition Knowledge, and Dietary Taurine Intake After 8-Week Nutrition Education Program in Low-Income Korean Children

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Abstract Taurine has been reported to play a key role in the growth and development of children's brains and nerves. Incorrect dietary habits and unbalanced nutrient intakes may be caused by socio-environmental and economic factors in low-income children. This study was conducted to investigate changes in blood lipid profiles, nutrition knowledge, dietary attitudes, and intakes of dietary taurine and nutrients after an 8-week nutrition education program (NEP) in low-income Korean children. In this intervention study, nutrition education, exercise, and nutrition counseling were conducted for 8-weeks in 22 low-income children (11–13 years old, 9 males and 13 females) at community child center located in Incheon, Korea. Changes after the NEP were evaluated using a one group pretest-posttest design. Statistical analysis was performed using SPSS 20.0. After the 8-week NEP, there was a significant decrease in the blood triglyceride level of female students ($p < 0.01$). As for nutrition knowledge, there were significant increases in the subscore of sugars and sodium in foods consumed by male students ($p < 0.05$), total score of nutrition knowledge ($p < 0.01$), subscore of sugars and sodium in foods ($p < 0.01$), and fat content of foods and adequate dietary intake in female students ($p < 0.05$). Dietary attitudes did not change. There were significant increases in intakes of dietary taurine, vitamin B6 ($p < 0.01$), and dietary fiber ($p < 0.05$) in female students after the NEP. There were significantly positive correlations between changes in dietary taurine intake and dietary attitudes as well as between changes in carbohydrate intake and total cholesterol level among all the subjects. Therefore, nutrition education to promote balanced nutrient intake and dietary attitudes for optimal growth and development of low-income children is needed.

Keywords Dietary taurine intake · Blood lipid profile · Nutrition knowledge · Nutrition education program · Low-income Korean children

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Abbreviations

NEP	nutrition education program
TG	triglyceride
TC	total cholesterol
LDL-C	low-density lipoprotein cholesterol
HDL-C	high-density lipoprotein cholesterol
CAN-Pro	computer aided nutritional analysis program
SE	standard error

1 Introduction

Taurine is synthesized from sulfur-containing amino acids methionine and cysteine in the body and plays an important role in the development of the nervous system and brain (Guidotti 1978). It is necessary to intake taurine through food for growth and development of body tissues during childhood (Hayes and Young 1988; Yoon et al. 2015). Therefore, growing children should consume animal protein containing taurine.

Children's unhealthy dietary habits are reported to be associated with inequalities in parental socioeconomic determinants such as parental low education level and social class, low-skill occupation, and low-income level (Nilsen et al. 2009; Rydén and Hagfors 2011). Low-income families with low socioeconomic factors may contribute to nutritional imbalance and obesity in children due to their inability to purchase expensive meat, fish, fruits, and vegetables (Kim et al. 2007; Novaković et al. 2014; Kim and Kim 2017).

The community child center is a representative children welfare institution for Korean low-income children. Community child centers have rapidly grown from 895 institutions (23,347 children) in 2004 to 4107 institutions (106,668 children) in 2016, and it has consisted of an average of 30 children per center (Headquarters for Community Child Center 2018). Approximately 86% of children at community child centers are from low-income families. Therefore, differentiated health care programs should be provided to low-income children at social welfare organizations.

Therefore, the purpose of this study was to investigate changes in blood lipid profiles, nutrition knowledge, dietary attitudes, and dietary taurine and nutrient intakes after an 8-week nutrition education program (NEP) "Smart 100 Years! Children's Health Care Program" in low-income Korean children.

2 Methods

2.1 Subjects

Subjects were recruited from elementary school students aged 9–11 years (average age: 10.3 years old) at three community child centers located in Incheon Metropolitan City, Korea. Among 33 total students, 84.8% (n = 28) agreed to participate and returned signed consent forms; 5 subjects refused blood sampling. Statistical analysis was conducted for 22 students (9 male students and 13 female students); 4 subjects were unable to complete the posttest since they were often absent, and 2 male students moved to other community child centers.

2.2 Study Design

This study was approved by the institutional review board of Inha University, Korea (150224-1). In this intervention study, one group pretest-posttest design was used to assess the effectiveness of the NEP (Campbell and Stanley 1966; Herbert et al. 2013). The framework of this NEP is shown in Fig. 1. Topic and titles of the NEP are shown in Table 1. This NEP was conducted on a small group targeting low-income elementary students at community child centers. We collected baseline data to check the health and nutritional status as well as nutrition knowledge before applying this NEP. We used variables such as anthropometric measurement, blood lipid profiles, nutrition knowledge, dietary attitudes, and dietary taurine and nutrient intakes in a pretest. Data were examined using a self-reported questionnaire. This NEP was conducted for 8 weeks from July to September 2015. Posttest evaluation

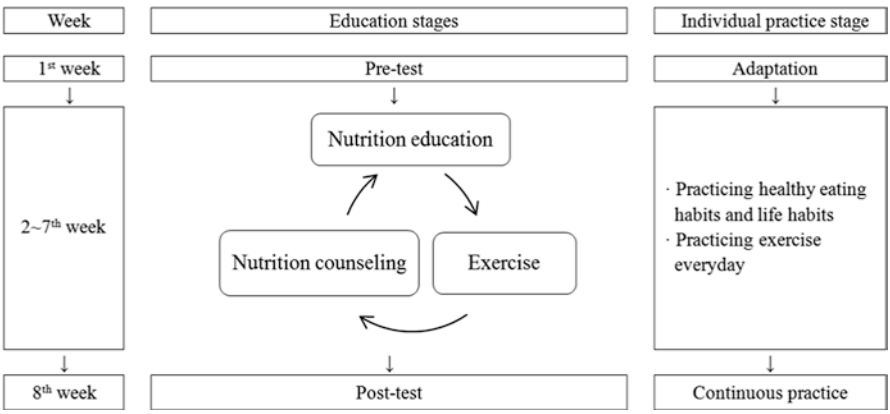

Fig. 1 Framework of the nutrition education program

Table 1 Topic and title of the nutrition education program

Weeks	Topic	Title
1st	Pre-test	Am I healthy?
2nd	Self-esteem	Hope you cotton up
3rd	Sugars and sodium	The secret of a healthy taste
4th	Fat	Do you prefer bacon or mackerel?
5th	Processed food	Selecting healthy snacks
6th	Adequate dietary intake	Preparing healthy meals
7th	Healthy growth and development	I am a health keeper chef!
8th	Post-test	Smartly health up!

was performed similar to the pretest, and subjects' satisfaction with the program was assessed.

2.3 Anthropometric Measurements and Blood Lipid Profiles

Each subject's height and body weight (Inbody BSM 330 and Inbody 620, Biospace Co., Korea) were measured to the nearest 0.1 cm and 0.1 kg, respectively. Value of BMI was calculated while height was entered manually on Inbody 620.

Blood was collected from each subject in the morning after 12 h of fasting overnight. Blood triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were determined by using Lipido Pro (Impopia, Korea) technology. In general, healthy blood lipid profiles in children and adolescents are as follows: TG <150 mg/dL, TC <170 mg/dL, LDL-C <110 mg/dL and HDL-C ≥35 mg/dL (University of Rochester Medical Center 2016).

2.4 Nutrition Knowledge and Dietary Attitudes

Nutrition knowledge was measured using 20 'Yes', 'No', or 'Do not know' questions regarding contents of nutrition education, which were modified from knowledge scales in Lee's study (2011) and Jang's study (2012). A correct answer was given 1 point. The questions were of five categories (sugars and sodium, fat, processed foods, adequate dietary intake, and healthy growth and development), including four items each (out of 4 points).

Dietary attitudes were assessed for six items, including interest in health and nutrition, attitude to improve dietary habits and attempt to reduce sugar and sodium intakes. Items were measured using a Likert 5-point scale. For each question, the score was given 5 points "strongly agree", 4 points "agree", 3 points "so-so", 2 points "disagree", or 1 point "strongly disagree".

2.5 Dietary Taurine and Major Nutrient Intakes

Dietary taurine and major nutrient intakes were determined using the 24-h dietary recall method. Subjects were asked to write all foods (meals and snacks) eaten over 1 day under the guidance of trained staff. Food intake of each subject was converted into nutrient intakes by the Computer Aided Nutritional Analysis Program 4.0 version (CAN-Pro, The Korean Nutrition Society, Seoul, Korea).

2.6 Statistic Analysis

All data were presented as the mean and standard error (SE) or frequency and percentage. SPSS 20.0 version was used for analysis. Paired t-test was conducted to examine changes in the variables of interest during the program. A value of $p < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Changes in Anthropometric Measurements and Body Composition

As shown in Table 2, average height ($p < 0.01$) and body weight ($p < 0.05$) significantly increased from 143.9 to 145.4 cm and 40.4–40.9 kg in male students and from 143.9 to 144.9 cm and 38.7–39.2 kg in female students, respectively. Average height and body weight of subjects were above those of children aged 9–11 years reported by KDRIs (Dietary Reference Intakes for Korean) in 2015 (142.9 cm and 38.2 kg in males and 142.9 cm and 35.7 kg in females, respectively) (Ministry of Health and Welfare, The Korean Nutrition Society 2015).

Table 2 Changes in anthropometric measurements and blood lipid profiles by gender

	Male student (n = 9)		Female student (n = 13)	
Variables	Before	After	Before	After
Height (cm)	143.9 ± 2.4	145.4 ± 2.4∗∗∗	143.9 ± 2.1	144.9 ± 2.0∗∗∗
Body weight (kg)	40.4 ± 2.3	40.9 ± 2.3∗	38.7 ± 2.6	39.2 ± 2.5∗
Body mass index (kg/m^2)	19.4 ± 0.8	19.2 ± 0.7	18.5 ± 0.9	18.5 ± 0.8
Triglyceride (mg/dL)	96.5 ± 7.5	94.5 ± 11.5	140.7 ± 1.7	103.6 ± 19.1∗∗
Total cholesterol (mg/dL)	155.6 ± 12.1	139.9 ± 12.6	135.4 ± 10.5	136.4 ± 8.8
LDL-cholesterol (mg/dL)	118.5 ± 1.5	102.0 ± 15.0	86.0 ± 11.5	98.7 ± 16.3
HDL-cholesterol (mg/dL)	40.9 ± 6.2	46.7 ± 4.0	40.7 ± 4.9	41.3 ± 3.4

Mean ± SE, p value was analyzed by paired t-test
∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001

There was a significant decrease in the blood TG level of female students during the NEP (from 144.6 to 103.6 mg/dL, $p < 0.05$). Although there were no significant differences in average blood LDL-C and HDL-C levels of male students, some male subjects did show alterations after the NEP; three out of nine males showed a reduced blood LDL-C level, whereas four out of nine males showed an increased blood HDL-C level (data not shown). Our findings are similar to those of a previous study by Sin et al. (2004) in which NEP significantly reduced blood TG level in obese female students (from 131.8 to 95.8 mg/dL) but not obese male students (from 103.0 to 74.9 mg/dL) (8–12 years old). This could be due to female students showing greater concerns about body image and thus participating more in the health care program compared to male students. Further, a previous study reported that a 12-week health exercise program in obese female higher elementary students significantly reduced blood TG level (Jung et al. 2009).

3.2 Changes in Scores of Nutrition Knowledge and Dietary Attitudes

Changes in the scores of nutrition knowledge and dietary attitudes during the NEP are presented in Table 3. In female students, the total score of nutrition knowledge significantly increased during the NEP (11.5–14.7 points, $p < 0.01$). Regarding nutrition knowledge score for sugars and sodium, significant increases were observed in both male students (2.4–3.2 points, $p < 0.05$) and female students (1.8–3.1 points, $p < 0.01$). In female students, nutrition knowledge scores regarding fat (1.8–2.9 points, $p < 0.05$) and adequate dietary intake (2.5–3.0 points, $p < 0.05$) significantly increased during the NEP.

Table 3 Changes in scores of nutrition knowledge and dietary attitudes by gender

Variables	Male student (n = 9)		Female student (n = 13)	
	Before	After	Before	After
Total score of nutrition knowledge (out of 20 points)	15.1 ± 1.1	14.4 ± 1.2	11.5 ± 0.8	14.7 ± 0.9**
Sugars and sodium	2.4 ± 0.5	3.2 ± 0.4*	1.8 ± 0.3	3.1 ± 0.2**
Fat	3.1 ± 0.4	2.6 ± 0.5	1.8 ± 0.3	2.9 ± 0.3*
Processed food	3.0 ± 0.2	2.4 ± 0.4	2.5 ± 0.3	2.7 ± 0.3
Adequate dietary intake	3.2 ± 0.2	3.2 ± 0.2	2.5 ± 0.2	3.0 ± 0.3*
Healthy growth and development	3.3 ± 0.3	3.0 ± 0.3	2.8 ± 0.2	3.0 ± 0.3
Total score of dietary attitudes (out of 30 points)	18.4 ± 0.9	19.0 ± 0.6	18.4 ± 1.0	19.0 ± 0.7

Mean ± SE, p value was analyzed by paired t-test
*$p < 0.05$, **$p < 0.01$

Dietary attitudes did not show a significant difference after the NEP (18.4–19.0 points in both male and female students).

3.3 Changes in Dietary Taurine and Nutrient Intakes

As shown in Table 4, there were significant increases in intakes of dietary taurine (81.8–270.0 mg, $p < 0.01$), dietary fiber (14.3–17.3 g, $p < 0.05$), and vitamin B6 (1.2–1.7 mg, $p < 0.01$) in female students after the NEP. During nutrition education, protein intake was significantly increased tendency ($p = 0.084$), which is thought to have affected the increase in dietary taurine intake. It has been reported previously that protein intake increased after applying the nutrition education program in children at community child centers (Kwon and Yeoh 2016).

3.4 Correlation Between Changes in Dietary Taurine and Nutrient Intakes

As shown in Table 5, there was a significant positive correlation between changes in dietary taurine intake and dietary attitudes. Dietary attitudes are attitudes related to eating. Intake of dietary taurine may play a significant role in mental well-being (Stapleton et al. 1997). It may be that an increase in dietary taurine intake has a positive relationship with improvement of dietary attitudes.

Table 4 Changes in dietary taurine and nutrient intakes

	Male student (n = 9)		Female student (n = 13)	
Variables	Before	After	Before	After
Taurine (mg)	84.7 ± 13.9	104.2 ± 29.4	81.8 ± 19.3	270.0 ± 47.0**
Energy (kcal)	1139.5 ± 29.4	1365.8 ± 127.6	1366.1 ± 148.8	1317.8 ± 112.3
Carbohydrates (g)	156.3 ± 20.3	200.7 ± 26.3	185.0 ± 22.7	175.8 ± 16.4
Protein (g)	51.7 ± 6.4	59.8 ± 5.3	64.1 ± 6.5	73.1 ± 5.1
Fat (g)	38.5 ± 3.0	39.7 ± 3.0	44.2 ± 5.0	39.5 ± 4.5
Dietary fiber (g)	15.6 ± 2.0	14.9 ± 1.7	14.3 ± 1.2	17.3 ± 1.5*
Vitamin A (μg RE)	910.5 ± 108.6	1064.3 ± 257.1	909.5 ± 136.4	1119.7 ± 286.4
Vitamin C (mg)	73.2 ± 9.9	67.5 ± 14.3	69.9 ± 10.7	87.2 ± 12.0
Vitamin B_6 (mg)	1.5 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.7 ± 0.1**
Folate acid (mg)	457.1 ± 62.0	431.2 ± 63.7	411.1 ± 54.6	447.1 ± 47.9
Calcium (mg)	384.3 ± 59.7	313.9 ± 45.0	334.2 ± 43.3	431.4 ± 80.1
Sodium (mg)	4330.1 ± 637.3	4414.9 ± 321.1	4076.5 ± 540.8	4682.8 ± 398.5
Iron (mg)	12.6 ± 2.1	14.2 ± 2.6	11.0 ± 1.3	12.5 ± 0.8

Mean ± SE, p value was analyzed by paired t-test
*$p < 0.05$, **$p < 0.01$

Table 5 Correlation between changes in blood lipid profiles, nutrition knowledge, dietary attitudes, and dietary taurine and nutrient intakes

Changes in variables	Changes in dietary taurine and major nutrient intakes						
	Taurine	Energy	Carbohydrate	Protein	Fat	Dietary fiber	Vitamin B_6
Triglyceride	−0.117	−0.009	−0.192	0.334	0.195	0.258	0.007
Total cholesterol	−0.031	0.422	0.557∗	0.222	0.080	0.137	0.068
LDL-cholesterol	0.761	0.678	0.804	0.379	0.353	−0.066	−0.384
HDL-cholesterol	−0.104	−0.135	0.003	−0.070	−0.422	−0.063	−0.099
Nutrition knowledge score	−0.447	0.131	0.161	−0.061	0.127	−0.094	0.104
Dietary attitudes scores	0.499∗	−0.172	−0.159	−0.032	−0.149	0.256	0.285

Pearson's correlation coefficient, $*p < 0.05$

Table 6 Children's satisfaction score with the nutrition education program

Variables	Male student (n = 9)	Female student (n = 13)	Total
Satisfaction with nutrition education	3.8 ± 0.3	4.1 ± 0.2	4.0 ± 0.2
Satisfaction with exercise	4.1 ± 0.3	4.2 ± 0.2	4.2 ± 0.1
Interest in activities	4.2 ± 0.2	4.3 ± 0.2	4.3 ± 0.2
Utilization of the workbook	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2
Need for a continuous program	3.6 ± 0.4	4.1 ± 0.3	3.9 ± 0.2

Mean ± SE

There was a significant positive correlation between changes in carbohydrate intake and TC level. Increased dietary carbohydrate intake was reported to be associated with an increase in TC level (Grundy and Denke 1990). Thus, nutrition education to promote correct dietary carbohydrate intake is needed since excessive intake of dietary carbohydrates may be a cause of childhood obesity (Ebbeling et al. 2002).

3.5 Satisfaction Score with the NEP

Subjects' satisfaction scores with NEP are shown in Table 6. For the low-income children's satisfaction questionnaire, most subjects responded 'very satisfied' or 'satisfied'. However, some students (22.2% of males, 9.1% of females) were dissatisfied with the 'need for a continuous program'. Similarly, according to previous studies (Lee and Kim 2013), a variety of educational methods such as games, experiments, activities, cooking, etc. increased the satisfaction score and effect of nutrition education.

4 Conclusions

Continuation and propagation of this NEP should be considered for low-income Korean children. As a further study, an intervention study on dietary taurine supplementation is needed to confirm a correlation between dietary taurine intake and dietary attitudes in a larger Korean children group.

Moreover, any nutrition program for children should increase interest by utilizing an activity-based curriculum along with a variety of educational methods since higher NEP satisfaction can increase effectiveness. Since female students showed more positive results compared to male students, the NEP could be modified to suit gender-specific characteristics. This may be due to significant differences in multidimensional self-conception and academic achievement according to gender (Kim and Lee 2015), although the ineffectiveness of the NEP in male students may be due to the group size being insufficient to detect any difference. Therefore, any subsequent evaluation of the effectiveness of the NEP intervention should add more community child centers and enlarge sample size.

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Relation of Taurine Intake During Pregnancy and Newborns' Growth

You-Mi Jung and Mi-Ja Choi

Abstract Taurine is a free amino acid and exhibits various biological functions such as brain development, retinal photoreceptor activity, reproduction, normal growth development and antioxidant activity. Taurine is mainly contained in meat and fish foods. Although taurine is inferred to be implicated in the development of the fetus, there are few reports of taurine intake and neonatal growth in pregnant women. Therefore, the purpose of this study was to evaluate the nutrient and taurine intake of pregnant women during the late gestation period using the food intake frequency method. The study was approved by the Institutional Review Board of the Keimyung University. The daily taurine intake of the pregnant women was 104.2 mg. The daily taurine intakes of the pregnant women were divided into three groups for analysis; low, middle, high (<60 mg, 60–120 mg, and >120 mg). The body weight of the top taurine group (3.37 kg) was significantly higher than that of the low and middle groups (3.16 kg, 3.20 kg). Also, the heights of the infants were 49.9 cm, 49.8 cm, and 51.1 cm for each group, showing significantly high measure in the group of taurine intake more than 120 mg/day. There was a positive correlation between the taurine intake of the pregnant women and the height of the newborns. In conclusion, weight and height at birth were significantly higher in the high taurine intake group of the pregnant women. There was a positive correlation between maternal taurine intake and birth length.

Keywords Taurine intake · Pregnancy women · Infant weight · Ponderal index · Growth

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Abbreviations

PI	Ponderal index
SQFFQ	Semi-Quantitative Food-Frequency Questionnaire
IRB	Institutional review board
BMI	Body mass index
PUFAs	polyunsaturated fatty acids
CAD	coronary artery disease
T1	1st tertile
T2	2nd tertile
T3	3rd tertile
n-3	omega-3 fatty acid

1 Introduction

There is taurine in forms of high concentrations in the brains of fetal and mammalian neonatal newborns and slowly decreases after birth to equal to the level of adult animals until birth (Sturman and Hayes 1980). Taurine is especially important during the period of pregnancy and lactation because fetus or neonates are undergoing rapid brain growth (Chesney. 1985). Demand for taurine increases during pregnancy and lactation to support fetal and neonatal growth and development (Wharton et al. 2004). Taurine is a normal constituent of the human diet (Park et al. 1998). Taurine in food can be found in seafood and meat products, and some in plant-derived foods (Waldron et al. 2018; Park et al. 1998). Taurine is found in anchovies, short-necked clams, oysters, octopuses, shellfish, fish, bread, cakes, seaweeds, eggs, and a little in vegetables (Waldron et al. 2018). The amount of taurine in 118 plant-derived foods that Koreans commonly consume is very small, about 1/100–1/1000 of what's in fish, shellfish, and meat products (Park et al. 1998).

Pregnancy nutrition is essential factors affecting fetal growth. During the last trimester of pregnancy, sulfur amino acids tend to accumulate in tissues (Holm et al. 2018). Taurine is considered an essential amino acid for the fetus and it is believed that fetal taurine supply depends entirely on placental transfer from maternal plasma (Holm et al. 2018). Vegetarians who chronically consume low levels of taurine can inhibit brain development (Cofnas 2018). It has also been proposed that taurine insufficiency in a fetus may be causally associated with fetal growth, such as low birth weight and development disorder (Holm et al. 2018). Taurine is involved in the growth of the fetus, and there are few studies of taurine intake and birth size. Hence, an adequate taurine intake during pregnancy is an essential determinant of fetus or infant development.

Therefore, the purpose of this study was to evaluate the taurine intake of pregnant women during the late gestation period and infant growth.

2 Methods

We assessed the diet of pregnant women aged 25–40 years old living in the city of Daegu, Korea. Face-to-face interviews were completed with women at 30–40 weeks of pregnancy by trained dietitians. Data were collected on maternal age, weight and height, gestation. The weight gain during pregnancy of the subjects was calculated by subtracting the body weight before pregnancy from the whole body weight. Dietary intake for women was assessed with the use of a 106-item Semi-Quantitative Food-Frequency Questionnaire (SQFFQ) that was specifically designed to include taurine sources (National Research Institute of Health 2014). Weight at birth (g) and length at birth (cm) were collected, and PI (kg/m^3), an estimate of infant body fatness, at birth was calculated as birth weight (kg/m^3). Estimates of taurine included in each reported food were obtained from the Korean Food Composition Table on Agriculture Food Composition (Rural Development Administration 2016). Approval was obtained from the Institutional Review Board (IRB) of the Keimyung University (40525-201506-BR-46-03). All of the women provided written informed consent. We obtained informative consent from study participants for the research.

2.1 Statistical Analysis

All data analyses were conducted using SAS version 9.3 (SAS Institute Inc., Cary, NC). Statistical significance was defined as a p-value <0.05 in 2-sided tests. Student's t-tests were used to statistical comparisons among different pregnant women's age groups and their significant differences, comparing three taurine intake groups which were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Pearson correlation was used to analyze correlated variables.

3 Results and Discussion

Intrauterine life may be a critical period for human health through life (Holm et al. 2018). The probability of a normal birth was reduced for women who were older (Sauer 2015). Advanced maternal age increases the risk of pregnancy complications such as fetal growth restriction, hypertension, and premature birth (Eriksson et al. 2000; Choi et al. 2006). Women are delaying childbearing to pursue educational and career goals in higher numbers than ever before (Jahromi and Husseini 2008). Delaying motherhood is an ongoing trend in industrialized countries worldwide (Niessen et al. 2017). Higher the mother's age, the higher the risks for fertility, pregnancy, childbearing, and for newborns (Min and Jeong 2015). The criterion was the

perception of reproductive age risks of motherhood at an advanced age, defined as >35 years (Min and Jeong 2015).

Descriptive characteristics of maternal weight gain during pregnancy, age at pregnancy, weight, and height are presented in Table 1. The prevalence of pregnant women with advanced maternal age (35–40 years old) was 24.0% in this study. Pregnant women were classified according to age (normal vs. advanced maternal age). The mean maternal age was 31.0 ± 2.2 and 36.9 ± 1.9 years old. There was no statistically significant difference in weight gain and gestational period during pregnancy. Late pregnancy has been linked to impaired glucose tolerance, insulin resistance, low birth weight, and complications of pregnancy (Jahromi and Husseini 2008; Choi et al. 2006).

We compared between maternal pregnancy age and offspring length and weight at birth (Table 2). There were no statistically significant differences between age groups in terms of length at birth, and PI.

It uses Rohrer's Ponderal Index (PI), which is used as an indicator of growth according to nutritional status during the fetal period. Rohrer's Ponderal Index (PI) was calculated using the newborns' weight and length (kg/m^3). A PI of <23.2 is regarded as indicative of disproportionate fetal growth, while 23.2–28.5 is normal and greater than 28.5 is obese (Falade et al. 2010). The infants in this study had PI in their normal range. There were no significant differences in size of infants' at birth according to age.

Pregnant women were classified according to taurine intake as low, middle, and high intake group (Table 3). Previous research showed that fish foods consumption is associated with gestational weight gains (Mortensen et al. 2010). Infants' weight

Table 1 Characteristics of pregnant women by age

Variable	<35 years (n = 57)	35 years ≤ (n = 18)	p-value
Age (year)	31.0 ± 2.2	36.9 ± 1.9	<.001
Gestational period (day)	275.6 ± 14.3	276.9 ± 6.4	.712
Height (cm)	161.7 ± 5.4	162.3 ± 3.7	.649
Weight (kg)	54.6 ± 6.5	53.4 ± 2.7	.450
Full term weight (kg)	68.5 ± 7.1	68.4 ± 4.9	.935
Pre-pregnancy BMI (kg/m^2)	20.9 ± 2.3	20.3 ± 1.1	.288
Weight gain during pregnancy (kg)	13.9 ± 3.8	15.0 ± 3.8	.316

Data are presented as Mean ± SD
BMI Body mass index

Table 2 Characteristics of infant by pregnant women's age

Variable	<35 years (n = 57)	35 years ≤ (n = 18)	p-value
Birth length (cm)	50.1 ± 2.0	50.8 ± 1.7	.249
Birth weight (kg)	3.2 ± 0.4	3.3 ± 0.4	.505
PI (kg/m^3)	25.7 ± 2.4	25.3 ± 2.7	.549

Data are presented as Mean ± SD
PI Ponderal index

Table 3 Characteristics of infants by taurine intake during pregnancy

Variable	T1[1] (n = 23) <60 mg	T2 (n = 25) 60–120 mg	T3 (n = 27) 120 mg <
Pregnant women's age (year)	33.3 ± 2.8[a]	31.8 ± 3.6[a]	32.2 ± 3.3[a]
Weight (kg)	3.16 ± 0.71[a]	3.20 ± 0.51[a]	3.37 ± 0.41[b]
Length (cm)	49.9 ± 5.3[a]	49.8 ± 6.1[a]	51.1 ± 4.3[b]
PI (kg/m^3)	25.4 ± 5.6[a]	26.0 ± 7.1[a]	25.3 ± 6.2[a]

Data are presented as Mean ± SD

[ab]Values with different superscripts within the row are significantly different at $p < .05$ by Duncan's multiple range test

[1]Taurine intake was divided into three. T1, 1st tertile (<60 mg/day); T2, 2nd tertile (60–120 mg/day); T3, 3rd tertile (120 mg/day <)

PI Ponderal index

and height were significantly higher in the high taurine group than both medium and low taurine groups and did not differ between low and medium taurine groups despite taurine intake was significantly higher in the medium taurine group assigned to 60–120 mg/day group. To our knowledge, these associations between maternal taurine status and height and weight in the offspring at birth have not been previously addressed.

Small size at birth and early rapid growth during childhood were found to be related to increased risk of coronary artery disease and other diseases in adult age (Gunnarsdottir et al. 2002; Chapman et al. 1993). The increase in size at birth was observed to have a protective effect against coronary artery disease (Barker et al. 2005). Even though coronary artery disease was inversely related to the size of men's birth (p for trend = .029). There was no significant relationship with birth weight or PI (kg/m^3) (Barker et al. 2005).

Although the potential effects of maternal taurine deficiency on offspring growth have been recognized in previous research, there are few published studies with which we can compare our findings directly. We found that lower taurine status was associated with lower weight and length at birth (Table 3).

Nutrient intake of pregnant women by taurine intake during pregnancy was shown in Tables 4 and 5. Women in the low taurine group had significantly lower protein, total cholesterol, phosphorus, potassium, vitamin A, B1, B2, B6, C, E and calcium intake than women in the middle or high taurine intake group. Despite several campaigns to promote milk and dairy products consumptions, most pregnant women are still not consuming enough milk and dairy products that results in reducing bone health. Calcium insufficiency is common in Korea. A particular concern is that almost a third of women have low calcium status. Women are recommended to take calcium 800 mg/day during pregnancy, yet low calcium condition is prevalent. In the context of current concerns about the high prevalence of calcium insufficiency in pregnant women, the recommended levels of calcium are used in clinical practice in Korea (Ahn and kim 2011). Women in Korea are mostly unaware of these guidelines (Lee et al. 2017). During pregnancy, pregnant women are recommended to take in an additional 340 kcal/day starting in the second trimester and

Table 4 Daily nutrient intake of pregnant women by taurine intake during pregnancy

Variable	T1[1)] (n = 23) <60 mg	T2 (n = 25) 60–120 mg	T3 (n = 27) 120 mg <
Taurine (mg)	33.6 ± 11.5[a]	83.2 ± 13.3[b]	183.8 ± 17.4[c]
Energy (kcal)	1813.2 ± 754.0[a]	2270.6 ± 625.3[b]	2545.7 ± 807.2[b]
Carbohydrate, % of energy	67.2 ± 7.5[a]	66.9 ± 5.5[b]	60.6 ± 9.0[b]
Protein, % of energy	13.5 ± 2.6[a]	13.7 ± 2.2[a]	15.5 ± 2.9[b]
Fat, % of energy	17.5 ± 5.8[a]	18.8 ± 5.0[a]	23.2 ± 6.8[b]
Carbohydrate (kcal)	1216.1 ± 503.3[a]	1506.5 ± 395.5[b]	1518.9 ± 446.3[b]
Protein (kcal)	240.3 ± 104.2[a]	310.6 ± 94.0[a]	401.3 ± 170.7[b]
Fat (kcal)	328.7 ± 188.5[a]	442.6 ± 193.9[a]	609.4 ± 298.7[b]
Carbohydrate (g)	304.0 ± 125.8[a]	376.6 ± 98.9[b]	379.7 ± 111.6[b]
Protein (g)	60.1 ± 26.1[a]	77.6 ± 23.5[a]	100.3 ± 42.7[b]
Fat (g)	36.5 ± 20.9[a]	49.2 ± 21.5[a]	67.7 ± 33.2[b]
Cholesterol (mg)	221.1 ± 118.9[a]	297.4 ± 117.9[a]	411.2 ± 214.9[b]

Data are presented as Mean ± SD

[abc]Values with different superscripts within the row are significantly different at $p < .05$ by Duncan's multiple range test

[1)]Taurine intake was divided into three. T1, 1st tertile (<60 mg/day); T2, 2nd tertile (60–120 mg/day); T3, 3rd tertile (120 mg/day <)

Table 5 Daily nutrient intake of pregnant women by taurine intake during pregnancy

Variable	T1[1)] (n = 23)	T2 (n = 25)	T3 (n = 27)
Vitamin A (μgRE)	334.3 ± 218.9[a]	560.8 ± 288.3[b]	597.3 ± 279.8[b]
Vitamin B_1 (mg)	1.1 ± 0.6[a]	9a1.3 ± 0.5[a]	1.7 ± 0.7[b]
Vitamin B_2 (mg)	0.9 ± 0.4[a]	1.3 ± 0.4[b]	1.6 ± 0.7[c]
Niacin (mg)	13.8 ± 7.1[a]	17.5 ± 6.6[a]	22.5 ± 10.7[b]
Vitamin B_6 (mg)	1.4 ± 0.7[a]	1.9 ± 0.8[a]	2.2 ± 0.8[b]
Folic acid (μg)	167.3 ± 105.9[a]	241.6 ± 113.6[b]	255.7 ± 94.9[b]
Vitamin C (mg)	105.7 ± 149.9[a]	120.5 ± 131.2[a]	141.5 ± 63.1[a]
Vitamin E (mg)	7.6 ± 4.5[a]	10.4 ± 4.2[ab]	12.4 ± 6.5[b]
Calcium (mg)	412.9 ± 261.2[a]	617.3 ± 241.1[b]	746.7 ± 407.9[b]
Phosphorus (mg)	889.2 ± 392.2[a]	1163.9 ± 341.4[b]	1429.4 ± 580.5[c]
Iron (mg)	9.3 ± 4.8[a]	12.2 ± 4.9[b]	14.9 ± 5.0[b]
Potassium (mg)	1880.9 ± 1064.9[a]	2613.6 ± 1207.5[b]	3042.9 ± 1295.0[b]
Sodium (mg)	1583.9 ± 970.7[a]	2384.0 ± 1029.4[b]	2876.6 ± 1464.8[b]
Zinc (mg)	7.6 ± 3.1[a]	10.1 ± 3.1[b]	11.8 ± 4.0[b]

Data are presented as Mean ± SD

[abc]Values with different superscripts within the row are significantly different at $p < 0.05$ by Duncan's multiple range test

[1)]Taurine intake was divided into three. T1, 1st tertile (<60 mg/day); T2, 2nd tertile (60–120 mg/day); T3, 3rd tertile (120 mg/day <)

450 kcal/day in the third trimester to meet the demands of the pregnancy state in Korea (The Korean Nutrition Society 2015). Thus, compared to non-pregnant women, pregnant women consume more energy, including calcium, yet their calcium intake is still significantly low. The daily taurine intake of the pregnant women was 104.2 ± 69.4 mg, as 33.6 ± 17.7 mg in the low group, 83.2 ± 17.3 mg in the middle group, and 184.5 ± 479.6 mg in the high group. At birth a small size accelerates childhood growth and increases the risk of coronary heart disease and other diseases in adult life (Cooke et al. 2018; Azuma et al. 1982). There is a protective effect of birth length increases in CAD (Churchill et al. 2008). The incidence of coronary artery disease was inversely related to the length of infant at birth, but was not significantly associated with birth weight or PI (Roysommuti et al. 2009). Increased intake of n-3 fatty acids can increase pregnancy and birth weight Fish and other seafood are healthful foods that are the primary dietary source for taurine and elongated omega-3 (n-3) polyunsaturated fatty acids (PUFAs) (Keisuke 2017).

Long-chain polyunsaturated n-3 fatty acids food contamination can be a source of concern for the safety of fish intake during pregnancy (Holm et al. 2018). Fish are known to be helpful for fetal growth and development, and are rich in polyunsaturated n-3 fatty acids, protein, selenium, iodine, and vitamin D (Olsen et al. 1995). In contrast, fish can be exposed to dioxins, polychlorinated biphenyls, methylmercury, and other heavy metals, which can adversely affect fetal growth and pregnancy (Holm et al. 2018; Food and Drug Administration 2001).

The average fish intake for the Japanese and Koreans are 73 g/day and 82 g/day, respectively. Koreans consume nearly 70% more shellfish on a daily basis (22.7 g/person) than the Japanese (13.5 g/person) (Tsuchiya et al. 2008). Japanese consumed three times as much salmon on a daily basis (1820 g/cohort) as did Koreans (581 g/cohort), whereas Koreans consumed about four times the amount of squid per day (1461 g/cohort), compared to Japanese (356 g/cohort) (Tsuchiya et al. 2008). Since different countries eat different kinds of fish, it seems that the effect of eating fish is different as well. And the survey on the consumption of taurine of 70 married women in Seoul and Gyeonggi Province said that 202.0 mg/day in Seoul and 85.5 mg/day in Gyeonggi Province (Yim et al. 2004), which are correlated to offspring length.

The consumption of taurine in Jeju Island (Kim et al. 2003) were found as 115 mg/day in urban areas and 216 mg/day in rural areas. It is contrary to the earlier report (Yim et al. 2004), which showed high intake tendency in urban areas. Accordingly, both residential areas and individual diet habits seem to affect taurine intake. The US Food and Drug Administration and Environmental Protection Agency have issued warnings, recommending that pregnant women limit their fish consumption to avoid excessive mercury exposure (Food and Drug Administration 2001; US Department of Health and Human Services 2004). Pregnant women in the US have consumed less fish after the guidelines were published (Oken et al. 2003).

Table 6 Correlation coefficients between maternal characteristics and infants' characteristics

	Age	Birth weight	Birth length	PI	Taurine intake	Gestational period
Birth weight	.004					
Birth length	.032	.620***				
PI	−.027	.413***	−.455***			
Taurine intake	−.098	.170	.196*	−.037		
Gestational period	.044	.180	.143	.022	.070	
Weight gain during pregnancy	.062	.248*	.317**	−.087	.021	.213*

PI Ponderal index
*p < .05, **p<.01, ***p<.001

Presumably, the participants of this study may also have reduced the fish intake due to the fear of mercury ingestion, but more studies are necessary. Considering recent study results of fish intake of various ethnic groups in America suggest that Koreans should increase their fish intake (Jensen 2006). According to the cases of nutrients intake of pregnant women in relation to taurine intake, the middle and high group had consumed significantly more than the low group, such as energy, carbohydrate, protein, vitamin A, vitamin B2, vitamin B6, phosphorus, and zinc. On the other hand, as taurine intake increases, consumptions of lipid, cholesterol, vitamin B1, and iron are also significantly increased ($p < .05$).

The results of correlation analysis are shown in Table 6. There was a positive association between maternal taurine intake and infant length at birth ($r = .196$, $p < 0.05$). There was a positive correlation between maternal weight gain during pregnancy and weight ($r = .248$, $p < .05$) and length ($r = .317$, $p < .01$) at birth. There were also strong positive associations between weight and length at birth.

Birth weight and length of newborns were significantly higher in the high taurine intake group of the pregnant women. There was a positive correlation between the taurine intake of the pregnant women and the height of the newborns. We found that lower taurine status was associated with lower length and weight at birth.

4 Conclusion

We examined how maternal taurine status in late pregnancy relates to the body growth of the offspring at birth. Birth weight and height of newborns were significantly higher in the high taurine intake group of the pregnant women.

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Relationship Among Dietary Taurine Intake, Dietary Attitudes, Dietary Behaviors, and Life Stress by Depression in Korean Female College Students

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Abstract This study was conducted to investigate the correlation between dietary taurine intake, nutrients intake, dietary attitudes, dietary behaviors, and life stress by depression in Korean female college students. Depression was measured by self-reported symptoms of depression on the CES-D (Center for Epidemiologic Studies Depression) scale. The subjects of this cross-sectional study included 56 female college students with depression (depression group, DG) and 122 female students without depression (control group, CG). Self-reported life stress score was determined using the life stress scale developed for Korean college students. Intakes of dietary taurine and nutrients were assessed using 3-day food records (2 weekdays and 1 weekend day) and evaluated using the computer aided nutrition program 4.0 version. Statistical analysis was performed using SPSS 24.0. We observed no significant difference in the average dietary taurine intake between DG (87.6 mg/day) and CG (92.3 mg/day). The average dietary intakes of vitamin A and calcium in DG were significantly lower as compared to CG ($p < 0.05$). The average total scores of dietary attitudes ($p < 0.01$) and dietary behaviors ($p < 0.05$) in DG were significantly lower as compared to CG. The average total life stress score ($p < 0.001$) and all stress categories were significantly higher in DG as compared to CG. No significant correlation was observed between the CES-D scale score and dietary taurine intake. However, there were significant negative correlations between the CES-D scale

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score and vitamin A, folic acid, vitamin C, and calcium intakes ($p < 0.05$), dietary attitudes, and dietary behaviors ($p < 0.01$). Scores of the CES-D scale and life stress showed a significantly positive correlation ($p < 0.01$). Therefore, continuous nutrition education and counseling for good dietary attitudes and behaviors are required. Future studies need to be undertaken to confirm the correlation between dietary taurine intake and depression by intervention with taurine.

Keywords Dietary taurine intake · Dietary behaviors · Life stress · Depression · Korean female college students

Abbreviations

CES-D	center for epidemiologic studies depression
DG	depression group
CG	control group
CAN-Pro	computer aided nutritional analysis program
SE	standard error

1 Introduction

Incessant negative thinking during depression degrades the overall quality of life (Becker 1974). One of the mental health disorders associated with human life, depression consequently leads to changes in food attitudes and eating habits. It was reported that depressed patients had poorer dietary attitudes and dietary habits compared to non-depressed people (Kim et al. 1993; Lee and Kim 2011). Thus, the overall nutritional status and nutrient intake are associated with depression, which may lead to poor dietary quality and eating disorders such as anorexia and bulimia (Bodnar and Wisner 2005; Claudat et al. 2016).

Taurine, an inhibitory neurotransmitter, plays an important role in depression (Mauri et al. 1998; Okamoto et al. 1983). Depression level of Korean female college students was reported to be correlated with a taurine intake (Sung and Chang 2007). Recently, it was reported that taurine levels in depressed rats were significantly decreased in the hippocampus (Cui et al. 2017), and taurine in mild stress-induced depressive rats showed an antidepressant effect (Wu et al. 2017).

Depression level of college students is increasing due to stresses related to employment, study, and social adjustment (Park and Kim 2014). However, few studies are available on the relationship of dietary attitude, dietary habits, a degree of life stress, and dietary taurine with the level of depression in Korean college students. Therefore, this study was conducted to investigate the relationship among dietary taurine intake and nutrients intakes, nutrition knowledge, dietary attitudes, dietary behaviors, and life stress with the level of depression in Korean female college students.

2 Methods

2.1 Subjects

A survey was conducted in September-October 2017 using an anonymous self-administered questionnaire. This cross-sectional study enrolled 200 female college students residing in Gyeonggi, Korea. Except for 22 questionnaires which include incomplete data, vegetarian subjects, and female students doing a fasting cure, 178 questionnaires were included in the final analysis. Depression was measured by the self-reported symptoms of depression based on the Korean version of the CES-D scale (Center for Epidemiologic Studies Depression) (Cho and Kim 1993). As shown in Table 1, the subjects were divided into 56 female college students with depression (depression group, DG) and 122 female students without depression (control group, CG). The CES-D scale is composed of 20 items and scored on a 4-point scale of 0–3 points, with the total score ranging from 0 to 60 points. This study was approved by the institutional review board of Inha University, Korea (170821-3A).

2.2 Dietary Taurine and Nutrient Intakes

Intakes of dietary taurine and nutrients were determined using 3-day food records (2 weekdays and 1 weekend day) and estimated using the Computer Aided Nutritional Analysis Program 4.0 version (CAN-Pro, The Korean Nutrition Society, Seoul, Korea) nutrient analysis software. Dietary taurine intake of the subject was calculated using 361 food items in 17 food groups that were inputted into the CAN-Pro database (Kim and Kim 1998; Park et al. 1998; Yoon et al. 2015).

2.3 Life Stress

Life stress score was determined using the life stress scale developed for Korean college students (Chon et al. 2000). A self-administered questionnaire containing 50 questions regarding the frequency and importance of life stress was evaluated using

Table 1 Depression level of the subjects by a score of the CES-D scale

Variables	Score of the CES-D scale		n (%)	
Depression group	Severe	25≤	19 (10.7)	56 (31.5)
	Moderate	21≤ – <25	18 (10.1)	
	Mild	16≤ – <21	19 (10.7)	
Control group	Normal	>16	122 (68.5)	122 (68.5)

the 4-point scale ranging from 0 to 3. Life stress scale of Korean college students considered eight areas: future, different gender, economic, family, friend, professor, value, and study-related problems.

2.4 Questionnaires

Nutrition knowledge was assessed by considering 20 items related to general health nutrition knowledge (Yoon and Choi 2002). Each question was answered with "Right", "Wrong", and "Do not know"; the total score of 20 points was calculated as 1 point for correct answers and 0 points for the wrong answer and unknowingness.

Dietary attitudes and dietary behaviors for healthy dietary life were assessed for 5 and 20 items, respectively (Korean Society of Community Nutrition 2000). The questions were measured using a 5-point Likert scale ranging from 1 (strongly disagree) to 5 (strongly agree). Total scores of dietary attitudes and dietary behaviors were measured as the summated scales of each question, totaling 25 and 100 points, respectively; a higher score indicated better dietary attitudes and dietary behaviors.

2.5 Statistic Analysis

Statistical analysis was performed using the SPSS 24.0 program (SPSS Inc., IBM corp., NY, USA). All variables were presented as the mean and standard error (SE) or frequency and percentage. Student t-test was conducted to compare the differences between DG and CG. Pearson's correlation analysis determined the relationship between variables and score of CED-S scale. Statistical significance was set at a level of $p < 0.05$.

3 Result and Discussion

3.1 Age and Anthropometric Data

The average age of the subjects was 22.1 years old and 21.8 years old in DC and CG, respectively (Table 2). There were no significant differences in height, body weight, and body mass index between DG (161.7 cm, 54.9 kg, and 21.0 kg/m^2, respectively) and CG (162.0 cm, 54.8 kg, and 20.9 kg/m^2, respectively).

Table 2 Age and anthropometric data of the subjects

Variables	Depression group (n = 56)	Control group (n = 122)	t-value
Age (years)	22.1 ± 0.4	21.8 ± 0.2	−0.737
Height (cm)	161.7 ± 0.6	162.0 ± 0.5	−0.352
Body weight (kg)	54.9 ± 0.9	54.8 ± 0.6	−0.058
Body mass index (kg/m^2)	21.0 ± 0.3	20.9 ± 0.2	−0.275

Mean ± SE, p-value was analyzed by the Student t-test

Table 3 Intake of dietary taurine and nutrients of the subjects

Variables (per day)	Depression group (n = 56)	Control group (n = 122)	t-value
Taurine (mg)	87.6 ± 11.2	92.3 ± 12.6	−0.277
Energy (kcal)	1308.5 ± 70.1	1324.0 ± 37.9	−0.212
Carbohydrates (g)	169.6 ± 9.5	179.6 ± 4.9	−1.033
Total fat (g)	45.4 ± 2.8	43.3 ± 1.6	0.653
Total protein (g)	50.5 ± 3.0	48.6 ± 1.7	0.543
Vitamin A (μg RE)	447.9 ± 32.4	552.5 ± 29.7	−2.378∗
Vitamin B_1 (mg)	1.1 ± 0.1	1.0 ± 0.0	0.941
Vitamin B_2 (mg)	0.8 ± 0.0	0.9 ± 0.0	−0.782
Vitamin B_6 (mg)	0.9 ± 0.1	1.0 ± 0.0	−0.962
Folic acid	243.8 ± 18.1	265.3 ± 9.8	−1.047
Vitamin C (mg)	43.4 ± 4.7	49.8 ± 3.1	−1.135
Calcium (mg)	250.1 ± 16.9	295.8 ± 13.7	−2.102∗
Iron (mg)	8.6 ± 0.5	9.4 ± 0.4	−1341
Zinc (mg)	6.3 ± 0.4	6.4 ± 0.2	−0.202
Potassium (mg)	1469.7 ± 100.8	1562.8 ± 59.1	−0.797
Sodium (mg)	2574.2 ± 173.8	2625.5 ± 111.5	−0.249

Mean ± SE, p value was analyzed by the Student t-test
∗$p < 0.05$

3.2 Intake of Dietary Taurine and Nutrients

As shown in Table 3, there was no significant difference in average dietary taurine intake between DG (87.6 mg/day) and CG (92.3 mg/day). The average daily taurine intake was 96.9 mg for female college students residing in the Seoul and Incheon areas (Sung and Chang 2009). The daily taurine intakes of health functional taurine-containing food consumer and non-consumer items were 157.3 mg and 166.2 mg, respectively, in 2015 (Na et al. 2015), which were higher than observed in this study.

The average dietary intakes of vitamin A (447.9 μg RE in DG and 552.5 μg RE in CG, $p < 0.05$) and calcium (250.1 mg in DG and 295.8 mg in CG, $p < 0.05$) were significantly lower in DG as compared to CG.

3.3 Scores of Nutrition Knowledge, Dietary Attitudes, and Dietary Behaviors

There was no significant difference in the average total scores of nutrition knowledge between DG and CG (13.4 and 12.6 points, Table 4). However, dietary attitudes ($p < 0.01$) and dietary behaviors ($p < 0.05$) in DG (18.6 and 53.2 points, respectively) were significantly lower as compared to CG (20.0 and 56.1 points, respectively).

A previous study has reported similar observations that the depressed group had poor dietary behaviors (Kim et al. 1993).

3.4 Life Stress Level

Table 5 shows the causes of life stress specified by the subjects. Total life stress score was calculated as the sum of the experience frequency multiplied by importance and was converted into out of 100 points. We found that the total score and all variables scores of life stress in DG were significantly higher compared to that observed in CG; future, family, friend, and value problem and total life stress ($p < 0.001$), economy, faculty, and grade problem ($p < 0.01$), and lover problem ($p < 0.05$).

Table 4 Scores of nutrition knowledge, dietary attitudes, and dietary behaviors of the subjects

Variables	Depression group (n = 56)	Control group (n = 122)	t-value
Nutrition knowledge (out of 20 points)	13.4 ± 0.6	12.6 ± 0.3	−1.322
Dietary attitudes (out of 25 points)	18.6 ± 2.2	20.0 ± 0.2	3.418**
Dietary behaviors (out of 100 points)	53.2 ± 1.2	56.1 ± 0.9	2.040*

Mean ± SE, p-value was analyzed by the Student t-test
*p < 0.05, **p < 0.01

Table 5 Life stress scores by life stress category

Variables	Depression group (n = 56)	Control group (n = 122)	t-value
Future problem	23.0 ± 3.2	8.3 ± 1.0	−4.459***
Lover problem	6.1 ± 1.8	3.0 ± 0.6	−1.644*
Economy problem	9.5 ± 2.2	4.4 ± 0.7	−2.842**
Family problem	8.9 ± 1.7	3.0 ± 0.6	−3.952***
Friend problem	5.5 ± 1.7	0.8 ± 0.2	−3.855***
Faculty problem	11.1 ± 2.6	3.9 ± 0.8	−3.495**
Value problem	23.1 ± 3.3	6.0 ± 0.9	−6.565***
Grade problem	32.7 ± 3.4	23.3 ± 1.7	−2.762**
Total life stress score	75.5 ± 7.7	37.8 ± 2.7	−5.808***

Mean ± SE, p value was analyzed by Student t-test
*p < 0.05, **p < 0.01, ***p < 0.001

Table 6 Pearson's correlation coefficients between dietary taurine and nutrient intakes, nutrition knowledge, dietary attitudes, dietary behaviors, life stress scores, and a score of the CES-D scale by depression

Variables	**Taurine**	**Energy**	**Carbohydrate**	**Total fat**	**Total protein**
Score of the CES-D scale	−0.055	−0.051	−0.142	0.066	−0.004
Variables	**Vitamin A**	**Vitamin B_1**	**Vitamin B_2**	**Vitamin B_6**	**Folic acid**
Score of the CES-D scale	−0.161∗	0.064	−0.062	−0.141	−0.154∗
Variables	Vitamin C	Calcium	Iron	Zinc	Potassium
Score of the CES-D scale	−0.171∗	−0.192∗	−0.137	−0.061	−0.142
Variables	**Sodium**	**Nutrition knowledge**	**Dietary attitudes**	**Dietary behaviors**	**Life stress**
Score of the CES-D scale	−0.089	0.011	−0.233∗∗	−0.233∗∗	0.551∗∗

Pearson's correlation coefficient, $*p < 0.05$, $**p < 0.01$

3.5 Correlation Between Changes of Dietary Taurine and Nutrient Intakes

There was no significant correlation between dietary taurine intake and scores of the CES-D scale (Table 6). Significantly negative correlations were observed between the CES-D scale score and intake of vitamin A, folic acid, vitamin C, and calcium ($p < 0.05$) whereas significantly negative correlation was found between the CES-D scale score and dietary attitudes and dietary behaviors ($p < 0.01$).

Scores of the CES-D scale and life stress showed a significantly positive correlation ($p < 0.01$). It has previously been reported that higher the level of depression, higher is the life stress (Lee 2004). However, although we observed no significant correlation between dietary taurine intake and life stress (data not shown), a previous study (Sung and Chang 2009) has reported that life stress of female college was negatively correlated with the dietary taurine intake.

4 Conclusions

These results showed that Korean female college students with depression had poor dietary attitudes and behaviors and an unbalanced nutrition status. Therefore, continuous nutrition education and counseling for good dietary attitudes and behaviors are required urgently. Furthermore, future studies are required to confirm the correlation between dietary taurine intake and depression by intervention with dietary taurine supplementation.

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Relationship Between Taurine Intake and Cardiometabolic Risk Markers in Korean Elderly

Hyosun Jun and Mi-Ja Choi

Abstract Taurine can suppress the creation of lipid hydroperoxide and decrease the level of total cholesterol and triglyceride in the blood. This study aimed to analyze the relationship between biochemical indicators associated with cardiometabolic disease and taurine intake in elderly people. Subjects of this study were those participated in the Korea National Health and Nutrition Examination Survey (KNHANES) 2016, aged over 75 years old. Data of dietary intake were collected through 24-h dietary recall method, and for nutrient analysis, the taurine intake was divided into quadrants. Mean values of taurine intake were 237.8 ± 17.5 mg/day in men and 157.5 ± 12.4 mg/day in women. Both men and women in the quadrants with the higher level of taurine intake appeared to show high intake levels of energy, protein, fat, total cholesterol, calcium, phosphorus, sodium, potassium, riboflavin, and niacin. As a result of relationship analysis between taurine intake and cardiometabolic risk markers (total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, blood pressure, blood glucose, and atherogenic index), taurine intake showed a significantly negative relation with AI in men but showed a positive relation in women with DBP.

Keywords Taurine intake · Blood lipids · Elderly men and women · Blood pressure · Blood glucose

Abbreviations

KDRIs	Dietary Reference Intakes for Koreans
BMI	body mass index
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
FBS	fasting blood sugar

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TG	Triglyceride
TC	Total cholesterol
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein
AI	atherogenic index

1 Introduction

Life expectancy of Koreans has increased sharply, and metabolic syndrome and cardiovascular diseases are increasing, mainly among the elderly. Also, cardiovascular and metabolic disease are one of the major causes of death and one of the factors that degrade the quality of life for elderly in Korea (KCDC 2017; KOSIS 2017). Taurine (2–aminoethanesulfonic acid) is a type of sulfur-containing amino acid that mainly present in animal sources. This amino acid is well known for its antioxidant effect and various functions in the body, such as reducing oxidative damage of unsaturated membrane lipids under many conditions and improving dyslipidemia by cholesterol degradation and promoting excretion of bile acid (Ogasawara et al. 1993; Huxtable 1992; Mochizuki et al. 1998). Because taurine is relatively limited in its source, it is affected by dietary intake, and elderly are more likely to have poor intake than younger groups and have a high prevalence rate of cardiovascular disease (Yoon et al. 2015; Kim et al. 2013).

Therefore, the aim of this study is to examine taurine intake, nutritional status and biochemical indicators associated with cardiometabolic risk markers and to analyze the relationship between taurine intake and risk markers for Koreans aged 75 and older.

2 Subjects and Methods

2.1 Study Population

The Korea National Health and Nutrition Examination Survey (KNHANES) is a national scale cross-sectional research of Korea Centers for Disease Control and Prevention (KCDC), to collect data on the health and nutritional status of Koreans annually. KNHANES includes noninstitutionalized Korean residing in Korea, and uses a multi-stage cluster sampling design. In this research, anthropometric measurements, social, and nutritional status were analyzed by health examination, health interview, and nutritional survey. This study used the data of KNHANES VII-I (2016). Five hundred sixty two elder citizens, aged 75 and older, were targeted out of total 8150 participants. Among them, 540 people who participated in the 24-h dietary recall were examined concerning taurine intake analysis.

2.2 Clinical Measurements and Health Examinations

Measured height and weight were used to calculate BMI (Body mass index) as weight divided by squared height (kg/m^2). Blood pressure was measured three times by sphygmomanometer after 5 min in a sitting position. Blood samples were collected in the overnight fasting state and were analyzed for total cholesterol (TC), triglyceride (TG), HDL-cholesterol, LDL-cholesterol, and FBS (Fasting blood sugar). Normal range standard of respective body and biochemistry factor was referred to the standards of metabolic syndrome diagnosis by NCEP-ATP III (2002) and Korean Society for the Study of Obesity (2018). AI (atherogenic index) was calculated with the following formula (Freedman et al. 1987; Lauer et al. 1988).

$$AI\left(Antherogenic\ Index\right) = \frac{Total\ Cholesterol - HDL\,Cholesterol}{HDL\,Cholesterol}$$

2.3 Dietary Assessment

The food intake inquiries of the participants were calculated from the 24-h dietary recall data collected through individual interviews. Except for taurine, energy and nutrient intake were analyzed using the database of the KCDC. To evaluate the nutritional status of participants' different taurine intake, they were divided into four groups according to their taurine intake to obtain the average nutritional intake per group. We used Dietary Reference Intakes for Koreans (KDRIs 2015) as a basis for assessing the state of nutrients intake in each group; Estimated Energy Requirement (EER) was applied for energy and either Recommended Nutrient Intake (RNI) or Adequate Intake was applied for another nutrients (EER for Energy; RNI for Protein, Vitamin A, Vitamin C, Vitamin B1, Vitamin B2, Niacin, Ca, P, and Fe; Adequate Intake for Dietary fiber, Vitamin D, Na, and K). Nutrients without these criteria were excluded from the KDRIs assessment, such as carbohydrate, fat, and cholesterol. The taurine intake was analyzed with the 24-h dietary recall data based on the database that reflected previous papers (Gormley et al. 2007; Kim et al. 1999; Ozawa et al. 1984; Food safety commission of Japan 2008; Rural Development Administration Korean Food Composition Table, 9th revision 2016).

2.4 Statistic Analysis

Statistical analysis was performed by IBM SPSS software (version 25.0; IBM SPSS Inc., Chicago, IL). Due to the complex sample design of KNHANES, the data was analyzed using a composite sample analysis method concerning sample weight. The

data of descriptive statistic analysis was marked with Mean ± Standard Error, such as nutrients intake of the participants. To assess the correlations between taurine intake and BMI, blood pressure, blood index, and nutrients intake, for the complex samples general linear model was used. All statistical significance levels in this study are set to $p < 0.05$.

3 Results and Discussion

3.1 Basic Characteristics, Dietary Taurine Intake and Biochemical Markers of Subjects

The basic sociodemographic characteristics of the study subjects are shown in Table 1. About 43.9% (n = 241) of the participants were men and 56.1% (n = 321) of them were women. Of all the participants, 36.4% of them do a walking exercise, and 24% of them do regular aerobic workouts. 61.8% of the people consumed more

Table 1 Sociodemographic status of the study subjects

Variables	Item	Frequency	WFP[a]
Sex	Men	241	43.9%
	Women	321	56.1%
Education level	Elementary school	354	66.3%
	Middle school	49	9.9%
	High school	67	14.1%
	University	47	9.7%
Income level	Low	139	24.2%
	Low middle	145	24.8%
	Upper middle	136	22.8%
	High	136	28.2%
Residence	Urban	393	75.8%
	Rural	168	24.2%
Employed	Yes	108	19.2%
	No	410	80.8%
Walking	Yes	191	36.4%
	No	322	63.6%
Aerobic physical activity	Yes	120	24.0%
	No	395	76.0%
Alcohol intake	Yes	225	61.8%
	No	130	38.2%
Smoking status	Smoker	55	30.3%
	Never smoker	149	69.7%

[a]*WFP* weight frequency percent

Table 2 Average dietary taurine intake and cardiometabolic risk markers by sex

Variables	Men (n = 235)	Women (n = 305)
Taurine intake (mg/day)	237.8 ± 17.5[a]	157.5 ± 12.4
BMI (kg/m^2)	23.1 ± 0.2	24.2 ± 0.3
SBP (mmHg)	128.9 ± 1.1	132.3 ± 1.2
DBP (mmHg)	69.1 ± 0.7	70.3 ± 0.6
FBS (mg/dL)	108.3 ± 2.1	107.3 ± 2.0
TG (mg/dL)	131.9 ± 4.0	140.5 ± 4.8
TC (mg/dL)	178.7 ± 2.3	191.3 ± 2.7
HDL-C (mg/dL)	46.2 ± 0.7	47.9 ± 0.8
LDL-C (mg/dL)	109.4 ± 4.1	109.5 ± 6.9
AI	3.1 ± 0.1	3.2 ± 0.1

BMI body mass index, *SBP* Systolic blood pressure, *DBP* Diastolic blood pressure, *FBS* fasting blood sugar, *TG* Triglyceride, *TC* Total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein, *AI* atherogenic index

[a]Mean ± Standard Error

than one drink a month, and 38.2% of them didn't drink. The smokers were 30.3% and the never smokers were 69.7%.

The mean values of dietary taurine intake and biochemical cardiometabolic risk markers of the participants are shown in Table 2. The average taurine intake among the whole participants (n = 540) was 193.1 ± 11.1 mg/day, as 237.8 ± 17.5 mg/day for men, 157.5 ± 12.4 mg/day for women, respectively. The taurine intake by men was 34% higher than the counterpart by women. In a prior study about taurine intake of Koreans, Park et al. (2001) reported that the taurine intake rates of adolescents and adults in Seoul was 177 ± 18.1 mg/day for adults, as for men and women, 216 ± 21.1 mg/day, and 181 ± 14.3 mg/day, respectively. And Kim et al. (2003) reported the average taurine intake in Jeju Island was 163.9 ± 150.2 mg/day. Also, Yim et al. (2004) reported the average taurine intake of adult women in Seoul was 202.0 ± 204.9 mg/day, in Kyunggi was 85.5 ± 67.2 mg/day. Thus, the intake levels examined in this study were at a higher level than the previous studies. Whereas, Kabayashi et al. (2000) reported the average consumption of taurine in Japan was 194 mg/day, as 225.5 mg/day for men, and 162.6 mg/day for women, which is close to this result. The result of the higher rate of taurine intake compared to the previous studies, close to the outcome of Japan, despite the fact that Japanese consumes more fish than Korean, seem influenced by the revision of the food composition tables that causing more taurine content and expansion of its database.

The average BMI was 23.1 kg/m^2 for men, and 24.2 kg/m^2 for women and both considered as overweight, according to the Korean Academy of Obesity standard of overweight (23.0–24.9 kg/m^2). Systolic blood pressure (SBP) was 128.9 mmHg for men, and 132.3 mmHg for women. Both results exceeded the normal value of 120 mmHg. However, diastolic blood pressure (DBP) was 69.1 mmHg, and

70.3 mmHg, respectively, that both fall within the normal range of under 80 mmHg. Regarding FBS, both men and women exceeded the normal value of 100 mg/dL, indicating impaired fasting glucose, TG values were both under the normal range of 150 mg/dL. HDL-C was 46.2 mg/dL for men, and 47.9 mg/dL for women, the result of men was normal with more than 40 mg/dL, but the result of women was below the normal value of 50 mg/dL. LDL-C were both around 109 mg/dL, that fall within the normal range of 100–129 mg/dL, and AI was 3.1 for men, and 3.2 for women, both of which were within normal range, but the outcome of women was a bit higher.

3.2 Nutrient State and the Intake Rate Compared with KDRIs by Taurine Intake Levels

The participants were divided into four groups (Q1, Q2, Q3, and Q4, for men and women) according to their taurine intake and gender, and were compared to each other. The average taurine intake in men Q1 was about 31.1 mg/day, 105.6 mg/day in Q2, 249.7 mg/day in Q3, and 656.8 mg/day in Q4. In the case of women, the average taurine intake was 28.3 mg/day in Q1, 96.6 mg/day in Q2, 261.1 mg/day in Q3, and 611.6 mg/day in Q4.

As shown in Table 3, compared the nutrients intake in men according to their taurine intake, Q4, which had the highest taurine intake, consumed a sufficient energy intake, 100.5% of the KDRIs. Compared to other groups, Q4 also had a higher level of intake of calcium and potassium, which the elderly were deficient in. However, they barely met 77% and 88% of KDRIs, respectively. Also, Q4 showed the highest intake rate of vitamin A, yet again only fulfilled 58% of KDRIs, suggesting the intake level of vitamin A in elderly men was significantly insufficient. Q1, the lowest taurine intake group, had a deficient intake of calcium, potassium, vitamin A, vitamin B2, niacin, and vitamin C, which meets only half of the KDRIs standard. Accordingly, groups with relatively higher taurine intake consumed, close to or satisfied with KDRIs, regarding calcium, potassium, vitamin B2, and vitamin C, however, intake levels of vitamin A were significantly insufficient in all groups.

As shown in Table 4, compared the nutrients intake in women according to their taurine intake, the highest taurine intake group Q4 consumed 93.7% of KDRIs energy standard, and even though their levels of intake of calcium and potassium were the highest among groups, the intake levels for calcium and potassium were 77% and 70.2%, respectively. The intake of Vitamin A was also taken the most, yet the intake level was 71.3%, indicating that elderly women took vitamin A at a significantly inferior level. For those with low taurine intake, Q1 and Q2, the intake for vitamin B2 were 46.2% and 66.4% respectively, and it showed an even more insufficient result for niacin, as they were only 51.7% and 61.6% respectively. Overall, groups with higher taurine intake were closer or satisfied with KDRIs standard with calcium, potassium, vitamin B2, and vitamin C, but levels of vitamin A intake were insufficient in general. Also, all women groups showed poor nutrition status of less than 75% of KDRIs intake rates of calcium, potassium, and vitamin A.

Table 3 The nutrient intake and the percentage of KDRIs by taurine intake levels for men

	Q1	Q2	Q3	Q4	*P*-value
Energy (kcal)	1489 ± 69[a] (74.5)[b]	1686 ± 94 (84.3)	1942 ± 84 (97.1)	2010 ± 66 (100.5)	<0.001
Protein (g)	39.0 ± 2.2 (70.8)	51.2 ± 2.8 (93.0)	66.7 ± 3.7 (121.2)	73.2 ± 4.0 (133.1)	<0.001
Fat (g)	16.8 ± 2.6	25.6 ± 2.9	34.4 ± 3.2	36.7 ± 2.8	<0.001
Cholesterol (mg)	62.3 ± 11.6	148.8 ± 29.3	239.4 ± 32.9	272.6 ± 26.8	<0.001
Carbohydrate (g)	275.8 ± 11.8	289.8 ± 15.3	325.9 ± 15.8	323.6 ± 13.6	0.032
Dietary Fiber (g)	17.8 ± 1.5 (71.1)	22.3 ± 1.3 (89.3)	26.6 ± 1.7 (106.4)	24.7 ± 1.5 (99.0)	<0.001
Ca (mg)	280.6 ± 24.1 (40.0)	404.4 ± 35.8 (57.8)	500.2 ± 41.4 (71.5)	539.0 ± 44.5 (77.0)	<0.001
P (mg)	642 ± 35 (91.8)	822 ± 40 (117.5)	1048 ± 60 (149.8)	1120 ± 52 (160.0)	<0.001
Fe (mg)	12.3 ± 0.9 (136.6)	15.2 ± 1.0 (168.6)	18.0 ± 1.0 (199.8)	17.9 ± 0.8 (198.7)	<0.001
Na (mg)	2563 ± 190 (233.0)	3643 ± 483 (331.2)	3700 ± 189 (336.4)	3978 ± 405 (361.7)	<0.001
K (mg)	2046 ± 138 (58.5)	2460 ± 143 (70.3)	3285 ± 237 (93.9)	3099 ± 194 (88.6)	<0.001
Vitamin A (μgRE)	208.9 ± 42.5 (29.8)	310.1 ± 39.2 (44.3)	394.7 ± 62.5 (56.4)	410.0 ± 42.7 (58.6)	0.013
Vitamin B_1 (mg)	1.4 ± 0.1 (114.8)	1.5 ± 0.1 (128.6)	2.0 ± 0.1 (165.0)	2.0 ± 0.1 (163.3)	<0.001
Vitamin B_2 (mg)	0.7 ± 0.1 (43.5)	1.0 ± 0.1 (65.7)	87.91.3 ± 0.1 (87.9)	1.4 ± 0.1 (95.9)	<0.001
Niacin (mg)	8.9 ± 0.5 (55.7)	11.1 ± 0.8 (69.2)	14.7 ± 0.9 (92.1)	16.3 ± 1.3 (102.1)	<0.001
Vitamin C (mg)	59.0 ± 7.7 (59.0)	86.9 ± 9.9 (86.9)	137.7 ± 19.0 (137.7)	132.0 ± 21.6 (132.0)	<0.001

Dietary Reference Intakes for Koreans 2015

[a]Mean ± Standard Error

[b]Percentage of KDRIs 2015. EER for Energy; RNI for Protein, Vitamin A, Vitamin C, Vitamin B_1, Vitamin B_2, Niacin, Ca, P, and Fe; Adequate Intake for Dietary fiber, Vitamin D, Na, and K

3.3 Relationship Between Taurine Intake and Cardiometabolic Risk Markers

The relationship between taurine intake and cardiometabolic risk markers are shown in Table 5 for men and Table 6 for women. AI was significantly increased in accordance with taurine intake (p = 0.024) in men.

In women, on the other hand, DBP increased significantly with taurine intake (p = 0.022). The average taurine intake for people aged 75 and older was 237.8 ± 17.5 mg/day for men and 157.5 ± 12.4 mg/day for women. Comparing the

Table 4 The nutrient intake and the percentage of KDRIs by taurine intake levels for women

	Q1	Q2	Q3	Q4	*P*-value
Energy (kcal)	1180 ± 39[a] (73.8)[b]	1266 ± 59 (79.1)	1378 ± 56 (86.2)	1498 ± 72 (93.7)	<0.001
Protein (g)	31.2 ± 1.3 (69.3)	40.0 ± 1.8 (88.9)	48.2 ± 2.8 (107.1)	56.0 ± 3.3 (124.4)	<0.001
Fat (g)	12.6 ± 1.1	18.8 ± 1.5	25.0 ± 2.0	31.4 ± 3.6	<0.001
Cholesterol (mg)	40.6 ± 10.0	121.6 ± 21.7	174.4 ± 34.9	203.4 ± 27.1	<0.001
Carbohydrate (g)	232.1 ± 7.7	230.9 ± 11.4	237.6 ± 13.0	239.5 ± 12.5	0.930
Dietary Fiber (g)	15.1 ± 0.8 (75.4)	17.6 ± 1.2 (88.2)	18.9 ± 1.4 (94.7)	20.4 ± 2.0 (102.1)	0.007
Ca (mg)	232.2 ± 16.9 (29.0)	278.0 ± 17.4 (34.8)	351.1 ± 21.2 (43.9)	423.5 ± 36.7 (52.9)	<0.001
P (mg)	521.8 ± 20.1 (74.5)	644.1 ± 31.0 (92.0)	783.5 ± 31.7 (111.9)	869.8 ± 45.8 (124.3)	<0.001
Fe (mg)	10.2 ± 0.7 (146.2)	11.4 ± 0.6 (162.4)	12.5 ± 0.8 (178.5)	14.1 ± 1.3 (200.9)	0.030
Na (mg)	2136 ± 161 (194.2)	2181 ± 141 (198.3)	2546 ± 198 (231.5)	2799 ± 271 (254.5)	0.082
K (mg)	1680 ± 84 (48.0)	1905 ± 122 (54.5)	2327 ± 141 (66.5)	2458 ± 207 (70.2)	<0.001
Vitamin A (μgRE)	201.4 ± 26.9 (36.6)	235.0 ± 37.0 (42.7)	306.5 ± 54.6 (55.7)	392.1 ± 69.9 (71.3)	0.062
Vitamin B_1 (mg)	1.1 ± 0.0 (103.1)	1.3 ± 0.1 (116.0)	1.4 ± 0.1 (126.8)	1.4 ± 0.1 (127.7)	0.002
Vitamin B_2 (mg)	0.6 ± 0.0 (46.2)	0.8 ± 0.1 (66.4)	1.1 ± 0.2 (89.4)	1.2 ± 0.1 (101.7)	<0.001
Niacin (mg)	7.2 ± 0.3 (51.7)	8.6 ± 0.5 (61.6)	10.6 ± 0.6 (75.7)	12.9 ± 1.4 (91.8)	<0.001
Vitamin C (mg)	51.9 ± 6.0 (51.9)	98.3 ± 15.2 (98.3)	96.5 ± 13.6 (96.5)	112.0 ± 35.8 (112.0)	0.001

Dietary Reference Intakes for Koreans 2015

[a]Mean ± Standard Error

[b]Percentage of KDRIs 2015. EER for Energy; RNI for Protein, Vitamin A, Vitamin C, Vitamin B_1, Vitamin B_2, Niacin, Ca, P, and Fe; Adequate Intake for Dietary fiber, Vitamin D, Na, and K

nutritional status of the elderly according to the taurine intake, the group with the highest taurine intake was better than the other group when it comes to nutritional intakes, such as energy, protein, calcium, phosphorous, potassium, vitamin B2, niacin, and vitamin C. On the other hand, although the highest taurine intake group also showed higher intake for vitamin A, all groups were found to be insufficient vitamin A intake compared to KDRIs. And all women groups had poor calcium intake as well. As a result after assessing the relations between taurine intake and cardiometabolic risk markers, there was a significant increase of DBP in women, while in men,

Table 5 Complex samples general linear model for the association between taurine intake and cardiometabolic risk markers in men

Parameter	Estimate	Standard error	*t* value	Pr > |*t*|	*F* value	*Pr* > *F*
Intercept	1,691.454	808.427	2.092	0.038	3.300	0.072
Age	−18.639	10.260	−1.817	0.072		
Intercept	152.512	109.760	1.390	0.167	0.600	0.440
BMI (kg/m^2)	3.699	4.776	0.775	0.440		
Intercept	327.559	150.169	2.181	0.031	0.363	0.548
SBP (mmHg)	−0.685	1.138	−0.602	0.548		
Intercept	274.228	114.008	2.405	0.018	0.099	0.753
DBP (mmHg)	−0.509	1.617	−0.315	0.753		
Intercept	335.537	55.345	6.063	0.000	2.574	0.111
FBS (mg/dL)	−0.779	0.485	−1.604	0.111		
Intercept	289.077	42.452	6.809	0.000	0.937	0.335
TG (mg/dL)	−0.285	0.295	−0.968	0.335		
Intercept	414.413	94.960	4.364	0.000	3.031	0.084
TC (mg/dL)	−0.911	0.523	−1.741	0.084		
Intercept	211.241	69.572	3.036	0.003	0.396	0.531
HDL (mg/dL)	0.881	1.400	0.629	0.531		
Intercept	391.475	172.428	2.270	0.030	0.835	0.367
LDL (mg/dL)	−1.240	1.357	−0.914	0.367		
Intercept	351.348	45.925	7.651	0.000	5.188	0.024
AI	−32.448	14.246	−2.278	0.024		

as their taurine intake increasing, AI decreases and it appeared to be beneficial to the prevention of heart disease. We assume that the taurine intake was not effective on cardiometabolic indicators in women is related to the fact that women's average taurine intake is lower than men's, only about 67%. And which requires further detailed research.

Taurine intake only has effects in men because it may be a greater intake for taurine; taurine intake was high compared with women. Also calcium intake is very low in women compared to men and calcium intake is associated with coronary heart disease and blood pressure (Bostick et al. 1999; Reid et al. 2002; Umesawa et al. 2006).

4 Conclusion

In conclusion, taurine decreased cardiometabolic disease risk in athrogenic index (AI) in male elderly, but did not in female elderly. More research is necessary to explain why the effectiveness of taurine in reducing the AI depends on gender.

Table 6 Complex samples general linear model for the association between taurine intake and cardiometabolic risk markers in women

Parameter	Estimate	Standard error	*t* value	Pr > |*t*|	*F* value	*Pr* > *F*
Intercept	751.850	611.323	1.230	0.221	0.961	0.329
Age	−7.592	7.744	−0.980	0.329		
Intercept	138.437	74.322	1.863	0.065	0.061	0.806
BMI (kg/m^2)	0.792	3.216	0.246	0.806		
Intercept	53.648	78.633	0.682	0.496	1.737	0.190
SBP (mmHg)	0.784	0.595	1.318	0.190		
Intercept	−29.519	77.380	−0.381	0.703	5.382	0.022
DBP (mmHg)	2.662	1.147	2.320	0.022		
Intercept	81.565	54.959	1.484	0.140	2.003	0.159
FBS (mg/dL)	0.759	0.536	1.415	0.159		
Intercept	160.949	25.446	6.325	0.000	0.007	0.932
TG (mg/dL)	0.014	0.169	0.085	0.932		
Intercept	71.661	48.369	1.482	0.141	3.668	0.058
TC (mg/dL)	0.476	0.248	1.915	0.058		
Intercept	154.631	56.472	2.738	0.007	0.008	0.931
HDL (mg/dL)	0.093	1.070	0.087	0.931		
Intercept	169.577	87.037	1.948	0.060	0.028	0.867
LDL (mg/dL)	−0.129	0.767	−0.168	0.867		
Intercept	126.418	30.386	4.160	0.000	1.075	0.302
AI	10.271	9.907	1.037	0.302		

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Relationships Between the Purposes of Taurine-Contained Nutritional Drink Intake and Lifestyle Habits: A Cross-Sectional Survey of Workers in Japan

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Abstract This study surveyed that the relationship between the frequencies of intake of taurine-contained nutritional drinks (TCND), and lifestyle and the purposes of intake it. The study was conducted a cross-sectional survey using 265 people (203 male, 62 female) aged 18–64 worked in two companies in Mie Prefecture, Japan between December 2017 and February 2018. The questionnaires gathered characteristics, demographic, socioeconomic, lifestyle habits and purpose of TCND intake. We divided the frequency of intake of TCND of at least a few times every month as the high-frequency TCND (HF-TCND) group, and the remaining as the low-frequency TCND (LF-TCND) group. Multivariate logistic regression analysis was used to investigate the relationship between characteristics, demographic, socioeconomic, lifestyle habits and purpose of TCND intake and HF-TCND after

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controlling for individual variables. Of all participants, 13.4% was evaluated as HT-CND. 16.3% for male or 4.3% for female were evaluated as HF-TCND ($p < 0.05$). The most reason for frequent choosing a TCND was fatigue recovery. Logistic regression analysis showed that sex, occupation, purpose of TCND intake and stressful are related to HF-TCND. Our study indicates that purpose of TCND intake, such as fatigue recovery and reducing stress, may partly affect the frequency of intake of TCND. Therefore, we must continue to show scientific evidence for taurine by enlightenment activity etc.

Keywords Taurine-contained nutritional drinks · Cross-sectional survey · Workers · Lifestyle habits · Fatigue recovery

Abbreviations

TCNB	Taurine-contained nutritional drinks
HF-TCND	High-frequency TCND
HF-TCND	Low-frequency TCND
OR	Odds ratios
CI	Confidence intervals

1 Introduction

In the modern society, energy drink typified by Red Bull and other is very popular (Nowak and Jasionowski 2015; Breda et al. 2014). Each energy drink contains many ingredients, including: caffeine, taurine, B group vitamins and many others. These energy drinks are sold in a form including taurine in other countries excluding Japan. However, because manufactured and released under the Japanese standard of soft drinks, taurine cannot be used in Japan. Synthetic products of taurine are treated as medicines, mainly used as the main ingredient of nutritional drinks including quasi-drugs.

The following indicators can be displayed for quasi-drugs of nutritional drinks. (1) Maintenance and improvement of physical strength and resistance and concentration. (2) Recovery from and prevention of fatigue. (3) Improvement and prevention of health problems associated with the weak constitution (including age-related physical weakness). (4) Improvement and prevention of poor health caused by malnutrition in daily life. (5) Supply of nutrients in the following cases: decreased physical strength during or after illness, exhaustion with fever, loss of appetite, during pregnancy or lactation. These indicators have sub-divided by the effectiveness of the many ingredients contained in nutritional drinks. Taurine has been classified as “tired easily, exhausted fatigue, poor physical fitness, heavy body and heavy body” or “declines in appetite due to a hangover, dullness” (The Ministry of Health

Labor and Welfare 2017). Among them, "fatigue recovery" is the most commonly cited reason for purchasing nutritional drinks, and most nutrition drinks contain taurine in Japan.

Synthetic products of taurine suggest the effectiveness of improvement of liver function in hyperbilirubinemia (excluding obstructive jaundice) and congestive heart failure (Azuma et al. 1985; Matsuyama et al. 1983). However, the effects of taurine supplementation on the fatigue recovery remain to be not defined in humans (National Institute Health and Nutrition 2018).

The purpose of this study is to provide basic data on the epidemiological features of the frequencies of intake of taurine-contained nutritional drinks (TCND) in young and middle-aged adults including characteristics, demographic, socioeconomic, lifestyle habits and purpose of TCND intake.

2 Methods

A cross-sectional study was conducted on the employees of two companies, who were recruited by the public health department of Mie Prefecture, Japan between December 2017 and February 2018. These companies consisted of various white-collar (85.4%) and blue-collar (14.6%) departments at a chemical company (Company A, 141 day-shift employees) and electronics company (Company B, 201 day-shift employees). These companies notified their employees of the present study, provided the measurement rooms used for the study, and facilitated the conduct of the study. Therefore, these companies and their employees actively participated in the study. All participants provided written informed consent prior to participating in this study, which was approved by the Institutional Review Board of Suzuka University of Medical Science (approval No. 241). This study was conducted according to the principles of the Declaration of Helsinki.

Data on frequency intake of TCND, demographic, socioeconomic, lifestyle habits and the purposes of TCDN intake were collected using paper-based questionnaires. The independent variables included; age (<45 or ≥45), body mass index (BMI) (<18.5 or 18.5–24.9 or ≥25.0), sex (male or female), education (<13 years or ≥13 years), occupation (white – collar or blue – collar), income (<5 million yen/year or ≥5 million yen/year), smoking (none, past smoker, current smoker), alcohol drinking (none, a few times/month, a few times/week, daily), purpose of TCND intake (other or fatigue recovery) and stressful (Yes or No).

Participants were classified as the frequency of intake of TCND of at least a few times every month as the high-frequency TCND (HF-TCND) group, and the remaining as the low-frequency TCND (LF-TCND) group. Differences in categorical variables between the HF-TCND and the LF-TCND groups were evaluated using the chi-square test. Multivariate logistic regression analysis was used to investigate the relationship between the purpose of TCND intake and, lifestyle, and the frequency of intake of TCND after adjusting for sex. The independent variables included sex, age, BMI, occupation, income, smoking, alcohol drinking, purpose of TCND intake

and stressful. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated. All statistical analyses were conducted using JMP 9.0.2 (SAS Institute Inc., Cary, NC, USA).

3 Result

In total, 265 participants responded to the questionnaire (203 male and 62 female) (mean age ± standard deviation = 43.5 ± 11.6 years; range = 18–64 years), and the response rate was 77.5% (265 of 342 eligible employees). The response rate of Company A rate was 70.9% (100 of 141 eligible employees), Company B rate was 82.1% (165 of 201 eligible employees). Of all participants, 13.4% was evaluated as HF-TCND. 16.3% for male or 4.3% for female were evaluated as HF-TCND ($p < 0.05$).

In response to the question "What kind of effect do you expect in TCND?" 71.6% of the participants answered "Recovery from and prevention of fatigue" (Fig. 1).

The distributions of the participant characteristics according to the HF-TCND or LF-TCND group are shown in Table 1. Sex, occupation, the purpose of TCND intake and stressful significantly differed between groups, whereas age, education, income, smoking, and alcohol drinking did not significantly differ between groups.

Table 2 shows the results of logistic regression analysis of associated factors for HF-TCND in all participants. The odds rate of HF-TCND was 4.335 (95% Cl 1.127–22.124) for male compared with female, and 3.479 (1.270–9.484) for occu-

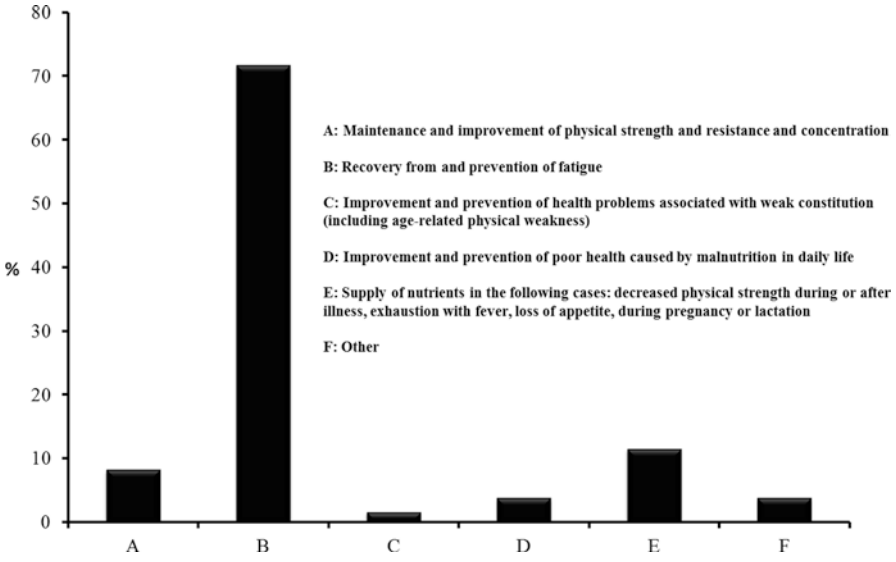

Fig. 1 What kind of effect do you expect on taurine-contained nutritional drinks?

Table 1 Characteristics of the LF-TCND and HF-TCND groups

Characteristics	Total	LF-TCND	HF-TCND	*P*-value
Age, n (%)				
<45 years	126 (47.5)	111 (88.1)	15 (11.9)	
≥45 years	139 (52.5)	118 (84.8)	21 (15.2)	0.4334
Sex, n (%)				
Male	203 (76.6)	170 (83.7)	33 (16.3)	
Female	62 (23.4)	59 (95.2)	3 (4.8)	0.0216
BMI, n (%)				
<25.0	187 (70.6)	163 (87.2)	24 (12.8)	
≥25.0	78 (29.4)	66 (84.6)	12 (15.4)	0.5808
Education, n (%)				
<13 years	116 (43.8)	103 (88.8)	13 (11.2)	
≥13 years	149 (56.2)	126 (84.6)	23 (15.4)	0.3188
Occupation, n (%)				
White – collar	222 (84.7)	196 (88.3)	26 (11.7)	
Blue – collar	40 (15.3)	30 (75.0)	10 (25.0)	0.0246
Income, n (%)				
<5 million yen	55 (20.9)	46 (83.6)	9 (16.4)	
≥5 million yen	208 (79.1)	183 (88.0)	25 (12.0)	0.3931
Smoking, n (%)				
None	163 (61.5)	143 (87.7)	20 (12.3)	
Past smoker	40 (15.1)	34 (85.0)	6 (15.0)	
Current smoker	62 (23.4)	52 (83.9)	10 (16.1)	0.7225
Alcohol drinking, n (%)				
None	87 (32.8)	74 (85.1)	13 (14.9)	
A few times/month	42 (15.9)	39 (92.9)	3 (7.1)	
A few times/week	76 (28.7)	64 (84.2)	12 (15.8)	
Daily	60 (22.6)	52 (86.7)	8 (13.3)	0.5851
Consumption purpose, n (%)				
Fatigue recovery	190 (71.7)	158 (83.2)	32 (16.8)	
Other	75 (28.3)	71 (94.7)	4 (6.3)	0.0150
Stressful, n (%)				
Yes	194 (73.2)	162 (83.5)	32 (16.5)	
No	71 (26.8)	67 (94.4)	4 (5.6)	0.0223

Data were expressed as n (%)
TCNB taurine-contained nutritional drinks, *HF-TCND* High-frequency TCND, *LF-TCND* Low-frequency TCND, *BMI* body mass index

pation (blue-collar) compared with occupation (white – collar), and 4.878 (1.559–21.811) for consumption purpose (fatigue recovery) compared with the purpose of TCND intake (other), and 3.269 (1.146–11.766) for stressful (yes) compared with stressful (no).

Table 2 Logistic regression analysis of factors associated with HF-TCND

Variable	OR	95% Cl		*P*-value
		Lower	Upper	
Age				
<45 years	Ref			
≥45 years	1.656	0.725	3.923	0.2329
Sex				
Female	Ref			
Male	4.335	1.127	22.124	0.0319
BMI				
<25.0	Ref			
≥25.0	1.053	0.434	2.455	0.9061
Education, n (%)				
<13 years	Ref			
≥13 years	1.450	0.589	3.764	0.4238
Occupation, n (%)				
White – collar	Ref			
Blue – collar	3.479	1.270	9.484	0.0159
Income, n (%)				
<5 million yen	Ref			
≥5 million yen	0.851	0.334	2.330	0.7442
Smoking				
None	Ref			
Past smoker	1.050	0.353	3.436	0.9319
Current smoker	1.068	0.403	2.952	0.8965
Alcohol drinking				
None	Ref			
A few times/month	0.323	0.064	1.231	0.1009
A few times/week	0.812	0.287	2.274	0.6906
Daily	1.536	0.512	4.748	0.4439
Consumption purpose				
Other	Ref			
Fatigue recovery	4.878	1.559	21.811	0.0046
Stressful				
No	Ref			
Yes	3.269	1.164	11.766	0.0230

TCNB taurine-contained nutritional drinks, *HF-TCND* High-frequency TCND, *BMI* body mass index, *CI* confidence interval, *OR* odds ratio

4 Discussion

The purpose of this study was to investigate the correlation between the intake of TCND and lifestyle in young and middle-aged adult. The purpose of consuming of TCND was also analyzed in this study. Our study showed that sex, occupation, the purpose of TCND intake and stressful are related to HF-TCND. In this cross-sectional observational study of Japanese young and middle-aged adults, fatigue recovery and reducing stress were significantly associated with HF-TCND in the purpose of TCND intake.

Taurine is a derivative of the amino acid cysteine and is found in high quantities in heart and skeletal muscle (Eley et al. 1994). According to WHO-CARDIAC study of Yamori et al. reported that people who consume a lot of seafood, which contains taurine in abundance, have a lower risk of developing metabolic diseases such as obesity, diabetes, and hypertension (Yamori et al. 2001, 2010; Sagara et al. 2015). In Japan, taurine is added in a large number of ND as Lipovitan D™ and Tiovita Drink™. Although taurine is considered a conditionally essential nutrient for humans and is thought to play a key role in several human diseases (Kendler 1989; Huxtable 1992; Sturman 1993), clinical studies evaluating the effects of taurine are limited.

In our study, the most reason for frequent choosing TCND was fatigue recovery. Moreover, the HF-TCND group showed that a significant fatigue recovery or stressful as the purpose of TCND intake compared with the LF-TCND group. Oxidative stress is involved in the mechanism of exercise-induced fatigue (Barclay and Hansel 1991) and mental stress (Sivoňová et al. 2004) in healthy humans, as well as the pathophysiology of prolonged fatigue (chronic fatigue syndrome) (Logan and Wong 2001; Manuely et al. 2001; Vecchiet et al. 2003). Several studies in clinical studies have shown nutrients to reduce oxidative stress (Takemoto et al. 2015; Hongo et al. 2017), but taurine has not been clearly. Each nutritional drink contains many ingredients other than taurine, including caffeine, B group vitamins, and many others. While a synergistic effect between each ingredient in nutritional drinks has been expected, few clinical reports are available on showing synergistic effect with taurine against recovery from fatigue.

However, more than 71.6% of participants chose fatigue recovery on the effect of TCND, and fatigue recovery and stressful as the purpose of TCND intake were related to HF-TDNC. In the result of this study, participants might be causing mislead that taurine is restoring fatigue. Therefore, we must continue to show scientific evidence for taurine by enlightenment activity etc.

This study has several limitations. First, this study was occupational field-based, not population-based. Moreover, because the participants in this study were workers, care should be taken when generalizing these results to a general population of the same generation. Second, due to the ratio of male and female greatly varied between companies, we calculated which lumped all the companies together. Third, data from cross-sectional studies are not sufficient to determine whether a causal relationship exists between sex, occupation, the purpose of TCND intake and stress-

ful, and HF-TCND. Therefore, it is crucial to perform longitudinal studies to clarify the causal relationships among these factors. Finally, a female employee was more difficult to recruit for this study. Future research should be conducted using a larger and more diverse sample group including both genders.

5 Conclusion

We conducted a cross-sectional study to investigate the relationship between the frequencies of intake of TCND, and lifestyle and the purposes of consuming it of young and middle-aged adults in two companies. As a result, the most reason for frequent choosing a TCND was fatigue recovery, and sex, occupation, the purpose of TCND intake and stressful were an important associated factor for HF-TCND. Fatigue recovery and reducing stress were an important factor to increase the TCND intake. However, participants might be causing mislead that taurine is restoring fatigue. Therefore, we must continue to show scientific evidence for taurine by enlightenment activity etc.

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Taurine-Related Nutritional Knowledge Has a Positive Effect on Intake of Taurine and Cognitive Function in the Elderly

Mi Ae Bae, So Hyun Park, So Hee Han, Kyung Ja Chang, and Sung Hoon Kim

Abstract The purpose of this study was to investigate the correlations among taurine-related nutritional knowledge (TNK), taurine intake frequency (TIF), and cognitive function (CF) in Korean elderly. Subjects of this cross-sectional study were 278 elderly persons in Korea without dementia (men 76, women 202). The subjects were divided into two groups: a group with a lower than average TNK score (LAG) and a group with a higher than average TNK score (HAG). Data were obtained via questionnaires and 1: 1 interviews. Correlation analysis available in SPSS 20.0 was used to analyze associations among the three factors. Average TNK score of all subjects was 4 out of 10 points, while that of the LAG (1.9 points) was significantly lower than that of the HAG (6.6 points) ($p < 0.001$). Average TIF score in the HAG (195.3 points) was significantly higher than that of the LAG (180.8 points) ($p < 0.001$). Compared to the LAG, the HAG members more frequently consumed foods such as webfoot octopus, whelk, dried anchovy, dried shrimp, mackerel, anglerfish, skate, short-necked clam, shrimp, and dried squid ($p < 0.01$). There was no significant difference in CF scores between the LAG and HAG. There was positive correlation between TNK and TIF scores in all the subjects ($p < 0.01$). There was no significant difference, but there was a tendency for a positive correlation, between TNK and CF scores ($p = 0.072$). These results suggest that nutritional education of the elderly about taurine is needed, and it is strongly recommended that the elderly frequently consume taurine-containing foods and supplements to prevent dementia.

Keywords Nutritional knowledge · Cognitive function · Intake of Taurine · Taurine · Elderly

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Abbreviations

TNK	Taurine-Related Nutritional Knowledge
TIF	Taurine Intake Frequency
CF	Cognitive Function
LAG	Group Lower than Average TNK Score
HAG	Group Higher than Average TNK Score
AD	Alzheimer's disease
MMSE-DS	Mini-Mental State Examination-Dementia Screening
SE	Standard Error

1 Introduction

Around the world, the proportion of the population that is the elderly increasing while the younger proportion is decreasing (United Nations Population Division 2017). In Korea, elderly people aged 65 or older account for approximately 14.2% of the total population, and Korea became an 'aged society' in 2017 (Ministry of the Interior and Safety 2017). Compared to other countries, the entry into an aging society in Korea was slow, but Korean societal aging is proceeding very rapidly (Choi 2007).

Korea is experiencing various problems due to rapid aging of its population. Among them, the mental health problems related to aging, dementia is rapidly increasing (World Health Organization News 2017a).

Dementia is a diverse brain disease that affect memory, language, thinking, emotion, and behavioral ability. Alzheimer's disease (AD) is the most common form of dementia in the elderly (World Health Organization News 2017b). According to data from the Central Dementia Center (2018) in Korea, the number of patients with dementia was 724,000 in 2018.

Taurine is known to play an important role in brain function and to be effective in neuroprotection and cognitive improvement (Carlo et al. 2012; Idrissi 2008). Moreover, animal experiments have shown that taurine has a positive role in recover memory (Kim et al. 2014). Taurine is mostly supplied from fish, shellfish, and animal protein (Benrabh et al. 1995; Stapleton et al. 1997). Previously, we investigated the correlation between cognitive function (CF) and the frequency of past taurine intake in elderly subjects with dementia and normal elderly subjects. The results, suggested that past taurine intake may have a positive effect on present CF in the elderly (Bae et al. 2017). Thus, there was a need to study the factors affecting taurine intake of the elderly.

Therefore, the purpose of this study was to investigate the correlations among assessment scores for taurine-related nutritional knowledge (TNK), taurine intake frequency (TIF), and cognitive function (CF) in Korean elderly subjects.

2 Methods

2.1 Subjects

As this was a human study, the study was conducted after obtaining the approval of the Institutional Review Board of Inha University (171120-9A). The subjects in this cross-sectional study were 278 elderly people (men 76, women 202) without dementia and aged over 65 years who were attending welfare facilities for the elderly and senior community centers in Incheon, a metropolitan city in Korea. Subjects were divided into two groups: a group with lower than average (LAG) taurine-related nutritional knowledge (TNK) scores and a group with higher than average (HAG) TNK scores.

2.2 Data Collection

Data were collected by using questionnaires and face-to-face interviews of elderly subjects in January to March, 2018. We investigated the subjects' general characteristics, TNK scores, intake frequency scores for taurine-containing foods and supplements (TIF), and cognitive function (CF) scores.

2.2.1 Taurine-Related Nutritional Knowledge

Assessment of TNK included ten questions based on the contents and results of previous studies as shown in Table 2 (Yoon et al. 2015). Construct validity of the questionnaire was confirmed through question analysis and factor analysis. The confidence coefficient of TNK in this study was 0.796.

The data were collected by the researcher directly by asking each question to each subject. Responses were given 1 point for the correct answer, and 0 points for the wrong or unknown answer. The total score range was 0–10 points and the higher the score, the higher the taurine-related nutritional knowledge.

2.2.2 Frequency of Taurine Intake

The TIF was evaluated by using the taurine index method developed in my previous study, and it was scored by 40 kinds of taurine-containing foods and supplements, which are easy to contact or consume for the elderly in Korea (Bae et al. 2017).

Each food and supplement was given 1–3 points according to their taurine content per 100 g. Foods and supplements containing less than 100 mg of taurine (low content) were given 1 point, while those containing 100–499.9 mg (medium content) were given 2 points, and foods and supplements containing more than 500 mg

(high content) were given 3 points. Taurine low content foods included butterfish, eel, mudfish, sea cucumber, warty sea squirt, green laver, sea tangle, seaweed, cod, yellow corvine, hairtail, agar-agar, and dried squid. Taurine medium content foods included squid, cuttlefish, octopus, mackerel, Spanish mackerel, pacific saury, atka mackerel, anglerfish, skate, flatfish, pollack, cockle, short-necked clam, and shrimp. Taurine high content foods and supplements included webfoot octopus, long-arm octopus, oyster, abalone, manila clam, mussel, whelk, crab, dried anchovy, dried shrimp, energy drink, and taurine supplement tablet.

The intake frequency of each food and supplement was assessed directly by each subject by using a Likert 5 – point scale, and the scores were assigned as follows: never eat (1 point), eat one time per 2–3 month (2 points), eat one to two times per month (3 points), eat one to two times per week (4 points), and eat everyday (4 points). The TIF score was formed by multiplying the score of taurine content (1–3 points) and the score of TIF (1–5 points) for each food and supplements was 79–395 points.

2.2.3 Cognitive Function

CF was evaluated by using the MMSE-DS, which is an easy, simple, and widely used method of assessing dementia (Arevalo-Rodriguez et al. 2015; Han et al. 2010).

There are eight categories in the MMSE-DS. The categories include time orientation (5 points), place orientation (5 points), memory (6 points), attention (5 points), language (3 points), ability to execute (3 points), visuospatial construction (1 point), and judgment and abstract thinking (2 points). The total maximum score is 30 points, and the higher score is indicative of better CF (Han et al. 2010).

2.3 Statistical Analysis

The statistical analysis of all data was conducted by using the SPSS 20.0 program. Data are expressed as percentages or as a mean ± standard error (SE). Statistical significance of differences was determined by Student's t-test, and correlations of scores of TNK, TIF, and CF were determined by using Pearson's correlation analysis. A significant difference was set at $p < 0.05$.

3 Results and Discussion

3.1 General Characteristics Data

The results summarizing the general characteristics of the subjects are shown in Table 1. The proportion of males and females in all subjects were 27.3% and 72.7%, respectively. The average age of all subjects was 71.0 years, and that in the LAG

Table 1 General characteristics of the subjects

Variables (units)	Total (N = 278)	Taurine-related nutritional knowledge level	
		LAG (N = 152)	HAG (N = 126)
Gender			
Male	76 (27.3)	46 (30.3)	30 (23.8)
Female	202 (72.7)	106 (69.7)	96 (76.2)
Age (years)	71.0 ± 0.5	72.6 ± 0.7	69.0 ± 0.7***
Education			
≤ Elementary school	84 (30.2)	50 (32.9)	34 (27.0)
≤ Middle school	37 (13.3)	24 (15.8)	13 (10.3)
≤ High school	94 (33.8)	51 (33.6)	43 (34.1)
≥ University	63 (22.6)	27 (17.8)	36 (28.6)
Family members (person)			
Alone	59 (21.2)	33 (21.7)	26 (20.6)
With spouse	81 (29.1)	40 (26.3)	41 (32.5)
With children	42 (15.1)	28 (18.4)	14 (11.1)
With spouse and children	80 (28.8)	43 (28.3)	37 (29.4)
Etc.	16 (5.7)	8 (5.3)	8 (6.4)
Household income (won)[a]			
⟨1 million	81 (29.1)	49 (32.2)	32 (25.4)
1 million -1.99 million	36 (12.9)	21 (13.8)	15 (11.9)
2 million -2.99 million	44 (15.8)	22 (14.5)	22 (17.5)
3 million -3.99 million	54 (19.4)	30 (19.7)	24 (19.0)
4 million ≤	63 (22.7)	30 (19.8)	33 (26.2)

N (%), Mean ± SE, $***p < 0.001$ by Student's t-test
[a]1125.9 KRW = 1 USD as of July 2018

(72.6 years) was significantly higher than that in the HAG (69.0 years). This result indicates that differences in knowledge levels may be related to age. However, there was no significant difference in the education level of the two groups.

Regarding the assessment of living with family members, there was no significant difference between LAG and HAG. The percentage of people living alone was 21.2% of the all subjects, and 29.1% and 15.1% were living with their spouses or children, respectively. In addition, 28.8% of all subjects were living together with their spouses and children.

Monthly average household income was not significantly different between the two groups. However, subjects of low-income with a household income of less than one million (1,000,000 KRW, 888.18 USD as of July 2018) represented 32.2% and 25.4% of the LAG and HAG, respectively. Taurine is mainly contained in seafood, shellfish, and meat, and typically, such foods are more expensive than vegetables and other foods. Therefore, even though taurine is effective in preventing dementia, subjects may hesitate to buy such a food item because it may be expensive. Thus, it is necessary to develop substitute foods or supplements to replace expensive ones.

3.2 Taurine-Related Nutritional Knowledge

The TNK scores and percentages of correct answers of all subjects are shown in Table 2 and Fig. 1. The average TNK score of all subjects was 4.0 out of 10 points, and that of the LAG (1.9 points) was significantly lower than that of the HAG (6.6 points) ($p < 0.001$). The percentage of correct answers among all subjects was higher than 50% for only three questions: 'squid, long-arm octopus, and webfoot octopus contain a lot of taurine' (70.1%), 'taurine helps to recover from fatigue' (62.2%), and 'taurine is mainly contained in fish and meat' (52.2%). However, the percentage of correct answers in the LAG was lower than 50% in all questions.

Table 2 Score of taurine-related nutritional knowledge of the subjects

Variables (units)	Total	Taurine-related nutritional knowledge level	
		LAG	HAG
Total average	4.04 ± 1.06	1.92 ± 1.02	6.60 ± 1.01***
1. Squid, long-arm octopus and webfoot octopus contain a lot of taurine	0.70 ± 0.03	0.49 ± 0.04	0.95 ± 0.02***
2. The white powder on the dried squid skin is taurine	0.47 ± 0.03	0.23 ± 0.03	0.76 ± 0.04***
3. Vegetables and fruits contain very little taurine	0.26 ± 0.03	0.12 ± 0.03	0.42 ± 0.04***
4. Taurine helps to recover from fatigue	0.62 ± 0.03	0.36 ± 0.04	0.94 ± 0.02***
5. Taurine is mainly contained in fish and meat	0.52 ± 0.03	0.25 ± 0.04	0.85 ± 0.03***
6. Lack of taurine can damage the eye tissue	0.33 ± 0.03	0.09 ± 0.02	0.63 ± 0.04***
7. A lot of taurine is contained in protein food	0.41 ± 0.03	0.13 ± 0.03	0.75 ± 0.04***
8. Our body needs more than 3000 mg of taurine a day	0.09 ± 0.02	0.03 ± 0.01	0.17 ± 0.03***
9. Excessive ingestion of taurine-containing beverages can cause caffeine poisoning	0.15 ± 0.02	0.03 ± 0.01	0.30 ± 0.04***
10. Taurine has s a role in the physiological activity of inhibiting sympathetic nerves in the brain, preventing obesity and diabetes, and antioxidant function	0.49 ± 0.03	0.20 ± 0.03	0.83 ± 0.03***

Mean ± SE, ***$p < 0.001$ by Student's t-test

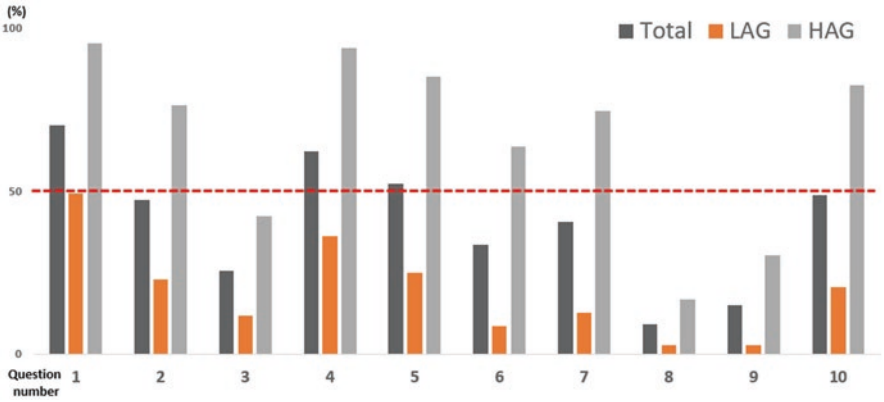

Fig. 1 The percentage of correct answers to each question among all subjects

3.3 Frequency of Taurine Intake

As summarized in Table 3, the TIF score in the HAG (195.3 points) was significantly higher than that of the LAG (180.8 points) ($p < 0.001$). In particular, short arm octopus, whelk, corb shell, shrimp, dried anchovy, dried shrimp, mackerel, anglerfish, skate fish, and dried calamari were foods that, compared to LAG members, HAG members ate more frequently ($p < 0.01$).

Among low taurine content foods, eel, sea tangle, cod, yellow corvine, hairtail, and dried squid were consumed more frequently in the HAG than in the LAG ($p < 0.05$). Among medium taurine content foods, squid, mackerel, Spanish mackerel, anglerfish, skate, short-necked clam, and shrimp were more frequently consumed in the HAG than in the LAG ($p < 0.05$). In case of high taurine content foods and supplements, webfoot octopus, long-arm octopus, oyster, abalone, manila clam, whelk, crab, dried anchovy, dried shrimp were more frequently consumed in the HAG than in the LAG ($p < 0.01$). However, there was no difference in the consumption of taurine supplements such as energy drink and tablet between the two groups.

Taurine is mainly supplied from fish and shellfish and animal foods. Many studies have shown that a fish and seafood diet that contains a lot of taurine has a positive effect on CF (BarbergerGateau et al. 2002; Ripps and Shen 2012; Ryota et al. 2012; Yamori et al. 2009). Thus, we recommend eating seafood intake including fish with a lot of taurine that can lower the risk of dementia.

3.4 Cognitive Function

The results of the CF assessment are summarized in Table 4 and Fig. 2. In the case of the CF, the total average scores of the LAG and HAG were 24.3 points and 25.6 points, respectively. There was no significant difference between the two groups.

Table 3 Score of taurine intake frequency of the subjects

Variables (units)	Total	Taurine-related nutritional knowledge level	
		LAG	HAG
Total score of TIF (points)[a]	187.4 ± 2.0	180.8 ± 2.8	195.3 ± 2.8***
Score of TIF[b]			
Low taurine content foods			
Eel	2.1 ± 0.0	2.0 ± 0.1	2.2 ± 0.1*
Mudfish	2.1 ± 0.0	2.1 ± 0.1	2.0 ± 0.1
Sea cucumber	2.0 ± .0.0	1.9 ± 0.1	2.2 ± 0.1
Warty seasquirt	2.1 ± 0.0	2.1 ± 0.1	2.2 ± 0.1
Green laver	2.6 ± 0.1	2.6 ± 0.1	2.7 ± 0.1
Sea tangle	3.1 ± 0.1	3.0 ± 0.1	3.3 ± 0.1*
Seaweed	2.9 ± 0.1	2.9 ± 0.1	3.0 ± 0.1
Cod	2.2 ± 0.0	2.1 ± 0.1	2.3 ± 0.1*
Yellow corvine	2.6 ± 0.0	2.5 ± 0.1	2.8 ± 0.1*
Hairtail	2.6 ± 0.0	2.5 ± 0.1	2.7 ± 0.1*
Agar-agar	1.8 ± 0.1	1.7 ± 0.1	1.9 ± 0.1
Dried squid	2.2 ± 0.1	2.0 ± 0.1	2.4 ± 0.1***
Medium taurine content foods			
Squid	2.5 ± 0.0	2.4 ± 0.1	2.6 ± 0.1*
Cuttlefish	1.8 ± 0.0	1.7 ± 0.1	1.9 ± 0.1
Octopus	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.1
Mackerel	2.9 ± 0.1	2.8 ± 0.1	3.1 ± 0.1**
Spanish mackerel	2.6 ± 0.1	2.5 ± 0.1	2.7 ± 0.1*
Pacific saury	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
Atka mackerel	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
Anglerfish	2.1 ± 0.0	2.0 ± 0.1	2.3 ± 0.1**
Skate	1.9 ± 0.0	1.8 ± 0.1	2.1 ± 0.1**
Flatfish	2.4 ± 0.0	2.3 ± 0.1	2.4 ± 0.1
Pollack	2.7 ± 0.0	2.6 ± 0.1	2.7 ± 0.1
Cockle	2.3 ± 0.0	2.3 ± 0.1	2.4 ± 0.1
Short-necked clam	2.1 ± 0.0	2.0 ± 0.1	2.3 ± 0.1**
Shrimp	2.5 ± 0.0	2.3 ± 0.1	2.7 ± 0.1***
Dried laver	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
High taurine content foods			
Webfoot octopus	2.3 ± 0.0	2.2 ± 0.1	2.4 ± 0.1**
Long-arm octopus	2.4 ± 0.0	2.3 ± 0.1	2.5 ± 0.1*
Oyster	2.5 ± 0.0	2.4 ± 0.1	2.6 ± 0.1*
Abalone	2.2 ± 0.0	2.1 ± 0.1	2.3 ± 0.1*
Manila clam	2.6 ± 0.0	2.5 ± 0.1	2.7 ± 0.1*
Mussel	2.3 ± 0.0	2.3 ± 0.1	2.4 ± 0.1
Whelk	2.1 ± 0.1	1.9 ± 0.1	2.3 ± 0.1***
Crab	2.4 ± 0.0	2.3 ± 0.1	2.5 ± 0.1*

(continued)

Table 3 (continued)

Variables (units)	Total	Taurine-related nutritional knowledge level	
		LAG	HAG
Dried anchovy	3.5 ± 0.1	3.4 ± 0.1	3.7 ± 0.1**
Dried shrimp	2.6 ± 0.1	2.4 ± 0.1	2.8 ± 0.1**
Taurine supplement (energy drink)	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Taurine supplement (tablet)	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.1

Mean ± SE, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Student's t-test

[a]Total score calculated by the frequency of intake of 40 kinds of taurine-containing foods and supplements

bThe score calculated by the frequency of intake of each food or supplement

Table 4 Cognitive function of the subjects

MMSE-DS (point)	Taurine-related nutritional knowledge level	
	LAG	HAG
Average total	24.3 ± 0.6	25.6 ± 0.6
Time orientation	4.3 ± 0.2	4.6 ± 0.1
Place orientation	4.5 ± 0.1	4.6 ± 0.2
Memory	5.2 ± 0.1	5.2 ± 0.2
Attention	2.8 ± 0.2	3.3 ± 0.3
Language	2.8 ± 0.1	2.8 ± 0.1
Ability to execute	2.5 ± 0.1	2.6 ± 0.1
Visuospatial construction	0.3 ± 0.1	0.5 ± 0.1
Judgment and abstract thinking	1.9 ± 0.4	1.9 ± 0.6

Mean ± SE

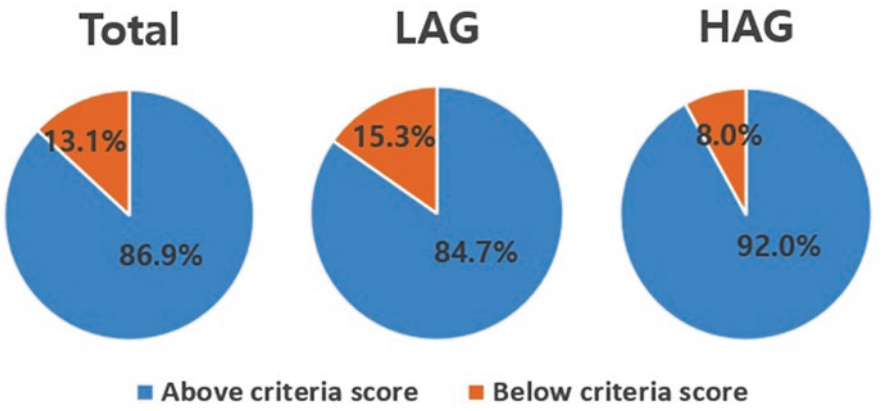

Fig. 2 The proportion of subjects in each group with above and below the CF criterion score

Table 5 Correlations of taurine-related nutritional knowledge, taurine intake frequency and cognitive function of the subjects

Variables	TNK score (p-value)
TIF score	0.169[a] (0.005)
CF score	0.197 (0.072)

[a]Obtained by using Pearson's correlation

Nonetheless, the percentage of people who had lower scores than the criterion score (i.e., those with the potential for cognitive impairment) was 13.1% in all subjects, 15.3% in the LAG, and 8.0% in the HAG.

According to the results of a survey on the elderly conducted by the Ministry of Health and Welfare Republic of Korea, the average MMSE-DS score was 25.2 points, and the percentage of people with a cognitive decline was 14.5% (Ministry of Health and Welfare Republic of Korea 2017). In particular, that percentage increases as age increases. The proportion (13.1%) of people with a cognitive decline among the subjects in this study was lower (14.5%) than that reported in a survey by the Ministry of Health and Welfare. However, it was higher in LAG (15.3%).

Appropriate maintenance of CF in the elderly is important for improving their health and quality of life. Cognitive stimulation such as education can delay or even prevent the degeneration processes in the brain, and such stimulation is positive because it improves cognitive status. Therefore, it is necessary to provide education and support during early screening for the prevention of dementia in elderly people with cognitive decline.

3.5 Correlations of Taurine-Related Nutritional Knowledge, Taurine Intake Frequency and Cognitive Function

Among all subjects, there was a positive correlation between TNK and TIF scores ($p < 0.01$). Although there was no significant difference, there was a tendency toward a positive correlation between TNK and CF scores ($p = 0.072$) (Table 5).

4 Conclusion

These results suggest that taurine-related nutritional knowledge had a significant positive effect on elderly subjects' taurine intake. Although there was no significant difference, taurine-related nutritional knowledge showed a tendency toward its positive effect on cognitive function. Therefore, nutritional education of the elderly about taurine is necessary. Moreover, it is strongly recommended that the elderly frequently consume taurine-containing foods and supplements to prevent dementia.

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The Development of Taurine Supplementary Menus for the Prevention of Dementia and Their Positive Effect on the Cognitive Function in the Elderly with Dementia

Mi Ae Bae, Ranran Gao, Won Cha, Hyung Chul Sang, Kyung Ja Chang, and Sung Hoon Kim

Abstract This study was conducted to investigate the effects on the cognitive function of the elderly with dementia, after consumption of menus developed to prevent dementia. For the purpose of this study, we developed two menus incorporating lotus seeds and taurine which are known to be effective in preventing dementia: tea supplemented with taurine and lotus seed (TATL) and scorched glutinous rice water supplemented with taurine and lotus seed (SATL). The most optimized supplement was determined through sensory evaluation, and was served with the normal diet for 4 weeks. The subjects of this study were 46 elderly women with dementia, divided into three groups: 16 subjects in the taurine supplement group (TG), 15 subjects in the taurine and lotus seeds supplement group (TLG), and 15 subjects in the control group (CG). Cognitive function was assessed by comparing the scores of MMSE-DS (Mini-Mental State Examination-Dementia Screening) before and after dietary supplementation, with higher scores indicating better cognitive functions. Statistical analysis was performed using SPSS 20.0 for Windows. The total score of MMSE-DS before supplementation SATL was not significantly different between CG (14.1 points), TG (14.2 points), and TLG (13.8 points). However, after consuming the SATL supplement, the total score of TG (16.7 points) and TLG (16.9 points) significantly increased ($p < 0.01$). In particular, in the case of TG, a significant increase was observed in the score for 'Judgment and abstract thinking' ($p < 0.05$). An increased tendency was also observed for scores of 'Place orientation' ($p = 0.071$) and 'Ability to execute' ($p = 0.054$), although statistically not significant. In the case of TLG, score of 'Place orientation' and 'Judgment and abstract thinking' was significantly increased ($p < 0.05$). These results show that dietary taurine supplementation

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has positive effects on the cognitive function (MMSE-DS) of elderly women with dementia. There-fore, it is necessary to include dietary taurine supplementation for the treatment and prevention of dementia. In addition, it is necessary to develop and supply a variety of menus containing taurine.

Keywords Taurine Supplementary Menu · Cognitive Function · Taurine · Dementia · Elderly

Abbreviations

TATL	Tea supplemented with Taurine and Lotus Seed
SATL	Scorched Glutinous Rice Water supplemented with Taurine and Lotus seed
TG	Taurine Supplement Group
TLG	Taurine and Lotus Seed Supplement Group
CG	Control Group
BMI	Body Mass Index
MMSE-DS	Mini-Mental State Examination-Dementia Screening
EEG	Electroen-cephalography

1 Introduction

With an increasing aged society, Korea has recently started focusing on the health problems of the elderly. In particular, the rapid upsurge of dementia in the elderly has increased the interest in nutrition management for them (Park et al. 2007). Since most of the seniors suffering from dementia have dietary problems and nutritional disorders, it is necessary to formulate an appropriate countermeasure (Christina et al. 2010).

There is no complete treatment for dementia. However, studies that delay the progress of dementia or have a positive effect on improving the cognitive function are continuing (Clare et al. 2006; Woods et al. 2012). Taurine is known to be effective in nerve transfer and animal experiments have revealed the positive effects of taurine on improving memory (Kim et al. 2014). Another study showed that the normal elderly consumed more taurine than the elderly with dementia. It has also been found that in addition to various other reported functions, taurine is efficient in lipid metabolism improvement, nerve cell development, exercise capacity improvement, and immunity enhancement, and exerts anti-inflammatory, antioxidant and anti-cancer effects in humans (Yoon et al. 2015). The natural mature seeds of lotus are neurostable and are known to be effective in dementia in oriental medicine (Imana and Purnuma 2015).

The elderly-friendly menus for the aged are being actively developed in accordance with the recent changes to the aging society in Korea (Kim and Kim 2016).

Due to a lack of customized menus to help prevent dementia this study was conducted to investigate the effects of two optimized menus on the cognitive function of the elderly with dementia after consuming menus supplemented with taurine.

2 Methods

2.1 Design of the Study

This was a nonequivalent control group pretest-posttest design study that developed elderly-friendly menus with taurine and lotus seeds and investigated the effects of two optimized menus on the cognitive function of the elderly with dementia.

2.2 Development of Elderly-Friendly Menus with Taurine

2.2.1 Main Material

The various ingredients used to develop the taurine supplementary menus were lotus seed, glutinous rice, taurine, and bottled water. Two menus were developed for the study: tea supplemented with taurine and lotus seeds (TATL), and scorched glutinous rice water supplemented with taurine and lotus seeds (SATL). Lotus seeds cultivated at Siheung City, Gyeonggi in Korea (2016) were procured in the frozen state. Glutinous rice was purchased from Korea, taurine powder was supplied by Dong-A Pharmaceutical, and bottled water was manufactured by Nongshim, Korea.

2.2.2 Production of Tea Supplemented with Taurine and Lotus Seeds (TATL)

The process development was based on the preliminary experiments of previous studies (Kim et al. 2014; Kwon 2015); the combination ratio of the main ingredients (taurine, lotus seeds, and water) was determined as given in Table 1. Although there are no reported side effects on the excess intake of taurine (Parvez et al. 2008), after duly considering the high age and poor health of the subjects, we limited the amount to 3 g by referring to a previous study in humans (Cha et al. 1999).

Table 1 Formula for TATL

No	Materials (g)		Water (L)	Taurine (g)
357	Raw lotus seeds with shell	200	2	3
583	Raw lotus seeds without shell			
405	Baked and crushed lotus seeds			

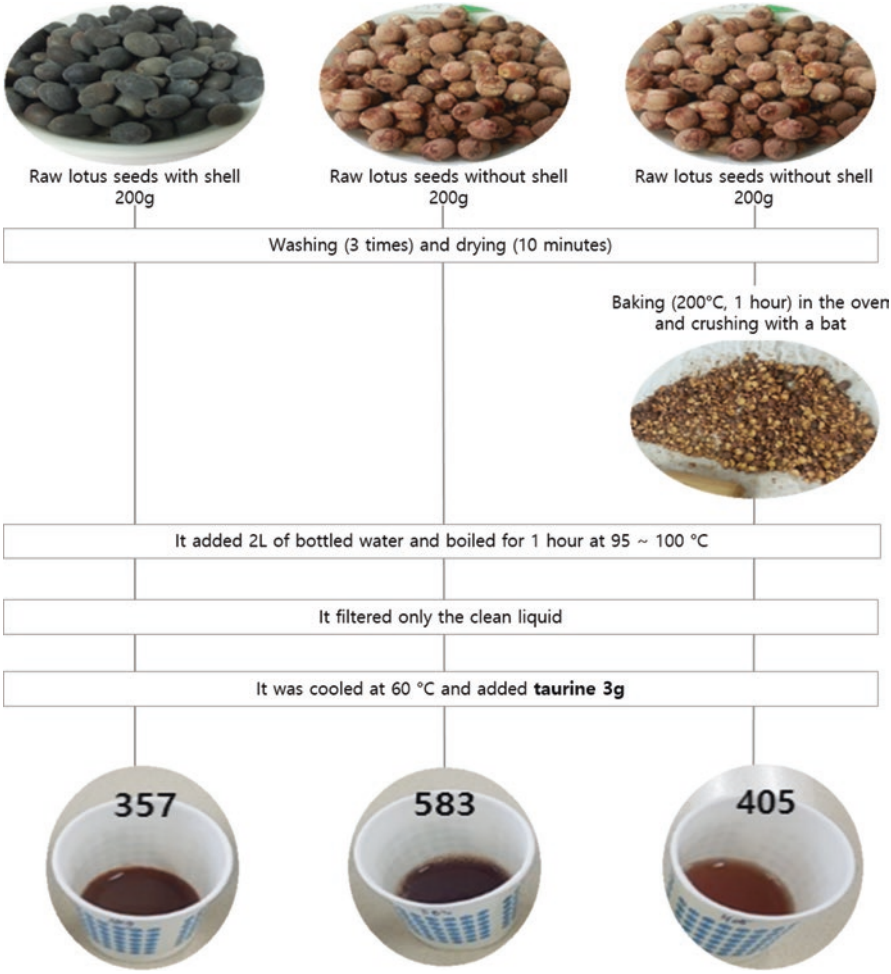

Fig. 1 Development process of TATL

The production steps of TATL was are presented in Fig. 1. Briefly, raw lotus seeds with shell (200 g) and without shell (400 g) were washed three times and dried at room temperature for about 10 min. Next, half of the prepared raw lotus seeds without shells (200 g) were baked in oven (200 °C, 1 h) and crushed. The three differently preprocessed materials were then added to 2 L of bottled water and boiled for 1 h at 95 – 100 °C. Finally, the solution was filtered; the clean liquid was collected and cooled to 60 °C, after which taurine (3 g) was added to it.

2.2.3 Production of Scorched Glutinous Rice Water Supplemented with Taurine and Lotus Seeds (SATL)

The combination ratio of the main ingredients (lotus seed powder, glutinous rice powder, water, and taurine) was determined as given in Table 2. The development process for SATL is presented in Fig. 3. The lotus seed powder and glutinous rice powder are first prepared as per the flow chart in Fig. 2. Next, ten times the amount of main ingredients mentioned in Table 2 were boiled at 95–100 °C for 15–20 min, stirring with a wooden spatula to prevent burning at the base. The mixture was then covered with a lid for about 20 min to promote gelatinization of glutinous rice. Finally, it was divided into ten equal parts, cooled to 60 °C, and supplemented with 3 g taurine each (Fig. 3).

2.2.4 Sensory Evaluation

Researchers evaluated the preferences (color, flavor, taste, texture, and overall perception) of TATL and SATL visiting the hospital and directly interviewing the subjects. Totally, 21 subjects were evaluated for sensory perception (5 patients with

Table 2 Formula for SATL

No	Materials (g)			Water (L)	Taurine (g)
	Lotus seed powder	Glutinous rice powder	Mixing ratio		
533	20	10	2:1	0.3	3
301	7.5	15	1:2		
101	10	10	1:1		

Fig. 2 Preparation of lotus seeds and glutinous rice powder

Fig. 3 Development process of SATL

dementia over 65 years, 5 patients without dementia over 65 years, and 11 hospital workers). Evaluation was done using the 7-point scale (1 point is very dissatisfied, 4 points is usually, and 7 points is very satisfied).

2.3 Subjects and Dietary Taurine Supplement

This study was approved by the Institutional Review Board (IRB) of the Inha University (161010-8AR). The subjects of this study were 46 elderly women with dementia who were hospitalized at a single geriatric hospital in Incheon, Korea. They were divided into the taurine supplement group (TG, 16 people), the taurine and lotus seeds supplement group (TLG, 15 people), and the control group (CG, 15 people).

The three groups were matched such that there were no significant differences in age, number of diseases, height, weight, BMI, and total score of cognitive function. Nevertheless, there was a decrease in the number of subjects evaluated due to their discharge from the geriatric hospital during the study period (1, 2, and 4 people, respectively, in TG, TLG, and CG).

In this study, we decided to use SATL as the dietary supplementary menu since subjects in the preliminary sensory evaluation preferred SATL to TATL. The CG was provided with SATL not supplemented with taurine and lotus seed powder for placebo effect. The dietary supplement was provided once a day (with lunch) for 4 weeks. The researchers directly monitored the intake and side effects every day.

2.4 Data Collection

This study measured the general characteristics (age, care period, number of diseases, and type of dementia), anthropometric data (height, weight, BMI, and blood pressure), and changes of cognitive function (MMSE-DS and brain wave) of the subjects. The sensory evaluation of the developed menus was conducted in March 2017, and pretests were conducted from April 21–27, 2017. Supplements of the optimized menu began were commenced from May 8, 2017, and the post-tests were conducted 4 weeks later.

The data of the general characteristics and height, weight, and blood pressure were collected from recent individual clinic records of the hospital to reduce stressing the subjects, and BMI was measured directly by the researcher using the In-Bodys10 (G-Tech International, Korea), with the subject in a lying down position.

MMSE-DS is the most widely used method for screening dementia; we used it by a face-to-face interview with the subjects to investigate change of cognitive function. The electroencephalography (EEG) were measured by gamma (γ, 30 Hz $\leq$), beta (β, 12-29.99 Hz), alpha (α, 8-11.99 Hz), and theta (θ, 4–7.99 Hz) waves by using the OMNIFIT (Omni C&S. Inc., Korea), and the researcher was completely trained and familiarized with the measurement method from the nurse of hospital.

All tests related to the measurement of the pre- and the post-test were conducted at the same time by a single researcher to minimize errors.

2.5 Statistical Analysis

The statistical analysis of all data was conducted using SPSS 20.0 pro-gram. The values were expressed as the percentage or mean ± standard error (SE). The statistical significance in changes of MMSE-DS scores was determined by Wilcoxon's signed-rank test. The significant difference of results was found at $p < 0.05$.

3 Results and Discussion

3.1 Development of the Elderly-Friendly Menus Supplemented with Taurine

3.1.1 Sensory Evaluation of Tea Supplemented with Taurine and Lotus Seeds (TATL)

The results of the sensory evaluation showing preference for TATL by the subjects are presented in Fig. 4. The preference of color was the highest at 405, flavor was similar for all teas, whereas the taste and overall preference were lowest at 357.

3.1.2 Sensory Evaluation of Scorched Glutinous Rice Water Supplemented with Taurine and Lotus Seeds (SATL)

The results of the sensory evaluation showing preference for SATL by the subjects are presented in Fig. 5. The preference for color and flavor was the lowest in 301, whereas taste, texture, and overall preference was lowest in 533. The preference for 101 was high in color, flavor, taste, texture, and overall preference.

Fig. 4 The result of sensory evaluation of TATL

Fig. 5 The result of sensory evaluation of SATL

Table 3 General characteristics and anthropometric data of the subjects

Variables			Total (N = 46)	CG (N = 15)	TG (N = 16)	TLG (N = 15)
General characteristics	Age (years)		80.7 ± 0.9	82.3 ± 1.2	80.8 ± 2.1	79.1 ± 1.1
	Care period (months)		26.6 ± 2.7	27.1 ± 5.7	26.1 ± 4.2	26.8 ± 4.5
	Number of diseases (except dementia)		1.9 ± 0.1	1.7 ± 0.3	1.7 ± 0.3	2.1 ± 0.2
	Types of dementia	Alzheimer's	23(50.0)	8(53.3)	8(50.0)	7(46.7)
		Other	23(50.0)	7(46.7)	8(50.0)	8(53.3)
Anthropometric data	Height (cm)		152.4 ± 0.7	151.0 ± 1.1	152.6 ± 1.1	153.7 ± 1.3
	Weight (kg)		53.5 ± 1.5	50.3 ± 2.8	54.4 ± 2.6	55.6 ± 2.7
	BMI (kg/m^2)[6)]		22.9 ± 0.6	22.0 ± 1.2	23.3 ± 1.0	23.5 ± 0.8
	Blood pressure (mmHg)	Systolic	126.1 ± 2.5	130.0 ± 4.8	121.3 ± 3.8	127.3 ± 4.3
		Diastolic	76.7 ± 1.5	74.7 ± 2.6	75.6 ± 2.6	80.0 ± 2.4

Mean±SE, N(%)

3.2 The Effects of Optimized Menus on the Elderly with Dementia

3.2.1 General Characteristics and Anthropometric Data of Subjects

The general characteristics and anthropometric data of the subjects are presented in Table 3. The average age of all subjects was 80.7 years, with an average hospitalization for 26.6 months. Half the subjects (50.0%) suffered from Alzheimer's disease with 1–2 chronic diseases. In addition, other types of dementia (50.0%) include vascular dementia, Parkinson's dementia, unexplained dementia, and mixed forms of dementia.

The average height, weight, and BMI of the all subjects were 152.4 cm, 53.5 kg, and 22.9 kg/m^2, respectively. Although within the normal range, the body weight and BMI were found to be slightly lower compared to the 2016 Korea National Health and Nutrition Examination Survey (151.5 cm, 56.5 kg, 24.6 kg/m^2 for average height, weight, and BMI, respectively). In addition, the average systolic and

diastolic blood pressures were 126.1 mmHg and 76.7 mmHg, respectively, which were within the normal range and similar to the 2016 Korea National Health and Nutrition Examination Survey (130.2 mmHg and 72.0 mmHg, respectively).

3.2.2 Effects of Optimized Menus on the Cognitive Function of Subjects

Mini-Mental State Examination-Dementia Screening (MMSE-DS)

Cognitive function was assessed by comparing the scores of MMSE-DS before and after the dietary supplementation; a higher score is indicative of better cognitive function. As shown in Table 4 and Fig. 6, the total score of MMSE-DS before dietary taurine supplementation did not differ significantly between CG (14.1

Table 4 The changes of MMSE-DS scores

MMSE-DS (point)	Group	Pre-test	Post-test
Average total	CG	14.1 ± 2.1	15.6 ± 1.8
	TG	14.2 ± 1.8	16.7 ± 2.0**
	TLG	13.8 ± 1.0	16.9 ± 0.9**
Time orientation	CG	2.3 ± 0.5	1.8 ± 0.5
	TG	2.1 ± 0.5	2.1 ± 0.5
	TLG	1.7 ± 0.3	2.2 ± 0.4
Place orientation	CG	2.4 ± 0.4	2.6 ± 0.5
	TG	2.1 ± 0.5	2.7 ± 0.4
	TLG	1.9 ± 0.2	2.8 ± 0.2**
Memory	CG	3.2 ± 0.5	4.0 ± 0.4
	TG	3.4 ± 0.5	3.7 ± 0.4
	TLG	3.7 ± 0.3	4.2 ± 0.2
Attention	CG	1.1 ± 0.4	1.1 ± 0.5
	TG	1.4 ± 0.5	1.9 ± 0.5
	TLG	1.1 ± 0.3	1.4 ± 0.3
Language	CG	2.3 ± 0.3	2.6 ± 0.2
	TG	2.3 ± 0.2	2.5 ± 0.1
	TLG	2.7 ± 0.1	2.8 ± 0.1
Ability to execute	CG	1.6 ± 0.3	1.9 ± 0.3
	TG	1.8 ± 0.3	2.2 ± 0.2
	TLG	1.3 ± 0.3	1.8 ± 0.3
Visuospatial construction	CG	0.0 ± 0.0	0.0 ± 0.0
	TG	0.1 ± 0.1	0.1 ± 0.1
	TLG	0.0 ± 0.0	0.0 ± 0.0
Judgment and abstract thinking	CG	1.3 ± 0.2	1.6 ± 0.2
	TG	1.2 ± 0.2	1.5 ± 0.2*
	TLG	1.4 ± 0.2	1.7 ± 0.1*

Mean ± SE, by Wilcoxon's signed-rank test
*p < 0.05, **p < 0.01

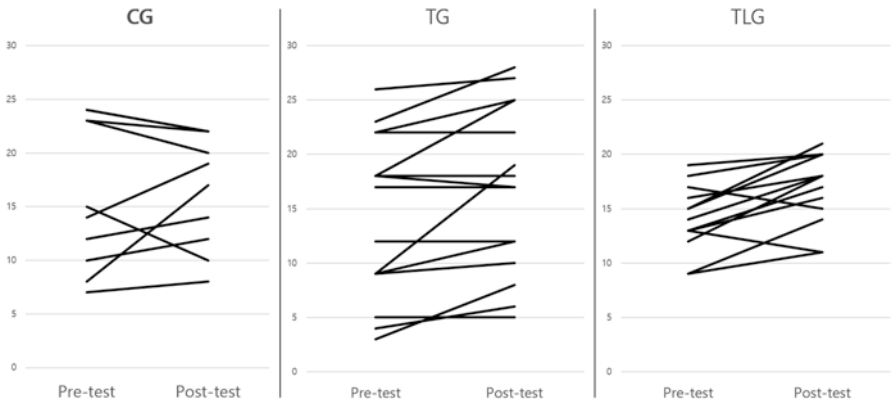

Fig. 6 The changes of total score of MMSE-DS in three groups

points), TG (14.2 points), and TLG (13.8 points). However, after dietary taurine supplementation, a significant increase was observed in the total score of TG (16.7 points) and TLG (16.9 points) ($p < 0.01$). In particular, in the case of TG, score of 'Judgment and abstract thinking' was significantly increased ($p < 0.05$). Although there was no significant difference, there was a tendency for increased scores of 'Place orientation' ($p = 0.071$) and 'Ability to execute' ($p = 0.054$). In the case of TLG, score of 'Place orientation' and 'Judgment and abstract thinking' was significantly increased ($p < 0.05$).

The Electroencephalography (EEG)

The results of electroencephalography (EEG) showed no significant difference between the three groups (Fig. 7). The pre-test showed a large distribution of results in each group, whereas the differences were small in the post-test. These changes are confirmed to be only in the lower value of the alpha wave than at other frequencies. However, since identical changes are observed in all groups, it cannot be attributed to the supplementation of dietary taurine. The degree of dementia and value of EEG analysis are closely related, and the value may therefore be weak in early dementia (Szelies et al. 1992). Hence, it is possible that the change could not be confirmed after only 4 weeks of taurine supplementation. EEG is a useful and highly sensitive method to objectively assess the function of the cerebrum (Kwon and Cho 2007). The limitation of this study remains that the errors during the measurement cannot be minimized by a researcher. Also, as reported in a previous study, Alzheimer's dementia exhibits lower alpha and beta-wave and higher theta wave as compared to the normal elderly (Kim et al. 2013). The low value of alpha wave in the cur-rent study was therefore attributed to the dementia of the subjects. We believe that a comparative study with normal elderly subjects is required in the future.

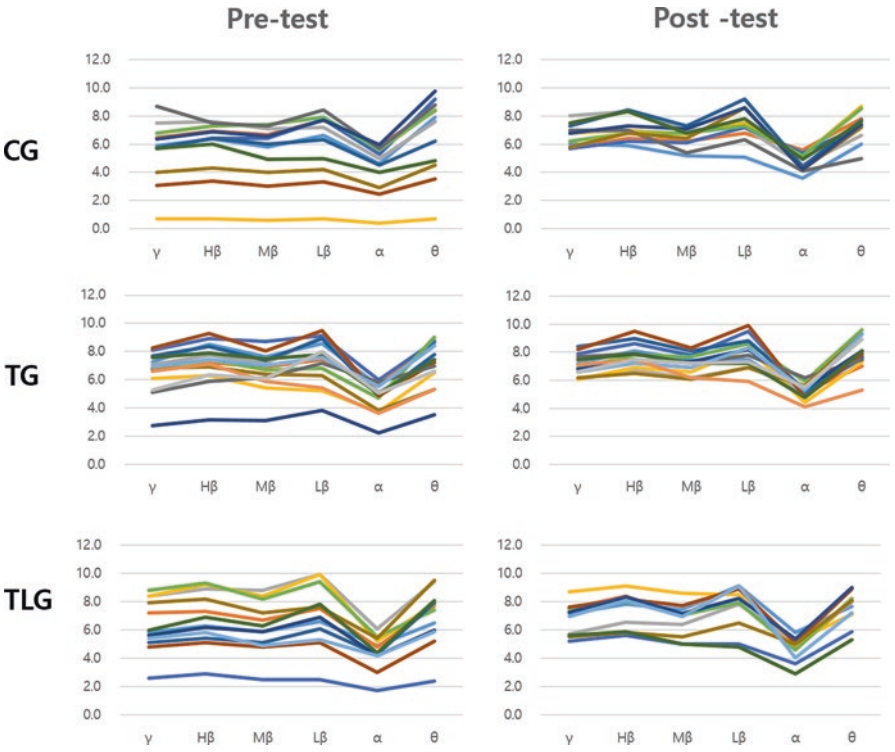

Fig. 7 The changes of EEG in three groups

4 Conclusion

These results show that dietary taurine supplementation has positive effects on the cognitive function (MMSE-DS) of elderly women with dementia. Therefore, it is necessary to utilize dietary taurine supplementation for the treatment and prevention of dementia. In addition, it is necessary to develop and distribute a variety of palatable menus containing taurine.

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The Intake of Taurine and Major Food Source of Taurine in Elementary School Children in Korea

Ji-Seon Jeong and Mi-Ja Choi

Abstract Taurine is a β-amino acid found most broadly distributed in human body, abundant in animal foods, and has an antioxidative function. Current nutritional intake and dietary habits of children in elementary schools show low level of the intake of vegetable foods and high level of the intake of processed foods and fast foods; this necessitates the emphasis of the intake of antioxidative nutrients for children. On account of the less consumption of vegetable foods as a main source of antioxidative nutrients for elementary school children, animal foods containing abundant amount of taurine can be preferably taken as an alternative foods therefor. Many previous studies have reported the protein intake of the children in elementary schools so far. However, the studies, reported the intake of taurine of elementary school children, are few. Thus, this study analyzed taurine and nutrients intake for children in Daegu, Korea. The average daily energy intake of the children was 153 ± 155 mg/day. The mean taurine intake values are followed; 27.6 ± 11.6 mg/day in the Q1 group, 61.2 ± 10.0 mg/day in the Q2 group, 137.7 ± 51.1 mg/day in the Q3 group, and 385.9 ± 123.6 mg/day in the Q4 group ($p < .001$). Q3 and Q4 groups showed significantly higher level of the intake of vitamin D, vitamin B12, Calcium, and folate than those of Q1 and Q2 groups. In the study, foods that affected the intake of taurine were as followed; fish and shellfish (79%), meat (14%), seaweed (5%), and other food products (2%).

As a consequence, Taurine intake appears to be affected by seafood intake, and if seafood is consumed primarily, the amount of energy intake would be appropriate and will contribute to the increase of intakes of taurine, calcium and vitamin D.

Keywords Taurine Intake · School children · Nutrient intake · Major food of taurine

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Abbreviations

RNI	Recommended Nutrient Intake
AI	Adequate Intake
EER	Estimated energy requirements
IRB	Institutional review board
BMI	Body mass index
Q1	1st quadrant
Q2	2nd quadrant
Q3	3rd quadrant
Q4	4th quadrant
LEG	Low energy group
REG	Recommended energy group
HEG	High energy group

1 Introduction

Taurine promotes growth and development, collagen synthesis, bone matrix formation in addition to that immune system maintenance, and antioxidation function (Aruoma et al. 1988; Huxtable 1992; Pasntes-Morales and Cruz 1985), enhance bone cell formations (Lubec et al. 1997; Chung 2001). The nutritional imbalance affects growth and development in the rapid growth period of school-age children (Lee et al. 2003). According to the 2016 National Health Statistics (Korea Health Statistics 2016) and Dietary Reference Intakes for Korean (The Korean Nutrition Society 2015), Korean elementary school students took 105.2% of the required amount of energy (Estimated Energy Requirements, EER) and 192.5% of the recommended amount of protein (Recommended Nutrient Intake, RNI). However, they had an insufficient amount of other nutrients (Korea Health Statistics 2016), such as 79.4% (RNI) of vitamin A, 62.3% (RNI) of calcium, 82.2% (Adequate Intake, AI) of potassium. It is crucial for children in their school years to consume nutrients that raise calcium and calcium metabolism due to lack of calcium intake. Since taurine is known to improve bone formation in calcium metabolism and help suppress bone loss (Chung 2001; Yoon et al. 2015; Kim et al. 2002; Park et al. 2001a; Chen et al. 2003), it suggests that eating enough taurine in childhood will help bone formation.

Students ate a lot of processed and fast food, but low amounts of vegetables that contain a lot of antioxidant nutrients (Ministry of Education 2016). Korean adolescents aged 10–18 took vitamin A, at a rate of 50.4% of their recommended intake, and vitamin C at 79.6%, which are significantly low amounts (Korea Health Statistics 2016). For children who lack antioxidants and calcium intake, taurine rich food intake is assumed to complement the insufficient consumption of calcium and

antioxidant nutrients. When it comes to taurine intake studies in Korea, there are some reports for teenagers and adults (Kim et al. 2003; Park et al. 2001b; Yim et al. 2004), but few studies on children. Therefore, this study analyzed the status of taurine and nutrients intake of children in Korea.

2 Methods

2.1 Study Population and Period

This research was conducted from October 18th to December 22nd, 2016, with 153 elementary school students aged 11. All elementary students were interviewed by trained dietitian, using a structured questionnaire. The questionnaire included questions on mothers' sociodemographic factors, children's weight, height, and food intake. The study was by Institutional Review Board, IRB at Keimyung University (IRB. No. 40525–201606–HR–94–01), and written informed consent was obtained from all participants and parents.

2.2 Taurine and Nutrient Intake

With the 24-h recall method, the participants were asked to record all food intake, food ingredients name, and consumption amounts a day before the food intake survey date. The nutrients intake was analyzed using CAN-Pro 4.0 (Computer aided nutritional analysis program for professionals 4.0) and compared the nutrient intake to the Dietary Reference Intakes for Korean (The Korean Nutrition Society 2015). Taurine intake was calculated using Food composition table, 9th edition (Rural Development Administration 2016).

2.3 Statistic Analysis

The statistical analysis of this study was assessed using SPSS (PASW version 18.0; SPSS Inc., Chicago, IL), with a significant probability of significance of $p < .05$ for all analyses. For general items, the frequency and percentage were obtained, and the average and standard deviation were calculated for each nutrient and taurine intake. The inter-group comparisons were analyzed using the t-test, $\chi 2$-test, and Analysis of variance (ANOVA), and the Scheffe method was used for post-verification. Pearson's correlation coefficients were obtained to determine the correlation between taurine intake and other nutrients intake.

3 Results and Discussion

3.1 Characteristics of the Study Subjects

General characteristics of participants are presented in Table 1. All participants and their mothers were informed of the study purpose, procedures, and risks, and signed the consent/assent forms. The study population was composed of 81 (59.2%) boys and 72 (47.1%) girls with the average age of 11 years old for both. The average height of children was 142.9 ± 5.9 cm, as 142.7 ± 6.0 for males, and 143.1 ± 5.7 for females, respectively. While the average weight was 37.8 ± 7.7 kg, as 38.5 ± 7.9 kg for boys, and 37.0 ± 7.5 kg for girls. The average BMI (Body Mass Index) of children was 18.4 ± 3.0, as 18.8 ± 3.0 for males, and 18.0 ± 2.9 for females, respectively. The average age of mothers was 41.9 ± 2.9 years old, 59.5% of them had jobs.

The nutrient intakes of elementary school students in Daegu, Korea were analyzed. According to the analysis of the nutrient intake by elementary school students in Daegu, the energy consumption was 1, 684 kcal/day (86.2%, EER), and the protein intake was 66.7 g/day (166.9%, RNI), as 24.2 g/day of vegetable protein, and 42.6 g of animal protein, respectively. The average daily intake of taurine was 153.0 ± 155.2 mg/day (Table 1). Using the 24-h recall method, Park et al. (2001a, b) reported high school students in Seoul consumed 219 mg/day of taurine, and adults consumed 177 mg/day.

3.2 The Intake of Taurine

Table 2 shows how much taurine and nutrients are consumed depending on the level of energy intake. Participants were classified by energy intake as followed; less than 75% of EER group (Low Energy Group, LEG), 75–125% group (Recommended

Table 1 General characteristics of the study subjects and taurine intake per day

	Q1[1] (n = 38)	Q2 (n = 38)	Q3 (n = 39)	Q4 (n = 38)	Total (n = 153)
Weight (kg)	38.8 ± 9.0[2,a,3]	37.5 ± 7.6[a]	39.0 ± 7.4[a]	36.0 ± 6.7[a]	37.8 ± 7.7
Height (cm)	143 ± 6[a]	143 ± 6[a]	144 ± 6[a]	142 ± 6[a]	143 ± 6
BMI[4] (kg/m^2)	18.8 ± 3.3[a]	18.2 ± 2.9[a]	18.8 ± 3.0[a]	17.9 ± 2.6[a]	18.4 ± 3.0
Mother's mean age (year)	41.5 ± 2.4[a]	41.9 ± 3.0[a]	41.2 ± 3.5[a]	42.5 ± 2.7[a]	41.9 ± 2.9
Taurine (mg)	27.6 ± 11.6[a]	61.2 ± 10.0[a]	137.7 ± 51.1[b]	385.9 ± 123.6[c]	153.0 ± 155.2

[1]Taurine intake level. *Q1* 1st quadrant, *Q2* 2nd quadrant, *Q3* 3rd quadrant, *Q4*, 4th quadrant. Q1 < 44.2 mg/d; 44.2 ≤ Q2 < 79.2 mg/d; 79.2 ≤ Q3 < 243.5 mg/d; 243.5 mg/d < Q4

[2]Mean ± SD

[3]One-way analysis of variance and Scheffe's multiple range test

[4]BMI; Body mass index (kg/m^2)

[a,b,c]Same letters on the shoulder of each values indicate no significant difference (P > 0.05), different letters on the shoulder of each value indicate significant differences (P < 0.05)

Table 2 Daily nutrients intake and the percent for KDRIs by the energy intake levels

	LEG[1] (n = 55)	REG (n = 55)	HEG (n = 13)
Energy (Kcal)	1178 ± 228	1849 ± 272	2750 ± 502
Taurine (mg)	104 ± 112[2,a,3]	189 ± 168[b]	121 ± 173[a]
Carbohydrate (g)	175 ± 42[a]	263 ± 55[b]	330 ± 95[c]
Protein (g)	47.6 ± 11.9[a]	71.7 ± 17.5[b]	115.2 ± 54.2[c]
	(60.0)[4]	(94.4)	(143.0)
Plant protein (g)	18.4 ± 5.4[a]	26.3 ± 7.6[b]	34.4 ± 12.7[c]
Animal protein (g)	29.2 ± 10.7[a]	45.4 ± 18.4[b]	80.8 ± 59.2[c]
Fat (g)	32.2 ± 10.9[a]	57.5 ± 21.1[b]	107.0 ± 39.7[c]
Cholesterol (mg)	271 ± 174[a]	373 ± 188[a]	726 ± 460[b]
Saturated fatty acid (g)	7.6 ± 5.2[a]	13.4 ± 9.1[a]	23.4 ± 20.8 [b]
C : P : F[5]	59 : 16 : 25	57 : 15 : 28	49 : 16 : 35

[1]*LEG* Low energy group (< 1427 Kcal), *REG* Recommended energy group (1427–1840 Kcal), *HEG* High energy group (1840 Kcal <)
[2]Mean ± SD
[3]One-way analysis of variance and Scheffe's multiple range test
[4]% of Dietary Reference Intakes for Koreans 2015
[5]C: P: F; Percentage of energy from Carbohydrate, Protein and Fat
[a,b,c]Same letters on the shoulder of each values indicate no significant difference (P > 0.05), different letters on the shoulder of each value indicate significant differences (P < 0.05)

Energy Group, REG), and more than 125% group (High Energy Group, HEG). The mean energy intake was; 1178 ± 228 kcal/day for LEG, 1849 ± 272 kcal/day for REG, and 2750 ± 502 kcal/day for HEG. Regarding the taurine intake depending on the level of energy intake, REG marked 189 ± 168 mg/day, HEG marked 121 ± 173 mg/day, and LEG scored 104 ± 112 mg/day. Taurine intake was significantly higher in REG compared to LEG and HEG. Animal protein intake was significantly higher in HEG at 80.8 ± 59.2 mg/day, compared to 45.4 ± 18.4 mg/day in REG. Cholesterol intake was significantly higher in HEG at 726 ± 460 mg/day, compared to 373 ± 188 mg/day in REG. Saturated fatty acid intake was significantly higher in HEG at 23.4 ± 20.8 mg/day, compared to 13.4 ± 9.1 mg/day in REG. These results beg the question as to whether high taurine intake is related to dietary habit or mothers' status, and which food group affects taurine consumption in Korean children.

In our study, ironically, the high energy consumption group had a lower taurine intake. HEG consumed significantly higher total protein and animal protein than REG. However, HEG had a significantly lower taurine intake, and they had high saturated fatty acid intake, suggesting they take in protein by high consumption of meat with high lipids.

3.3 The Levels of Taurine Intake and the Intake of Nutrient

Taurine intake was divided into four groups to compare the intake of nutrients according to the levels of taurine intake (Tables 3 and 4). The mean taurine intake values are followed; 27.6 ± 11.6 mg/day in the Q1 group, 61.2 ± 10.0 mg/day in the

Table 3 Energy and nutrients intake by the taurine intake levels per day

	Q1[1] (n = 38)	Q2 (n = 38)	Q3 (n = 39)	Q4 (n = 38)
Energy (Kcal)	1653 ± 600[2,a,3] (85.4)[4]	1637 ± 619[a] (84.2)	1686 ± 488[a] (85.4)	1,762 ± 413[a] (89.7)
Carbohydrate (g)	234 ± 83[a]	222 ± 81[a]	226 ± 56[a]	266 ± 67[a]
Protein (g)	63.2 ± 28.2[a] (158.0)	67.6 ± 37.5[a] (168.9)	71.9 ± 25.9[a] (179.8)	64.1 ± 18.4[a] (160.3)
Fat (g)	51.3 ± 29.1[a]	53.2 ± 29.2[a]	55.2 ± 36.9[a]	50.6 ± 17.4[a]

[1]Taurine intake level. *Q1* 1st quadrant, *Q2* 2nd quadrant, *Q3* 3rd quadrant, *Q4* 4th quadrant. Q1 < 44.2 mg/day; 44.2 ≤ Q2 < 79.2 mg/day; 79.2 ≤ Q3 < 243.5 mg/day; 243.5 mg/day < Q4

[2]Mean ± SD

[3]One-way analysis of variance and Scheffe's multiple range test

[4]% of Dietary Reference Intakes for Koreans 2015

[a]Same letters on the shoulder of each values indicate no significant difference (P > 0.05), different letters on the shoulder of each value indicate significant differences (P < 0.05)

Q2 group, 137.7 ± 51.1 mg/day in the Q3 group, and 385.9 ± 123.6 mg/day in the Q4 group (Table 1). Q3 and Q4 groups had significantly higher consumptions of vitamin D, folic acid, vitamin B12, and calcium than the first and second group (Table 4). The Q1 group consumed 415 ± 217 mg/day of calcium, and 578 ± 260 mg/day in the Q4 group, showing significantly higher levels of calcium intake in groups with high taurine intakes. The result of vitamin D intakes also suggests that more taurine intake groups showed significantly higher consumptions of vitamin D; the Q1 group consumed 2.2 ± 1.9 μg/day (44%, AI) of vitamin D, 4.9 ± 3.8 μg/day (97.7%, AI) in the Q3 group, and 4.1 ± 3.4 μg/day (81.8%, AI) in the Q4 group. It was reported that vitamin D had been insufficiently consumed by children in many countries, such as Indonesia, the US, Spain, and other European nations (Spiro A and Buttriss 2014; Au et al. 2012; Soesanti et al. 2013; López-Sobaler et al. 2017). Calcium intake was higher than the previous study suggests, 42.9% (RNI) (Kim et al. 2011), and similar to the intake of 61% (RNI) (Kim and Lee 2008). In particular, as the levels of intake of calcium and vitamin D were higher in high taurine intake groups, having foods with abundant taurine for school-aged children is important to supplement the lack of calcium and vitamin D in many children.

The correlation between taurine and other nutrients intakes showed that taurine has a significant positive correlation with vitamin K, folic acid, vitamin B12, and calcium (Table 5). In this study, foods affected taurine intake of children were as followed; fish and shellfish (79%), meat (14%), seaweed (5%), and other food products (2%) in Fig. 1. Thus, seafood appeared to be the most significant foods for taurine intake. REG was found to have a protein intake of 71.7 ± 17.5 mg/day (94.4%, RNU), which is considered as moderate intake, and taurine intake was high. Kim et al. (2003) reported that taurine intake has a significant correlation with the consumption of seafood, yet there was no significant correlation when it comes to meat and protein intake. It seems increasing seafood intake, as the source of protein, is necessary to obtain higher taurine intake in children. Overall, taurine intake was positively associated with calcium, vitamin K, folic acid, and vitamin B12 intakes.

Table 4 Daily Nutrients intake by the taurine intake levels

	Q1[1] (n = 38)	Q2 (n = 38)	Q3 (n = 39)	Q4 (n = 38)
Vitamin A (μgRE)	593 ± 449[2,a,3] (104.3)[4]	655 ± 331[a] (114.3)	703 ±v457[a'] (120.9)	710 ± 62 5[a](123.1)
Vitamin D (μg)	2.2 ± 1.9[a] (43.0)	3.4 ± 2.3[ab] (68.3)	4.9 ± 3.8[b] (97.7)	4.1 ± 3.4[b] (81.8)
Vitamin E (mg)	13.9 ± 9.8[a] (154.7)	16.4 ± 8.3[a] (181.8)	15.1 ± 9.1[a] (167.7)	16.4 ± 5.6[a] (182.2)
Vitamin K (μg)	84.9 ± 57.8[a] (154.4)	111 ± 71[a] (200.1)	142 ± 103[a] (258.0)	154 ± 384[a] (280.3)
Vitamin C (mg)	61.4 ± 46.0[a] (81.9)	76.0 ± 90.2[a] (100.3)	88.0 ± 109.5[a] (121.1)	83.3 ± 62.9[a] (111.1)
Thiamin (mg)	1.5 ± 0.7[a] (164.2)	1.2 ± 0.6[a] (134.7)	1.3 ± 0.7[a] (148.6)	1.4 ± 0.4[a] (150.2)
Riboflavin (mg)	1.1 ± 0.7[a] (104.9)	1.3 ± 0.7[a] (120.0)	1.4 ± 0.7[a] (121.1)	1.4 ± 0.6[a] (125.3)
Niacin (mg)	14.3 ± 6.5[a] (119.2)	12.9 ± 6.2[a] (107.4)	14.3 ± 6.2[a] (119.4)	13.0 ± 5.3[a] (108.4)
Vitamin B_6 (mg)	1.5 ± 0.7[a] (138.0)	1.4 ± 0.7[a] (128.2)	1.4 ± 0.5[a] (127.2)	1.3 ± 0.5[a] (121.3)
Folate (μg)	299 ± 134[a] (99.7)	334 ± 140[a] (111.2)	397 ± 132[a] (132.3)	412 ± 267[a] (137.4)
Vitamin B_{12} (μg)	3.9 ± 2.4[a] (227.7)	5.6 ± 2.4[ab] (326.9)	8.4 ± 3.6[b] (494.8)	14.2 ± 10.8[c] (833.4)
Calcium (mg)	415 ± 217[a] (51.9)	469 ± 224[ab] (58.7)	572 ± 250[b] (71.5)	578 ± 260[b] (72.3)
Sodium (mg)	2876 ± 1,357[a] (205.4)	2750 ± 882[a] (196.5)	3191 ± 1155[a] (227.9)	3065 ± 943[a] (218.9)
Potassium (mg)	1994 ± 876[a] (66.5)	1971 ± 596[a] (65.7)	2287 ± 800[a] (76.2)	2194 ± 756[a] (73.1)
Iron (mg)	11.0 ± 5.3[a] (110.2)	10.7 ± 4.5[a] (107.1)	11.6 ± 3.9[a] (116.4)	11.5 ± 4.2[a] (114.5)
Zinc (mg)	8.9 ± 3.6[a] (111.3)	9.2 ± 4.7[a] (114.7)	9.9 ± 3.4[a] (123.7)	9.6 ± 2.9[a] (120.3)
Cholesterol (mg)	263 ± 186[a]	384 ± 202[ab]	451 ± 282[ab]	366 ± 276[b]

[1]Taurine intake level. *Q1* 1st quadrant, *Q2* 2nd quadrant, *Q3* 3rd quadrant, *Q4* 4th quadrant. Q1 < 44.2 mg/day; 44.2 ≤ Q2 < 79.2 mg/day; 79.2 ≤ Q3 < 243.5 mg/day; 243.5 mg/day < Q4

[2]Mean ± SD

[3]One-way analysis of variance and Scheffe's multiple range test

[4]% of Dietary Reference Intakes for Koreans 2015

[a,b]Same letters on the shoulder of each values indicate no significant difference (P > 0.05), different letters on the shoulder of each value indicate significant differences (P < 0.05)

3.4 Mothers' Employment Status and Taurine Intake

The consumption of taurine in accordance with mothers' occupational status is shown in Table 6. When mothers are full-time homemakers, the Q3 group, high taurine intake group scored the highest at 35.5% of frequency. While when mothers were employed, the Q1 group, low taurine intake group scored the highest at 31.9% of frequency. When mothers were full-time homemakers, their children's taurine intake rates were significantly higher (p < .05) than those with working mothers,

Table 5 Correlation between taurine intake and other nutrient intake

Nutrients	Correlation coefficients (r)	Nutrients	Correlation coefficients (r)
Energy	0.140	Vitamin B_6	−0.050
Protein	0.050	Folate	0.276**
Vitamin A	0.119	Vitamin B_{12}	0.630***
Vitamin D	0.141	Calcium	0.190*
Vitamin E	0.141	Phosphorus	0.127
Vitamin K	0.195*	Sodium	0.148
Vitamin C	0.055	Potassium	0.130
Thiamin	< 0.001	Iron	0.108
Riboflavin	0.140	Zinc	0.144
Niacin	−0.014	Cholesterol	0.152

∗Significantly correlated by pearson correlation
*p < .05, **p < .01, ***p < .001

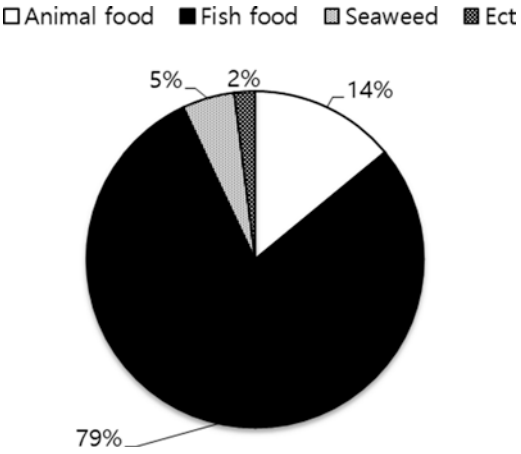

Fig. 1 Percentage of the food contributions to intake of taurine

Table 6 The level of the taurine intake by mother's employment status

	Q1[1] (n = 38)	Q2 (n = 38)	Q3 (n = 39)	Q4 (n = 38)	χ^2 (P–value)
Not employed	9 (14.5)[2]	14 (22.5)	22 (35.5)	17 (27.4)	9.048 (.029)
Employed	29 (31.9)	24 (24.8)	17 (25.5)	21 (24.8)	

[1]Taurine intake level. *Q1* 1st quadrant, *Q2* 2nd quadrant, *Q3* 3rd quadrant, *Q4* 4th quadrant. Q1 < 44.2 mg/day; 44.2 ≤ Q2 < 79.2 mg/day; 79.2 ≤ Q3 < 243.5 mg/day; 243.5 mg/day < Q4

suggesting the former children would have higher seafood intake in general. An apparent association was found between children's taurine intake and mothers' employment status.

4 Conclusion

This study analyzed taurine and nutrients intake for children in Daegu, Korea. The average daily energy intake of the children was 153.0 ± 155.2 mg/day. The taurine intake of the energy intake group with 75–125% (EER) was 189 ± 168 mg/day, which is significantly higher than the other groups, as 121 ± 173 mg/day in more than 125% (EER) group, and 104 ± 112 mg/day in less than 75% (EER) group, respectively.

The intake of vitamin D, vitamin B12, calcium, and folic acid was significantly higher among those with the high taurine intake. Taurine intake appears to be affected by seafood intake, and if seafood is consumed primarily, the amount of energy intake would be appropriate and will contribute to the increase of intakes of taurine, calcium and vitamin D in children.

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Taurine Enhances Stretch Reflex Excitability

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Abstract The purpose of this study was to characterize the effects of taurine (supplementation and acute injection) on the stretch reflex in the ankle muscles, and in particular to compare the effects of chronic taurine supplementation versus acute injection on the muscle tension, amplitude of electromyogram and velocity of muscle response. Stretch reflex responses were evoked using a specialized stretching device designed for mice. The triceps surae muscle of an awake mouse was stretched at various speeds ranging from 500 to 500,000° per second. A transducer recorded the muscle resistance at each velocity and the corresponding EMG. We found that at each velocity, the taurine-fed mice generated more tension and exhibited a higher EMG response. Acute taurine injection did not affect the tension but significantly reduced the EMG. To evaluate if the enhances response was due to neuronal excitability of changes in the passive properties of the muscles, we anesthetize the mice to eliminate the central component of the reflex. Under these conditions, taurine-fed mice still exhibited an enhanced stretch reflex response. We have previously shown that taurine-fed mice have reduced expression of $GABA_A$ receptors and other biochemical changes in the GABAergic system that are consistent with hyperexcitability. $GABA_A$ receptor is a major component of the inhibitory (GABAergic) system and its reduced expression probably contributes to the enhanced stretch reflex in these mice through biochemical mechanisms that involve alterations not only at the spinal level but also at the cortical level.

Keywords Stretch reflex · Taurine supplementation · Motor neuron · Electromyogram · Muscle spindle

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Abbreviations

TAU	taurine
EMG	electromyogram
GAD	glutamic acid decarboxylase
CSAD	cysteine sulfonic acid decarboxylase

1 Introduction

Taurine, a sulfur-containing amino acid, is present in high concentrations in mammalian plasma and tissues, plays a vital role in various essential biological processes such as development of the central nervous system (CNS) and the retina, glucose regulation, calcium modulation, anti-oxidant activity, membrane stabilization, reproduction, and immunity (Huxable 1992; Sturman 1993; Schuller-Levis and Park 2003, 2006; Stephen et al. 2010). It is clear that taurine induces a multitude of cellular and physiological actions directly or indirectly through compensatory mechanisms. An interesting aspect of taurine actions is its ability to mediate short- and long- term effects on brain physiology and biochemistry depending on the length of exposure. Short-term effects are mediated mainly through activation of the $GABA_A$ and glycine receptors, which are inhibitory in nature and lead to a general depression of neuronal activity. However, chronic dietary intake of taurine results in long lasting biochemical and behavioral changes characterized by increased neuronal excitability. Taurine-fed mice for example, showed increased susceptibility to KA-induced seizures (El Idrissi et al. 2003). Associated with this increased state of neuronal excitability, there are biochemical changes in the GABAergic system. Chronic supplementation of taurine in drinking water causes an increase in the levels of glutamate and GABA as well as the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD). Additionally, there was an upregulation in the expression levels of the NR1 subunit of the NMDA receptors and a reduced expression of the β3 subunit of $GABA_A$ receptors (El Idrissi et al. 2013).

There are two pathways for taurine biosynthesis. The conversion of cysteine into hypotaurine and taurine is mediated by CSAD. Supplementation of taurine to mice has been shown to inhibit CSAD both at the mRNA and protein level, leading to a buildup of cysteinsulfinate. The alternative pathway for taurine biosynthesis involves the production of cysteamine (Pitari et al. 2000). The cysteamine moiety is derived from cysteine during co-enzyme A degradation (Pitari et al. 2000). Pantetheinase is a ubiquitous enzyme that recycles pantetheine (vitamin B5) and produces cysteamine. Vanin-1 gene, which encodes pantetheinase, is widely expressed in mouse tissues (Pitari et al. 2000). Vanin-1 null mice have no detectable free cysteamine, indicating the importance of this pathway in cysteamine production and taurine biosynthesis (Pitari et al. 2000). Thus, supplementation of taurine to mice leads to a

build-up of two intermediate potentially important metabolites: cysteinsulfinate and cysteamine. Cysteinsulfinate, is an agonist for NMDA receptors and has been shown to accumulate in the spinal cord in taurine-fed mice (Dominy et al. 2007). Therefore, we tested the effect of this metabolite on spinal cord excitability by measuring the stretch reflex in taurine-fed mice. Consistent with this, we found that taurine dietary supplementation increased spinal cord excitability as demonstrated by the exaggerated stretch reflex.

2 Materials and Methods

2.1 Animals

All mice used in this study were 2-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 0.05%, and this solution was made available to the mice in place of drinking water for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Measurement of Spinal Stretch Reflex

Spinal stretch reflex was evoked using a specialized stretching device designed for mice. The triceps surae muscle of an awake mouse was stretched at varying speeds (3.125, 31.25, 312.5 and 3125 cm/s). Three consecutive measurements were performed at each speed. A transducer recorded the muscle resistance and an electrode recorded the EMG.

2.3 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 EMG and Tension Were Correlated to the Speed of Leg Displacement

Prior to measuring the stretch reflex, we correlated the velocity of leg displacement with the amplitude of the EMG and the tension produced by the muscle. We found that the EMGs were highly correlated with the speed of leg displacement (Fig. 1). Lower speeds (3.15 cm/s) yielded no recordable electrical activity from the muscle fibers. Whereas higher speeds (3125 cm/s) resulted in maximum firing and higher amplitude EMGs (Fig. 1).

3.2 Taurine Induced Hyper-Excitability in Spinal Cord

To further investigate the functional role of taurine on spinal cord excitability e measured the stretch reflex in taurine-fed mice. We used the high speed leg displacement (3125 cm/s) that yielded the highest amplitude EMGs. We found that

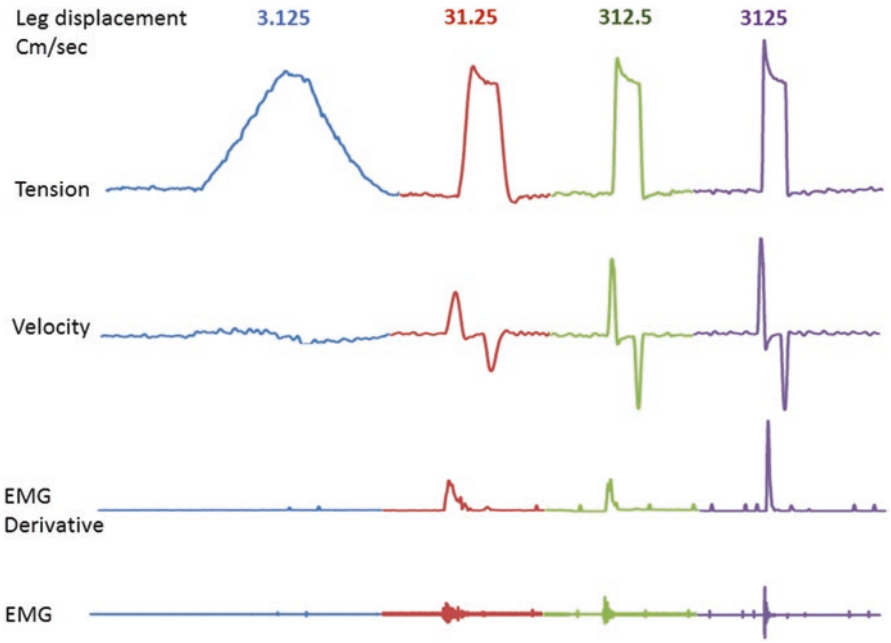

Fig. 1 Spinal stretch reflex was evoked using a specialized stretching device designed for mice. The triceps surae muscle of an awake mouse was stretched at varying speeds. A transducer recorded the muscle resistance. In controls, there was a correlation between speed of leg displacement and tension and EMG amplitude

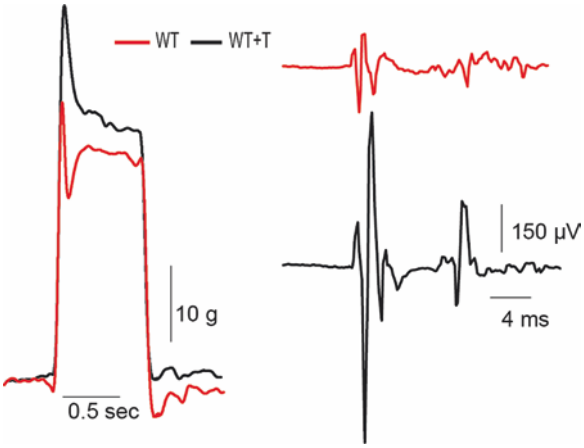

Fig. 2 Taurine in the diet of wild type mice increased spinal excitability. Spinal stretch reflex was evoked using a specialized stretching device designed for mice. The triceps surae muscle of an awake mouse was stretched at a speed of 3125 cm/s. A transducer recorded the muscle resistance. On the left of the graph, stretch responses from a wild type (WT) mouse (red) and from a mouse that was fed taurine (black). On the right are the corresponding EMG. The stretch reflex was exaggerated in taurine fed mice

mice treated with taurine in the drinking water showed a significant increase in spinal cord excitability and an exaggerated stretch reflex compared to controls (Fig. 2).

4 Discussion

The spinal cord mediates several reflex actions through local circuits. The simplest of these reflex arcs involves the response to muscle stretch, which provides direct excitatory feedback to the motor neurons innervating the muscle that has been stretched. The sensory signals for the stretch reflex originate from muscle spindles which is comprised of eight to ten intrafusal fibers embedded within the extrafusal muscle fibers. When the muscle is stretched, the intrafusal muscle fibers are in turn stretched, which results in initiation of action potentials. The afferent sensory neurons form monosynaptic excitatory connections with the α motor neurons in the ventral horn of the spinal cord that innervate the same muscle, leading to rapid muscle contraction. The α motor neurons innervating the antagonistic (heteronymous) muscles are inhibited via local inhibitory circuit inter neurons. This reciprocal innervation results in a rapid contraction of the stretched muscle and a simultaneous relaxation of the antagonist muscle leading to a rapid and efficient responses to changes in the tension and length in the muscle. The selectivity, accessibility and simplicity of connections in this circuit makes it an excellent system to study the principles underlying the mechanisms affecting stretch reflex excitability.

Several lines of evidence suggest that taurine may function as a potent inhibitory neuromodulator that regulate neuronal activity in many cerebral areas (Haas and Hosli 1973; Hussy et al. 1997; Huxtable 1989; Jiang et al. 2004). There is increasing evidence supporting the existence of functional interactions between GABA and taurine (El Idrissi and Trenkner 2004; Kuriyama and Hashimoto 1998). Taurine has been shown to increase plasma membrane chloride conductance by affecting bicuculine-sensitive chloride channels (del Olmo et al. 2000; Mellor et al. 2000; Wang et al. 1998). Taurine has also been shown to act as a partial agonist of $GABA_A$ receptors in synaptic membranes (Quinn and Harris 1995), and to activate Cl- influx through GABAA receptors in cerebellar granule cells in vitro (El Idrissi and Trenkner 2004). The interaction of taurine with $GABA_A$ receptors can also be shown in vivo. Subcutaneous injections of taurine (43 $mg.kg^{-1}$) reduces seizure severity in mice injected with kainic acid (El Idrissi et al. 2003), suggesting that the anti-convulsive effects of taurine might be mediated by direct interaction with the $GABA_A$ receptors in vivo. Furthermore, the chronic interaction of taurine with $GABA_A$ receptors induces a variety of alterations to the GABAergic system that encompasses key proteins involved in synaptic transmission at the inhibitory synapse. These alterations include increased hippocampal and cortical GAD expression, decreased hippocampal expression of the beta 2/3 subunits of the $GABA_A$ receptor (El Idrissi and Trenkner 2004), and an increase in the number of somatostatin-positive neurons (El Idrissi 2006; Levinskaya et al. 2006). We suggest that these biochemical changes to the inhibitory GABAergic system induced by taurine supplementation would affect the efficacy of the inhibitory system within the brain.

In this study we showed that chronic taurine supplementation led to an increase neuronal excitability at the spinal cord level. Such an increase in excitability was illustrated by an exaggerated stretch reflex in taurine-fed mice. This indicate that similar to the brain, taurine induces biochemical changes in the spinal cord that lead to hyperexcitability.

5 Conclusion

Taurine supplementation to mice (0.05% for 8 weeks) significantly enhanced the stretch reflex excitability. Spinal stretch reflex was evoked using a specialized stretching device designed for mice. The triceps surae muscle of an awake mouse was stretched at varying speeds. Stretch responses from control mice showed a velocity-dependent recruitment of motor neurons and the amplitude of the EMG was correlated to the tension produced by the muscle.

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Part III
Taurine and Organ Dysfunction

Modification by Ethanol and Taurine, Singly and in Combination, of Changes in Indices of Renal Dysfunction Caused by Diabetes in Rats

Sanket N. Patel and Cesar A. Lau-Cam

Abstract The present study was carried out in diabetic rats to examine the effects of ethanol (EtOH) and taurine (TAU), singly and in combination, in reducing the changes of laboratory test values indicating renal dysfunction. For this purpose, male Sprague-Dawley rats, 250–280 g in weight and in groups of 6, were made diabetic with a single, 60 mg/kg intraperitoneal dose of streptozotocin in 10 mM citrate buffer pH 4.5. On day 15 and for the remaining 14 days of the study, the diabetic rats (a) started to drink 5% EtOH in place of water, (b) received a single daily 2.4 mM/kg oral dose of TAU or (c) were allowed to drink 5% EtOH after receiving a dose of TAU. Starting from day 28 and ending on day 29, a 24 h urine sample was collected, its volume was measured, and then used to measure glucose (GLC), total protein (TP) and electrolytes (Na^+, K^+, Ca^{++}, Mg^{++}). Blood samples collected immediately thereafter via cardiac puncture were processed for the plasma fractions which were analyzed for their creatinine (CRT) and urea nitrogen (UN) contents. In comparison to normal (control) rats, diabetic ones showed a higher output of urine (+5.6-fold), a massive increase in plasma GLC (+473%), passed more GLC (+73.8-fold) and TP (+8.2-fold) in the urine, showed higher plasma CRT (+241%) and UN (+74%) levels, a lower plasma UN/CRT ratio (−47%) and a greater output of electrolytes in the urine (by at least twofold). By themselves both EtOH and TAU were found to markedly lower the effects of diabetes, with EtOH generally appearing more effective than TAU. However, the concurrent availability of EtOH and TAU was found to be more protective than either treatment alone.

Keywords Diabetes · Rats · Ethanol · Taurine · Plasma · Urine · Glucose · Creatinine · Urea nitrogen · Total proteins · Electrolytes

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Abbreviations

Ca^{++}	Calcium
CRT	Creatinine
EtOH	Ethanol
GLC	Glucose
K^{+}	Potassium
Mg^{++}	Magnesium
Na^{+}	Sodium
UN	Urea nitrogen
STZ	Streptozotocin
TAU	Taurine
TP	Total protein

1 Introduction

Diabetic kidney disease (DKD) is a common microvascular complication of patients with type 1 or type 2 diabetes mellitus (DM). Two of the most prominent risk factors for this condition are hyperglycemia and hypertension, with obesity also playing a causative role (Alicic et al. 2017). Characteristic manifestations of DKD are proteinuria, electrolyte abnormalities, renal anatomical and structural changes, lipid peroxidation and the progressive loss of renal function (Trachtman et al. 1995). If allowed to progress, end-stage renal disease (ESRD) requiring dialysis and transplantation, and a fatal outcome may eventually ensue (Zoccali et al. 2009).

On the other hand, the human consumption of ethanol (EtOH) has been found to influence the risk of type 2 DM depending on the amount consumed, with a moderate consumption reducing the risk (He et al. 2007; Koppes et al. 2005), and an excessive consumption increasing the risk (Koppes et al. 2005). Moreover, evidence gathered with naive rats has suggested that at low doses EtOH sensitivity to insulin increases but without affecting the body weight gain or the activity of enzymatic indices of hepatocellular injury (Furuya et al. 2003).

Several lines of evidence in support of a beneficial role for TAU in renal disease are available. Indeed, studies in rats have found that making this amino acid available as part of the drinking water (1%) will limit the extent of renal injury, the progressive decline in renal function and the disruption of renal structure that accompany a periodical 12-weeks treatment with the nephrotoxic aminonucleoside puromycin (Trachtman et al. 1992). Again, making TAU available on a daily basis as part of the drinking water and for 52 weeks resulted in a reduction of total proteinuria and albuminuria by nearly 50% while limiting glomerular hypertrophy, glomerulosclerosis and tubule-interstitial fibrosis in rats made diabetic with streptozotocin (Trachtman et al. 1995). In our laboratory, supplementing a rat diet with TAU was shown to attenuate the azotemia, hyperkalemia and proteinuria associated with streptozotocin-induced DM in rats (Pandya et al. 2015; and to lower the blood

glucose and HbA_{1c} while raising the plasma insulin (Budhram et al. 2013; Pandya et al. 2015). Interestingly, while Lin et al. (2010) reported that in streptozotocin-treated rats the oral administration of TAU, as a 70% suspension, for 10 weeks reduced the blood glucose to a value not significantly different from that of control rats, Ha et al. (1999) indicated that allowing the diabetic rats to drink water with 1% TAU in it for 4 weeks had no effect on the diabetic blood glucose even though it reduced proteinuria and renal lipid peroxidation.

Taking into account the reported effects of EtOH and TAU on experimental DM, the present study was undertaken with streptozotocin-treated rats to evaluate the effects of these compounds on DM-induced changes of markers of renal function when available singly and in combination with each other.

2 Materials and Methods

2.1 Chemicals

All the chemicals used in the study were purchased from commercial sources in the United States. STZ was obtained from A.G. Scientific, Inc., San Diego, CA; 200 proof ethanol was purchased from VWR International, West Chester, PA; and TAU was from Sigma-Aldrich, St. Louis, MO.

2.2 Animals

Male Sprague-Dawley rats, 250–280 g in weight, obtained from Taconic Farms Inc., Germantown, NY, USA, housed in a temperature- (23 ± 1 °C) and humidity controlled room on a 12 h light-12 h dark cycle, and used after a 7 day acclimation period, during which they had free access to a commercial rodent diet (LabDiet® 5001, PMI Nutrition International, Brentwood, MO, USA) and filtered tap water. The rats were randomly assigned to groups of 6 each, and were cared in accordance with guidelines described in the Animal Care Blue Book from the United States Department of Agriculture (2013).

2.3 Treatment Solutions

They were prepared each day just before an experiment. The ethanol (EtOH) solution, 5% v/v in concentration, was made by placing 50 mL of 200 proof EtOH, one envelope of sugar-free and caffeine-free Wyler's lemonade (Jel Sert Company, West Chicago, IL, USA), five envelopes of Splenda™ (McNeil Nutritionals, LLC, Fort Washington, PA. USA) and about 200 mL of tap water in a 1000 mL

volumetric flask. After dissolving the powders with the help of gentle shaking, the solution was brought to volume with additional tap water. The solution of STZ was prepared in 10 mM citrate buffer pH 4.5 to a concentration of about 67 mM (or 18 mg/mL); and that of TAU in distilled water to provide a solution 0.3636 M (or 45.44 mg/mL) in concentration.

2.4 Treatments

Diabetes was induced on day 1 with STZ (60 mg/kg, i.p. route); TAU (2.4 mM/kg/2 mL) was administered by oral gavage, once daily, starting from day 15 and continuing for the next 27 days. The EtOH solution was placed in a feeding tube and replaced each day. The amount consumed daily, from days 15 to 28, was recorded.

2.5 Samples

A 24 h urine sample was collected from day 27 to 28 after placing the rat in a metabolic cage. On day 28 the rats were anesthetized with isoflurane, and their bloods collected by cardiac puncture into heparinized tubes. Each blood sample was processed for its plasma fraction, which was analyzed without delay.

2.6 Assays

Urine samples were used to measure glucose (GLC), total protein (TP) and electrolytes (Na^+, K^+, Ca^{++}, Mg^{++}). The plasma samples were used to measure creatinine (CRT) and urea nitrogen (UN). All the assays were performed using assay kits purchased from Stanbio Laboratory, Boerne, TX, USA.

2.7 Statistical Analysis of the Experimental Data

The results are reported as mean ± SEM for $n = 6$; and they were analyzed for statistical significance using Welch's t-test and a commercial statistical software package (GraphPad Prism 3.02, La Jolla, CA, USA). Values were considered to be significantly different at $p \leq 0.05$.

3 Results

3.1 Urine Flow Rate

The effects of the various treatment agents on the diabetic urinary flow rate are shown in Table 1. Diabetic rats passed much more urine per hour than rats in the control group (by 5.6-fold, $p < 0.001$). While the consumption of EtOH by the diabetic rats led to a small, although significant, decrease of the diabetic urine flow rate (−18%, $p < 0.05$ vs. DM), feeding TAU resulted in only an insignificant reduction (−7%). In contrast, providing EtOH plus TAU reduced the diabetic urine outflow by about one-half ($p < 0.001$ vs. DM). In naive rats, EtOH and TAU, singly or as a combination, did not have a significant effect on the urinary output rate.

3.2 Urine GLC Rate of Elimination

As indicated in Table 2, diabetic rats passed much more GLC into the urine per hr than control rats (+73.8-fold, $p < 0.001$). Providing EtOH to the diabetic rats reduced the amount of rate of GLC elimination to a significant extent (+59.2-fold, $p < 0.001$ vs. control); and providing TAU had little effect (+68.2-fold). However, when EtOH and TAU were available concurrently, the rate of GLC elimination in the urine was much lower than of untreated diabetic rats (−43%, $p < 0.001$ vs. DM group). None of the treatment agents, alone or as a combination, altered the baseline value of GLC flow rate to a significant extent.

Table 1 Rates of urine flow, glucose (GLC) and total protein (TP) for control (CTRL), diabetic (DM) and DM rats treated with EtOH, TAU or EtOH-TAU[a]

Group	Urine flow rate mL/h	Urine GLC mg/h	Urine TP mg/h
CTRL	0.68 ± 0.03	0.05 ± 0.00	1.09 ± 0.05
DM	3.83 ± 0.08***	3.69 ± 0.29***	8.96 ± 1.06***
EtOH	0.53 ± 0.05	0.04 ± 0.00	0.83 ± 0.08
TAU	0.60 ± 0.03	0.04 ± 0.01	0.91 ± 0.04
EtOH-TAU	0.63 ± 0.06	0.05 ± 0.01	0.99 ± 0.10
DM-EtOH	3.15 ± 0.12$^{**,+}$	3.29 ± 0.16***	5.83 ± 0.35$^{***,+}$
DM-TAU	3.59 ± 0.13**	3.41 ± 0.17***	6.22 ± 0.33$^{***,+}$
DM-EtOH-TAU	1.90 ± 0.06$^{**,+++}$	2.09 ± 0.05$^{***,++}$	3.66 ± 0.12$^{***,++}$

[a]Values are given as the mean ± SEM for n = 6 rats
Differences were significant at $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. CTRL group; and at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. DM group

Table 2 Rates of excretion of sodium (Na^+), potassium (K^+), calcium (Ca^{++}) and magnesium (Mg^{++}) in the urine for control (CTRL), diabetic (DM) and DM rats treated with EtOH, TAU or EtOH-TAU[a]

Group	Urine Na^+ mEq/h	Urine K^+ mEq/h	Urine Ca^{++} mEq/h	Urine Mg^{++} mEq/h
CTRL	7.74 ± 1.27	2.11 ± 0.15	0.21 ± 0.01	0.012 ± 0.001
DM	42.09 ± 4.81***	4.61 ± 0.57***	3.19 ± 0.41***	0.101 ± 0.011***
EtOH	9.61 ± 2.64*	1.62 ± 0.33*	0.18 ± 0.02	0.012 ± 0.003
TAU	6.96 ± 1.44	1.73 ± 0.20*	0.26 ± 0.03	0.016 ± 0.002
EtOH-TAU	7.98 ± 0.96	1.10 ± 0.23**	0.30 ± 0.05	0.017 ± 0.001
DM-EtOH	15.84 ± 4.00$^{***,+++}$	2.83 ± 0.28$^{*,++}$	2.14 ± 0.30$^{***,++}$	0.106 ± 0.010***
DM-TAU	19.84 ± 3.59$^{***,+++}$	2.62 ± 0.31$^{*,++}$	2.72 ± 0.27$^{***,+}$	0.102 ± 0.006***
DM-EtOH-TAU	16.94 ± 4.02$^{***,+++}$	2.19 ± 0.19^{+++}	1.78 ± 0.29$^{***,++}$	0.059 ± 0.002$^{***,+++}$

[a]Values are given as the mean ± SEM for n = 6 rats
Differences were significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. CTRL group; and at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. DM group

3.3 Urine TP Rate of Elimination

From the results shown in Table 2, DM markedly increased the rate of elimination of TP into the urine (by 8.2-fold, $p < 0.001$). This effect was attenuated by both EtOH and TAU, with the former providing a greater effect than the latter (by 35%, $p < 0.01$ and 31%, $p < 0.05$, respectively, vs. DM). However, a combined treatment with EtOH and TAU produced a greater attenuating effect than either treatment alone (by 59%, $p < 0.001$ vs. DM). In naive rats none of the treatment agents or their combination showed a significant effect on the control value.

3.4 Urine Electrolytes

The effects of EtOH and TAU on the urinary levels of Na^+, K^+, Ca^{++} and Mg^{++} are shown in Table 2. In all instances, DM was found to significantly ($p < 0.001$) enhance the elimination of these electrolytes in the urine, although to different extents. Overall, the increases of Na^+ and K^+ were much lower than those of Ca^{++} and Mg^{++}.

As shown in Table 2, the amount of urinary Na^+ in diabetic rats was much higher than in control rats (+5.4-fold, $p < 0.001$). Both EtOH and TAU were found to attenuate this effect to a significant extent, with the former providing a greater effect than the latter (64% and 53% reductions, both at $p < 0.001$ vs. DM). On the other hand providing EtOH plus TAU together did not enhance the effect seen with EtOH alone further. In naive rat, none of the treatments agents exerted a significant effect on the baseline Na^+ value.

Although DM also raised the urine K^+ significantly (by 118%, $p < 0.001$ vs. control), the change was less pronounced that for the urine Na^+ (Table 2). Providing

EtOH to the diabetic rats significantly reduced the elevation (+34%, $p < 0.01$), although not as much as with TAU (+24%, $p < 0.05$). A treatment with EtOH-TAU virtually normalized the K+ level of the diabetic rats (only +3%). In contra, in naive rats the urine K+ was lowered by both EtOH (−23%, $p < 0.05$) and TAU (−18%, $p < 0.05$), especially when they were given together (−48%, $p < 0.001$ vs. control).

In diabetic rats, the urine Ca^{++} level was significantly higher than in control ones (by 15.2-fold, $p < 0.001$) (Table 2). This effect was attenuated by both EtOH and TAU ($p < 0.001$), with the effect of EtOH (+10.2-fold) being greater than that of TAU (+13.2-fold, $p < 0.001$. A combined treatment with EtOH and TAU was more potent than either compound alone (only +8.5-fold, $p < 0.001$ vs. control). None of the treatment agents affected the urine Ca^{++} of naive rats to a significant extent.

In common with the other electrolytes, the urine Mg^{++} was also elevated in diabetic rats (by 8.4-fold, $p < 0.001$ vs. control) and in diabetic rats treated with either EtOH (+8.8-fold) or TAU (+9.3-fold) (Table 2). However, feeding the diabetic rats with EtOH plus TAU reduced the diabetic Mg^{++} level by almost one-half (+4.9-fold, $p < 0.001$). While EtOH did not alter the Mg^{++} level of naive animals, TAU and EtOH-TAU exerted a significant reducing effect (by 33%, $p < 0.05$ and 42%, $p < 0.01$ vs. control, respectively).

3.5 Plasma CRT and UN

The effects of EtOH and TAU on the plasma CRT and UN values are shown in Table 3. In diabetic rats the plasma CRT was much higher than in control rats 241%, $p < 0.001$). The consumption of EtOH by the diabetic rats lowered the plasma CRT significantly (+84%, $p < 0.001$ vs. control) and to a greater extent than

Table 3 Levels of plasma creatinine (CRT), urea nitrogen (UN), plasma UN to CRT and urine to plasma CRT of control (CTRL), diabetic (DM) and DM rats treated with EtOH, TAU or EtOH-TAU[a]

Group	Plasma CRT mg/dL	Plasma UN mg/dL	Plasma UN to CRT ratio	Urine to plasma CRT ratio
CTRL	1.16 ± 0.09	20.18 ± 1.75	17.4	8.6
DM	3.96 ± 0.41***	35.06 ± 4.98***	8.9***	0.9***
EtOH	1.50 ± 0.11*	21.29 ± 2.14	14.2*	6.5$^{*,+++}$
TAU	1.03 ± 0.07	19.89 ± 1.97	15.3	7.3^{+++}
EtOH-TAU	1.10 ± 0.15	21.37 ± 2.40	15.4	6.3$^{**,+++}$
DM-EtOH	2.13 ± 0.17$^{***,++}$	27.14 ± 1.66$^{*,+}$	12.7$^{*,++}$	2.3$^{***,+}$
DM-TAU	2.59 ± 0.29$^{***,++}$	29.71 ± 2.11$^{*,+}$	11.5$^{**,+}$	1.7$^{***,+}$
DM-EtOH-TAU	2.25 ± 0.14$^{***,++}$	24.65 ± 3.58$^{*,+}$	11.0^{**+}	6.1$^{**,+++}$

[a]Values are given as the mean ± SEM for n = 6 rats

Differences were significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. CTRL group; and at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{++}p < 0.001$ vs. DM group

a treatment with TAU (+123%, $p < 0.001$ vs. control) or EtOH plus TAU (+94%, $p < 0.001$ vs. control) (Table 3). In naive rats, none of the treatment while EtOH raised the basal plasma CRT value to a significant extent (+29%, $p < 0.05$), neither TAU nor OH-TAU showed a significant effect. By analogy to the results for the CRT, diabetes also raised the plasma UN to a significant extent (+74%, $p < 0.001$ vs. control), an effect that was lowered by both EtOH (to +34%, $p < 0.01$) and TAU (to +47%, $p < 0.001$) (Table 3). Providing EtOH and TAU together led to a greater effect than with the individual agents (+22%, $p < 0.05$ vs. control). None of the treatment agents had an effect on the basal plasma UN value.

3.6 Plasma UN/CRT and Urine CRT/Plasma CRT Ratios

From the results graphically shown in Table 3, it is evident that diabetes had a significant negative effect on both the plasma UN/CRT and urine CRT to plasma CRT ratios and that these effects were counteracted to different extents by EtOH and TAU. First, diabetes reduced the plasma UN/CRT ratio by almost one-half of the basal value (−47%, $p < 0.001$); but a treatment with EtOH, TAU or EtOH-TAU reduced the effect significantly (decreases of 24%, 35% and 35%, respectively, $p < 0.05$ vs. control) (Table 3). Similarly, diabetes had a profound lowering effect on the urine to plasma CRT ratio (−90%, $p < 0.001$ vs. control). While providing EtOH to the diabetic rats resulted in a significant protection (−63%, $p < 0.001$), providing TAU did not (−80%, $p < 0.001$) (Table 3). On the other hand, a combined treatment with EtOH-TAU increased the urine to plasma CRT ratio to a greater extent (only −29%, $p < 0.05$) than either treatment agent alone. In naive rats, EtOH, TAU and EtOH-TAU exerted a small, although statistically, significant lowering effect on the basal urine to plasma CRT ratio (by 21%, 15% and 27%, all at $p < 0.05$ vs. control).

4 Discussion

The evaluation of EtOH as a protectant against DM-induced renal impairment was suggested by the results of earlier studies indicating that it can positively influence the kidney by lowering the incidence (Hsu et al. 2013) and extent of diabetes-related organ damage as a result of chronic kidney disease (Cheungpasitporn et al. 2015). However, for EtOH to demonstrate its protective effects it needs to be consumed in moderation inasmuch as a greater consumption can lead to increased serum levels of CRT, a common indicator of renal dysfunction (Savdie et al. 1984). Similarly, several epidemiological studies have uncovered an inverse association between alcohol consumption and the risk of renal dysfunction (Reynolds et al. 2008) and between the daily amount of alcohol consumption and the risk of developing proteinuria based on the hazard ratio (Yamagata et al. 2007). On the other hand, TAU

has been found to decrease the changes of markers of renal damage, plasma CRT and UN, urinary albumin and blood GLC and proinflammatory cytokines associated with STZ-induced diabetes (Das and Sil 2012).

Taking into account the beneficial effects associated with EtOH intake, the present study was undertaken to determine its role on the urine flow rate, the urinary rate of elimination of both GLC and TP, and the changes in serum CRT and UN values of diabetic rats. Finding that diabetic rats excreted much more GLC than normal rats agrees with the results of a study by Ying et al. (2018) in Zucker diabetic fatty rats and in which the urinary GLC output increased 10 weeks earlier than an increase in plasma GLC. Moreover, the greater than normal urinary excretion of proteins seen in the present study parallels the results of Smith et al. (2000) in Zucker diabetic rats. While the increased passage of GLC into the urine may be in part due to tubular damage (Chow et al. 2004), the accompanying proteinuria may be a sign of diabetic glomerular dysfunction (Arakawa et al. 2001) as a result of changes to the glomerular basement membrane structure (Koh et al. 2014). Indeed, a work carried out with Wistar albino rats made diabetic with STZ uncovered severe tubular degeneration, degeneration of glomeruli, focal necrosis of tubules, cystic dilatation of tubules, and fatty infiltration in the kidney tissue were observed, to which dilatation of the Bowman's capsule and hyaline casts were added following a daily oral treatment with 2 g/kg of EtOH for 30 days (Shanmugam et al. 2011). Of the two treatment agents evaluated, EtOH was found to be more effective than TAU in lowering the rate of GLC elimination in the urine, but a combined treatment with EtOH-TAU was much more effective than either agent alone. In this connection, there are studies showing that moderate EtOH consumption attenuates progression and loss of GFR in patients with primary kidney disease, lowers proteinuria and raises the creatinine clearance compared to abstainers and heavy drinkers (Schaeffner and Ritz 2012). While the present work has not looked into the mechanism accounting for the increased effect of moderate concentrations of EtOH in the presence of TAU, there is evidence to indicate that it may be related to the intrinsic antioxidant action of TAU against acute kidney injury by an inflammatory cell infiltrate or even by a potentially inflammatory chemical like EtOH (Latchoumycandane et al. 2014).

Diabetes altered the urinary excretion of Na^+, K^+, Ca^{++} and Mg^{++}, all of which were elevated in the urine. The generalized loss of electrolytes in the urine is most likely the result of a marked decline of renal function (Araki et al. 2015), specifically due to tubular interstitial changes seen both in humans and STZ-treated rats (Ziyadeh and Goldfarb 1991). However, Lee et al (2006) have related the increased urinary excretion of Ca^{++} and Mg^{++} to an increased renal Ca^{++} and Mg^{++} transporter abundance in STZ-induced diabetic rats, which may represent a compensatory adaptation for the increased load of Ca^{++} and Mg^{++} to the distal tubule. As established for the excretion of TP in the urine, EtOH-TAU was more effective than either EtOH or TAU, in that order, in preventing the renal excretion of electrolytes into the urine. The only exception was the effect on the urinary Na^+ excretion and for which EtOH was insignificantly more effective than EtOH-TAU.

Measurement of the circulating levels of both CRT and UN are routinely used as indicators of renal function and as a reflection of the glomerular filtration rate, with

the former being less affected by dietary and physiologic conditions unrelated to renal function (Hosten 1990). In the present study the plasma values of both CRT and UN were grossly elevated, with the elevation of the CRT being about three-fold higher than that of the UN. These results confirm those of Arikawe et al. (2012) who also found both parameters to be elevated in STZ-treated rats. In contrast, providing EtOH or TAU reduced the elevation by more than one-half, especially when they were given together. In the case of EtOH, these results have been bee related to an enhancement of both CRT clearance and glomerular filtration rate (Chung et al. 2005). Furthermore, at low doses EtOH may protect the kidney against morphological damage, cytoskeletal derangement, inflammatory mediators and the injurious effects of free radicals, thus contributing to the maintenance of renal function (Ma et al. 2016; McCarthy et al. 2015).

Although upward changes in the blood UN/CRT ratio is of help in differentiating between prerenal acute kidney injury (AKI) and intrinsic AKI (Manoeuvrier et al. 2017), in the present study the diabetic rats showed a significant decrease of the ratio relative to that of normal rats, a finding that is likely to be the result of severe hepatocellular damage (Hosten 1990). While both EtOH and TAU elevated the plasma UN/CRT ratio significantly relative to the diabetic ratio, by itself EtOH was insignificantly more potent than either TAU or EtOH-TAU. Overall, these results are taken as an indication of protection of hepatic cells against the damaging effects of diabetes by both EtOH and TAU. Similarly, diabetic rats exhibited a much lower urine CRT/plasma CRT ratio than control rats, a finding that is taken as an indication of renal dysfunction (Zhang et al. 2017). Providing the diabetic rats with EtOH, but not with TAU, raised the diabetic ratio to a significant extent, an effect that was even greater when EtOH and TAU were available together. In naive rats, EtOH and TAU, singly and in combination had a small although significant effect on the control urine CRT/plasma CRT ratio.

5 Conclusion

The results of this investigation indicate that both EtOH and TAU can improve the glycemic state and renal function of diabetic rats, with EtOH appearing more potent than TAU, and with the combination EtOH-TAU providing a greater protection than either treatment alone.

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Effect of Taurine on Thymus Differentiation of Dex-Induced Immunosuppressive Mice

Jun Piao, Fanpeng Meng, Hui Fang, Fengyuan Piao, Bo Jin, Ming Li, and Wenzhe Li

Abstract Taurine (2-aminoethanesulfonic acid) has positive effects on the formation of immune systems. In this study, we evaluated the effects of taurine on the development of T lymphocyte subpopulations in thymus of immunosuppresive mice. The immunosuppressed mice model was established by intraperitoneal injection of dexamethasone (Dex) for 7 days. Mice (male, Kunming strain) were randomly divided into three groups, the normal control group (Cont.), the Dex-induced immunosuppressive model group (Dex + PBS), and the taurine intervention group (Dex + TAU). Taurine was administered at a dose of 200 mg/kg for 30 days or until euthanasia. Total cell numbers in the thymi of mice were evaluated by cell count, and the flow cytometry was used to determine the proportion of different cell subsets. Our results showed that the size and weight of thymi of Dex + PBS group were significantly smaller than those of Cont. group, and taurine administration efficiently increased the thymus index. Taurine also significantly increased the number of CD4- CD8- double negative (DN), CD4+ CD8+ double positive (DP), CD4+ single positive (CD4+) and CD8+ SP (CD8+) cells compared with the Dex + PBS group, but did not affect the CD4+/CD8+ cell ratio in thymus of Dex-induced immunoseppressive mice. Our results suggested that taurine has a positive effect on thymus differentiation in Dex-induced immunosuppressive mice.

Keywords Taurine · Immunosuppressive · Thymus · T cell differentiation

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing organic acid (Fig. 1a), which has various beneficial physiological functions, including membrane stabilization, anti-oxidative activity, neuroprotection against neurotoxicity and modulation of intracellular calcium levels (Tochitani 2017). In addition, taurine can promote the secretion of pituitary hormones and activate pancreatic function so as to regulate the body's metabolism, thus preventing cardiovascular disease, improving endocrine status and enhancing immunity (Lambert et al. 2015; Wu and Prentice 2010; Yu et al. 2016). In literature, as a natural compound, taurine is a potent agent for metabolic correction of many diseases (Sapronov et al. 2001). For example, taurine can form taurine chloramine (TauCl) with HOCL. This haloamines share anti-inflammatory and anti-oxidant properties by inducing the expression of heme oxygenase-1 (HO-1) (Kim and Cha 2009; Marcinkiewicz et al. 2000; Muhling et al. 2008). Taurine was also found to effectively enhance the immune function of mice with T-cell lymphoma during chemotherapy (Dong et al. 2017). Thymus is the

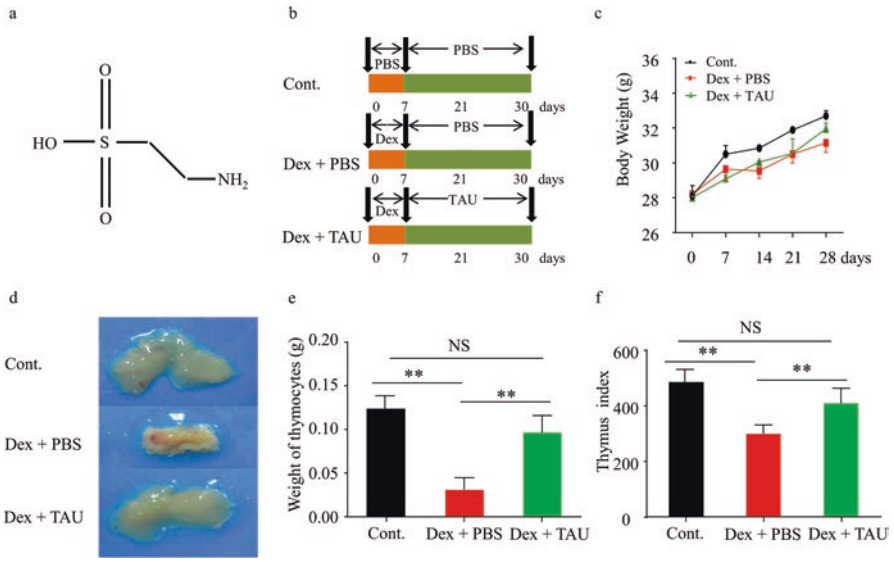

Fig. 1 Taurine increased the number of thymocytes in Dex-induced immunosuppresive mice. (**a**) The structural formula of taurine. (**b**) Modeling method: Cont. group: PBS was injected for 7 consecutive days (Dex equal volume of PBS), and then PBS was injected every 2 days until the 30th day or until euthanasia. Dex + PBS: Dex was injected for 7 consecutive days, and Dex was injected every 2 days after model until the 30th day or until euthanasia. Dex + TAU: Dex was injected for 7 consecutive days, Dex was injected every 2 days after model, and taurine was administered daily until the 30th day or until euthanasia. (**c**) Changes in the average weight of mice in 4 weeks. (**d**) The comparison of the thymus morphology between the normal group and the taurine model group. (**e**) Weight change of thymus. Data are representative of the mean ± SD from three independent experiments (∗∗$p < 0.01$). (**f**) Change of thymus index. Data are representative of the mean ± SD from three independent experiments (∗∗$p < 0.01$)

initial site for development of T cell immunological function, which is essential for T cell development and maturation (Majumdar and Nandi 2018). The earliest populations of progenitor thymocytes lack cell surface expression of CD4 and CD8, and are therefore referred to as double negative (DN) cells (Anderson et al. 1996). The DN population can be further subdivided by surface expression of CD44, and CD25 (Godfrey et al. 1993), through the stages DN1 (CD25-CD44+), DN2 (CD25+CD44+), DN3 (CD25+CD44-), and DN4 (CD25-CD44-). DN4 has undergo β selection and initiated the process of differentiating to the CD4+ CD8+ (DP) cells, which give rise to both CD4 single positive (CD4+) and CD8 single positive (CD8+) populations (Solanki et al. 2018).

Although there are many studies on taurine, the effect of taurine on thymus differentiation of the immunocompromised model is not clear. In this study, dexamethasone (Dex) was used as an inducer to establish an immunocompromised model. By comparing the normal group, the model group and taurine administration model group, we will study the effect of taurine on thymus differentiation.

2 Materials and Methods

2.1 Chemical and Material

Taurine was purchased from Sigma Chemical Company (St. Louis, USA). When used, it was dissolved in water at a concentration of 0.1 g/ml. Dex was purchased from laboratory of Dr. Ehrenstorfer in Augsburg-Germany and was dissolved in phosphate buffered solution (PBS) at a concentration of 4 mg/ml.

2.2 Mice

Thirty-six Kunming male mice (18–22 g) were gained from the Major Gene Engineering and Disease Model Animal Research Institute of Dalian medical university. Mice were maintained in a room illuminated for 12 h (08:00–20:00) and kept at 24 ± 1 °C with free access to food and water under specific pathogen–free (SPF) condition. All animal procedures complied with the institutional animal protocol.

2.3 Grouping and Modeling

The mice were randomly divided into three groups, the normal control group (Cont.), the Dex-induced immunosuppressive model group (Dex + PBS), and the immunosuppressive + taurine group (Dex + TAU), each containing 12 mice

(Fig. 1b). Intraperitoneal injection of Dex was adopted to establish a model of immunosuppression in two groups except the Cont. group. Mice in the Cont. group were injected every day with equal volumes of PBS intraperitoneally for 7 days, then, injected once every 2 days until the 30th day or until euthanasia; mice in the Dex + PBS group were intraperitoneally administered with 20 mg/kg body weight of Dex daily for consecutive 7 days, after the model was established, injected once every 2 days until the 30th day or until euthanasia; mice in the Dex + TAU group were injected for 7 consecutive days, Dex was injected every 2 days after model generation, and taurine was administered daily until the 30th day or until euthanasia.

2.4 Processing of Thymic Tissue

After euthanasia, each mouse's thymus was collected and weighed. The thymus index was calculated according to the following formula: Thymus weight (g)/body weight (kg) × 100. The single cell suspension was prepared by grinding the thymus with a scrub slider and then passing it through a 300-mesh nylon mesh.

2.5 Antibodies

The monoclonal antibodies (mAbs) used in this study are anti-CD16/32 (2.4G2), PE-Cy5-labeled anti-mouse CD8a (L3T4), and APC-labeled anti-mouse CD4 (53–6.7). All Abs were purchased from BD Biosciences.

2.6 Flow Cytometric Analysis

For phenotypic analysis, 1 × 106 thymocytes were first incubated with an anti-CD16/CD32 (2.4G2) mAb to block Fc receptors and then stained on ice for 40 min with several combinations of mAbs. Flow cytometry was performed on a FACS-Calibur (Becton Dickinson, Mountain View, CA), and the data were analyzed with the BD Accuri C6 software or Flowjo software (Treestar, San Carlos, CA). Cell debris was excluded by appropriate two-dimensional gating methods. For cell sorting, the thymocytes were stained with PE-Cy5-labeled anti-CD8a, APC-labeled anti-CD4 and sorted with a BD FACS Aria TM II (Becton Dickinson) instrument. DN, DP, CD4+ and CD8+ cells were collected then proceed to the following experiments.

2.7 Statistical Analysis

Statistical analyses were carried out using the ANOVA test by GraphPad prism 6. All statistical analyses were performed using the general linear model procedure. P values of less than 0.05 was considered statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3 Results

3.1 Effects of Taurine on Body Weight and Thymus Morphology of Immnosupressive Mice

The average body weight of mice in different experimental groups during the treatment time is shown in Fig. 1c. The body weight of mice in the Cont. group increased from 28.1 ± 0.8 to 32.7 ± 0.4 g from the beginning to the end of the treatment. In Dex + PBS group, the body weight of mice showed a mild increase from 28.2 ± 0.1 to 31.1 ± 0.8 g, which is lower than that of the control group. Treatment with taurine showed an improving effect on the body weight of immnosuppressive mice, with an average body weight increase from 28 ± 1.1 to 31.9 ± 0.5 g.

Dex + PBS group resulted in significant histological changes in the thymus of mice. As can been seen in Fig. 1d, in thymus of mice in Dex + PBS group, the normal lymphoid tissue was replaced by fat, the medulla disappeared, resulted in an unidentifiable tissue structure. In contrast, the thymi of mice in Dex + TAU group did not show obvious change in morphology.

Figure 1e shows the changes in the thymus weight of normal and treated mice. The average weight of total thymus was 0.128 ± 0.008, 0.025 ± 0.004 and 0.100 ± 0.004 g in Cont., Dex + PBS and Dex + TAU groups, respectively. Injection of Dex to mouse resulted in a remarkable weight loss of thymus. But no statistically significant differences were detected between the Cont. group and the Dex + TAU group. Also, the thymus/body weight ratio (thymus index) reflects the analysis of thymus weight loss (Fig. 1f), with Cont. and Dex + TAU group being significantly different from group Dex + PBS and no difference from group Cont. and Dex + TAU. This suggests that taurine plays a key role in thymic differentiation.

3.2 *Effects of Taurine on Cell Differentiation of Thymus in Immunoseppressive Mice*

When measuring the total cell count in thymus, we observed that the number of cells in the Cont. group and the Dex + TAU group was significantly different from that of the Dex + PBS group, and there was no difference between the Cont. group and the Dex + TAU group.

To examine a potential regulatory role of taurine during thymocyte development, we stained thymocytes with anti-CD4 and anti-CD8 Abs. The subpopulations of DN, DP, CD4+ and CD8+ cells were sorted using the markers CD4 and CD8. Analysis of subpopulation of thymocytes in the Dex + PBS model group revealed a marked reduction in numbers of DP thymocytes (2.1%) compared to those of Cont. group (75.7%), while those were restored by administration of Taurine (60%) (Fig. 2b, c). Conversely, the numbers of total DN thymocytes were significantly increased in the Dex + PBS group (54.7%) than that of the Dex + TAU group (26.7%) and Cont. group (5.5%).

The absolute cell number of thymocytes was further evaluated. Injection of Dex resulted in a significant increase in the proportion of DN cells in the thymus of mice,

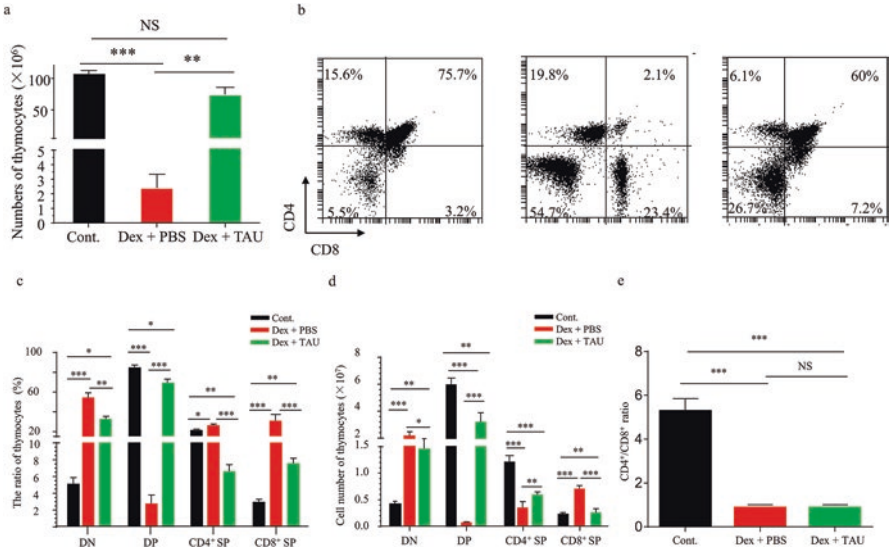

Fig. 2 Taurine regulates thymocyte differentiation. (**a**) Cell numbers of thymus. Data are representative of the mean ± SD from three independent experiments (***p < 0.001; **p < 0.01). (**b**) FCM analyses of thymocytes population. Numbers of the major subsets of DN, DP, CD4+, and CD8+ cells were calculated. Data are presented as mean ± SD from six mice per group. (**c**) Percentage of the immune cells of thymocytes. Data are representative of the mean ± SD from three independent experiments (***p < 0.001; **p < 0.01; *p < 0.05). (**d**) Absolute cell number of immune cells in thymus. Data are representative of the mean ± SD from three independent experiments (***p < 0.001; **p < 0.01; *p < 0.05). (**e**) The CD4+/CD8+ ratio was analyzed. Data are representative of the mean ± SD from three independent experiments (***p < 0.001)

and after treatment with taurine, the proportion of these cells decreased (Fig. 2d). The number of DP cells in the Cont. group was very high, but there was a very significant decrease in the Dex + PBS group, in contrast, we found a major recovery of DP cell proportion in the thymus of Dex + TAU group, which was close to the Cont. group. The proportion of CD8+ cells in the Dex + PBS group was similar to that of the Cont. group, and it decreased significantly in the Dex + TAU group (Fig. 2d). These findings implied that taurine could enhance the transition of DN to DP stage.

Compared with the Cont. group, the absolute number of CD4+ cells decreased significantly in the Dex + PBS group and re-increased by taurine treatment (Fig. 2d). The absolute number of CD8+ cells in the Dex + PBS group increased significantly compared with Cont. group, and after administration of taurine, this number reduced largely but still higher than the Cont. group (Fig. 2d). Next, when measured the CD4+/CD8+ ratio, we found no difference between Dex + PBS and Dex + TAU group, but they were obviously lower than that of the Cont. group (Fig. 2e).

4 Discussion

Taurine is a necessary amino acid for human body and has positive effects on immune systems (Dong et al. 2017; Kim and Cha 2009; Marcinkiewicz et al. 2000; Muhling et al. 2008). As a primary (also called central) lymphoid organ in most mammal, thymus is required for the development of lymphocyte effector cells and for the elaboration of the primary lymphocyte repertoire; primary lymphoid organs thus serve a non-redundant function in the immune system (Boehm and Bleul 2007). To our knowledge, this is the first study that was conducted to observe the effect of taurine on the thymus differentiation of mice with Dex-induced immunosuppression.

As a prescription for anti-inflammation and anti-allergic drug, Dex is often used to make immunosuppressive model by reducing the quantity of antibody produced by lymphocytes, then down-regulating immunologic function (Li et al. 2017). Administration of high dose Dex impairs the function of thymus cells (Ichiyoshi et al. 2003). As demonstrated by our results, Dex treatment significantly reduced the weight and total cell numbers of mice thymus, in contrast, administration of taurine could effectively prevent Dex-induced weight and cell loss of mouse thymus, suggesting that it has a critical role in thymus development and function.

Taurine is present in lymphocytes and can modulate the functions of these immune cells, such as protection against antioxidants and regulation of inflammatory aspects of the immune response (Fazzino et al. 2010). Lymphocytes are one of the main immunocytes in our body, and their proliferation and differentiation are important for immune response. The proliferation and differentiation state demonstrate the function of lymphocytes to some extent (Gorosito Serran et al. 2015; Rajasekaran et al. 2016). As can be seen, the lymphocytes' proliferation in the thymus of mice declined significantly when body immunity is suppressed by Dex. Experiment of lymphocytes' proliferation revealed that taurine could improve the

growth of T cells and B cells in Dex-treated mice. These results indicate that taurine can regulate the nonspecific immunity of mice.

T cell subset plays an important role in cellular and humoral immunity. The percentage of CD4+ and CD8+ cells indicates the proportion of T helper (Th) cells and T cytotoxic (Tc) cells (Taniuchi 2018). We therefore investigated the effects of taurine treatment on the distribution of subset of lymphocytes. Results showed that Dex injection had significantly suppressed the T cell proportions, including not only the CD4+ and CD8+ cells, but also the DP cells, in contrast, the proportion of DN cell in thymus of Dex-treated mice increased dramatically, suggested that Dex induced the DN stage arrest. Post taurine treatment, it significantly increased the number of DN, DP, CD4+ and CD8+ cells compared with the Dex+PBS group. These results suggested that taurine has a positive effect on the development of thymic cells in Dex-induced immunosuppressive mice. On the other hand, under normal condition, the number of T cells as well as the proportion of CD4+/CD8+ cell is maintained in a certain level. The immune condition could be generally estimated by calculating the proportion. The reduced proportion of CD4+/CD8+ cell is usually associated with severe illnesses and poor prognosis (Yin et al. 2015). We therefore investigated the effects of taurine on the ratio of CD4+/CD8+ cell in thymus of Dex-treated mice. However, the results showed that taurine did not affect the proportion of CD4+/CD8+ cell in the Dex + PBS mice. Since DP thymocytes undergo positive or negative selection (Solanki et al. 2018), administration of taurine could influence CD4 and CD8 fate decision by positive or negative selection. The study how taurine regulates the T cell selection needs to be further investigated.

5 Conclusion

In conclusion, our experiments proved that taurine could enhance immunity of immunosuppressive mice through adjusting the development and nonspecific immunity of thymus. These provide a theoretical basis for application of taurine in enhancing the immune function of elders, children, or patients with a poor health. Furthermore, at present, the clinical treatments of diseases, such as T-cell lymphoma, mainly include radiotherapy, chemotherapy and immunotherapy. Although chemotherapy drugs can effectively kill tumor cells, they exhibits more inhibition on immune system, severely affecting the chemotherapy efficacy and leading to poor prognosis (Marcinkiewicz 2010; Sapronov et al. 2001; Sartori et al. 2017; Zhang et al. 2015). Therefore, application of taurine may effectively enhance the immune function of patients undergoing chemotherapy treatment to improve the efficacy of the treatment.FundingThis work was supported by the National Nature Science Foundation of China (31570797, 81571498), the China Postdoctoral Science Foundation (2016M601317, 2018T110225), and the Research Foundation from the Department of Education, Liaoning Province, China (L2016003).

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Effects of Taurine on Broiler Aortic Endothelial Apoptosis Induced by Heat Stress

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Abstract Heat stress is an environmental factor that causes severe economic loss to the current intensive breeding industry and induces huge impact on the long-term growth in livestock and poultry industry. Many animal experiments confirmed that heat stress is a major cause of heat stroke death, which is due to severe damage to endothelial cells. In order to provide a theoretical basis for the treatment or mitigation of heat stress related diseases in broilers, the effect of taurine on injury and apoptosis of aortic endothelial cells in broilers under heat stress was investigated in the present study. Ten days healthy broilers were sacrificed, then aortic tissue was used to isolate and cultivate primary broiler aortic endothelial cells. The third to the fifth generations of cells were used in the experiment. The cells were randomly divided into five groups, including control group (C), heat stress group (HS), low taurine (HS+LTau) group, mild taurine (HS+MTau) group and high taurine (HS+HTau) group. Cells in all groups were cultivated for 24 h in cell incubator (37 °C, 5% CO_2). Then the heat stress group cells were cultivated in a 43 °C thermostatic water bath for 6 h under heat stress, and then re-incubated under 37 °C for 1 h. The results showed that compared with the control group, expression levels of Bax, Caspase-9, Caspase-3, Cyt-c, P53 and other pro-apoptosis factors in HS groups were significantly increased ($P < 0.05$), while expression levels of anti-apoptosis factor Bcl-2 showed a significant decrease ($P < 0.05$). Compared with HS group, expression levels of Bcl-2 in endothelial cells were significantly increased by taurine administration ($P < 0.05$), while expression of Bax, Caspase-9, Caspase-3, Cyt-c and P53 were significantly increased by taurine ($P < 0.05$). In summary, the

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present data indicated that taurine could protect against injury and apoptosis of aortic endothelial cells under heat stress by inhibiting the activation of mitochondria-mediated apoptotic pathways.

Keywords Taurine · Apoptosis · Heat stress · Broiler aortic endothelial cells

1 Introduction

Taurine, known as beta-amino ethanesulfonic acid, was originally isolated in bezoar. Taurine is colorless or white crystal, tasteless, stable in nature and soluble in water (Xiaoqiong and Deyi 2011). Widely distributed in animals' tissues and organs in the form of free amino acids and fulfills many physiological functions. There are two different pathways for the death of eukaryotic cells, one is necrosis and the other is programmed cell death (PCD), also known as apoptosis (apoptosis).

Taurine serves as cytoprotective agent by improving mitochondrial damage, cytotoxicity and apoptosis in body (Devi and Anuradha 2010). After rats suffered from intestinal ischemia-reperfusion (IR) injury in rat, Tau-treated rats showed significantly lower jejunal and ileal apoptotic index with high Bcl-2/Bax ratio compared to IR rats, indicating that Tau Treatment could prevent intestinal mucosal injury and inhibit intestinal epithelial cell apoptosis in IR rats (Sukhotnik et al. 2016)

Vascular endothelial cells (VEC) are single-layered flat cells that cover blood vessels' surface and are arranged longitudinally. They provide a smooth inner surface for blood vessels to maintain physiological functions. Due to their wide coverage, they are considered a tissue with extensive biological activity in the body. As one of the most obvious tissue cells in early traumatic injury (Zhengtao 2014), they take active part in inflammatory reactions and apoptosis.

Heat stress destroys vascular integrity and permeability, and aggravates injury of endothelial cells (Plumier et al. 1996). Death from heat stroke is mainly caused by massive hemorrhage, injury and thrombosis of endothelial cells. These physiological changes are related to intense heat stress-induced cytotoxicity, inflammatory response and coagulation mechanism, leading to severe damage to endothelium cells and affecting the normal work of multiple organ tissues. Li (Li et al. 2016) showed that heat stress can induce endothelial cells apoptosis of human umbilical veins, and that cells treated with intense heat stress can induce the production of ROS. Meanwhile mitochondrial apoptotic pathways are activated, indicating apoptosis as an important pathological feature of heat stress.

Taurine has become a focus of study due to its extensive physiological functions and pharmacological effects. However, fewer reports have focused on the protective mechanism of Tau against heat stress-induced endothelial cell damage. Our experiment will study different levels of taurine as protection against heat stress-induced injury of broilers' endothelial cells at 43 °C, analyze effects of Tau on apoptosis-related factors of endothelial cells, and further elucidate protective mechanism of Tau on aortic endothelial cell injury, thus providing references for treatment or relief

of heat stress-induced diseases in broilers, for changes in traditional preventive measures, and for improvement of broiler performance and disease resistance.

2 Methods

2.1 Instrument

CO_2 incubator was purchased from Hong Kong Heraeus Co., Ltd.; PCR amplification instrument, iQ5 fluorescence quantitative PCR instrument, Bio-Rad Gel Doc XR gel imaging system, and chemiluminescence gel imaging system were from Bio-Rad company of the United States.

2.2 Main Reagents

DMEM/F12 medium and PBS solution were purchased from Hyclone, USA; TRNzol total RNA extract (100 mL), SYBR Premix Ex TaqII, and reverse transcription kit were from Dalian Bao Biological Reagent Co., Ltd.; Western blotting related reagent was from Beijing Plylai Co.; Bcl-2 polyclonal antibody, Bax polyclonal antibody, Caspase-3 polyclonal Antibody, Caspase-9 polyclonal antibody, and goat anti-rabbit secondary antibody were from Abcam, USA; Cyt-c monoclonal antibody, P53 monoclonal antibody, GAPDH monoclonal antibody, and goat anti-mouse secondary antibody were from Santa Inc., USA.

2.3 Study Design and Sample Collection

The passages of endothelial cells in the logarithmic growth phase of three to five generations were divided into normal control (C) group, heat stress (HS) group, and heat stress groups with low, medium and high levels of Tau (LTau, MTau, HTau). Among them, normal control cells were always cultured in cells incubator (at 37 °C, with 5% CO_2), and the cell culture medium concentration of Tau in each heat stress group was 0 mmol/L Tau (HS group), 20 mmol/L Tau (LTau group), 40 mmol/L (MTau group) and 80 mmol/L (HTau group) respectively.

Each group of cells were placed in a cell culture incubator for 24 h, and those in heat stress groups were placed in a constant temperature water bath at 43 °C. After 6 h of heat stress stimulation, Incubated in the incubator for 1 h, and the cells and culture fluids were collected as follow-up indicators (Zhan Jinshun et al. 2014). Please refer to heat stress treatment methods (Wu et al. 2016).

2.4 Detection of Apoptosis-Related Genes in Aortic Endothelial Cells of Heat-Stressed Broilers

2.4.1 Primer Design and Synthesis

Based on the Gen Bank accession sequence of NCBI chicken and the Bax gene sequence of the canary, the GAPDH gene was used as references and all the primers were synthesized by Sheng gong (Shanghai) Co., Ltd. Primer sequences were shown in Table 1.

2.4.2 Extract Total RNA in Tissues and Prepare cDNA

The jejunal RNA was extracted according to the instructions of the RNA isolation kit, the concentration and purity of RNA were determined; cDNA was prepared based on the procedure of reverse transcriptase of reverse transcription reaction (HiScriptR II Reverse Transcriptase) (R223-01).

2.4.3 Real-Time Fluorescence Quantitative PCR Detection

Primers were designed using Primer Premier 5.0, the Gen Bank for accession sequence and GAPDH mRNA for reference. The results were shown in Table 1. The synthesis of primers was completed by Shanghai Shenggong Biological Engineering Co., Ltd.

Table 1 Design of primer

Gene	GeneBank	Primer sequence	Product length	Annealing temperature
GAPDH	NM_204305.1	F:GAGGGTAGTGAAGGCTGCTG	116 bp	59.8 °C
		R:CGCATCAAAGGTGGAGGAAT		
Bcl-2	NM_205339.2	F:GGATGCCTTTGTGGAATTGT	121 bp	56.8 °C
		R:ATAAGCGCCAAGAGTGATGC		
Bax	XM_009100858.2	F:CAGGATGATCGAGCAGGTG	128 bp	57.4 °C
		R:GCTTGCAGGCAAAGTAGAAGA		
Caspase-9	NC_006108.4	F:AGTCCTTGGTTCTGGGACCT	121 bp	59.8 °C
		R:CAGCACAGTTCGTGTGATCC		
Caspase-3	NM_204725.1	F:CATCGGGCATTCTTCTCAAT	119 bp	56.8 °C
		R:CGTGCTTCCAAAGGGTAATG		
P53	NM_205264.1	F:TCCTCACCATCCTTACACTGG	118 bp	58.5 °C
		R:CGGAAGTTCTCCTCCTCGAT		
Cytochrome c	NM_001079478.1	F:AGCACAAGACTGGACCCAAC	124 bp	59.7 °C
		R:CAGTGGCTCAGGAAAGCTG		

2.5 Detection of Apoptosis-Related Proteins in Aortic Endothelial Cells of Heat-Stressed Broiler Chickens

2.5.1 Total Cellular Protein Extraction

Plylai protein extraction kit was used to extract total protein. Protein concentrations were determined by BCA method.

2.5.2 Detection of Target Protein Expression Levels

SDS-PAGE electrophoresis was used to detect apoptosis-related proteins according to the procedure of Western blotting. Use ultrasensitive chemiluminescence imaging system for imaging.

2.6 Statistical Analysis

Excel was used for preliminary analysis of the test results, and SPSS 17.0 analysis software for statistical analysis. Differences between groups were analyzed by one-way ANOVA, and LSD methods were used for multiple comparisons. Measured data were expressed as mean ± standard deviation (x ± SD), and $P < 0.05$ was statistically significant.

3 Results

3.1 Effects of Taurine on Apoptosis Related Genes in Aortic Endothelial Cells in Heat Stressed Broilers

3.1.1 Effects of Taurine on the Relative Expression of Bcl-2, Bax and P53 Genes

As can be seen from Table 2, the relative expression level of Bcl-2 gene in the endothelial cells in HS group was significantly decreased ($P < 0.05$), which was 0.58 times lower than that of C group. In each group to which Tau was added, the relative expression levels of Bcl-2 gene were 0.87-fold, 0.90-fold and 1.27-fold higher than C group respectively, and there was no significant difference ($P > 0.05$). The relative expression levels of Bcl-2 gene in Tau groups were 1.5 times, 1.55 times, and 2.19 times respectively than that of HS group; the expression of Bcl-2 gene in HTau group was significantly higher than that in HS group ($P < 0.01$). The expression of Bcl-2 gene in LTau group and MTau group was not significantly different from that in HS group and significantly different from HTau group ($P < 0.05$).

Table 2 The mRNA relative expression of Bcl-2, Bax, P53 in endothelial cells

Group	Bcl-2	Bax	P53
C	1.00 ± 0.13^{ABbc}	1.00 ± 0.07^{ABab}	1.00 ± 0.12^{Aa}
HS	0.58 ± 0.16^{Aa}	1.83 ± 0.20^{Cc}	1.98 ± 0.22^{Bb}
HS+LTau	0.87 ± 0.08^{ABab}	1.23 ± 0.14^{Bb}	0.95 ± 0.06^{Aa}
HS+MTau	0.90 ± 0.02^{ABab}	0.65 ± 0.03^{Aa}	1.09 ± 0.09^{Aa}
HS+HTau	1.27 ± 0.08^{Bc}	1.14 ± 0.06^{ABb}	0.80 ± 0.11^{Aa}

Same letters denote insignificant differences between groups ($P > 0.05$). Different letters indicate significant difference between groups ($P < 0.05$). The data shown represent means ±standard deviation

As shown in Table 2, the relative expression level of Bax and P53 genes in HS group was significantly higher than that in group C ($P < 0.01$), which was 1.83 times and 1.98, respectively. The relative expression of Bax gene in each group was 1.23 times, 0.65 times and 1.14 times than that of group C respectively, and there was no significant difference ($P > 0.05$); the relative expressions of P53 gene in Tau groups were 0.95, 1.09 and 0.80 times than that of C group, respectively, and there was no significant difference from group C ($P > 0.05$).

In Tau groups, the relative expressions of Bax gene were 0.67 times, 0.36 times and 0.62 times than that of HS group, which was significantly different from HS group ($P < 0.01$). Among them, MTau group was significantly different from LTau group ($P < 0.01$), and the difference was significant compared with HTau group ($P < 0.05$). The relative expressions of P53 gene in Tau groups were 0.48, 0.55 and 0.40 times than that of HS group, respectively, which were significantly different from HS group ($P < 0.01$). There was no significant difference between Tau groups ($P > 0.05$).

The results showed that HS could down-regulate the expression of anti-apoptosis gene Bcl-2 in endothelial cells, up-regulate the expression of pro-apoptotic genes Bax and P53, and cause a large number of endothelial cell apoptosis. Adding Tau can increase the expression of Bcl-2 gene, decrease Bax and P53 gene expression levels, and play a role in inhibiting endothelial cell apoptosis. As for different apoptosis-regulating genes, the optimal concentration of Tau was also different. In contrast, MTau and HTau had a better effect on inhibiting apoptosis of aortic endothelial cells.

3.1.2 Effects of Taurine on Relative Expression of Caspase-9, Caspase-3 and Cytochrome c Genes

As shown in Table 3, compared with group C, the relative expression of Caspase-9 gene in HS group was significantly increased ($P < 0.01$), which was 2.62 times than that of group C, In Tau groups, the relative expression levels of Caspase-9 gene was 1.43, 0.77, and 1.22 times than that of group C, respectively, and there was no significant difference compared with group C ($P > 0.05$).

Table 3 The mRNA relative expression of Caspase-9, Caspase-3, Cyt-c in endothelial cells

Group	Caspase-9	Caspase-3	Cyt-c
C	1.00 ± 0.16^{ABab}	1.00 ± 0.17^{ABa}	1.00 ± 0.24^{Aa}
HS	2.62 ± 0.21^{Cc}	1.89 ± 0.29^{Bb}	2.17 ± 0.17^{Bc}
HS+LTau	1.43 ± 0.05^{Bb}	1.10 ± 0.09^{ABa}	1.50 ± 0.09^{Ab}
HS+MTau	0.77 ± 0.12^{Aa}	1.28 ± 0.15^{ABab}	0.88 ± 0.08^{Aa}
HS+HTau	1.22 ± 0.06^{ABb}	0.80 ± 0.26^{Aa}	1.48 ± 0.01^{Ab}

Same letters denote insignificant differences between groups ($P > 0.05$). Different letters indicate significant difference between groups ($P < 0.05$). The data shown represent means ±standard deviation

Compared with HS group, the relative expression levels of Caspase-9 gene in Tau groups were 0.55, 0.29 and 0.47 times, respectively, which were significantly different from those in HS group ($P < 0.01$). The difference between MTau group and LTau group was highly significant ($P < 0.01$), and the difference was significant from HTau ($P < 0.05$).

As seen in Table 3, the relative expression of Caspase-3 gene in endothelial cells in HS group was significantly higher than that of group C ($P < 0.05$), which was 1.89 times than that in group C; the relative expression levels of Caspase-3 gene in Tau groups were 1.10 times, 1.28 times and 0.80 times, respectively. There was no significant difference between Tau groups and group C ($P > 0.05$). Compared with HS group, the relative expression levels of Caspase-3 gene in Tau groups were 0.58 times, 0.68 times and 0.42 times respectively in HS group, among which HTau group showed highly significant difference compared with HS group ($P < 0.01$). LTau group and HS group had significant difference ($P < 0.05$), but there was no significant difference between MTau and HS group ($P > 0.05$).

It can be seen from Table 3 that the relative expression level of Cyt-c gene in HS group was significantly higher than that in group C ($P < 0.01$), which was 2.17 times than that of group C. The relative expressions of Cyt-c gene in Tau groups were 1.5, 0.88 and 1.48 times than that of group C. Among them, there was significant difference between LTau group and HTau group compared with group C ($P < 0.05$). There was no significant difference from group C ($P > 0.05$); the relative expression levels of the Cyt-c gene in Tau groups were 0.69, 0.41 and 0.68 times respectively than that in HS group. There was a significant difference from HS group ($P < 0.05$), among which there was a significant difference between MTau group and LTau group and HTau group ($P < 0.05$).

These results show that HS can up-regulate the expression of Caspase-9, Caspase-3 and Cyt-c in endothelial cells and induce apoptosis. The addition of Tau can down-regulate the expressions of these three pro-apoptotic genes and inhibit their activation. It may be that Tau improves HS-induced apoptosis of aortic endothelial cells by inhibiting related gene expression in mitochondrial apoptosis pathway. Among them, the optimal concentration of Tau was not the same as different apoptosis-regulating genes. In contrast, MTau was more effective than LTau and HTau.

3.2 *Effects of Taurine on Apoptosis-Related Proteins in Aortic Endothelial Cells of Heat-Stressed Broiler Chickens*

3.2.1 Effects of Taurine on the Expressions of Bcl-2, Bax and P53 Proteins

As shown in Fig. 1, the expression of Bcl-2 protein in endothelial cells was the lowest in HS group, and the expression of Bcl-2 protein in HS group was significantly lower than that in group C ($P < 0.05$). There were no significant differences in Tau groups ($P > 0.05$), indicating that HS caused a decrease in the level of Bcl-2, an anti-apoptotic protein in endothelial cells. Taurine addition can increase the expression of Bcl-2 protein, and was not significantly different from the normal level; compared with HS group, the levels of Bcl-2 protein in LTau and MTau groups were significantly increased ($P < 0.05$).

There was no significant difference in Bcl-2 protein level between LTau group and MTau group compared with HS group ($P > 0.05$), indicating that LTau and MTau had an active role in inhibiting HS-induced endothelial cells apoptosis. This effect by improving the expression of anti-apoptotic protein Bcl-2 did Tau inhibit the apoptosis of endothelial cells and improve the damage to aortic endothelial cells.

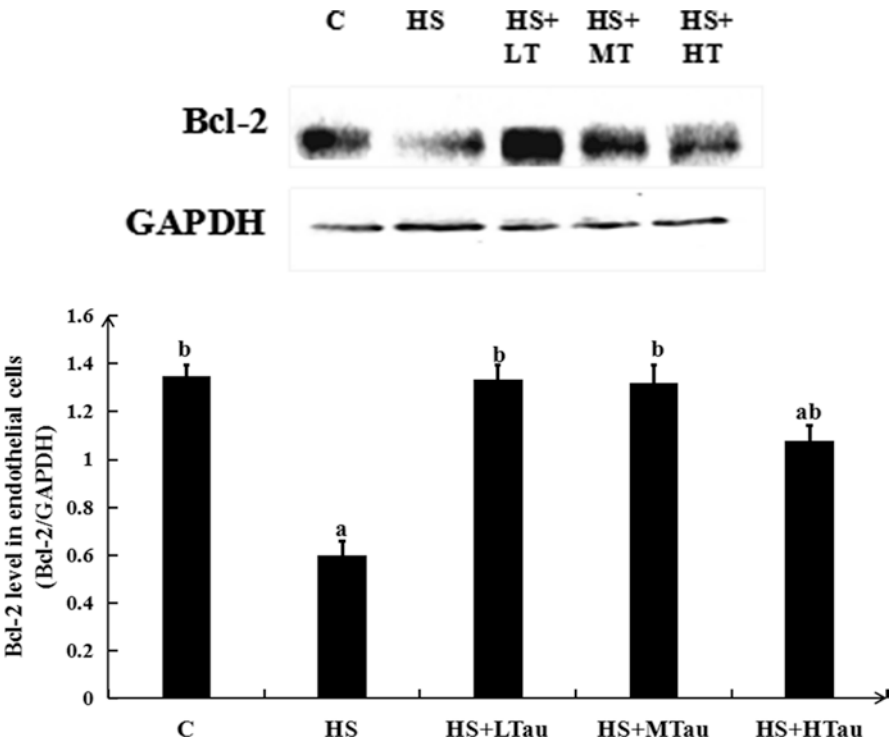

Fig. 1 The expression level of Bcl-2 protein in endothelial cells

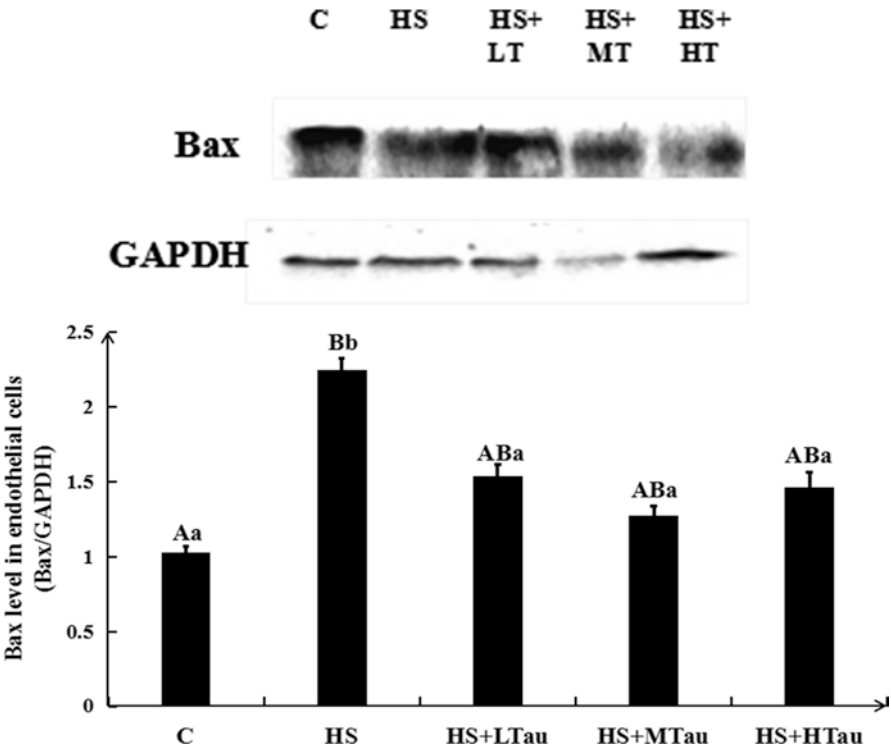

Fig. 2 The expression level of Bax protein in endothelial cells

There was no significant difference among Tau groups ($P > 0.05$), and LTau and MTau showed better effects than HTau.

As shown in Fig. 2, compared with group C, the level of Bax protein in the endothelial cells of HS group was significantly higher ($P < 0.01$), and there was no significant difference between Tau groups and group C ($P > 0.05$), indicating that hyperthermia led to activation of pro-apoptotic protein Bax in endothelial cells, high expression of Bax, and massive endothelial cell apoptosis. Although did not reduce the level of Bax protein to normal levels, the addition of tauine inhibited bax expression. Compared with HS group, Tauine groups can significantly reduce Bax protein level in endothelial cells ($P < 0.05$), indicating that the addition of Tau can inhibit the expression of Bax protein, thus inhibiting aortic endothelial cell apoptosis and easing cell damage. There was no significant difference between Tau concentrations ($P > 0.05$).

Figure 3 showed that HS significantly increased the expression of pro-apoptotic transcriptional protein P53 in aortic endothelial cells. Compared with group C, the expression of P53 protein in endothelial cells of HS group significantly increased ($P < 0.01$), and that in Tau groups was significantly higher than that in group C ($P < 0.05$). There was no significant difference between MTau and HTau compared with group C ($P > 0.05$), indicating that hyperthermia caused activation of P53, a

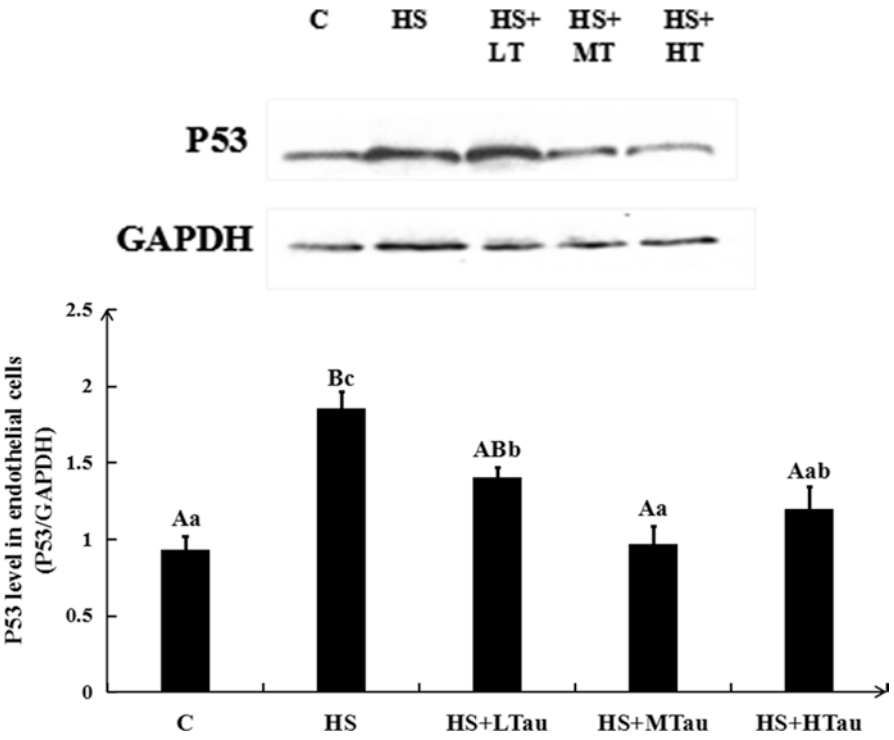

Fig. 3 The expression level of P53 protein in endothelial cells

pro-apoptotic transcription protein in endothelial cells, resulting in massive apoptosis of endothelial cells, Although it can reduce the level of P53 protein in endothelial cells, LTau did not significantly inhibit endothelial cell apoptosis. MTau and HTau inhibited the expression of P53 close to the normal level compared with HS group; LTau significantly reduced the level of P53 protein in endothelial cells ($P < 0.05$); MTau and HTau significantly reduced the P53 protein level in endothelial cells ($P < 0.01$). However, the difference between MTau group and LTau group was significant ($P < 0.05$), indicating that Tau addition can inhibit HS-induced activation of P53 protein in endothelial cells, and apoptosis of aortic endothelial cells, by inhibiting the expression of pro-apoptotic transcription protein P53. Among them, MTau group performed a stronger inhibitory effect of P53 protein.

3.2.2 Effects of Taurine on Expression of Caspase-9, Caspase-3 and Cytochrome c Proteins

As can be seen from Fig. 4, HS induced endothelial cells to significantly increase in Caspase-9 protein expression. Compared with group C, the Caspase-9 protein expression in endothelial cells of HS group was significantly higher ($P < 0.01$).

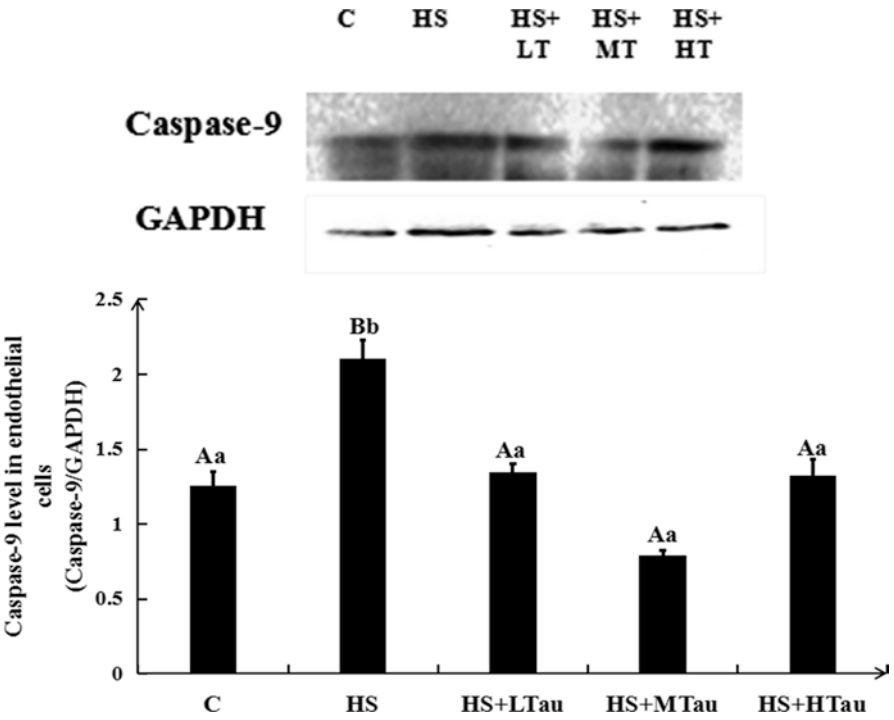

Fig. 4 The expression level of Caspase-9 protein in endothelial cells

There was no significant difference between Tau groups and group C ($P > 0.05$), indicating that HS would lead to the activation of Caspase-9 protein in the upstream of the Caspase family, and the high expression of Caspase-9 protein would in turn stimulate the mitochondria-mediated endogenous apoptotic pathway.

Caspase-9 activates downstream effector proteins and induces endothelial cell apoptosis. Tau addition can reduce Caspase-9 expression to near or below normal levels. Compared with HS group, Caspase-9 protein expression of endothelium cells in LTau, MTau, and HTau was extremely significantly decreased ($P < 0.01$). There was no significant difference between Tau concentrations ($P > 0.05$), indicating that the addition of Tau inhibited the expression of Caspase-9 protein, the Caspase family's transmission response, mitochondria-mediated endogenous apoptosis and apoptosis of aortic endothelial cells. Among them, MTau inhibited the expression of Caspase-9 significantly better than LTau group and HTau group.

As can be seen from Fig. 5, compared with the C group, Caspase-3 protein level in the endothelial cells of HS group was significantly higher ($P < 0.01$), and that in LTau group was significantly higher than that of group C ($P < 0.05$), while the levels of Caspase-3 in the MTau group and the HTau group were not significantly different from those in group C ($P > 0.05$), indicating that HS caused an increase in the level of Caspase-3 protein downstream of the Caspase family, and Caspase-3 was finally implemented and eventually performed mitochondria-mediated endogenous apop-

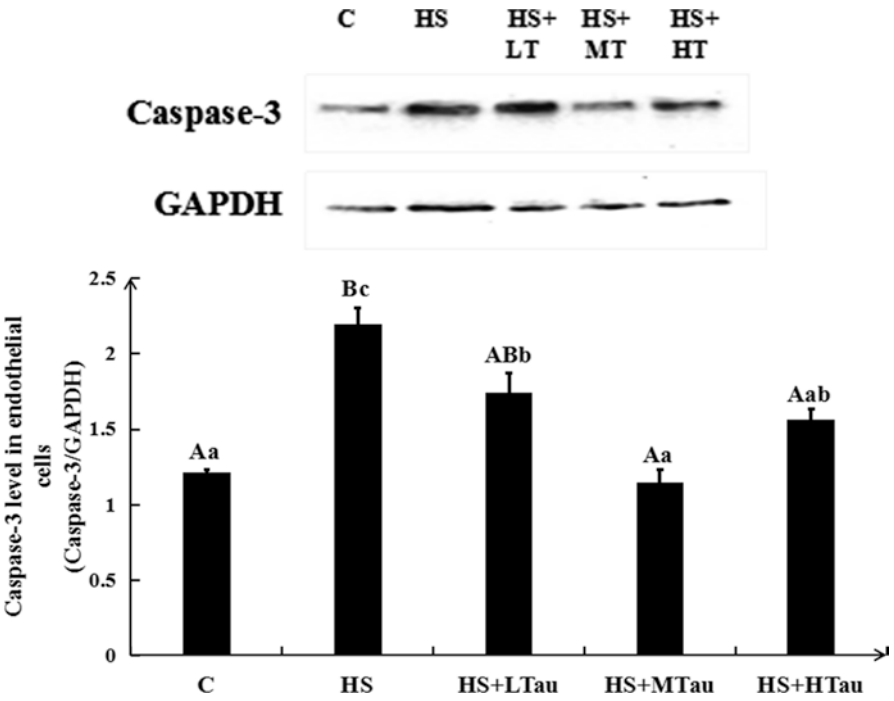

Fig. 5 The expression level of Caspase-3 protein in endothelial cells

tosis. The addition of Tau can reduce Caspase-3 levels, and the effect of LTau was not obvious, but MTau and HTau performed close to normal level; compared with HS group, LTau group can significantly reduce the level of Caspas-3 protein in endothelial cells ($P < 0.05$), the level of Caspase-3 protein was significantly decreased in MTau group and HTau group ($P < 0.01$), and the difference between MTau group and LTau group was significant ($P < 0.05$), indicating that Tau addition could inhibit the expression of Caspase-3 protein downstream of apoptosis, thereby inhibiting mitochondria-mediated apoptosis of aortic endothelial cells and improving apoptosis. In contrast, MTau group showed better effect.

As shown in Fig. 6, compared with group C, the expression of Cyt-c protein in endothelial cells in both HS group and LTau group was significantly higher ($P < 0.01$); there was significantly higher expression in HTau group ($P < 0.05$), while there was no significant difference in MTau group ($P > 0.05$).

It has been suggested that HS increased the expression of Cyt-c in mitochondria which may induce mitochondria-mediated endogenous apoptosis. Taurine addition can reduce the expression of Cyt-c protein, but only MTau group decreased to normal levels. LTau group and HTau group did not show obvious the effect.

Compared with HS group, MTau group could significantly reduce Cyt-c protein level ($P < 0.01$), and HTau group could significantly reduce Cyt-c protein ($P < 0.05$). The Cyt-c protein expression showed no significant difference between LTau group

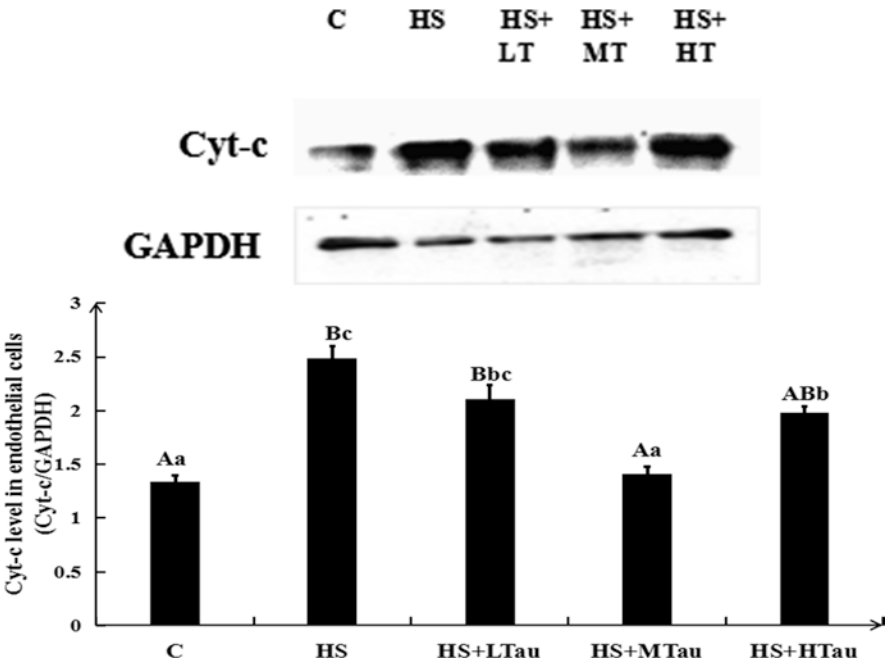

Fig. 6 The expression level of Cytochrome c protein in endothelial cells

and HS group ($P > 0.05$), highly significant difference between MTau group and LTau group ($P < 0.01$), but significant difference ($P < 0.05$) compared with HTau group, indicating that the addition of Tau can inhibit HS-induced high expression of Cyt-c.

Tau inhibited endogenous mitochondria-mediated apoptosis of aortic endothelial cells and played a role in inhibition of apoptosis of aortic endothelial cells. MTau was most effective.

4 Discussion

Studies have shown that HS induced apoptosis by activating the P53-mediated mitochondrial apoptosis pathways (Gu et al. 2014). Li (Li et al. 2014) found that HS could induce mitochondrial apoptosis in human umbilical vein endothelial cells. HS activated Caspase-9 and Caspase-3 to induce apoptosis, revealing that HS triggered mitochondria-mediated apoptotic pathways (Gu et al. 2015).

HS increased oxidative metabolism and promoted ROS formation, depleting cell clearance and damaging antioxidant enzymes (Liu et al. 2010), thereby damaging DNA and ultimately leading to cell resistance to oxidative failure and even inducing apoptosis. A large amount of ROS can also regulate MPTP, depolarizing mitochondrial membranes and release of mitochondrial pro-apoptatic factors, such as Cyt-c (Yu et al. 2015).

Cyt-c promoted activation of caspase-3 and eventually led to apoptosis. Tau played an important role in anti-cell stress and apoptosis. After suffering from intestinal ischemia-reperfusion (IR) injury, Tau-treated rats showed significantly lower jejunum and ileum apoptotic index and significantly increased Bcl-2/Bax ratio compared with IR rats, indicating that addition of Tau prevented intestinal mucosal damage and inhibited apoptosis of intestinal epithelial cells in IR rats (Sukhotnik et al. 2016).

Treatments combined with Tau can greatly reduce the area of infarct in ischemic-induced brain injury, significantly increasing the expression of Bcl-2 and reducing the expression of the pro-apoptotic protein Bax. The activity of caspase-12 and caspase-3 was reduced by inhibiting release of Cyt-c from mitochondria (Gharibani et al. 2015).

Tau had the effect on mitochondria and endoplasmic reticulum of rat cardiomyocytes in the glucose-deficient (GD) stage. It was reported that pretreatment with 80 mM exogenous Tau reduced the influence of GD in cardiomyocytes, indicating that Tau had beneficial effects on inhibiting mitochondria-dependent apoptosis and UPR-related apoptosis. Therefore, Tau may have clinical significance for acute myocardial infarction (Yang et al. 2013).

Tau can reduce the increase of Fas and Bax mRNA expressions after doxorubicin (DOX) exposure, and the formation of Caspase-3 protein in the group given DOX and Tau, compared with DOX group, indicating that the underlying mechanism of Tau as protection against DOX-induced damage to liver may be due to inhibition of apoptotic responses (Nagai et al. 2016).

Studies on the anti-apoptotic effects of Tau on exposure of human proximal tubular epithelial cells (HK-2) to oxidized low-density lipoprotein (oxLDL) found that oxLDL induced death and apoptosis of HK-2 cells, resulting in calcium accumulation, mitochondrial permeability instability, and Bax/Bcl-2 protein breakdown. Pretreatment with 100 μM Tau significantly attenuated oxLDL-induced cytotoxicity and inhibited epithelial cell apoptosis, which may be due to the antioxidant activity of Tau and its ability to regulate ERK and P53 apoptotic pathways (Chang et al. 2014). These findings are in agreement with those reported in this study.

QPCR and Western blot results showed that after HS stimulation, the expression levels of pro-apoptotic factors such as Bax, Caspase-9, Caspase-3, Cyt-c and P53 were significantly increased in HS group, where gene expression levels were 1.83 times, 2.62 times, 1.89 times, 2.17 times and 1.98 times, respectively, compared with C group ($P < 0.05$). Furthermore, there was a significant reduction in Bcl-2 expression in endothelial cells in HS group. Gene and protein expression levels were significantly decreased ($P < 0.05$), and Bcl-2 gene expression level was only 0.58 times that of the normal control group, indicating that these pro-apoptotic factors were activated by HS, inducing apoptosis of endothelial cells, which was consistent with the previous findings.

After adding Tau, the expression of Bcl-2 mRNA and protein in endothelial cells was significantly increased ($P < 0.05$). The expression of Bcl-2 in HTau group was significantly higher than that in HS group ($P < 0.01$); the level of Bcl-2 protein in LTau group and MTau group were significantly higher than that of HS group ($P < 0.05$). HTau induced an up-regulation of Bcl-2 gene expression at the gene level but not at the protein level. This may be related to the quality and quantity of protein, which was difficult to accurately reflect the storage, translocation, degradation and translation regulation of the mRNA itself. As the final product of the gene, protein also embodied gene function. Therefore, protein expression levels are more convincing. MTau had a better effects on Bcl-2.

After Tau was added, the expressions of Bax, Caspase-9, Caspase-3, Cyt-c, and P53 genes and protein were significantly lower than those of HS group ($P < 0.05$). Among them, the optimal concentration of Tau was not consistent with different pro-apoptotic factors.

MTau and HTau were more efficient than LTau in reducing the expression of Caspase-3 gene, while LTau was more effective than HTau and MTau in inhibiting the expression of P53, and Bax, Caspase-9 and Cyt-c. MTau showed better than HTau and LTau on inhibition of gene expression. Overall MTau better inhibit apoptosis of endothelial cells.

On the level of protein expression, LTau was better than HTau and MTau in inhibiting the expression of Bax protein, and MTau showed better inhibitory effects on the expression levels of Caspase-9, Caspase-3, Cyt-c, and P53. Overall, MTau had more prominent effects on apoptotic proteins.

Finally, by analyzing the overall level of genes and proteins, we found that hyperthermia stress induced high expression of pro-apoptotic genes and proteins, inhibited the expression of anti-apoptosis genes and proteins, and caused massive apoptosis of endothelial cells. We suggest that HS may induce mitochondria-mediated endogenous apoptosis in the upstream of Caspase family Caspase-9, downstream Caspase-3 and mitochondrial Cyt-c gene and protein expression. P53 factor may induce the Bax gene specifically, or translocate to the mitochondria to activate the mitochondrial pathway.

5 Conclusion

The addition of Tau can significantly reverse these changes, reducing expressions of pro-apoptotic factors such as Bax, Caspase-9, Caspase-3, Cyt-c by increasing expression of anti-apoptosis factor Bcl-2. P53 expression inhibited mitochondria-mediated endothelial cell apoptosis, thereby improving endothelial cell injury and exerting its anti-apoptotic effect. Among them, MTau has a good inhibitory effect on HS-induced apoptosis of endothelial cells.

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Effects of Taurine Supplementation on Vascular Endothelial Function at Rest and After Resistance Exercise

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Abstract High-intensity resistance exercise has been shown to increase arterial stiffness and reduce vascular endothelial function. Taurine supplementation has a favorable effect on maintaining vascular function. We had previously reported that taurine supplementation attenuated increases in resistance exercise–induced arterial stiffness. In the present study, we further investigate the effects of taurine supplementation on vascular endothelial function at rest and after resistance exercise.

Twenty-nine healthy men were recruited and randomly assigned to either the placebo supplement group (n = 14) or the taurine supplement group (n = 15) in a double-blinded manner. Subjects were required to ingest 6 g of either a placebo or the taurine supplement for 2 weeks prior to and 3 days following the exercise. Two weeks after the commencement of supplementation, the subjects were asked to perform 2 sets of 20 repetitive unilateral maximal-effort resistance exercise of the elbow flexors on a Biodex isokinetic dynamometer, with each contraction lasting 3 s, with 1 repetition performed every 9 s and 4 min rest in between sets. We evaluated the changes in brachial artery flow-mediated dilation (FMD) in the non-exercised arm as an index of vascular endothelial function. Relative and absolute FMDs were measured prior to supplementation, before exercise, and 24, 48, and 96 h after exercise.

Two weeks of taurine supplementation significantly increased both relative and absolute FMDs. Baseline diameter significantly increased at 96 h following the exercise in both groups. However, there was no change in the peak diameter. Consequently, both relative and absolute FMDs were significantly reduced at 96 h after the exercise in both groups. Taurine supplementation does not affect resistance exercise–induced reduction in FMD.

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Two weeks of taurine supplementation (6 g/day) significantly increased vascular endothelial function at rest; however, taurine supplementation did not improve resistance exercise–induced reduction in FMD.

Keywords FMD · Resistance exercise · Vascular endothelial function

1 Introduction

Central large arteries play a buffering role in lowering the blood flow and pressure; that is, increasing arterial stiffness can reduce the buffering action and lead to increasing blood pressure. Increased arterial stiffness has been identified as an independent risk factor for future cardiovascular disease (Najjar et al. 2005).

Increased arterial stiffness may be associated with reduced vascular endothelial function. Dysfunction of the vascular endothelium is one of the earliest events in cardiovascular disease (Ross 1993). Regular aerobic exercise has been known to reduce arterial stiffness (Tanaka et al. 2000) and promote improvement in vascular endothelial function (Pugh et al. 2014; Birk et al. 2012). In contrast, although resistance exercise training also has been shown to reduce arterial stiffness, it has been reported in previous studies that acute high-intensity resistance exercise could reduce vascular endothelial function assessed by brachial artery flow-mediated dilation (FMD) response (Choi et al. 2016; Stacy et al. 2013).

Nutritional strategies might minimize these detrimental effects of resistance exercise. Taurine (2-aminoethanesulfonic acid) is the most abundant semi-essential amino acid. Taurine can be synthesized in several mammalian tissues, including the skeletal muscle, but it is mainly acquired from diet, such as meat and seafood. Previous studies have shown a protective effect of taurine against endothelial dysfunction. Taurine treatment reverses diabetes-induced vascular endothelial dysfunction in rodents (Wang et al. 2008; Ikubo et al. 2011) and humans (Moloney et al. 2010). Further, we had previously reported that taurine supplementation attenuates resistance exercise–induced arterial stiffness by reducing circulating oxidative markers (Ra et al. 2016). In addition, taurine supplementation could attenuate muscle damage symptoms following high-intensity resistance exercise (Ra et al. 2015; da Silva et al. 2014; Silva et al. 2011; Zhang et al. 2004).

From previous evidence, we hypothesized that taurine supplementation may limit resistance exercise–induced reduction in vascular endothelial function. In the present study, we investigated the possible effects of taurine on vascular endothelial function at rest and after performing resistance exercise.

2 Methods

2.1 Participants and Supplement Protocol

The present study was a double-blind, randomized, placebo-controlled trial that was conducted in accordance with the principles of the Declaration of Helsinki. A total of 29 healthy young men (age, 20–33 years: BMI, 21.7 ± 0.3 m/kg^2) participated in the present study. None of the participants had any regular physical activity for at least 1 year prior to commencing the study. All the participants were normotensive and non-obese, and none of them was on any medication nor were they smokers. None of the participants was taking taurine or any other nutritional supplement prior to enrollment. The present study was approved by the Ethical Committee of the University of Tsukuba. Written informed consent was obtained from all the subjects prior to their participation in the study.

Participants in the taurine group were given 2 g of taurine powder and those in the placebo group acted as the controls and were given the same amount of lactose powder The participants had to orally ingest each supplement after every meal (thrice a day) for a total of 18 days (14 days supplementation prior to exercise day and 4 days following the initiation of exercise).

2.2 Experimental Procedures

All the participants assembled at the laboratory in the morning after fasting overnight. They had been asked to refrain from caffeine and alcohol consumption for at least 12 h, and intense exercise for at least 48 h prior to entering the study. The participants were made to rest in the supine position for 20 min in a quiet place. Brachial vascular endothelial function was assessed for each of the participants while they were in the supine position. During the study, we measured brachial vascular endothelial function on five different days: prior to starting supplementation (Pre), immediately before exercising (BEx, day 15), and 24, 48, and 96 h after exercise (day 16, 17, and 19).

2.3 Resistance Exercise

Following the 2 weeks of supplementation (on day 15), participants performed 40 maximal eccentric unilateral contractions of the elbow flexor, as previously reported (Ra et al. 2015, 2016). This resistance exercise was performed with the non-dominant arm. Each contraction was held for 3 s and repeated every 9 s, and consisted of maximal contraction through a range of motion from 90 to 180° of elbow flexion.

2.4 Assessment of Vascular Endothelial Function

Vascular endothelial function was assessed by endothelium-dependent FMD. Brachial artery FMD was assessed in the non-exercised arm using a novel stereotactic probe-holding device equipped with an edge-tracking system for 2D imaging and pulsed Doppler flow velocimeter for automatic measurement (UNEXEF; Unex Co. Ltd., Nagoya, Japan) as previously described (Choi et al. 2016). In the present study, both relative and absolute FMDs were calculated as percentage and absolute changes in the arterial diameter divided by the baseline diameter at maximal dilation after the cuff deflation (after 5 min inflation). Namely, relative FMD (%) = ([peak diameter – baseline mean diameter]/baseline mean diameter) × 100; absolute FMD (mm) = peak diameter – baseline mean diameter.

2.5 Statistical Analysis

Values are expressed as mean ± SE. Changes in measurements were analyzed for effects of time and supplement in a two-way ANOVA, and post hoc analysis was performed using the Bonferroni method. All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA) with statistical significance set at $p < 0.05$.

3 Results

3.1 Effects of the 2-Week Taurine Supplementation on Vascular Endothelial Function at Rest

Prior to starting supplementation (Pre), there were no statistically significant differences between the placebo and the taurine group in both relative and absolute FMDs. Both relative and absolute FMD significantly increased following the 2-week taurine supplementation (Fig. 1).

3.2 Effects of Taurine Supplementation on Vascular Endothelial Function After Performing Resistance Exercise

There were no significant interactions in any variables illustrated in Fig. 2. Although mean peak arterial diameters in both groups were not changed, mean baseline diameters in both groups significantly increased on 96 h after exercise compared with

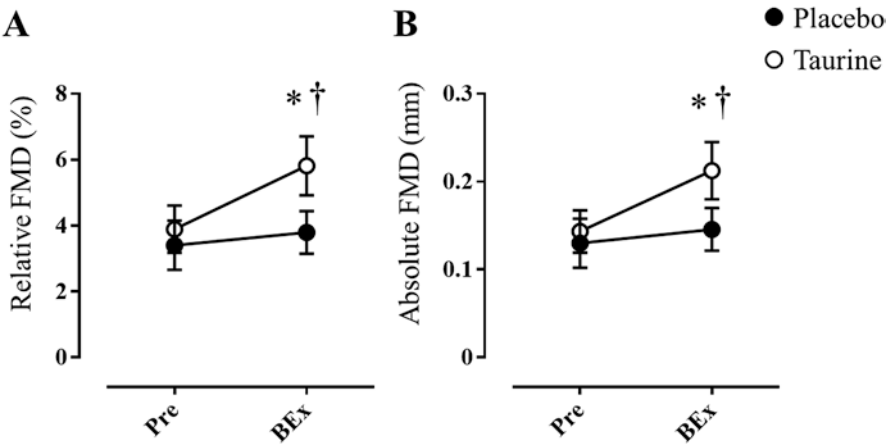

Fig. 1 Changes in (**a**) relative and (**b**) absolute FMDs by 2 weeks placebo or taurine supplementation. Two-way ANOVA revealed significant interaction (time∗supplement) in both relative and absolute FMD change. ∗$p < 0.05$ shows the significant difference between two times point in taurine group. †$p < 0.05$ shows the significant between-group difference on BEx (day 15)

BEx (Fig. 2a). In addition, both relative and absolute FMD values gradually decreased and significant differences were found on 96 h compared with BEx, respectively (Fig. 2c, d).

4 Discussion

It has been reported that taurine supplementation significantly improves both endothelium-dependent and -independent vascular function in pre-hypertensive (Sun et al. 2016) and type I diabetes patients (Moloney et al. 2010). In the present study, we confirmed that 2 weeks taurine supplementation (6 g/day) significantly increased brachial artery FMD value in healthy young men; it was found to be mainly dependent on increasing peak arterial diameter. Taurine supplementation may contribute to nitric oxide (NO) production and/or NO bioavailability due to increasing share stress. However, this relationship is not clear as there was no analysis performed of either NO concentration in the blood stream or its availability. Altogether, taurine supplementation can be a good nutritional strategy for maintaining vascular endothelial health even in healthy young populations.

We have already reported that the exercise-induced decrease in vascular endothelial function is associated with increasing central arterial stiffness (Choi et al. 2016). In addition, we previously confirmed that taurine supplementation in young men could attenuate exercise-induced arterial stiffening (Ra et al. 2016). However, in the present study, we did not observe the protective effect of taurine supplementation on reduction in exercise-induced vascular endothelial function. In the present study, reductions in both relative and absolute FMDs were mostly dependent on gradually

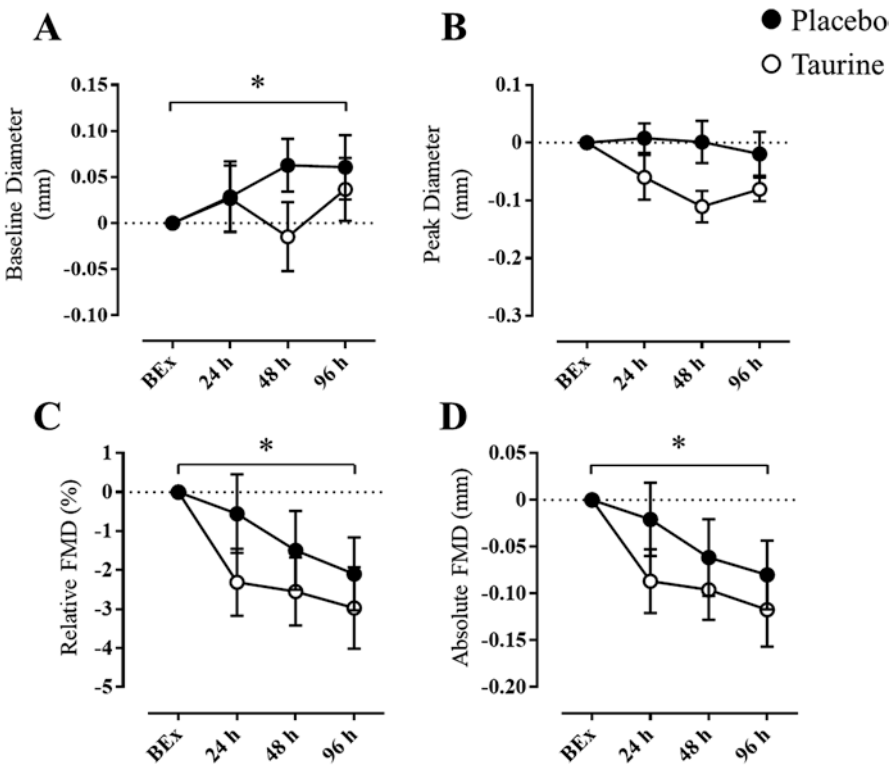

Fig. 2 Changes in (**a**) baseline diameter, (**b**) peak diameter, (**c**) relative FMD, and (**d**) absolute FMD after resistance exercise expressed as changes from BEx. There were no significant interactions in any variables. Significant time effects were found in (**a–d**) $*p < 0.05$ shows significant difference at 96 h compared with BEx ($p < 0.05$) in both group

increasing baseline arterial diameter (Fig. 2a). Skeletal muscle tissue damage due to high-intensity eccentric exercise has been shown to increase oxygen saturation (Ahmadi et al. 2008) and blood volume (Kano et al. 2005) in the muscle. We would like to focus attention on the effects of taurine supplementation on vascular endothelial function between resting state and after high-intensity resistance exercise.

The reduction in endothelial-dependent FMD value which we observed might indicate disruption of endothelial cells or the vascular smooth muscle cells, and it might lead to decreasing blood flow to the damaged muscle during vascular dilation. Severe eccentric types of resistance exercise can impair local microvascular function (Kano et al. 2005) and vasodilator response of the skeletal muscle (Heap et al. 2006). A reduction in vascular endothelial function after eccentric exercise associated with reduced share stress to endothelial cells (Stacy et al. 2013), increased central arterial stiffness (Choi et al. 2016), and skeletal muscle tissue damage (Barnes et al. 2010; Stacy et al. 2013). Taurine supplementation failed to prevent exercise-induced muscle damage symptoms (Ra et al. 2015, 2016). Collectively, the present results suggest that taurine supplementation improves vascular endothelial

function at rest but not after performing resistance exercise. Future research examining the precise mechanisms associated with taurine supplementation and the vascular endothelial health is warranted to elucidate our findings.

5 Conclusion

In summary, 2 weeks taurine supplementation (6 g/day) significantly increased vascular endothelial function at rest; however, taurine supplementation did not improve resistance exercise–induced reduction in endothelium-dependent FMD.

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Perinatal Taurine Supplementation Prevents the Adverse Effects of Maternal Dyslipidemia on Growth and Cardiovascular Control in Adult Rat Offspring

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Abstract Maternal dyslipidemia induces metabolic and cardiovascular disorders in adult offspring. This study tests the hypothesis that perinatal taurine supplementation prevents the adverse effects of maternal dyslipidemia on growth and cardiovascular function in adult rat offspring. Female Wistar rats were fed normal rat chow and water with (Dyslipidemia) or without dyslipidemia induction (Control) by intraperitoneal Triton WR-1339 injection, three times a week for 4 weeks. The female Control and Dyslipidemia rats were supplemented with (Control+T, Dyslipidemia+T) or without 3% taurine in water from conception to weaning. After weaning, male and female offspring were fed normal rat chow and water throughout the experiment. At 16 weeks of age, body weights significantly increased in male but not female Dyslipidemia compared to other groups, while visceral fat content significantly increased in both male and female Dyslipidemia groups. Further, both sexes displayed similar high fasting blood sugar and normal plasma leptin levels among the groups. While plasma total cholesterol and triglycerides significantly increased only in female Dyslipidemia, low-density lipoprotein cholesterol increased in both male and female Dyslipidemia groups. Mean arterial pressures and heart rates significantly increased, while baroreflex sensitivity decreased in male and female Dyslipidemia compared to all other groups. High-density lipoprotein cholesterol did not significantly different among male or female groups. These changes of the male

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and female Dyslipidemia group were ameliorated by perinatal taurine supplementation. The present study indicates that perinatal taurine supplementation prevents the adverse effects of maternal dyslipidemia on growth and cardiovascular function in both male and female, adult offspring.

Keywords Baroreflex · Hypercholesterolemia · Hypertension · Maternal dyslipidemia · Perinatal taurine

Abbreviations

BSHR	baroreflex sensitivity control of heart rate
Control+T	Control plus taurine supplementation
Dyslipidemia+T	Dyslipidemia plus taurine supplementation
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein cholesterol
PHE	phenylephrine
SNP	sodium nitroprusside

1 Introduction

Perinatal environments affect adult cardiovascular and metabolic functions. Among them, maternal dyslipidemia is reported to cause metabolic and cardiovascular dysfunction in adult offspring (Herrera and Ortega-Senovilla 2014; Szostak-Wegierek 2014). Higher triglyceride is linked to elevated cholesterol, which upon entrance into the intima leads to low-grade inflammation, foam cell formation, atherosclerotic plaques, and atherosclerosis, respectively (Ganda et al. 2018). Triglycerides is a main source of energy for the mother during pregnancy and do not freely cross maternal-placental membrane to fetus. However, free fatty acid, glycerol, ketone, glucose, and amino acid of the mother can be transported to the fetus for energy sources and building blocks. These micronutrients are very important for fetal growth and development (Ghio et al. 2011; Herrera and Ortega-Senovilla 2018). However, the prevalence of hypertriglyceridemia is increasing among young women and pregnancies (Mendelson et al. 2016). Many lines of evidence report that maternal dyslipidemia is a high risk of dyslipidemia, diabetes mellitus, and hypertension in the adult offspring (Herrera and Ortega-Senovilla 2014). This perinatal effect is rather epigenetic than genetic modulation, as supported by abnormal expression of lipid-regulatory genes in the intestine and liver of the affected offspring (Juritsch et al. 2017). Further, this epigenetic effect can transfer to the next generation (Herrera and Ortega-Senovilla 2018). In addition, statins-treated dyslipidemia pregnancies prevent the effects of maternal dyslipidemia on metabolic syndrome in the offspring (Elahi et al. 2008).

Cholesterol is important for fetal development but insufficiently synthesized in human and mice fetuses. It can be transported from mothers to the fetuses during pregnancy via the endothelial cells of the fetus-placenta to fetal circulation (Ghio et al. 2011; Herrera and Ortega-Senovilla 2014). Thus, maternal hypercholesterolemia can cause dyslipidemia and subsequently injuries in the fetus. The fetal hypercholesterolemia or dyslipidemia has long-term effects on adult metabolic and cardiovascular function in a sex-dependent manner (Predazzi et al. 2015). However, the underlying mechanism of fetal dyslipidemia on adult function and disease is not conclusive.

Lipid profiles depend on sex, race, age, and lifestyle. For the global average data, all lipid components, including total cholesterol, triglyceride, LDL-C, and HDL-C increase with advancing age in both men and women (Pan and Chiang 1995). Although total cholesterol levels with age are almost similar between men and women, men display higher triglyceride and LDL-C but lower HDL-C levels than premenopausal women do. Further, the menopausal women has higher triglyceride, LDL-C, and HDL-C levels than the men of same age do. The sex difference in incidence of cardiovascular disease is paralleled to the LDL-C and triglyceride levels. The adult lipid profiles are also dependent on perinatal environment as mentioned above. The effect of maternal dyslipidemia on adult metabolic and cardiovascular functions is generally, but not all, more severe in males than females (Pan and Chiang 1995; Predazzi et al. 2015). However, in male and female (6–13 years old) children, maternal hypercholesterolemia similarly increases hypercholesterolemia in both sexes independent of birthweights (Christensen et al. 2016). This may be underlain, at least in part, by the inflammatory imbalance between transforming growth factor-alpha and interleukin-10 activities (Narverud et al. 2011). Later in life, the maternal dyslipidemia amplifies the lipid profile differences between males and females (Predazzi et al. 2015).

Taurine is a small sulfur containing amino acid that plays many physiological roles from prenatal to adult life (Roysommuti and Wyss 2014). Like other micronutrients, fetuses and newborns need taurine supply from their mothers via placenta and milk. Taurine supplementation from food is recommended during pregnancy and lactation due to insufficient maternal biosynthesis. Taurine possesses many activities including anti-dyslipidemia (Murakami 2017). Taurine supplementation decreases body weight and fat mass in obese animals and humans. Epidemiologic studies also report a high incidence of metabolic syndrome and cardiovascular disease in people consuming low taurine diets (Yamori et al. 2010). However, perinatal taurine excess and depletion can induce arterial pressure and renal dysregulation, even at different degrees (Roysommuti and Wyss 2014). Previously, we have reported that perinatal taurine supplementation prevents maternal diabetic effects on cardiovascular disorders in adult offspring (Thaeomor et al. 2017). The present study tests the hypothesis that perinatal taurine supplementation prevents the effect of maternal dyslipidemia on dyslipidemia and hypertension in adult rat offspring.

2 Methods

2.1 Animal Preparation

Male and female Wistar rats were bred at the animal unit of Suranaree University of Technology and maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (06.00–18.00 h). All rats were fed normal rat chow and accessed to water ad libitum. Female rats were induced dyslipidemia by intraperitoneal injection of freshly prepared Triton WR-1339 (400 mg/kg of body weight), three times a week for 4 weeks (Dyslipidemia group) (Zarzecki et al. 2014). Forty-eight hours later, hypercholesterolemia (≥300 mg/dl) and hypertriglyceridemia (>130 mg/dl) were confirmed from the lateral tail venous blood samples. Then, these females were subjected to a mating procedure with normal males. The control group was similarly treated without dyslipidemia induction (Control). After conception, the pregnant rats were caged individually and supplemented with 3% taurine in water (Control plus taurine supplementation, Control+T; Dyslipidemia plus taurine supplementation, Dyslipidemia+T) or water alone until weaning (Dyslipidemia and Control groups). After weaning, both male and female offspring were fed the normal rat chow and water throughout the experiment (n = 7 each group).

All experimental procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health.

2.2 Experimental Protocol

At 16 weeks of age, fasting blood sugar and plasma leptin levels were determined from blood samples drawn from the lateral tail vein. Two days later, all rats were anesthetized with Nembutal (50 mg/kg of body weight, intraperitoneal) and their femoral arteries and veins were inserted with polyethylene tubes (PE-50 fused to PE-10). Each arterial cannula was connected to a pressure transducers and the PowerLab (ADIntruments, Bella Vista, New South Wales, Australia) for continuous recording of arterial pressure and heart rate, while the venous cannula was connected to an infusion pump for drug injection. After baseline data recording, each rat was subjected to a measurement of baroreflex sensitivity control of heart rate by an intravenous infusion of phenylephrine (to increase arterial pressure) and sodium nitroprusside (to decrease arterial pressure). Then, a blood sample was collected from the arterial catheter (about 2 ml) for measuring plasma lipid profiles. Finally, all rats were sacrificed by a high dose of anesthesia followed by collections of heart weight, kidney weight, and visceral adipose tissue.

2.3 Measurement of Visceral Adipocyte Size

Visceral adipose tissues were fixed in paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The stained sections were obtained with a ×40 objective lens, recorded on a digital camera (DS-Fi2 microscope camera, Nikon, Japan), displayed on a high resolution monitor (Eclipse Ci-L microscope, Nikon, Japan). An averaged diameter of adipose tissue was analyzed and measured by Image Pro-Plus 6.0 (10 adipocytes per stained section) (Silverio et al. 2017).

2.4 Data Analyses

Blood glucose was immediately measured by a glucometer (Accu-CHECK, Roche), plasma leptin by Rat Leptin ELISA kit # EZRL-83BK (Merck Millipore, Merck), and plasma lipid profiles by the Suranaree Hospital Chemical Analysis Unit (Suranaree University of Technology, Nakhon Ratchasima, Thailand). Mean arterial pressure and heart rate were analyzed by the PowerLap software, while BSHR was calculated from the ratio of a change in heart rate to a change in mean arterial pressure following phenylephrine (BSHR-PHE) or sodium nitroprusside infusion (BSHR-SNP).

2.5 Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons among the four male and female groups were performed by using one-way ANOVA followed by the *post hoc* Duncan's Multiple Range test (StatMost32 version 3.6, Dataxiom, CA, USA). The significant criterion is $p < 0.05$.

3 Results

3.1 Weights and Blood Chemistry

At 16 weeks of age, body weights significantly increased in male Dyslipidemia compared to other male groups while significantly decreased in female Dyslipidemia+T compared to other female groups. The Dyslipidemia significantly increased visceral adipocyte sizes compared to the other three male or female groups, while perinatal taurine supplementation significantly decreased these high fat cell sizes in both sexes (Table 1).

Table 1 Body weights and visceral adipocyte sizes in adult male and female rats

	Body weight (g)		Visceral adipocyte size (μm)	
Treatment	Male	Female	Male	Female
Control	359 ± 5	251 ± 3	410 ± 17	353 ± 18
Control+T	367 ± 7	249 ± 4	418 ± 17	317 ± 16
Dyslipidemia	507 ± 4*	250 ± 3	486 ± 13*	412 ± 22*
Dyslipidemia+T	408 ± 4	235 ± 2*	244 ± 9*	351 ± 12

Data are means ± SEM (*P < 0.05 compared to all other male or female groups
Control+T Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

Table 2 Fasting blood glucose and plasma leptin levels in adult male and female rats

	Fasting blood glucose (mg/dl)		Fasting plasma leptin (ng/dl)	
Treatment	Male	Female	Male	Female
Control	84.0 ± 1.9	84.9 ± 3.0	6.9 ± 0.5	7.0 ± 0.3
Control+T	85.3 ± 3.4	85.0 ± 2.3	6.6 ± 0.5	6.9 ± 0.5
Dyslipidemia	96.4 ± 2.2*	98.3 ± 4.8*	6.4 ± 0.5	6.4 ± 0.5
Dyslipidemia+T	82.2 ± 1.6	83.6 ± 3.8	6.7 ± 0.6	6.5 ± 0.5

Data are means ± SEM (*P < 0.05 compared to all other male or female groups
Control+T Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

Both male and female Dyslipidemia groups displayed significant increases in fasting blood glucose but not plasma leptin levels (Table 2). Further, these effects of maternal dyslipidemia were abolished by perinatal taurine supplementation in both sexes (male and female Dyslipidemia+T groups). Plasma cholesterol and triglyceride were significantly increased in female Dyslipidemia and were normalized by perinatal taurine supplementation, while they were not significantly different among the male groups. LDL significantly increased in both male and female Dyslipidemia, and this change was normalized by perinatal taurine supplementation (Table 3). In contrast to other lipid components, HDL did not significantly different among male or female groups, irrespective of taurine supplementation.

3.2 Cardiovascular Parameters

Compared to Control, mean arterial pressures (Fig. 1) and heart rates (Fig. 2) significantly increased in both male and female Dyslipidemia groups, and these changes were normalized by perinatal taurine supplementation (Dyslipidemia+T group). In addition, the perinatal taurine supplementation did not affects the mean arterial pressure and heart rate of the Control+T compared to Control groups.

In contrast to mean arterial pressures, the BSHR-PHE (Fig. 3) and BSHR-SNP (Fig. 4) significantly depressed in both male and female Dyslipidemia compared to

Table 3 Plasma lipid profiles in adult male and female rats

	Total cholesterol (mg/dl)		Triglyceride (mg/dl)		HDL (mg/dl)		LDL (mg/dl)	
Treatment	Male	Female	Male	Female	Male	Female	Male	Female
Control	80.6 ± 5.1	82.7 ± 3.9	122.1 ± 5.8	125.1 ± 4.3	62.9 ± 1.2	70.1 ± 3.1	102.4 ± 6.3	100.7 ± 4.4
Control+T	80.7 ± 2.7	77.3 ± 4.3	123.0 ± 4.4	115.6 ± 3.9	60.0 ± 1.7	68.3 ± 3.0	100.1 ± 6.1	97.7 ± 3.9
Dyslipidemia	95.0 ± 2.5	101.3 ± 3.2*	131.4 ± 5.7	143.7 ± 4.1*	60.7 ± 1.5	60.9 ± 2.5	137.0 ± 7.0*	133.1 ± 4.2*
Dyslipidemia+T	81.1 ± 2.2	83.3 ± 4.2	121.0 ± 5.3	109.9 ± 2.9	62.0 ± 1.7	68.9 ± 1.0	106.7 ± 4.9	103.0 ± 4.7

Data are means ± SEM (*$P < 0.05$ compared to all other male or female groups

Control+T Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein; n = 7 each group)

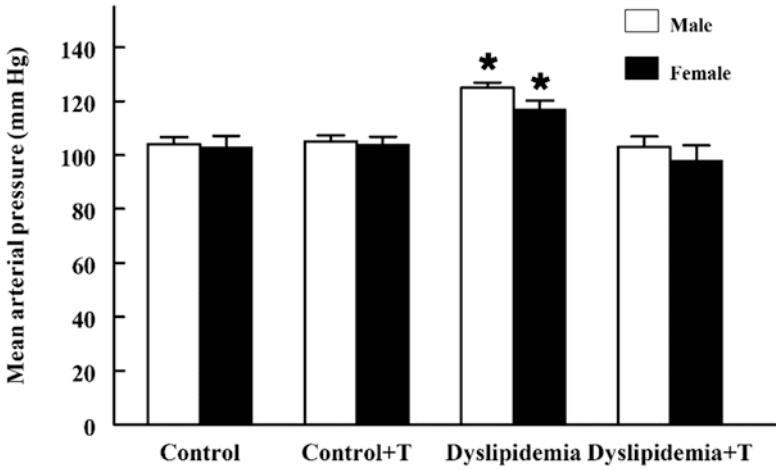

Fig. 1 Mean arterial pressures in adult male (open bars) and female rats (dark bars) ($^{*}P < 0.05$ compared to all other male or female groups; *Control+T* Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

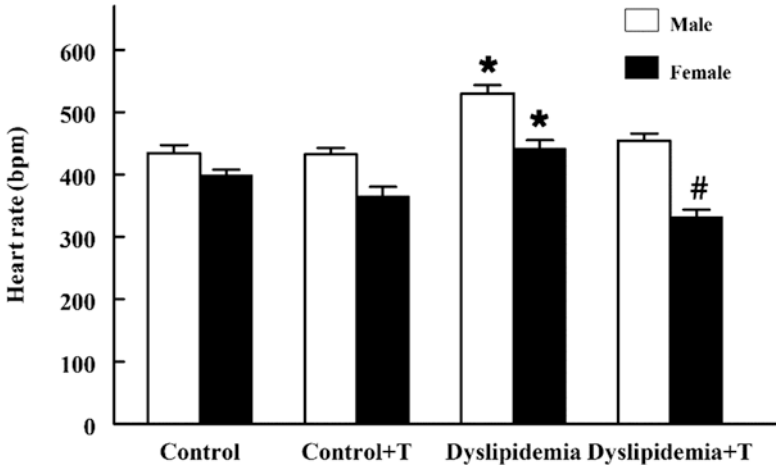

Fig. 2 Heart rates in adult male (open bars) and female rats (dark bars) ($^{*,\#}P < 0.05$ compared to all other male or female groups and Control, respectively; *Control+T* Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

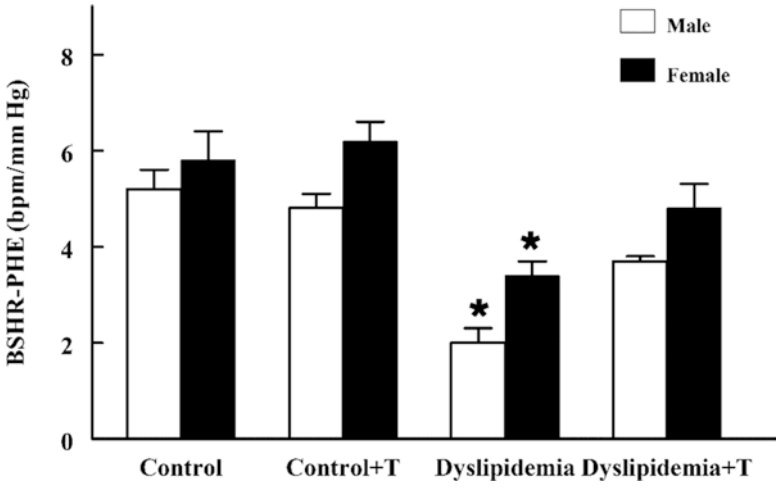

Fig. 3 Baroreflex sensitivity control of heart rate measured by phenylephrine infusion (BSHR-PHE) in adult male (open bars) and female rats (dark bars) ($^{*}P < 0.05$ compared to all other male or female groups; *Control+T* Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

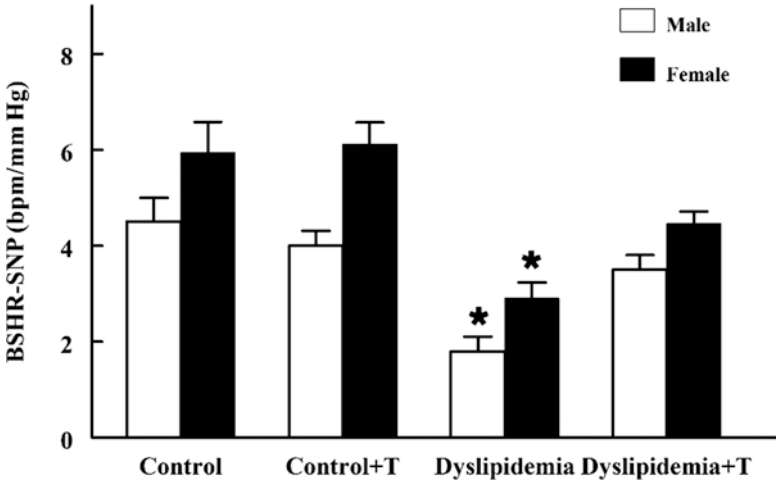

Fig. 4 Baroreflex sensitivity control of heart rate measured by sodium nitroprusside infusion (BSHR-SNP) in adult male (open bars) and female rats (dark bars) ($^{*}P < 0.05$ compared to all other male or female groups; *Control+T* Control plus taurine supplementation, *Dyslipidemia+T* Dyslipidemia plus taurine supplementation; n = 7 each group)

their Control groups. Further, perinatal taurine supplementation abolished these adverse effects of maternal dyslipidemia without any effect on the baroreflex sensitivity in the Control+T compared to Control groups.

4 Discussion

Maternal dyslipidemia affects fetal growth and development that program metabolic and cardiovascular functions in adult offspring (Christensen et al. 2016; Herrera and Ortega-Senovilla 2018; Szostak-Wegierek 2014). Thus, maternal lipids control can minimize the adverse effects of maternal dyslipidemia on dyslipidemia, diabetes mellitus, and hypertension in adult offspring (Elahi et al. 2008; Mendelson et al. 2016). Taurine possesses anti-dyslipidemia and anti-obesity, particularly in animal models (Murakami 2017). The present study indicates that in both male and female offspring, maternal dyslipidemia caused dyslipidemia, mild hyperglycemia, blunted baroreflex function, tachycardia, and hypertension; and these adverse effects were eliminated by perinatal taurine supplementation. Moreover, the present data suggest that the dyslipidemia is more severe in adult female than male offspring.

Several lines of evidence report that maternal dyslipidemia either by genetic obesity, diabetes mellitus, or high fat intake is related to excessive fetal growth and overweight newborns (Herrera and Ortega-Senovilla 2018). Low plasma angiopoietin-like protein 4 and high leptin levels secreted from adipose tissue and placenta during pregnancy increases free fatty acid transport from maternal circulation to fetuses (Herrera and Ortega-Senovilla 2014). The increased maternal free fatty acid and glucose availability facilitates fetal lipid accumulation and growth. This early life obesity can program adult metabolic function (Herrera and Ortega-Senovilla 2018; Szostak-Wegierek 2014). Compared between male and female children from maternal dyslipidemia, their fat contents and other risk factors of cardiovascular disease are similar (Christensen et al. 2016); thus, their lipid profile differences at later life may be dependent on sex steroids or other unknown factors (Predazzi et al. 2015). The present study indicate that compared to Control, maternal dyslipidemia increased body weights in male but not female offspring, while dyslipidemia was more severe in female than male offspring. Further, the maternal dyslipidemia increased visceral fat mass more in male than female offspring. These data support the sex-specific effects of maternal dyslipidemia in adult offspring (Predazzi et al. 2015). In addition, similar high fasting blood sugar and normal plasma leptin levels among sexes might exclude the difference role of insulin-glucose regulation among male and female Dyslipidemia groups. The possible roles of testosterone in males and estrogen in females need further studies.

Obesity and dyslipidemia is reported to increase sympathetic and decrease parasympathetic nerve activity in both human and animal models (Guarino et al. 2017). Hypertension and blunted baroreflex function are well correlated to body fat mass and hypercholesterolemia (Guarino et al. 2017; Serhiyenko and Serhiyenko 2018).

It is likely that hypertension and tachycardia in both male and female Dyslipidemia groups may result from dyslipidemia-induced sympathetic overactivity. Thus, perinatal taurine supplementation could normalized these adverse effects of maternal dyslipidemia. Statin treatment of maternal dyslipidemia also prevents dyslipidemia and hypertension in adult offspring (Elahi et al. 2008). It is possible that the underlain mechanism of taurine and statin treatment may be related to the normalization of maternal and fetal fat metabolism. In addition, renin-angiotensin system overactivity is reported to underlie hypertension and blunted baroreflex function in metabolic syndrome (Borghi et al. 2017; Petrie et al. 2018). Angiotensin II can stimulate sympathetic activity and blunt baroreflex function by acting at the brain areas related to arterial pressure control (Seravalle and Grassi 2016). Thus, the renin-angiotensin system activity has to be further investigated in the present model.

The anti-dyslipidemia of taurine may include modulation of lipid metabolism, anorexic effect, anti-oxidative stress, and anti-inflammation (Murakami 2017). However, taurine possesses several other physiological functions from prenatal to adult life, including growth and development (Roysommuti and Wyss 2014). Perinatal taurine supplementation promotes prenatal and postnatal growth and development and protects against adult diseases, including hypertension. In spontaneously hypertensive rats, sympathetic nerve and renin-angiotensin system overactivity contribute importantly to hypertensive development (Wyss et al. 1994, 1995); and taurine supplementation in perinatal life attenuates hypertension in these rats (Racasan et al. 2004). In contrast, perinatal taurine depletion induces several adverse effects in adults (Roysommuti and Wyss 2014). Taurine content is reported to decrease in patients and animal models of dyslipidemia (Murakami 2017); thus, the advantage of perinatal taurine supplementation against the adverse effects of maternal dyslipidemia on adult metabolic and cardiovascular disorders may include body taurine replenishment and other taurine activities not directly related to anti-dyslipidemia or anti-obesity.

5 Conclusion

Maternal dyslipidemia has long-term effects on growth and cardiovascular function in adult offspring. The present study demonstrates that maternal dyslipidemia induces dyslipidemia, blunted baroreflex function, and hypertension in adult male and female offspring, and these adverse effects are normalized by perinatal taurine supplementation. Thus, the taurine supplementation can be an alternative treatment of dyslipidemia and hypertension, particularly during pregnancy.

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Pleiotropic Effects of Taurine on Nematode Model for Down Syndrome

Hyunsook Chang and Dong Hee Lee

Abstract Taurine is traditionally used to treat Down Syndrome (DS); however, the actual foundation for this treatment is not well understood. DS patients suffer from disturbance of the proteostasis network (PN) due to aberrant calcium signaling, which eventually causes endoplasmic reticulum stress (ERS). Taurine has been suggested to play a role in modulating calcium homeostasis and ERS. This study examined whether taurine affects DS symptoms using *C. elegans* – a DS model in which calcineurins, Ca^{2+}/calmodulin-dependent protein phosphatase is mutated to null. The DS nematode model has short body length, slow growth, fertility defects, serotonin-resistant egg-laying defects, and faulty thermal sensing. This study focused on whether taurine may ameliorate the severity of DS at the whole-body level, including reduction in ERS. When treated with taurine, DS nematodes appeared to have lower levels of ERS and phenotypes closer to the wild type. DS nematodes also showed improved egg laying efficiency and thermal sensing index comparable to the wild type. Our findings offer a new perspective on the effectiveness of taurine in treating DS and designing therapeutic strategies to lower ERS and restore disrupted PN.

Keywords Calcineurin · C. elegans · Down syndrome · DSCR-1 · Taurine

Abbreviation

DS	Down syndrome
DSCR-1	Down syndrome critical region 1
RCN	Regulator of calcineurin
Can a(b)	Calcineurin A(B)
NGM	Nematode growth medium

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1 Introduction

Down syndrome (DS) is one of the most common chromosomal aberrations, causing mental retardation and innate heart abnormalities in humans. The condition is named after the British physician John Down, who described its features in 1866. DS is triggered by trisomy of chromosome 21 (karyotype of 46, 21+), or by an extra third segment of chromosome 21 (familial DS; 2N = 46). DS is often referred to as trisomy 21 syndrome among physicians. Taurine is an extremely important dietary supplement, especially during the infant stage when its biosynthesis is limited (Yamori et al. 2010). When female cats are deficient in taurine during pregnancy, the development of the visual cortex in kittens is adversely affected (Palackal et al. 1986). Taurine deficiency also causes abnormal brain development (Whittle et al. 2007). Indeed, significantly low levels of taurine are found in the cerebral cortex of fetuses and newborns with DS.

About 1 per 1000 newborns are affected by DS each year. The syndrome involves an unusual physique, reduced intelligence, and congenital malformations of the heart. DS is usually characterized by a physical delay in development, typical facial appearance, and moderate mental retardation. The average IQ of a young adult with DS is 50, comparable to that of 8 to 9-year-olds. DS is also associated with gastrointestinal abnormalities, and immune system malfunctions, along with pathological and neurochemical variations of Alzheimer's disease (Di Domenico et al. 2013; Cuervo 2008). Affected organs include the esophagus, nerve, heart, lung, blood, skeleton, muscle, eye, thyroid, large intestine, genitalia, ear, mouth, and nose (Esbensen 2010).

Approximately 200–250 genes are overexpressed in individuals with DS (Patterson 2009). Among these, DS critical region gene 1 (DSCR-1) is highly expressed in the brain of DS fetuses and inhibits calcineurin A, the catalytic subunit of the Ca^{2+}/calmodulin-dependent protein phosphatases (Fuentes et al. 2000; Strippoli et al. 2000). Aberrant calcium signaling causes endoplasmic reticulum stress (ERS) and subsequent malfunction within the proteostasis network (PN), which regulates protein translation, chaperone-assisted protein folding, and degradation pathways (Di Domenico et al. 2013). DS individuals experience comprehensive failure in the biogenesis, folding, and trafficking of proteins (Aivazidis et al. 2017).

Taurine can alleviate symptoms of DS and muscular dystrophy in children. Taurine primarily plays a neuroprotective role in the central nerve system. Taurine is often used to treat anxiety, epilepsy, and seizures. Taurine appears to have multiple effects on cellular functions, and has minimal toxicity. Despite the frequent use of taurine in treating DS individuals, the actual basis for this treatment is not well understood. This study examined the positive effects of taurine on DS individuals using a *C. elegans* model of DS, which has a null mutation in calcineurin A or

B. Calcineurins are the only Ca^{2+}-dependent phosphatase to be expressed in the mammalian brain (Baumgartel and Mansuy 2012). Calcineurin A is a serine/threonine protein phosphatase that has a catalytic phosphatase domain and a regulatory domain, which consists of three domains (calcineurin B binding domain, calmodulin-binding domain, and downstream autoinhibitory (AI) domain). Calcineurin B has four EF-hand motifs that bind to calcium ions. Calcineurin gain-of-function (gf) mutants for the tax-6 gene display a hyperactive pattern of movement. While null mutations in calcineurins appear to hinder motor performance and temperature regulation (Kuhara et al. 2002). Although Ca^{2+} modulation function is lower in mutants, they appear to manage the modulatory function when treated with taurine.

Indeed, taurine exerts beneficial effects on muscle atrophy, muscular dystrophy, and senescence via metabolic alterations in calcium homeostasis. Taurine can control sarcolemma excitability and muscle contractility by modulating pathological changes in Ca^{2+} in muscle tissues, providing an enhanced therapeutic solution. Taurine is known for its therapeutic potential to restore skeletal muscle function and performance in various pathological conditions. Evidence is accumulating for an association between the adjustment of intracellular taurine levels in skeletal muscle and diverse pathophysiological conditions. In addition, taurine treatment can lessen myolemmal hyper-excitability in myotonia-related disorders.

Caenorhabditis elegans is a frequently used animal model owing to its low number of cells, short life cycle, and hermaphroditic reproduction. It requires only simplicity in biological application. In addition, it can be easily maintained to study the pathology of DS. This study utilized two types of calcineurin mutant C. elegans to study the effects of taurine on DS. Cellular characteristics of the disease pathway are conserved in DS pathology. The mutant DS model can be easily utilized to study human cases of DS (Ahn et al. 2006; Kaletta and Hengartner 2006). In human DS cases, the expression of DSCR-1, the regulatory factor-1 of calcineurin, negatively modulates the expression of calcineurins (Fuentes et al. 2000; Klee et al. 1998). DSCR-1 and the RCN, the regulator of calcineurin in C. elegans, share more than 40% homology (Lee et al. 2003). C. elegans null mutants tax-6 and cnb-1 show similarity to RCN-1 overexpressing models and a series of phenotypes resembling human cases of DS (Lee et al. 2002).

The DS models have smaller and slender bodies than wild-type animals. They also show several other characteristic phenotypes comparable to human DS cases. For example, they have shorter body size, slower growth, lower fecundity, stronger serotonin-resistant egg-laying, and defective thermal sensing. Due to defects in motility, they move lethargically within shorter tracks than their wild-type counterparts. The effects of taurine on the DS models were assayed using molecular expression of key ER stresses at phenotypically. Various assays were performed to examine whether taurine had a significant effect on typical DS models, using assays of ER stress, lifespan, thermotaxis, egg-laying, and motility.

2 Methods

2.1 Nematode Strains and Culture

All nematodes were cultured at 20 °C on nematode growth medium (NGM) seeded with OP50. The developmental stages of the nematodes were synchronized by bleaching an overnight culture on OP50-free media. Three strains of *C. elegans* were used: N2, tax-6 (p675), and cnb-1 (jh103). The latter two strains are mutants for DS. Tax-6 is a null mutant of calcineurin A, while cnb-1 has the calcineurin B null mutation. All nematode strains were provided by the Developmental Biology Lab of Hanyang University (Seoul, Korea). Different concentrations of taurine were prepared by diluting a taurine stock solution of 10 mg/ml into double-distilled water. The taurine stock solutions were spread onto the surface of NGM-agar plates at concentrations of 0, 10, and 100 μg/ml in OP50.

2.2 Assay of Taurine Effects on ERS

The ERS conditions were prepared by treating the nematodes on tunicamycin-containing media (10 μg/ml). The induced ERS conditions were verified according to the ERS marker expression. After treatment for 12 h under ERS conditions, the nematodes were tested for the effect of taurine at each of the concentrations. Taurine was added to the OP50 stock by diluting a taurine stock solution of 10 μg/ml into double distilled water. The final taurine concentrations were 0, 10, and 100 μg/ml in OP50, and these mixtures were spread onto the NGM-agar media. Aliquots of normalized samples were electrophoresed on a 10% SDS PAGE. ER marker proteins were quantified and standardized to the expression of actin protein. The expression of xbp-1 and hsp-70 were detected via western blotting using antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blotting was carried out according to the manufacturer recommendations. The values of the relative expressions were calculated relative to the control treatments.

2.3 Assay of Lifespan Under ERS

The effect of taurine on the lifespan of ER-stressed nematodes was assessed following synchronization of nematodes by bleaching, as described above. Eggs were placed on NGM with OP50 and kept at 25 °C until the young adult stage (Hyun et al. 2016). Nematodes were selected for uniformity and transferred to a fresh plate that contained 5 μg/ml of tunicamycin. Following incubation for 1 h at 20 °C, 50 individuals were transferred to media containing 0, 10, or 100 μg/ml taurine. Nematode survival was monitored until they no longer responded to moderate touch

with a platinum wire. Assays were repeated twice for all lifespan experiments. Individuals were counted as dead or alive every other day until all were dead.

2.4 Egg-Laying Assay

Assays were performed to determine whether taurine affected resistance to egg-laying of the calcineurin mutants after treatment with serotonin. Serotonin stimulates egg-laying in wild-type nematodes (Hardaker et al. 2001). Individuals at the L4 stage were selected from stock plates for uniformity and grown for 72 h at 20 °C on NGM. They were transferred to plates with three different taurine concentrations (0, 10, and 100 μg/ml) and cultured for 6 h. Each nematode was placed into a well of a 96-well plate holding 12.5 mM serotonin creatinine sulfate (Sigma-Aldrich) in M9 buffer (N = 20). Plates were incubated for 60 min at 20 °C, and the eggs in each well were counted.

2.5 Thermotaxis (TTX) Assay

The thermotaxis medium was prepared by mixing Bacto agar in NG buffer (25 mM KH2PO4 [pH 6.0], 1 mM MgSO4, 1 mM CaCl2, and 0.3% NaCl). After autoclaving, the agar mixture was dispensed into 10-cm petri dishes and left with the lid open at room temperature until solidification. Then, the plates were turned upside down on a paper towel. To establish a temperature gradient, plates were chilled from the center on a 4-cm plate of ice for 20 min. Meanwhile, 10 nematodes were washed twice in NG by centrifugation for 90 s and pooled into an Eppendorf tube containing 1 ml NG buffer. The pooled nematodes were placed on the line at 0, after excess liquid was blotted with a piece of Kimwipe. Individuals were permitted to move for 60 min and counted every 8 spaces of the plate. Ice was replaced frequently to maintain the temperature at the center of the plate. The TTX index (TTXI) was calculated according to the following formula:

$$\mathrm{TTXI} = \sum_{N=-4}^{+4} N * X_N$$

2.6 Mobility Assay

The effect of taurine on mobility was assessed after treatment with taurine. Initially, nematodes were grown together on OP50 media in the absence of taurine for 12 h and three L4 worms were chosen for uniformity. Each of the three selected worms

was transferred onto media containing taurine at 0, 10, or 100 mg/ml. Mobility was calculated by visually comparing the total distance moved along the tracks made over 6 h.

3 Results

Positive effects of taurine were consistently observed in all five assays. According to the marker protein expressions, taurine significantly lowered the level of ERS. The assays focused on four different behaviors: life span, egg-laying, thermotaxis, and mobility. Taurine extended the lifespan for ER-stressed worms, adjusted TTXI, increased egg-laying, and augmented mobility.

3.1 Taurine Reduced ERS Marker Expression

When ERS was induced at the whole-body level, both tax-6 and cnb-1 mutants responded to the induced conditions. The level of hsp-70 significantly increased relative to the other ER stress markers. The level of hsp-70 expression showed a typical dose-dependent response to the amount of added tunicamycin (Fig. 1). Taurine reduced the development of ER stress, as the intensity of hsp-70 sharply decreased after individuals were treated with taurine, in all mutants. The significant down-grade of the ER stress markers suggests that ERS was reduced by taurine. When nematodes were incubated at various taurine concentrations, hsp-70 protein

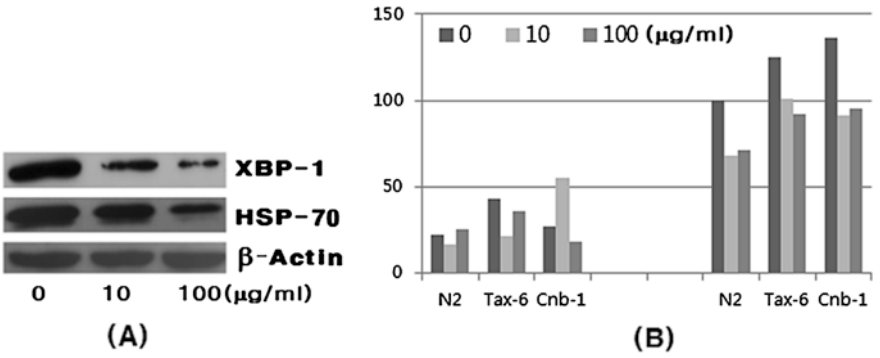


Fig. 1 Expression of ER stress marker proteins
After treatment for 4 h with 10 μg/ml of tunicamycin, worms were further cultured on media containing taurine at 0, 10, and 100 μg/ml. (**a**) The level of ER stress was compared according to the expression of marker proteins following immunoblotting for Tax-6 null mutant (**b**) The level of ER stress was quantitated following treatment with taurine according to the expression of hsp-70

expression was lower and related to the taurine concentration. These results imply that taurine diminished ERS caused by tunicamycin in the nematodes.

3.2 *Taurine Extended the Lifespan of ER-Stressed Mutants*

Surprisingly, mutants lived longer than N2. However, the mutant strains showed more sensitivity to tunicamycin, the ER stress inducer, than N2. Therefore, nematodes were treated with a consistent amount of taurine across age groups. As a result, calcineurin null mutants showed the longest lifespan after taurine treatment, and responded rapidly to taurine treatment (Fig. 2).

The nematodes responded sensitively to tunicamycin treatment applied at 1 day after the initiation of the culture. When treated with tunicamycin alone, half of the individuals died within 10 days of the start of the experiment. However, the nematodes in the taurine treatment showed increased survival. Difference was greatly reduced between the tunicamycin and the non-tunicamycin treatment groups. Depending on the concentration of taurine treatment, the nematodes increased their lifespan to that of individuals that did not receive tunicamycin, and responded

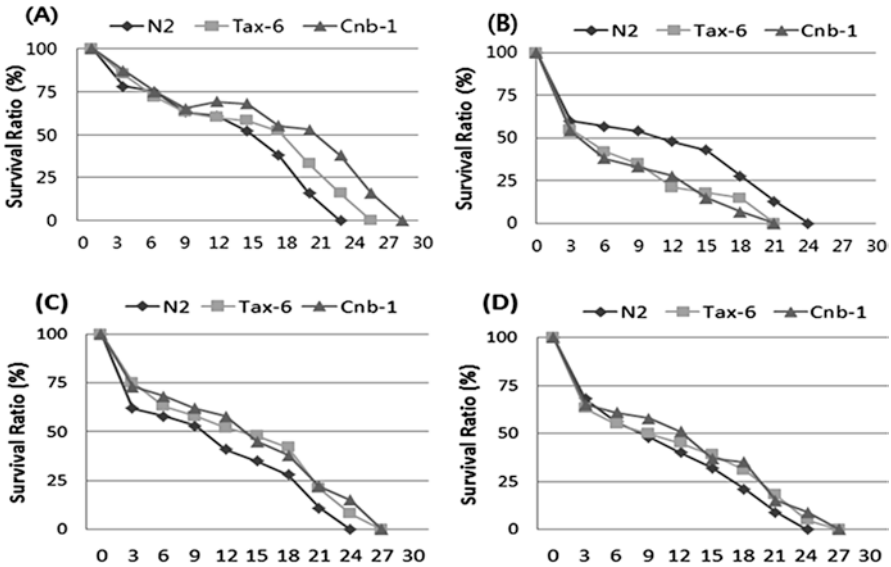


Fig. 2 Effects of taurine on lifespan of ER stressed C. elegans
Tunicamycin-treated worms were grown on media containing taurine. The survival ratio was compared between taurine-free and taurine-supplemented group. The survival ratio was calculated for 30 days according to the formula. Survival ratio = (# of living worms/# of initial worms) × 100(%) (**a**) Conditions of (−) tunicamycin and (−)taurine, (**b**) (+)tunicamycin and (−)taurine (**c**) (+)tunicamycin and (+)taurine (at 10 μg/ml) (**d**) (+)tunicamycin and (+)taurine (at 20 μg/ml)

promptly to taurine treatment. As a result, calcineurin null mutants showed an extended lifespan after taurine treatment.

3.3 Taurine Enabled Nematodes to Modulate TTX

Thermotaxis assays were performed to determine whether taurine enabled mutants to modulate thermotaxis. Comparison between taurine and taurine-free worms was used to examine the thermotactic behavior of *C. elegans*. When *C. elegans* were allowed to move along a thermal gradient, they appeared to pursue an optimal temperature and continued to seek this temperature. Wild-type worms appeared to move quickly along the temperature gradient. Mutant worms moved more slowly along the gradient, with a more random pattern of movement. When worms completed a circuit of all temperature zones after 60 min, TTX was assessed according to the distribution of the temperature zones.

The level of thermotaxis was quantitated as the TTXI using the formula described in Methods. The two DS mutants without pre-treatment with taurine had higher TTXI along the temperature gradient. Tax-6 mutants showed a significant change in thermophilicity, especially after treatment with 10 μg/ml of taurine (Fig. 3). CnB null mutants also showed significant difference after treatment with 10 μg/ml taurine. For both mutants, this taurine concentration was very effective in modulating the temperature specific responses.

Calcineurin mutants exhibited positive thermotaxis or thermophilic behavior. They persistently pursued warmer temperatures than their original conditioning temperature. When treated with taurine, their thermophilic behavior reduced slightly according to the TTXI, which reflected their ability to recall their initial cultivation temperature and conditions. Mutant nematodes showed lower TTXI after taurine treatment. Although there was no association between taurine treatment and

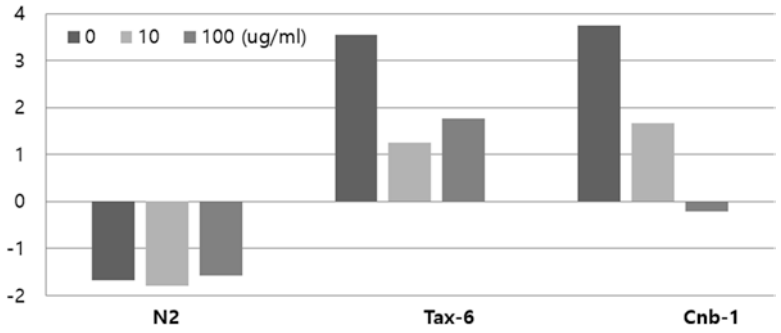


Fig. 3 Thermotaxis comparison
After various taurine treatments (0, 10, and 100 μg/ml) for 6 h, worms were allowed to move freely on thermotaxis medium for 60 min. The y-axis refers to thermotaxis index (TTXI) which was calculated as described in METHODS

thermotaxis, this result strongly indicates that they became temperature sensitive after taurine treatment, although calcineurin null mutants were somewhat unresponsive to the temperature gradient. Taurine is known to boost memory in many animal models. Taurine might enable nematodes to recognize their original cultivation temperature, which is associated with a source of food.

4 Taurine Enabled Nematodes to Recover Serotonin Sensitivity

Serotonin is known to stimulate nematode egg-laying muscles during a short period of time by promoting their motor nerves (Bastiani et al. 2003; Donohoe et al. 2009). The effect of taurine on egg-laying was assayed after treatment with serotonin. The calcineurin null mutants are resistant to serotonin and less responsive to muscle nerve signaling and are less productive than the wild-type. Both tax-6 and cnb-1 mutants are resistant to serotonin and they lay fewer eggs in the presence of serotonin than the wild-type (Bandyopadhyay et al. 2002; Lee et al. 2003, 2013). Following the serotonin treatment, the mutant nematodes still laid a smaller number of eggs. However, after exposure to taurine media, their egg-laying ability appeared to improve. Calcineurin null mutants showed a reduced resistance to serotonin when treated with taurine at 10 or 100 μg/ml (Fig. 4). This augmented level of egg-laying

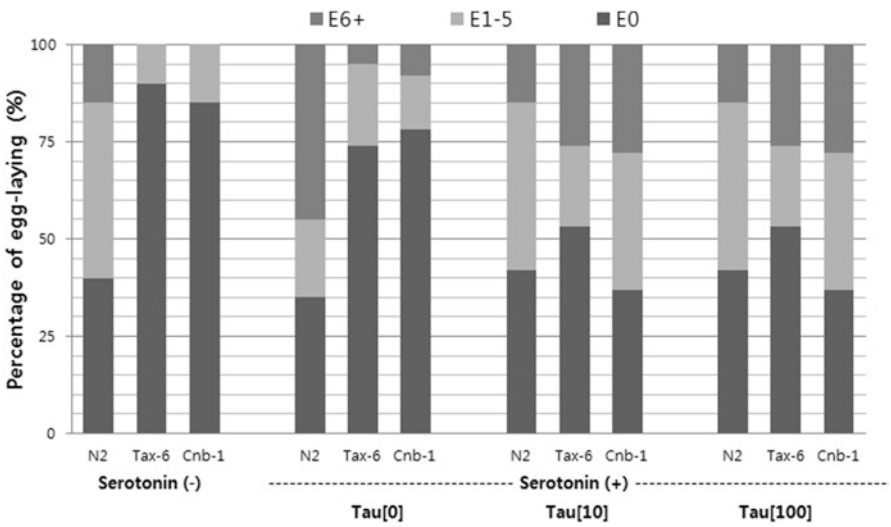
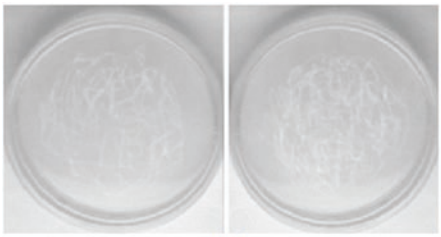


Fig. 4 Serotonin-mediated egg-laying assay
After taurine treatment (0, 10, and 100 μg/ml) for 6 h, eggs were treated with serotonin at 12.5 mM and their eggs were counted. The number of eggs laid was classified into three groups: E0, E1–5, and E6+, which represent no egg, 1–5 eggs, and more than 5 eggs, respectively. Y-axis refers to the percentage of egg-laying performance at different taurine concentrations



(A)

Strain	Taurine (μg/ml) 0	10	100
N2	**	***	***
Tax-6	*	**	**
Cnb-1	*	**	**

(B)

Fig. 5 Motility assay
Worms were treated with taurine at the three concentrations. Their motility was visually compared according to the strain and taurine concentration. They substantially enhanced their movement ability following treatment with taurine. The total moving distance was compared at different concentrations of taurine. The motility appeared to increase compared to non-taurine control. (**a**) Comparison between taurine-free (*left*) and taurine (*right*) groups in Tax-6 null mutants. (**b**) Total distance was visually compared among the strain and the taurine concentration and their performance was expressed as the numbers of asterisks

could be attributed to the reduced level of ERS. Tax-6 (can-1) null mutants recovered their resistance after treatment with 10 or 100 μg/mL taurine, as did the cnb-1 mutants.

5 Taurine Reinforced Nematode Motility

When treated with taurine, the mobility of nematodes increased significantly more than the taurine-free control. Worms substantially enhanced their movement ability. The total distanced moved was compared across individuals treated with different concentrations of media-applied taurine. Results showed substantial differences between taurine and non-taurine treatments of different doses (Fig. 5). This implied that taurine enabled individuals to increase their muscle strength. Mutants exhibited slightly more movement on recurrent tracks and increased amplitudes when treated with taurine.

6 Discussion

Positive effects of taurine were consistently verified in all five assays. When treated with taurine, the level of ERS was reduced. The decrease in ERS coincided with four types of behavioral tests. The calcineurin null mutants showed significant changes in the four behaviors tested after taurine treatment. The marker protein expressions also showed that taurine significantly lowered the level of ERS. Reduction in ERS coincided with improvement in behavior in the two strains of DS mutants. Deficiency in functional calcineurins tends to induce ERS. Most likely, a reduction

in the severity of ERS resulted in a decreased unfolded protein response and proteasomal degradation at the whole-body level. Improvement was evident following treatment with taurine: extended lifespan for ER-stressed individuals, adjusted TTXI, enhanced fecundity, and augmented motility. The effects of taurine depended on the concentration and duration of the treatment.

DS cases may experience ERS at a whole-body level. Many studies indicate that DS cases may experience chronic ERS due to oxidative damage and aberrant calcium signaling. Accumulation of unfolded/damaged protein aggregates may eventually lead to ERS as a consequence of improper protein folding, inadequate protein degradation or ER dysfunction. Recurrent ERS may result in further malfunction within the PN that regulates protein translation, chaperone-assisted protein folding, and degradation pathways. The unfolded protein response (UPR) may prompt chaperones to improve protein folding; however, this interferes with protein biosynthesis and provokes protein degradation if recurrent.

DS cases undergo a comprehensive failure in the biogenesis, folding, and trafficking of proteins. The fact that taurine diminished ERS in this study provides an explanation for the effective usage of taurine for DS cases. Taurine may restore ER function and the balance between protein degradation and protein biosynthesis/folding, unfolded or misfolded proteins activates three stress sensors (PERK, ATF6, and IRE1) that initiate distinct UPR pathways.

This study also examined the behavioral impact of taurine, including lifespan under ERS. Lifespan reflects a composite progression of chronic deterioration in the fitness of an organism (Yang et al. 2012). Accumulation of oxidized and misfolded macromolecules shortens the lifespan and continuously creates physiological damage at the cellular level throughout the life of an organism. Both tax-6 and cnb-1 mutants displayed longer lifespan in the absence of the ER stress inducer. After treatment with tunicamycin, the two mutants showed more sensitivity to tunicamycin and a reduction in lifespan. This implied that the two mutants experienced a chronic level of ERS. When subjected to ERS, the mutants responded sensitively to the presence of additional ER stress induction. In both CnA and CnB null mutants, lifespan significantly increased in the presence of taurine after ERS. These results strongly indicate that taurine enabled the calcineurin mutants to extend their lifespan under ERS. All biological pathways stimulating extended lifespan lead to enhanced autophagy (Dwivedi et al. 2009). Autophagy allows cells to eliminate dysfunctional or redundant components, or to recycle subcellular constituents. A greater level of autophagy was evident in the calcineurin null mutants than in N2. Following treatment with tunicamycin, the two calcineurin mutants had reduced autophagy; thus, negating the extended lifespan phenotype. However, taurine was able to recover the autophagy activity, as shown in a study on humans. Taurine may stimulate autophagic flux in the calcineurin null mutants, compensating for the adverse effects of ERS on lifespan. Alternatively, these results may represent a novel mechanism for the improvement of lifespan by taurine.

Egg-laying is a highly managed practice in nematodes that involves the coordination of 16 vulva muscles (Bandyopadhyay et al. 2002). In nematodes, serotonin prompts the motor nerves of the vulva to stimulate egg-laying. This serotonin effect

is less effective in both calcineurin null mutants, and they are resistant to serotonin, and lay fewer eggs than wild-types. Following treatment with taurine, the two mutants appeared to re-establish sensitivity to serotonin. Taurine is known to exert a beneficial effect on muscle performance, damage recovery, and growth. Studies on the function of taurine in skeletal muscle indicate it has a primary role in phospholipid stabilization and intracellular Ca^{2+} regulation, along with an increase in the rate of Ca^{2+} uptake into the sarcoplasmic reticulum and total storage capacity. This may have enabled worms to compensate for the loss in calcium metabolism.

C. elegans can sense the temperature at which their physical activities are adversely affected, and use temperature to find food based on the temperature of their former growth conditions. When placed on a thermal gradient, calcineurin mutants show erratic thermotaxis behavior. When worms moved along the temperature gradient, they appeared to move around a preferred isothermal track. The mechanisms involved in thermotaxis learning remain to be elucidated. The neural circuit for thermotaxis requires various gene products affecting calcium metabolism, oxidative stress genes, and the insulin/IGF-1/TGF-β pathway.

Calcineurin null mutants showed an uncoordinated and defective style of motion on the media (Bandyopadhyay et al. 2004). Conversely, wild-types moved speedily in a consistent pattern of an undulating wave. Especially, cnb-1 null mutants showed very inept motility, which greatly improved after taurine treatment. This indicated that taurine may promote a pathway that can compensate for the affected physiological pathway in the mutants.

This study provided an example of the multiple effects of taurine on calcineurin in *C. elegans*, which affected ERS, lifespan, thermotaxis, egg-laying, and motility. Analyses of calcineurins and their regulators in this animal model provide a unique opportunity to identify potential drug targets for many human diseases, particularly DS, neurodegenerative, and myocardial diseases. This study provides an important understanding of the behavioral effects of taurine on DS cases. These results may be helpful in elucidating the concentration of taurine that can be used for treating DS cases. Additional experiments are needed to understand the potential interactive mechanisms among sensory neurons, RCN-1, and calcineurin. Along with potential pleiotropic effects, taurine can improve DS and be further used to reduce DS in clinical treatments. Human clinical trials using taurine in various pathologies, such as diabetes, cardiovascular, and neurological maladies have been implemented and may provide guidelines for planning future studies for DS cases.

7 Conclusion

These results show that the symptoms of DS can be alleviated by treatment with taurine. In the nematode model of DS, typical DS traits were significantly improved. Taurine can be further evaluated as a treatment for DS. The physiological significance of calcineurin was confirmed, considering that many of the proteins that bind with calcineurin are critical for maintaining normal homeostasis. Since dysfunctional

calcineurins are associated with numerous diseases, they are suitable targets for drug development for disorders, including cardiac hypertrophy, Alzheimer's disease, and schizophrenia. Although further studies are necessary to extrapolate from nematodes to humans, prospective drug targets can be identified by further analyses of interactions between cna-1 and cnb-1 and regulation of phosphatase in this DS model. This approach also has significant potential to understand the basis for many human disorders, such as neurodegenerative and myocardial diseases.

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Protective Effects of Taurine on the Radiation Exposure Induced Cellular Damages in the Mouse Intestine

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Abstract There has been a growing interest in radiation effects as a result of the Fukushima nuclear power plant accident in 2011. Exposure to ionizing radiation causes oxidizing events to different organs such as the bone marrow, intestine, and kidney, which can result in radiation-induced injuries. Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid possessing several important physiological functions, including membrane stabilization, anti-oxidative activity, anti-inflammatory effects and modulation of intracellular calcium levels. Taurine appears to be an attractive candidate for use as a radioprotector and as a radiation mitigator, but its protection mechanism against radiation-induced cell damage is still unclear until now. In this review we describe some of the mechanisms explaining the radioprotective/mitigating effects of taurine on radiation-induced cellular damage and our recent findings on this subject.

Keywords Intestine · Radiation · Mitigator · Taurine transporter · Reactive oxygen species (ROS)

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Abbreviations

Tau	Taurine
TauT	Taurine transporter
ROS	Reactive oxygen species

1 Introduction

There has been a growing interest in radiation effects after the Fukushima nuclear power plant accident of 2011. Ionizing radiation used in cancer treatment or emanating from nuclear plant accidents are the two common causes of radiation exposure. Exposure to ionizing radiation generates reactive oxygen species (ROS) and free radicals which, in turn, can cause oxidative stress in the irradiated cells. Furthermore, ionizing radiation can cause radiation-induced injuries to organs such as the bone marrow, intestines, and skin (Painuli and Kumar 2016). While the mechanism responsible for radiation-induced injuries remain unclear, several reports have implicated an inflammatory process in which cytokines and ROS appear to be involved (Van et al. 2005; Gridley et al. 2007; Anscher 2010; Sato et al. 2015). Radio protectors and radiation mitigators are found to reduce the damage caused by irradiation on normal tissues (Citrin et al. 2010).

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid possessing several important physiological functions, including membrane stabilization, anti-oxidant activity, anti-inflammatory effects and modulation of intracellular calcium levels (Oliveira et al. 2010; Ma et al. 2010; Kato et al. 2015). Taurine appears to be an attractive candidate for use as a radioprotector and as a radiation mitigators, but it is not known how it protects against radiation induced cell damage. In this review, we describe examples of and mechanisms underlying radiation-induced cellular damages and work from this and other laboratories supporting a radioprotective/mitigating effect for taurine.

2 Radiation-Induced Cellular Damages

Exposure of DNA and water molecules to ionizing radiation leads to the production of ROS and free radicals (Bhilwade et al. 2014), which, in turn, can cause radiation-induced injuries in the exposed cells. ROS attack either directly or indirectly virtually all cellular components including DNA, proteins and lipids. ROS can also impair cellular functions and enhance inflammatory responses (Li et al. 2018). In cells, free radicals can cause either single stranded (SSBs) or double stranded

(DSBs) DNA breaks, and can promote apoptosis and mediate an inflammatory process (Duan et al. 2017; Smith et al. 2017). Particularly, radiation-induced injuries can easily induce single stranded or double stranded DNA breaks in organs such as the bone marrow, intestines and skin.

Exposure to ionizing radiation induces injury to organs with rapidly proliferating cells and results in an acute radiation syndrome such as gastrointestinal syndrome and hematopoietic syndrome (Suman et al. 2012). Gastrointestinal syndrome leads to death within 10–12 days after ionizing radiation exposure (Rosen et al. 2015). The loss of villus epithelial cells or crypt stem cells has been suggested to be responsible for gastrointestinal syndrome (Qiu et al. 2008). Hematopoietic syndrome leads to death within 30 days after ionizing radiation exposure. The depletion of hematopoietic progenitor cells for white blood cell and megakaryocyte lineages has been suggested to be responsible for hematopoietic syndrome, a condition that leads to lymphocytopenia, neutropenia and thrombocytopenia (Dainiak 2002; Rosen et al. 2015).

3 Radioportectors and Radiation Mitigators

Exposure to ionizing radiation generates ROS and free radicals in irradiated cells, which can result in radiation-induced injuries. Radioprotectors and radiation mitigators can reduce radiation-induced injuries. Due to their antioxidant activity, radioprotectors have an ability to reduce free radicals. Aamifostine (WR-2721) is the only radioprotectors approved by the United States Food and Drug Administration (US FDA) for clinical application (Szejk et al. 2016). It can act as a potent free radical scavenger (Chok et al. 2010) and protect normal tissues from damage by chemotherapy agents and radiation (Brown 1985; Wasserman and Brizel 2001). Amifostine is useful as a radioprotector, but it has some side effects (Kumar et al. 2002). Thus, a safe, effective and inexpensive radioprotector is needed. Owing to its anti-oxidative activity taurine appears to be an attractive candidate for use as a radioprotector against radiation-induced injuries and as a radiation mitigator (Oliveira et al. 2010). Radioprotectors are used to reduce injury of normal tissues from irradiation. A major mechanism of the protective effect of radioprotectors is to scavenge-free radicals induced by the ionizing radiation. Since free radicals are generated in the nanosecond to microsecond timeframe, radioprotectors need to be present at the time of an exposure to ionizing radiation for them to be effective (Citrin et al. 2010).

Unintentional exposure to ionizing radiation may be the result of an unexpected event such as that occurring from a nuclear plant accident. Hence, we need protective substances that could be administered promptly after irradiation to reduce radiation-induced injuries. Agents administered after irradiation are termed radiation mitigators (Singh et al. 2013). Radiation mitigators are used to accelerate recovery or repair injury caused by radiation.

4 Protective Effect of Taurine Against Organ Damage

4.1 Inflammation and Apoptosis

A radiation-induced injury is an inflammatory process in which cytokines and ROS play a role (Gridley et al. 2007; Anscher. 2010). The oxidative changes may continue for days and months after the initial radiation exposure (Petkau 1987). NF-κB activity increases after ionizing radiation exposure. It is one of the important regulators of proinflammatory gene expression (Veuger et al. 2008; Criswell et al. 2003). Taurine prevents organ damage associated with inflammation after undergoing metabolic conversion to taurine chloramine and taurine bromamine, two anti-inflammatory signaling substances. Taurine chloramine inhibits the production of inflammatory mediators through a mechanism that involves inhibition of NF-κB activation (Barua et al. 2001). Furthermore, taurine inhibits the secretion of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8, it has been suggested that it is a potent anti-inflammatory factor (Liu et al. 2017). As a result, taurine might contribute to the recovery from radiation-induced injuries.

Exposure to ionizing radiation induces injury by promoting gastrointestinal syndrome and hematopoietic syndrome. Gastrointestinal syndrome induces the loss of villus epithelial cells and crypt stem cells, and hematopoietic syndrome leads to lymphocytopenia, neutropenia and thrombocytopenia. Exposure to ionizing radiation induces apoptotic cells which are found to be associated with the loss of villus epithelial cells and crypt stem cells, and lymphocytopenia. The protective effect of taurine against organ damage may stem from its ability to suppress oxidative stress and apoptotic responses (Nagai et al. 2016). In a study carried out in mice, taurine was found to reduce the percentage of apoptotic cells or spermatocyte-derived cells (GC-2) subjected to ionizing radiation (Yang et al. 2017). Taurine also significantly suppressed UVB-induced cell apoptosis in the lens epithelial cells (Dayang and Dongbo 2017). Work from our laboratory found that post-exposure therapy with taurine at 3 and 7 days after radiation did not mitigate mouse blood lymphocytes suppression, but it helped blood lymphocytes to recover their function after 10 days (Yamashita et al. 2017). These effects could be related to the known functions of the amino acid as a growth factor for lymphocyte progenitor cells and lymphocytes (Fazzino et al. 2010).

It is reported that exposure to ionizing radiation raises the urinary taurine excretion (Goyer and Yin 1967; Johnson et al. 2012; Watson 1962). We have previously found that ionizing radiation exposure increases urinary taurine concentration in mice, an effect that could have resulted from the release of taurine from injured cells (Yamashita et al. 2017). It has also been found that the administration of taurine to mice increased survival to radiation exposure, but this effect was reduced when administered before the radiation exposure (Abe et al. 1968). Probably because of a reduction of taurine uptake after whole body irradiation. Taurine uptake into cells is associates with an increased expression of the taurine transporter (TauT) (Kwon and Handler 1995; Warskulat et al. 2004). Taurine is taken up into tissues via a taurine

transporter (TauT). In an experiment conducted in our laboratory using mice that had been subjected to 7Gy whole body X radiation and developed histologically evident small intestine injuries, the expression of taurine/taurine transporter in the small intestine was reduced (Yamashita et al. 2017). Conversely, the depletion of taurine will be harmful because it may lead to the inhibition of the recovery of physiological functions that are dependent on the immune system, the integrity and cellular growth of the intestinal mucosa.

4.2 Reactive Oxygen Species (ROS)

Exposure to ionizing radiation induces gastrointestinal syndrome and hematopoietic syndrome (Suman et al. 2012). Ionizing radiation exposure has been linked to alterations in mitochondrial membrane potential, the generation of ROS generation and mitochondrial membrane damage (Yoshida et al. 2012; Leach et al. 2001). Damage to the mitochondrial membrane by an exposure to ionizing radiation could enhance the production of ROS which, in turn, can contribute to the damage gastrointestinal tract and the activation of inflammatory pathways associated with radiation (Yoshida et al. 2012). The functional changes in mitochondria after exposure to ionizing radiation is caused by oxidative stress. The biochemical modifications, which occur shortly thereafter or during radiation exposure, are responsible for most of the effects seen in radiation-induced injuries. However, oxidative stress may continue to cause its harmful effects for days and months after the initial exposure to ionizing radiation – presumably because of continuous generation of ROS (Datta et al. 2012; Petkau 1987). Yoshida et al. (2012) have shown that the intracellular ROS disappeared within 30 min after 5 Gy gamma-ray irradiation. Furthermore, the oxidation of mitochondrial DNA and a significant increase of ROS levels in the mitochondria were observed in cells at 24 h or later after 5 Gy gamma-ray irradiation. Many studies have confirmed the antioxidant activity of taurine (Cetiner et al. 2005; Hansen 2001). Even though is not a radical scavenger and a regulator of the antioxidative defenses (Gurer et al. 2001). Jong et al. (2012) has reported that taurine is a regulator of mitochondrial protein synthesis, and a protector of the mitochondrion against excessive superoxide generation. Taurine is a regulator of mitochondrial protein synthesis, and protecting the mitochondria against excessive superoxide generation (Jong et al. 2012). Schaffer et al. (2009) reported that taurine may inhibit the production of ROS via regulation of mitochondria function. Taurine might contribute to recovery from radiation-induced small intestinal injuries by regulating the mitochondria. This assumption was tested in our laboratory by examining the effects of taurine on histologically evident small intestinal injuries of mice after 4.5 Gy whole body X-irradiation. X-irradiation decreased the expression of TauT in the small intestine, and the administration of taurine suppressed the effect of radiation on the small intestine. These results suggest that taurine from exogenous sources might compensate for the taurine losses via TauT and, in this way, contribute to recovery from radiation-induced small intestine injury, possibly by

suppressing an inflammation caused by cytokines and ROS. These results also point to the critical role played by the increase expression of TauT in the mitigating effect of taurine on radiation exposure.

5 Conclusion

Radiation-induced injuries occur through an inflammation-based process in which cytokines and ROS play a critical role. In such situation, taurine may be beneficial by inhibiting the secretion of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8-and by inhibiting ROS production through a regulatory role in mitochondria. In this way, taurine can contribute to recovery from radiation-induced injuries. Since the expression of the taurine transporter is reduced after high-dose radiation exposure, and taurine lose occur during irradiation, the recovery of physiological functions after radiation exposure will be impaired. Hence, the intake of taurine after an exposure to ionizing radiation will compensate for the taurine losses and, in this way contribute to recovery from radiation-induced injuries. Features such as low cost and a lack of side effects make taurine an attractive candidate for use as a radiation-mitigating agent.

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Taurine Prevented Hypoxia Induced Chicken Cardiomyocyte Apoptosis Through the Inhibition of Mitochondrial Pathway Activated by Calpain-1

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Abstract **Objective** To determine whether taurine has protective effects on chicken myocardial apoptosis induced by hypoxic condition through inhibiting calpain-1 derived mitochondrial apoptotic pathway. **Methods** Chicken primary embryonic myocardial cells were isolated and cultured at 37 °C under a 5% CO_2 atmosphere. Firstly the optimum concentration of taurine or PD150606 was chosen by detecting the cell viability. Chicken cardiomyocytes were cultured in 95% N_2-5% CO_2 atmosphere for 12 h to produce hypoxic conditions. Before hypoxic treatment, 10 mM taurine and 10 uM PD150606 (a specific calpains inhibitor) were added separately or together. The cell apoptosis was detected by acridine orange/ethidium bromide (AO/EB) double staining. Western blotting was used to determine the protein expressions of calpain-1, cytochrome c, Bcl-2, procaspase-9 and procaspase-3 in the cardiomyocytes. **Results** Taurine administration effectively attenuated the myocardial apoptosis under hypoxic condition, reduced the calpain-1 protein level. In addition, pre-treated taurine could up-regulate the protein expressions of Bcl-2 and procaspase-3 in hypoxic myocardial cells, down-regulate protein expression levels of cytochrome c and procaspase-9. Moreover, taurine exhibited same inhibition effect as PD150606 on the cell apoptosis and proteins express under hypoxic condition. **Conclusions** Taurine could attenuate the chicken cardiomyocyte apoptosis impaired by hypoxia through inhibiting calpian-1-derived mitochondrial apoptotic pathway in vitro.

Keywords Taurine · Apoptosis · Calpain-1 · Mitochondrial apoptotic pathway · Chicken primary myocardial cells

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1 Introduction

Myocardial ischemia is the leading cause of myocardial cell injury in heart disease, such as coronary heart disease (CHD), hypertension and congestive heart failure (CHF). Many experimental studies have shown that cardiomyocyte injuries induced by ischemic/hypoxia are closely related to the apoptosis (Kositprapa et al. 2000; Inserte et al. 2005; Bajaj and Sharma 2006; Li et al. 2009a; Zheng et al. 2015).

Calpains are a family of calcium-dependent cysteine proteases, which participate in cardiac patho-physiology. Among them, calpain-1 is one of the ubiquitous calpains and was studied extensively. Studies have reported calpain-1 plays an important role in promoting myocardial cells apoptosis under hypoxic conditions (Kositprapa et al. 2000; Zhang et al. 2015). The up-regulation of calpain-1 has also been observed in ischemic hearts (Inserte et al. 2005; Li et al. 2009b). Furthermore, transgenic mice with over-expression of calpian-1 are sufficient to heart failure (Galvez et al. 2007). Inhibitions of calpains could reduce ischemic cardiac injury in animal models of ischemia/reperfusion or myocardial infarction (MI) (Mani et al. 2009; Hernando et al. 2010; Ma et al. 2012; Zheng et al. 2015).

Taurine, 2-aminoethylsulfonic acid, presents a high concentration in mammalian heart and accounts for more than 50% of the total amino acid pool of heart tissues (Lombardini 1996). Studies have reported that taurine has myocardial protective effects, such as tissue structure and function maintenance, osmoregulation, membrane stabilization, antioxidant, modulation of ion movement and anti-apoptosis (Ito et al. 2008; Schaffer et al. 2010; Wojcik et al. 2013; Wang et al. 2018). Exogenous administration of taurine could inhibit myocardial cell apoptosis under hypoxia/ischemic conditions (Takahashi et al. 2003; Rivard et al. 2007; Yang et al. 2013; Setyarani et al. 2014; Wang et al. 2018).

However, the relationship between taurine and calpain-1 in the hypoxic cardiomyocytes is unknown. In this study, taurine and PD150606 were pre-incubation in the chicken primary myocardial cells and hypoxia was used to induced cell apoptosis. The protective effect of taurine was evaluated by detection the cell apoptosis and protein expressions of calpain-1, cytochrome c, Bcl-2, procaspase-9 and procaspase-3 to explore whether taurine could inhibit calpian-1-derived mitochondrial apoptotic pathway against myocardial hypoxic injury.

2 Material and Methods

2.1 Isolation of Chicken Primary Embryonic Cardiomyocytes

Firstly, AA broiler eggs were obtained from Shenyang Huamei Livestock and Poultry CO., LTD (Shenyang, China). Animal handling and experimental procedures followed the guidelines of the Animal Care and Use Committee of Shenyang

Agricultural University. Chicken embryos at 12 d were used to get the hearts under sterile conditions. The ventricular tissues were cut into pieces after being washed five times in pre-cooled phosphate buffered saline (PBS) solution and then were digested with 0.1% trypsin solution at 4 °C for 14–16 h to obtain a cell suspension. The reaction was terminated by addition of same volume Dulbecco's modified Eagle's medium (DMEM/F12; Hycloe, USA) containing 10% fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin and 100 units/mL streptomycin at 37 °C for 5 min. After discarding the supernatant, the cardiac tissues were washed by double volume DMEM/F12 medium containing 0.08% collagenase type II, 0.05% bovine serum albumin (BSA; Hycloe, USA), 100 units/mL penicillin and 100 units/mL streptomycin and then resuspended with three times volume of same medium at 37 °C for 15–20 min. Repeat this step 4–5 times. All the supernatant was filtered through a 200 μm nylon mesh cell strainer into a new tube, and then centrifuged at 1000 × g for 5 min. Discard the supernatant and resuspend the cells in DMEM/F12 medium containing 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin. Cell density was adjusted at 5×10^5/mL and cells were cultured in cell culture bottle at 37 °C under a 5% CO_2 atmosphere. Cells were transferred to new cell culture bottle after 1 h Repeat this step again. Cell density was adjusted at 4×10^5/mL and cells were cultured in DMEM/F12 medium containing 10% FBS, 0.1 mM 5-Brdu (Sigma, St. Louis, MO, USA), 100 units/mL penicillin and 100 units/mL streptomycin for 72 h.

2.2 Measurement the Myocardial Cell Activity by MTS

Cardiomyocytes viability was measured by using MTS. Briefly, isolated cells were plated in 96-well plates at a density of 4×10^4 cells per well. When the cell fusion got 80%, the cells were pre-treated with taurine (0, 5, 10, 20 mM; Sigma) or PD150606 (0, 10, 20 mM; Sigma; dissolved in 0.1% DMSO) for 12 h, 24 h. The cell viability was detected by adding MTS solution following the kit manufacture instruction (Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay kit, Promega, USA) and optical density was determined at 490 nm by a spectrophotometer (Infinite M200PRO; Tecan, Switzerland). Each administration repeated at least three times.

2.3 Experimental Treatment

Isolated chicken primary myocardial cells were plated in 24-well plates at a density of 2×10^5 cells per well. After cultured 60–72 h at 37 °C in the CO_2 incubator, the cells were divided into eight groups and treated as Table 1, each treatment repeated three times. Group 1 served as the normal control group (C), the cells were cultured

Table 1 The experiment design

Group	Taurine (10 mM)	PD150606 (10 μM)	Hypoxia (5% CO_2 + 95% N_2, 12 h)
Normal control (C)	–	–	–
Taurine control (CT)	+	–	–
Taurine +PD150606 control (CTP)	+	+	–
PD150606 control (CP)	–	+	–
Model (M)	–	–	+
Taurine +Model (MT)	+	–	+
Taurine +PD150606+Model (MTP)	+	+	+
PD150606+Model (MP)	–	+	+

at 37 °C for 24 h without any treatment. Group 2 was the taurine control group (CT), taurine was supplemented to the cell cultural medium with the final concentration at 10 mM after dividing group. Group 3 was taurine +PD150606 control group (CTP), PD150606 was added to the culture solution with 10 μM final concentration at 11.5 h after taurine supplement. Group 4 was the PD150606 control (CP), PD150606 was added to the culture solution at 11.5 h, and then the cells culture continued 12.5 h till the end. The cells in group 5 were cultured in 5% CO_2 and 95% N_2 at 37 °C for 12 h from 12 to 24 h after gouping, as the hypoxia model group (M). In group 6 (MT), 10 mM taurine was added to the cell culture medium at 12 h before hypoxia treatment. In group 7 (MTP), taurine and PD150606 was added respectively at 12 h and 0.5 h before hypoxia administration. In group 8 (MP), PD150606 was added at 0.5 h before hypoxia treatment.

2.4 Detection of Myocardial Cell Apoptosis by AO/EB Double Staining

After cells were cultured at 37 °C, 5% CO_2 and 95% N_2 for 12 h (the time was decided by previous experiment), removing culture medium completely, cells were washed three times with pre-cooled PBS and then fixed with neutral 4% paraformaldehyde buffered for 3 h at room temperature. The cells were washed three times by PBS again after discarding the fixed solutions. 0.2 mL 0.5% Triton X – 100 was added to each well. After 10 min, washing the cells with PBS for three times, dual fluorescent staining solution (AO/EB double staining kit, Beijing Solarbio Science & Technology Co., Ltd.) was added to each well. The apoptotic cells and the total cells were counted at 5 view in each well using a Leica DM4000B inverted fluorescent microscope (Leica, Germany). The percentage of apoptosis was calculated by the ration of the number of apoptotic cells to the number of total cells.

2.5 Western Blotting Analysis

The proteins in chicken primary myocardial cells were extracted according to kit instructions (Beyotime, China). After detection the protein concentration with a BCA protein assay kit (Beyotime, China, P0012S), the proteins were separated with SDS-PAGE electrophoresis and then transferred onto polyvinylidenedifluoride (PVDF) membranes (Beyotime, China). After blocking with 5% (W/V) non-fat milk in Tris buffer solution tween (TBST) (Applygen, China) for 3 h at room temperature, the membranes were incubated with antibodies against β-actin (anti rabbit β-actin, Abcam, Cambridge, UK, ab8227, dilution: 1:5000), calpain-1 (anti goat calpain-1, Abcam, Cambridge, UK, ab174683, dilution: 1:1000), cytochrome c (anti rabbit cytochrome c, BBI, UK, D220521; dilution: 1:1000), Bcl-2 (anti rabbit Bcl-2, Abcam, Cambridge, UK, ab174683, dilution: 1:1000), caspase-9 (anti rabbit caspase-9 P10, Bioss, UK, bs-8502R, dilution: 1:1000), and caspase-3 (anti rabbit caspase-3, ABclonal, USA, A11953, dilution: 1:1000) overnight at 4 °C. After being washed in TBST for three times, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, ZSGB-Bio, China, ZB-2305) for 45 min at room temperature. Signals were visualized by a Super ECL kit (New Cell & Molecular Biotech Co., Ltd. (Suzhou, China, P0018). The optical densities of protein bands were recorded by DNR bio-imaging system (Microchemi 4.2, Isreal) and quantified by Image Quant 5.0 software, the relative expressions were normalized to β-actin.

2.6 Statistical Analysis

All the data were expressed as means ± standard error and significant differences were determined by one-way ANOVA and Duncan's multiple -range test using SPSS 16.0 software. A difference was considered significant at the $p < 0.05$ level. A highly significant was considered at the $p < 0.01$ level.

3 Results

3.1 Optimum Dose of Taurine or PD150606

The effect of different concentrations of taurine on the myocardial cell viability at 12 h and 24 h were shown in Fig. 1a. Cell viability was not influenced by different taurine treatment at 12 h. While at 24 h, all taurine treatment increased the cell viability, especially when taurine was added at 10 mM, cell viability was elevated

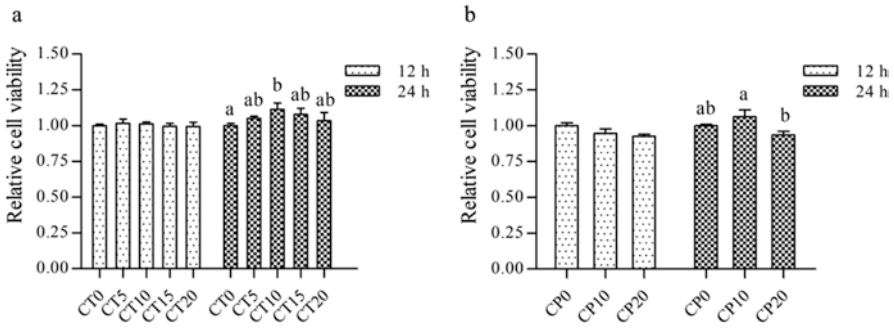

Fig. 1 The cell viability of myocardial cells at 12 h and 24 h. (**a**) Effect of taruine on cell viability. (**b**) Effect of PD150606 on cell viability. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$)

significantly compared with no taurine treatment, but there wasn't significant difference between the taurine treatment groups. From these results, 10 mM taurine was chosen for subsequent experiment.

The effect of different concentrations of PD150606 on the cell viability cells at 12 h and 24 h were shown in Fig. 1b. At 12 h and 24 h, no matter 10 μM PD150606 or 20 μM PD150606 had no effect on the cell viability compared with no adding group. However, the cell viability treated with 20 μM PD150606 were reduced significantly than that treated with 10 μM PD150606 ($p < 0.05$). In the following experiment, 10 μM PD150606 was used.

3.2 The Effect of Taurine on the Cardiomyocyte Apoptosis

The effect of taurine on the cardiomyocyte apoptosis was shown in Figs. 2 and 3. Under the normal culture condition, cell apoptosis also could be detected (Fig. 2a, b, c, d). Hypoxia treatment 12 h induced obvious apoptosis, not only the early-stage apoptotic cells but also the late-stage apoptotic cells were also increased (Fig. 2e). Pre-treated taurine, PD150606, taurine + PD150606 respectively could reduce the myocardial cell apoptosis induced by hypoxia (Fig. 2f, g, h). As shown in Fig. 3, compared with C group, the percentage of apoptosis was decreased by 23.42% ($P < 0.01$) (group CT), 20.49 ($P < 0.01$) (group CTP) and 23.42% ($P < 0.01$) (group CP) respectively. However there wasn't significant difference between the different treatment groups under normal culture. These outcomes indicated that taurine or PD150606 was contributed the anti-apoptosis at normal condition. The percentage of apoptosis in M group was increased significantly compared with the C group ($P < 0.01$). As compared with the M group, the percentage of apoptosis was decreased by 16.56% ($P < 0.05$) (group MT), 39.11% ($P < 0.01$) (group MTP) and

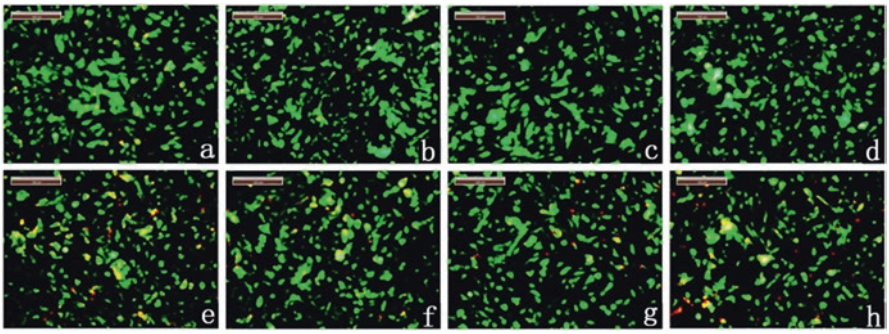

Fig. 2 The fluorescence micrographs of myocardial cells at 12 h after hypoxia treatment (100× magnification). (**a**) C group. (**b**) CT group. (**c**) CTP group. (**d**) CP group. (**e**) M group. (**f**) MT group. (**g**) MTP group. (**h**) MP group. Normal cells: the cells showed green. Early apoptotic cells: nucleus showed yellow-green fluorescence by AO staining and concentrated into a crescent or granular that located in 1 side of cells. Late apoptotic cells: the nucleus showed orange-green or red fluorescence and gathered in concentration and located in bias

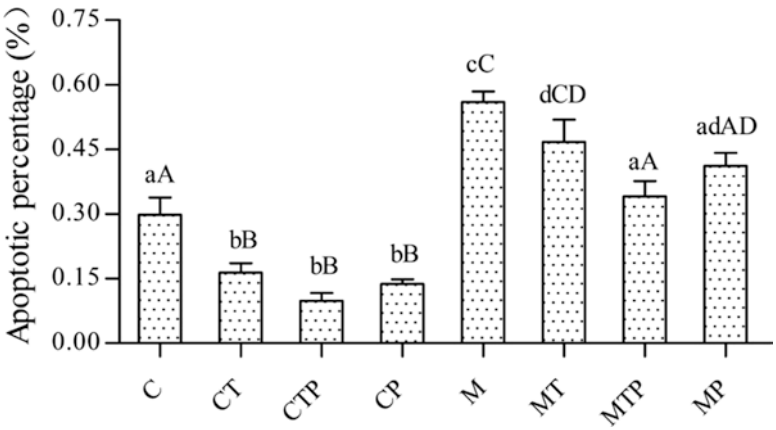

Fig. 3 Effect of taurine on the percentage of apoptosis in myocardial cells (%) Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

26.47% ($P < 0.01$) (group MP) respectively. Pre-treated taurine + PD150606 was significantly inhibited the cell apoptosis by 27.03% ($P < 0.01$) compared with taurine administration (group MT) and 17.21% ($P > 0.05$) compared with PD150606 (group MP) administration in hypoxia conditions. However, there was no significant difference between group MT and MP. These results implied taurine had the same effect as PD150606 in inhibiting myocardial cell apoptosis induced by hypoxia.

3.3 *The Effect of Taurine on the Protein Expression Levels of Calpain-1, Cytochrome c, Bcl-2, Procaspase-9 and Procaspase-3*

Figure 4 illustrated the protein level of calpain-1 under different treatments. Compared with the control group, the expression levels of calpain-1 in CT, CTP and CP groups were increased significantly ($P < 0.01$), but there was no significant difference between the treatment groups under normal conditions. The expression level of calpain-1 in M group were elevated significantly ($P < 0.01$) compared with the group C. Pre-treated taurine or PD150606 could significantly decrease the protein level of calpain-1 ($P < 0.01$), and there was no difference between group MT and group MP. The results indicated that calpain-1 was involved in the myocardial cell injury induced by hypoxia and taurine could down-regulate the calpain-1 protein expression against the injury. Besides, the inhibited effect of taurine was similar with the PD150606.

As shown in Fig. 5, compared with the control group, the expression levels of cytochrome c and procaspase-9 in model group (M) were increased significantly ($P < 0.01$), but the expression levels of Bcl-2 and procaspase-3 were decreased ($P < 0.01$). In contrast with the M group, the values in the group MT showed a significant decline in protein expressions of cytochrome c ($P < 0.01$), procaspase-9 ($P < 0.01$) and a notable elevation in the protein expression levels of Bcl-2 and

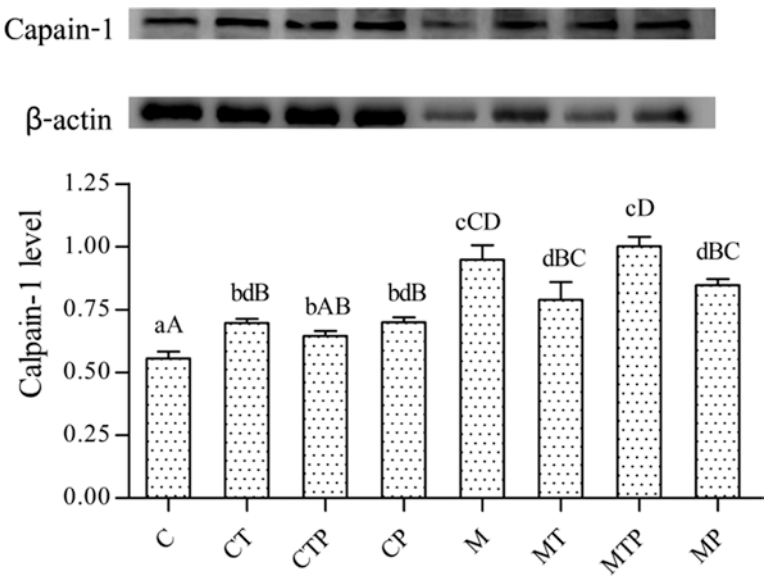

Fig. 4 The effect of taurine on the expression levels of calpain-1 in myocardial cells. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

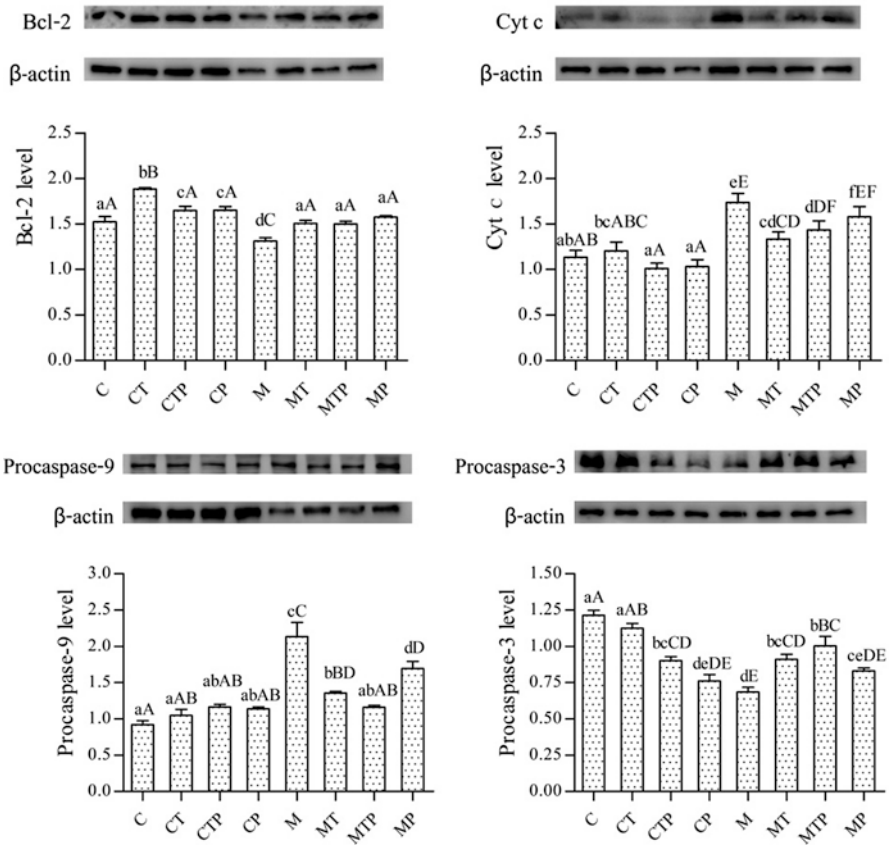

Fig. 5 The effect of taurine on the expression levels of cytochrome c, Bcl-2, procaspase-9 and procaspase-3 in myocardial cells. Data are the mean ± SEM (n = 3). The column with different lowercase letters on the top represent significant difference ($p < 0.05$), with different capitals represent significant difference ($P < 0.01$)

procaspase-3 ($P < 0.01$). The expression levels of the four proteins had similar changes in the MP group, while the values showed the regulation effect of pre-administrated PD150606 was lower than that of pre-administrated taurine on the cytochrome c ($P < 0.01$) and procaspase-9 ($P < 0.05$) expression. No significant difference was observed between MT and MTP groups on the four proteins, but obvious difference was found between MTP and MP groups. Compared with the MP group, the values in group MT showed significant decrease of the protein expressions of cytochrome c ($P < 0.05$) and procaspase-9 ($P < 0.01$) and a further increase of the protein level of procaspase-3 ($P < 0.01$). The results indicated that taurine had the similar regulation as PD150606 in hypoxia conditions.

4 Discussion

The major findings of the present study are as follows: (1) Taurine attenuated the myocardial cells apoptosis induced by hypoxia and exhibited the same inhibitory effect as PD150606. (2) Taurine inhibited the protein express of calpain-1 in hypoxic myocardial cells and the effect of inhibition was equal to the PD150606. (3) Pretreated taurine and/or PD150606 up-regulated the protein expressions of Bcl-2 and procaspase-3 and down-regulated protein levels of cytochrome c and procaspase-9 in hypoxic myocardial cells, the regulating effect of taurine on cytochrome c and procaspase-9 is more effective than that of PD150606.

Apoptosis, or programmed cell death, is an important way to maintain the cellular homeostasis between cell division and cell death. Myocardial cell is terminally differentiated cell, apoptosis could lead to a progressive reduction in overall heart function and possible heart failure (Gaballa and Goldman 2002). In modern broiler flocks, fast-growing broiler chickens are susceptible to heart disease, the right ventricular and heart failure was found easily in broilers with sudden death syndrome or ascites syndrome (Olkowski 2007). In this experiment, the apoptosis was significantly increased in chicken primary myocardial cells impaired by 12 h hypoxia. Taurine administration attenuated the myocardial cells apoptosis and reduced the calpain-1protein expression level under hypoxic condition. It also showed that taurine played the same effect of inhibition as PD150606. These results indicated calpain-1 involved the chicken primary myocardial cell injury induced by hypoxia, taurine played anti-apoptosis through inhibiting the protein express of calpain-1.

In this study, calpain-1 involved the chicken primary myocardial cell injury induced by hypoxia, which is consist with previous reports that activation and/or up-regulation expression of calpain-1 contributes to the cardiomyocytes injury under hypoxia/ischemic conditions (Kositprapa et al. 2000; Inserte et al. 2005; Li et al. 2009b; Zhang et al. 2015; Zheng et al. 2015). Taurine played the similar inhibition effect as PD150606 on the protein express of calpain-1. PD 150606 is a selective, cell-permeable, non-peptide and uncompetitive calpains inhibitor. It inhibits calpains by binding to the calcium-binding domain of the enzyme (Waters et al. 1997). As a calcium-dependent cysteine protease, the accumulation of intracellular Ca^{2+} could significantly increase apoptosis susceptibility by the activation of calpain-1 and unregulated over-activation of calpains led to the loss of Ca^{2+} homeostasis (Zatz and Starling 2005). Another researches reported calpains inhibits Na^{+}/K^{+} ATPase activity in the heart (Inserte et al. 2006) which may induce apoptosis via Ca^{2+} overload (Ramirez-Ortega et al. 2007). Numerous studies have demonstrated taurine could inhibit the increase of myocardial intracellular Ca^{2+} (Yang et al. 2013; Wang et al. 2018) and prevent the Ca^{2+} overload in cardiomyocytes under stress conditions (Xu et al. 2006).

It has been reported that mitochondria apoptosis pathway plays an important roles in the apoptosis of myocardial cells (Yang et al. 2013). Cytochrome c leads to caspase-3 activation which is an important mitochondrial apoptotic marker (Hu et al. 2018). Once cytochrome c was released into cytosol, cytochrome c, apoptosis activating factor-1 (Apaf-1) and procaspase-9 could form apoptosome complex in

the presence of dATP, and this complex could drive the activation of caspase-3. In the present study, we demonstrated that taurine prevented the hypoxia-induced apoptosis in cardiomyocytes, accompanied by the up-regulation of Bcl-2 and procaspase-3 protein expressions and down-regulation of cytochrome c and procaspase-9 protein levels. Taurine could effectively prevent myocardial ischemia-induced apoptosis by inhibiting the assembly of the Apaf-1/caspase-9 apoptosome and suppressing cleavage of caspase-9 and caspase-3 (Takatani et al. 2004). Our results also showed that taurine administration decreased the protein levels of cytochrome c and procaspase-9 under hypoxia-induced myocardial injury, which maybe reduce the form of cytochrome c/Apaf-1/procaspase-9 and inhibit the mitochondrial mediated apoptotic pathway. Bcl-2 family members could regulate apoptosis by modulating the release of cytochrome c from mitochondria to cytosol. In the study, taurine increased the Bcl-2 protein level under hypoxia. The increase of the Bcl-2 protein express could decrease the release of cytochrome c which could inhibit mitochondrial mediated apoptotic pathway.

5 Conclusion

In summary, this study shows that preventive administration of taurine effectively decreased hypoxia-induced cardiomyocyte apoptosis by inhibiting mitochondrial pathway activated by calpain-1 in chicken primary myocardial cells.

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Taurine Supplementation Ameliorates Arsenic-Induced Hepatotoxicity and Oxidative Stress in Mouse

Shuangxing Li, Bin Kai Wei, Jinhua Wang, Guangtao Dong, and Xiujie Wang

Abstract We previously reported that taurine treatment inhibited arsenic (As)-induced apoptosis in the liver of mice. This study was designed to explore the effect of taurine on liver function and its underlying mechanism in As-exposed mice. Mice were randomly divided into 3 groups, ten mice in each group. Group 1, control group, only orally received drinking water alone. Group 2, As intoxication group, was exposed to 4 mg/L As_2O_3 via drinking water for 60 days. Group 3, taurine protection group, was treated with 4 mg/L As_2O_3 and 150 mg/kg both. Taurine administration significantly revered the increases of alanine transaminase (ALT) and aspartate transaminase (AST) activities in serum. The decrease of glutathione (GSH) was inhibited with taurine treatment in the liver of As-exposed mice. At the same time, taurine significantly inhihibited As-induced enhancement of malondialdehyde (MDA) in the liver. Here we show that taurine protective effect on liver function in As-exposed mice maybe involve lipid peroxidation.

Keywords Taurine · Arsenic · Hepatoxicity · Oxidative stress

Abbreviations

ALT	alanine transaminase
As	arsenic
AST	aspartate transaminase
GSH	glutathione
MDA	malondialdehyde

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1 Introduction

Arsenic (As), a well known environmental toxicant, is widely distributed in the environment. As was found in food, water, ambient air and dust in organic form or inorganic form (Meliker et al. 2007; Das et al. 2010; Rodriguez-Lado et al. 2013). The inorganic form is a high risk factor for cancer of liver, skin, kidney, lung and bladder, and mediates injuries of a number of tissues, including the central nervous system, liver, and kidneys (Jain et al. 2011; Chen et al. 2011; Mathew et al. 2010; Anwar-Mohamed et al. 2012; Messarah et al. 2012). Human exposure to the toxicant is mainly through skin contact, contaminated drinking water or inhested food, industrial pollution inhalation, and interferes with all tissues and organ systems in body. There is increasing evidence that people around the world are at risk of chronic As toxic exposure and As poisoning has been a global health issue.

Epidemiological surveys show that chronic As exposure has a direct correlation with liver injury, including cirrhosis, fibrosis, and cancer (Bardach et al. 2015; Benbrahim-Tallaa et al. 2005). About 20 years ago, Ayala-Fierro et al. found that As compounds caused viability decline in primary rat hepatocyte in a time-dependent manner (Ayala-Fierro et al. 1999). Our group previously reported that As exposure was toxic to the liver by inducing disturbances in Bax, Bcl-2 and cytochrome c levels (Li et al. 2017). The hepatotoxicity of arsenic is considered a major hazard of As exposure.

Taurine, 2-aminoethanesulphonic acid, a sulfur-containing-β-amino acid, presents in many mammalian tissues being a major free intracellular amino acid (Batista et al. 2013). It was reported that taurine treatment relieved the harmful effects of hexavalent chromium and tamoxifen in mice (Bosgelmez et al. 2008; Tabassum et al. 2006), and lead, cadmium and copper and in rats (Hwang et al. 1998; Hwang and Wang 2001; Patrick 2006). It was also reported that taurine possesses several cytoprotective properties being as an intracellular calcium flux regulator, antioxidant and osmoregulator in hepatocyte (Das et al. 2010, 2012; Heidari et al. 2014). Warskulat et al. reported that in taurine transporter knockout mice, severe taurine limitation model, liver disease and apoptosis were induced (Warskulat et al. 2006, 2007). Taurine is considered an attractive candidate to preventing As-induced injury.

In the present study, the activities of alanine transaminase (ALT) and aspartate transaminase (AST) in serum were examined in As-exposed mice with or without taurine treatment. The level of glutathione (GSH) and malondialdehyde (MDA) in liver were also assessed in taurine/As mice. The aim of the study was to investigate the beneficial role of taurine on As-induced liver injury and its mechanism in mice.

2 Methods

2.1 Animal and Treatment

Thirty 19.2~24.7 g Kunming mice, male, were provided by the Experimental Animal Center, Dalian Medical University. The mice, five per cage, were kept in standard conditions, 12 h dark-light cycle (07:00–19:00) in 55% humidity, 20–24 °C with a standard diet and water ad libitum. After acclimation for 1 week, the animals were randomly divided into three groups, each of ten mice. Control group orally received ddH2O alone. As group orally received 4 mg/L As_2O_3 in drinking water for 60 days. Taurine groups received 4 mg/L As_2O_3 in drinking water and taurine (150 mg/kg) once daily by gavage for 60 days. All mice had free access to drinking water. All the experiments with animals were carried out according to the guidelines of the animal ethical committee of Dalian Medical University.

2.2 Liver Index Calculation

The liver index was assessed by the following formula: (liver weight/body weight) × 100%.

2.2.1 Liver Function Analysis

Blood were collected from the abdominal vena cava after anesthesia in EDTA-coated tubes and serum samples were prepared by centrifugation. To assess hepatotoxicity, serum levels of ALT and AST were examined by commercially kits according to the manufacturer's instructions.

2.3 GSH Level Measurement

GSH concentration were measured by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) method with commercially available kits according to the manufacturer's recommended protocol. The rate of formation of TNB was monitored and GSH levels were determined. Liver tissue from each mouse was excised rapidly, washed with PBS buffer, homogenized on ice using an Ultrathurax T25 Homogenisor, and then centrifuged at 1000 g for 10 min at 4 °C. Supernatant were used for the analysis of liver GSH.

2.4 MDA Level Assessment

The amount of MDA, an end product of lipid peroxidation, was examined by using a thiobarbiturate method with a commercial kit (Nanjing Jiancheng, China). Liver tissues from each mouse were washed with PBS, homogenized on ice, and then centrifuged at 3000 rpm for 10 min at 4 °C. Supernatant were used for the analysis of MDA in the liver. The levels of MDA were expressed as nmol/mg protein.

2.5 Statistic Analysis

Data were expressed asmeans ± SD and analyzed using the SPSS 11.0 statistical software. The comparisons between groups were analyzed using one-way ANOVA followed by LSK test, and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of Taurine Treatment on Liver Index

After 60 days experiment, all mice were weighed. Liver tissues were removed and weighed immediately after mice were sacrificed. The liver index was calculated accordingly (Table 1). There were no significant differences of liver indexes among the different groups, showing that no abnormal swelling was induced in liver.

3.2 Effect of Taurine Treatment on ALT and AST Activities

Serum ALT and AST, leaking from the damaged hepatocytes, are commonly used as biochemical indexes of the liver function. As shown in Fig. 1, the activities of AST and ALT in serum were increased in As group compared with control group, indicating liver dysfuntion in As group. In taurine protected group, ALT and AST activities were decreased compared to As group, indicating taurine could mitigate As-induced liver injury.

Table 1 Effect of taurine treatment on liver index of As-exposed mice

	Control group	As group	Taurine group
Liver index (%)	3.69 ± 0.41	4.21 ± 0.78	4.13 ± 0.55

Values were expressed as mean ± SD; n = 10 for each treatment group. Liver indexes were expressed as liver weight/body weight X 100

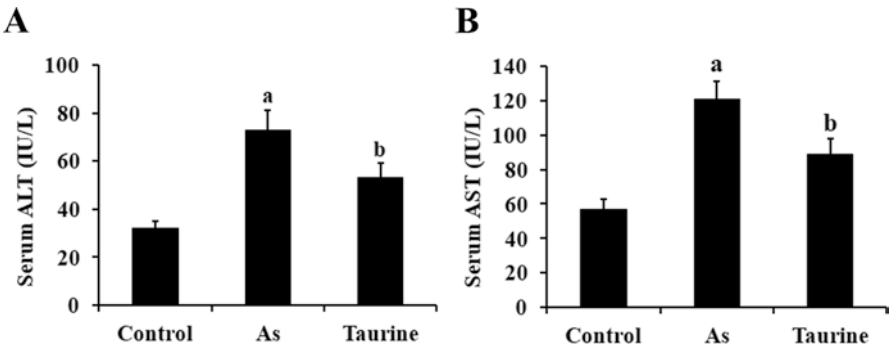

Fig. 1 Effect of taurine treatment on ALT (**a**) and AST (**b**) activities in serum
Data were presented as mean ± SD. $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with As group

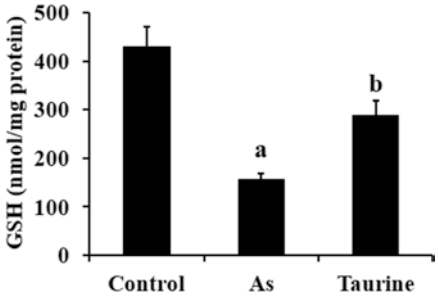

Fig. 2 Effect of taurine treatment on GSH level in liver
Data were presented as mean ± SD. $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with As group

3.3 Effect of Taurine Treatment on GSH

We assessed GSH level in liver with a commercial kit. Figure 2 shows that As exposure markedly reduced the level of GSH, whereas taurine treatment significantly opposed this reduction.

3.4 Effect of Taurine Treatment on MDA

Figure 3 shows the effect of taurine treatment on MDA level in liver. We found that As exposure markedly increased the level of MDA, while taurine treatment significantly attenuated the enhancement, indicating a beneficial role of taurine in lipid peroxidation.

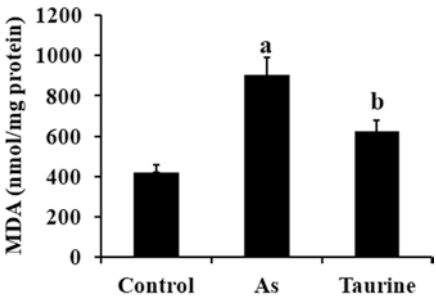

Fig. 3 Effect of taurine treatment on MDA level in liver
Data were presented as mean ± SD. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

4 Discussion

The present study explored the protective effects of taurine on As-induced liver injury and its underlying mechanisms in mice. The production of free radical and excessive oxidative stress play vital roles in the occurrence and development of the hepatotoxicity of toxicants (Zhang et al. 2014; Mershiba et al. 2013; Gao et al. 2013). In the livers of As-exposed mice, we found an increae in the end-product of lipid peroxidation MDA, as well as an induction of GSH level in liver. These findings indicate enhanced oxidative liver damage. Free radical-induced lipid peroxidation would result in cellular dysfunction. Conistent with this, ALT and AST levels in serum, markers for liver dysfunction, were markedly increased following As exposure, indicating As-related liver injury.

Taurine, a non-protein amino acid, is a conditionally essential amino acid which is one of the most abundant amino acid found in organs and tissues. An increasing number of evidence indicate that taurine possess protective properties owing to its antioxidant property (Higuchi et al. 2012; Budhram et al. 2013). Our results showed that taurine treatment inhibits As-induced increase of ALT and AST activities in serum, reverse the decrease of GSH level in liver and prevented the enhancement of MDA, despite that taurine has no significantly effect on liver index. It is suggest that taurine treatment has a protective effect on As-induced liver injury, which related with its antioxidant properties.

5 Conclusion

In summary, the present study shows that taurine treatment significantly inhibited the increases of ALT and AST activities in serum. Taurine also reversed As-induced disturbance of GSH and MDA in liver. Taurine has protective effect on liver function in As-exposed mice and its protective mechanism may involve lipid peroxidation.

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Taurine-Containing Hot Water Extract of *Octopus Ocellatus* Meat Prevents Methylglyoxal-Induced Vascular Damage

Seon-Heui Cha, Eui Jeong Han, Ginnae Ahn, and Hee-Sook Jun

Abstract Endothelial cell dysfunction (ECD) is a broad term, which implies dysregulation of endothelial cell functions. Several factors contribute to ECD including high blood pressure, high cholesterol levels, diabetes, obesity, hyperglycemia, and advanced glycation end products (AGEs). The highly reactive dicarbonyl methylglyoxal (MGO) is mainly formed as byproduct of glycolysis. Therefore, high blood glucose levels result in increased MGO accumulation. Taurine-rich foods are considered to protect against various diseases including vasculopathy and to exert antiaging effects. Here, we investigated the protective effect of hot water extract of *Octopus ocellatus* meat (OOM), which contains high amounts of taurine, on MGO-induced cell damage in human umbilical vein endothelial cells and zebrafish embryos. Hot water extract of OOMinhibited MGO-induced cytotoxicity and DNA damage, as well as AGEs accumulation. In addition, hot water extract of OOM protected against vascular damage in zebrafish embryos. These results suggest that hot water extract of OOM possesses protective activity against MGO-induced cytotoxicity in both umbilical vein endothelial cells and zebrafish embryos. Therefore, it could be used as a dietary source of an agent for the prevention of vascular diseases.

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Keywords Seaweed · *Octopus ocellatus* meat · *Octopus ocellatus* · Diabetic complication · Taurine · Vascular disease

Abbreviations

AGEs	advance glycation end products
ECD	endothelial cell dysfunction
EGFP	enhanced green fluorescent protein
HUVECs	human umbilical vein endothelial cells
MGO	methylglyoxal
OOM	Octopus ocellatus meat

1 Introduction

Diabetes mellitus is a characterized by hyperglycemia due to insufficient insulin, and is one of causal factor in the development of endothelial cell dysfunction (ECD). ECD is a broad term which comprises various vascular diseases, including impairment of endothelial cell barrier functions, vasodilation, proliferative capacities, tube formation properties, angiogenic properties, adhesion of white blood cells and diapedesis (Goligorsky 2005). In the diabetic condition, hyperglycemia causes increased protein glycation and the formation of advanced glycation end products (AGEs), which are generated by irreversible non-enzymatic reactions between carbohydrates and proteins. AGEs are increased in the extracellular matrix (Stitt et al. 1998; Horie et al. 1997) and can undergo auto-oxidation to generate other reactive intermediates, thereby resulting in vascular complications such as diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy (Fowler 2008).

Taurine is a sulfur-containing amino acid-like endogenous compound found in substantial amounts in mammalian tissues. Taurine plays an important role in biological and physiological functions, and taurine deficiency has been associated with various pathological conditions including cardiovascular regulation, antioxidation, diabetes and diabetic complications (Militante and Lombardini 2002; Militante et al. 2000; Hansen 2001; Jong et al. 2012). Thus, taurine-rich foods are considered to protect against various diseases including vasculopathy and to exert anti-aging effects. In particular, dietary intake of taurine enhances the population of endothelial cells in smokers (Moloney et al. 2003).

Octopus ocellatus is a marine mollusk that is widely consumed in Mediterranean, South American, and Asian countries as a food. Octopus ocellatus meat contains high amounts of taurine, however it is usually cooked at high temperatures. It is not known whether cooked *Octopus ocellatus* meat (OOM) might deliver enough taurine to ameliorate ECD. As a first step, we elucidated whether hot water extract of OOM prevents MGO-induced vascular damage.

2 Methods

2.1 *Preparation of Hot Water Extract of* Octopus ocellatus *Meat (OOM)*

Octopus ocellatus were purchased at a market and rinsed with freshwater to remove salt, epiphytes and sand. OOM (20 g) was solubilized with 1 L of distilled water for 4 h under continuous shaking at 100 °C, and then the extract was filtered through a Whatman No. 6 filter paper and freeze-dried. The extract was stored at −70 °C until further use and dissolved in cell culture media for cell treatment.

2.2 *Amino Acid Composition Analysis of Hot Water Extract of* Octopus ocellatus *Meat (OOM)*

The amino acid composition of hot water extract of OOM was analyzed using an amino acid analyzer (S433-H, Sykam GmbH, Germany). Hot water extract of OOM (50 mg) was hydrolyzed using 2 mL of 6.0 M HCl in a sealed vacuum ampule at 110 °C for 24 h. HCl was removed by a rotary evaporator and the final volume was adjusted to 10 mL with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated and detected using a cation separation column (LCA K06/Na, 4.6 × 150 mm) with a flow rate of 0.45 mL/min (buffer) and 0.25 mL/min (reagent) at wavelengths of 440 and 570 nm. For the determination of free amino acids, 2 g of OOM extract was homogenized at 12,000 rpm twice for 2 min with 75% ethanol, followed by centrifuging at 2000 × g for 30 min. The supernatant solvents were removed by using a rotary evaporator, and redissolved in 8.0 mL of distilled water containing 5-sulfosalicylic acid (0.2 g) at 4 °C for 1 h. Then, the mixture was centrifuged at 2000 × g for 30 min, and 2 mL of the supernatant was transferred to a new tube containing 1 mL of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were determined.

2.3 *Cell Culture*

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM (Welgene, Korea) supplemented with 10% FBS (Welgene, Korea), 100 U/ml penicillin and 100 μg/ml streptomycin (Welgene, Korea), and were maintained in a humidified incubator with 5% CO_2.

2.4 Assessment of Cell Viability

Cell viability was estimated using a cell counting kit (D-PlusTM CCK; Dongin LS, Korea) that measures mitochondrial dehydrogenase activity. For the D-PlusTM CCK assay, HUVECs (1×10^4 cells/well) were seeded onto 96-well plates. After 16 h, the cells were treated with hot water extract of OOM and/or methylglyoxal (MGO). We treated the cells with different concentrations of MGO (0.2, 0.4, 0.8, and 1 mM) for cell viability for 24 h and hot water extract of OOM (125, 250, 500, and 1000 μg/ml) for 24 h for toxicity. To study the protective effects of hot water extract of OOM, cells pretreated with vehicle (control) or 250 μg/ml hot water extract of OOM for 1 h, and subsequently incubated with or without 0.4 mM MGO for 24 h at 37 °C. The D-PlusTM CCK solution was then added to the wells to a total reaction volume of 110 μl. After 3 h of incubation, the absorbance was measured at a wavelength of 450 nm. The optical density of the formazan generated in the control cells was considered to represent 100% viability.

2.5 Determination of AGEs

HUVECs (5 × 105 cells/well) were seeded onto 6-well plates, and the cells were incubated with vehicle (control) or 250 μg/ml hot water extract of OOM for 1 h and then further incubated with or without 0.4 mM MGO for 24 h. The cells were incubated overnight in chloroform and methanol (2:1 v/v) mixture followed by homogenization in 0.1 N NaOH and centrifugation at 16,000 rpm for 15 min at 4 °C. The supernatant was analyzed for AGE content at an excitation/emission wavelength of 370/440 nm against 0.1 N NaOH blank on a spectofluorometer (Victor 3, Molecular Devices, San Jose, CA). A 0.1 mg/ml of BSA (bovine serum albumin, Sigma, St. Louis, MO) preparation in 0.1 N NaOH was used as a reference (arbitrary units/mg protein).

2.6 Assessment of DNA Strand Breaks

The alkaline comet assay for assessment of DNA strand breaks was conducted according to the method of Samarakoon et al. (2013). Briefly, the cell suspension was mixed with 75 μL of 0.5% low melting agarose, and added to slides pre-coated with 1% normal melting agarose. After solidification of the agarose, the slides were covered with another 100 μL of 0.5% low melting agarose, and then immersed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid, 10 mM Tris, and 1% sodium lauroylsarcosine; 1% Triton X-100 and 10% dimethyl sulfoxide) for 30 min at 4 °C. Slides were subjected to electrophoresis, stained DNA (20 μg/mL

ethidium bromide) was observed by fluorescence microscopy (BX-FLA; Olympus Optical Co. Ltd., Tokyo, Japan), and the image was analyzed using a Komet 5.0 imager (Liverpool, UK).

2.7 Treatment of Zebrafish Embryos with Hot Water Extract of OOM and Methylglyoxal

Transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) under the control of the flk1 promoter Tg(*flk1-egfp*) were obtained from Korean Zebrafish Organogenesis Mutant Bank and used in the experiment. Embryos approximately 3 days post-fertilization (n = 9~11) were transferred to a 24-well plate and maintained in 1 ml of embryo media. To determine the effect of hot water extract of OOM on vascularization, embryos were incubated with or without hot water extract of OOM (125, 250, 500 μg/ml) or MGO (0.5, 1, 1.5 mM) for 1 day. To determine the protective effect of hot water extract of OOM, embryos were incubated in the presence of hot water extract of OOM for 1 h prior to the addition of MGO (1 mM) for 12 h. After that, the embryos were fixed in 4% paraformaldehyde overnight at 4 °C and washed with PBS for 5 min at room temperature. After washing several times with PBS, the embryos stained with DAPI (Invitrogen, Carlsbed, CA) for 10 min, mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA), and observed with a confocal microscope (Zeiss, Germany). ImageJ software (https://imagej.nih.gov/ij/) was used to quantify the fluorescence. Zebrafish embryos were handled in accordance with the guidelines of Gachon University.

2.8 Measurement of Heart Rates

Zebrafish embryos were incubated with/without hot water extract of OOM and/or MGO, and heart rates were measured as an indicator of toxicity (Cha et al. 2017). Counting and recording of atrial and ventricular contractions were performed for 3 min under a microscope, and results were presented as the average heart rate per min.

2.9 Statistical Analysis

All measurements were carried out in triplicate and all values are represented as the mean ± S.E. The results were subjected to an analysis of variance (ANOVA) with the Two-way and Tukey test to analyze the differences.

3 Results

3.1 *Taurine-Containing Hot Water Extract of OOM Prevents MGO-Induced Toxicity in HUVECs*

Taurine is known for its cytoprotective ability in a variety of tissues (Ito et al. 2012). To determine whether hot water extract of OOM has taurine, we analyzed its amino acid content. Taurine accounted for 29.66% of the total amino acids, which was the highest portion of amino acids (Table 1). MGO is a highly reactive compound formed as a by-product of glycolysis. It can occur in high amounts in diabetic patients and can result in the formation of AGEs and reactive oxygen species (Desai et al. 2010). To examine whether hot water extract of OOM has a protective effect

Table 1 Free amino acid composition (%) of *Octopus ocellatus* meat (OOM) hot water extract

Amino acid name	% Amino acid
Taurine	29.66
Phosphoserine	0.23
Phosphoethanolamine	0.45
Aspartic acid	1.64
Threonine	0.79
Serine	0.87
Glutamic acid	4.10
Proline	1.40
Glycine	2.34
Alanine	4.41
Citrulline	2.85
α-Aminobutyric acid	0.12
Valine	4.70
Methionine	5.31
Isoleucine	4.45
Leucine	9.99
Tyrosine	4.99
Phenylalanine	5.50
β-Alanine	1.17
Histidine	0.33
Anserine	0.11
Tryptopan	0.00
Hydroxylysine	0.27
Ornitine	2.85
Lysine	9.01
Ethanolamine	0.04
Arginine	2.50
Total	**100.00**

on MGO-induced cytotoxicity, HUVECs were treated with either hot water extract of OOM or MGO alone or were pre-incubated with hot water extract of OOM for 1 h and then further incubated with MGO at various doses. Hot water extract of OOM alone did not show any cytotoxicity in HUVECs in the concentration range tested (125–1000 μg/ml) (Fig. 1a). A significantly lower cell viability was observed in HUVECs treated with MGO in a dose-dependent manner (Fig. 1b). Pretreatment with hot water extract of OOM in the presence of 0.4 mM MGO increased the cell viability similar to control (Fig. 1c). In addition, MGO treatment induced AGEs

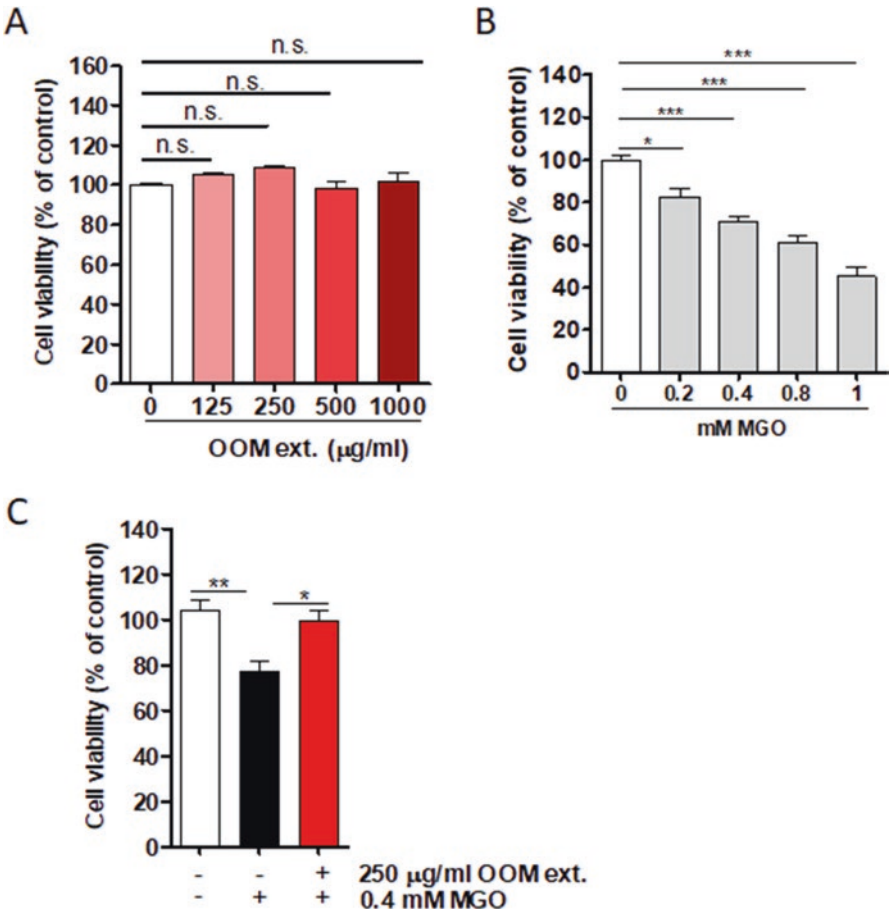

Fig. 1 Taurine-containing hot water extract of *Octopus ocellatus* meat (OOM) prevents MGO-induced toxicity in HUVECs. (**a**). HUVECs were incubated with the indicated concentrations of OOM extract (ext.) for 24 h. (**b**). HUVECs were incubated with the indicated concentrations of methylglyoxal (MGO) for 24 h. (**c**). HUVECs were incubated with 250 μg/ml OOM for 1 h and then further incubated with or without 0.4 mM MGO for 24 h. CCK-8 assays were subsequently performed. Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s. no significance

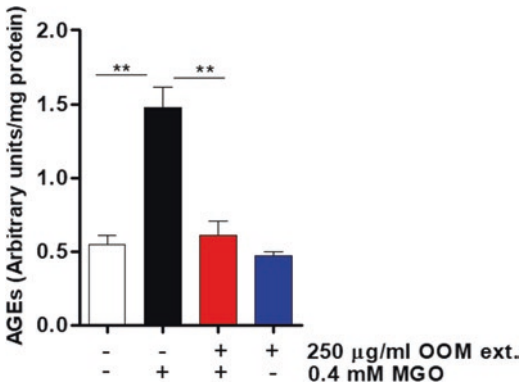

Fig. 2 Taurine-containing hot water extract of *Octopus ocellatus* meat (OOM) prevents MGO-induced AGEs accumulation in HUVECs. HUVECs were incubated with 250 μg/ml OOM extract (ext.) for 1 h and then further incubated with or without 0.4 mM methylglyoxal (MGO) for 24 h. AGEs was determined. Experiments were performed in triplicate. $**p < 0.01$

accumulation and pretreatment with hot water extract of OOM (Fig. 2) inhibited MGO-induced AGE accumulation, indicating that taurine-containing hot water extract of OOM possesses a cytoprotective effect against MGO-induced damage in HUVECs.

3.2 Taurine-Containing Hot Water Extract of OOM Prevents MGO-Induced DNA Damage in HUVECs

Taurine is a potent free radical scavenger that attenuates the damage caused by excessive oxygen free radicals (Sree and Sethupathy 2014). Therefore, we determined whether hot water extract of OOM can attenuate MGO-induced DNA damage. As expected, the DNA tail in the comet assay, an indicator of DNA fragmentation, was significantly increased by MGO treatment. Pretreatment with hot water extract of OOM significantly reduced the MGO-induced DNA tail (Fig. 3), suggesting that the hot water extract of OOM attenuates MGO-induced DNA damage.

3.3 Taurine-Containing Hot Water Extract of OOM Prevents MGO-Induced Vascular Damage in Zebrafish Embryo

Next, we determined whether hot water extract of OOM has a protective effect against MGO *in vivo*. We used transgenic zebrafish line where EGFP expression is controlled by the flk1 promoter, which results in endothelial-specific expression of EGFP. Incubation of embryos with hot water extract of OOM in the concentration

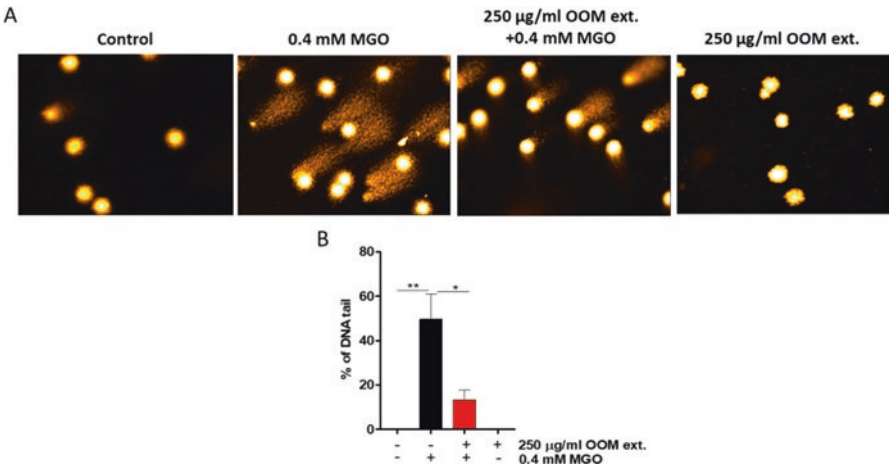

Fig. 3 Taurine-containing hot water extract of *Octopus ocellatus* meat (OOM) prevents MGO-induced DNA damage in HUVECs. HUVECs were incubated with 250 µg/ml OOM extract (ext.) for 1 h and then further incubated with or without 0.4 mM methylglyoxal (MGO) for 24 h. Thereafter, the cells were harvested in low melting point agarose. Comet assays were subsequently performed. (**a**). Image of DNA staining. (**b**). % of DNA tail from A. Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$

range tested (125–1000 µg/ml) for 24 h did not have toxic effects, as determined by heart rate measurements (data not shown), whereas heart rate was increased by MGO treatment in a dose-dependent manner (Fig. 4a) with reduction of EGFP expression (Fig. 4c). As expected, the elevated heart rate was partially rescued by hot water extract of OOM pretreatment (Fig. 4b), in addition, an increased intensity of EGFP was observed in hot water extract of OOM plus MGO-treated embryos compared with MGO (Fig. 4d). These data suggest that hot water extract of OOM protects against vascular damage in zebrafish embryos.

4 Discussion

Recent evidence indicates that reactive carbonyls including MGO cause diabetic complications including vasculopathy (Fukami et al. 2008; Singh et al. 2014; Ahmed 2005; Vulesevic et al. 2016). Therefore, attenuation of MGO toxicity is extremely important in treating diabetic vasculopathy. In the present study, we investigated the protective effects of hot water extract of OOM, which is rich in taurine, on MGO-induced vascular dysfunction both in HUVECs and in zebrafish embryos for the first time.

Previous studies suggested that reaction of MGO with amino acid leads to the production of free radicals (Kang 2003) and cytotoxicity due to DNA-protein cross-linkage (Brambilla et al. 1985). Our study demonstrated that MGO treatment caused cytotoxicity and DNA damage in HUVECs. Those effects were significantly

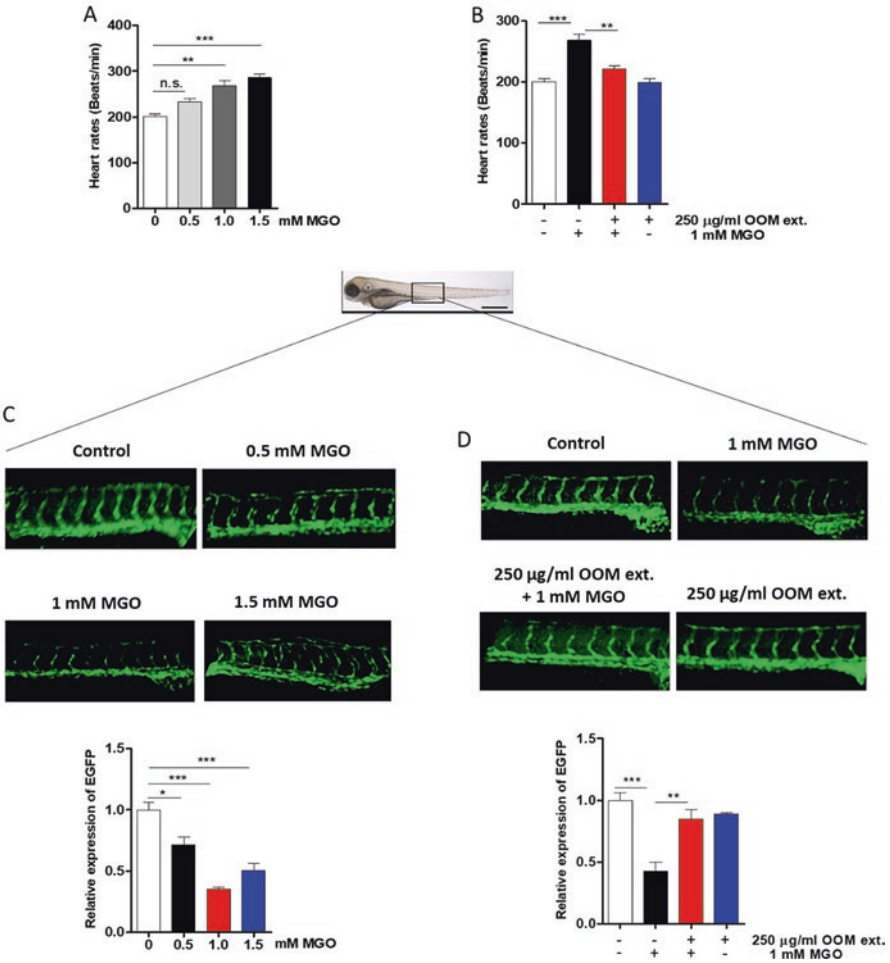

Fig. 4 Taurine-containing hot water extract of *Octopus ocellatus* meat prevents MGO-induced vascular damage in zebrafish embryo. (**a**). Embryos were incubated with the indicated concentrations of methylglyoxal (MGO) at 3 days post fertilization for 12 h. (**b**). Embryos were incubated with 250 μg/ml OOM ext. for 1 h and then further incubated with or without 1 mM MGO for 12 h. Heart rates of embryos were measured for 3 min. (**c**, **d**). Confocal microscopy images of zebrafish vessels. Scale bar: 100 **μ**m. (**c**). Embryos were incubated with the indicated concentrations of MGO at 3 days post fertilization for 12 h. (**d**). Embryos were incubated with 250 **μ**g/ml OOM extract (ext.) for 1 h and then further incubated with or without 1 mM MGO for 12 h. $n = 8$~12. $**p < 0.01$, $***p < 0.001$, n.s. no significance

attenuated by pretreatment with hot water extract of OOM. Interestingly, hot water extract of OOM pretreatment also protected MGO-induced vascular malformation in zebrafish embryos.

The activation of endothelial cells caused by proinflammatory and proapoptotic states leads to the inability of the endothelium to properly function (Xu and Zou 2009). In diabetes, endothelial cells are directly exposed to excessive blood glucose

levels, and hyperglycemia is a known causal factor to the loss of endothelial function (Hadi and Suwaidi 2007). Excessive blood glucose exposure can stimulate the generation of reactive oxygen species and MGO, leading to the formation of AGEs (Giacco and Brownlee 2010).

Taurine has been shown to be anti-diabetic and can reduce diabetic complications (Ito et al. 2012), particularly by inhibition of hyperglycemia-induced endothelial dysfunction by inhibiting AGEs production, oxidization of LDL, and scavenging malondialdehyde and hypochlorous acid. As well, taurine has been reported to exert detoxifying effects on MGO by increasing glyoxalase-1, which reduces MGO to D-lactate in the glyoxalase system and prevents MGO accumulation (Maclean et al. 2018). Treatment with hot water extract of OOM significantly decreased toxicity and DNA damage. This suggests that taurine obtained from a dietary source may provide a feasible means of maintaining endothelial cell function and thereby preventing vascular diseases in humans.

5 Conclusion

In conclusion, our study shows that high temperature extracts of OOM can effectively protect HUVECs from toxicity induced by MGO. Moreover, hot water extract of OOM attenuates DNA damage as well as AGEs accumulation in HUVECs and vascular malformation in zebrafish embryos caused by MGO treatment. These results suggest that hot water extract of OOM may be an ingredient for functional foods for diabetes and its vascular complications and a source of pharmaceutical agents for treating diabetes and its vascular complications.

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Taurine-Rich-Containing Hot Water Extract of *Loliolus Beka* Gray Meat Scavenges Palmitate-Induced Free Radicals and Protects Against DNA Damage in Insulin Secreting β-Cells

Seon-Heui Cha, Eui Jeong Han, Ginnae Ahn, and Hee-Sook Jun

Abstract The loss of pancreatic β-cells plays a central role in the pathogenesis of both type 1 and type 2 diabetes, and many studies have been focused on ways to improve glucose homeostasis by preserving, expanding and improving the function of β-cell. Elevated levels of free fatty acids such as palmitate might contribute to the loss of β-cells. A marine squid, *Loliolus beka* has long been used as a food in Korea, China, Japan and Europe due to its tender meat and high taurine content. Here, we investigated the protective effects of a hot water extract of *Loliolus beka* meat (LBM) against palmitate toxicity in Ins-1 cells, a rat β-cell line. Treatment with LBM extract protected against palmitate-induced cytotoxicity and scavenged overproduction of nitric oxide, alkyl, and hydroxyl radicals. In addition, LBM extract protected against palmitate-induced DNA damage and β-cell dysfunction. These findings suggest that LBM protects pancreatic β-cells from palmitate-induced damage. LBM could be a potential therapeutic functional food for diabetes.

Keywords Seaweed · *Loliolus beka* meat · *Loliolus beka* · Diabetes · Beta-cell · Taurine

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Abbreviations

DM diabetes mellitus
ROSs Reactive oxygen species

1 Introduction

It was estimated that 415 million people had diabetes mellitus (DM) in 2015, and that number is projected to rise to 642 million by 2040 (IDF diabetes atlas, 7th edition). DM is a group of chronic metabolic disorders characterized by a deficiency in circulating insulin levels, resulting in high blood sugar levels over a prolonged period. Insulin deficiency is caused by a reduction in the number of insulin-producing β-cells in both type 1 and type 2 diabetes (Weir and Bonner-Weir 2013). Thus, it is important to preserve the health of β-cells, and preventing β-cell degeneration is an essential approach in the treatment of DM.

Reactive oxygen species (ROSs) such as free radicals are essential signaling molecules that regulate physiological cell functions. They are continuously formed in aerobic living organisms by normal intracellular metabolism and by exogenous sources such as UV-radiation, redox-cycling drugs, carcinogenic compounds, and environmental factors. However, the overproduction of ROS in pathological conditions has deleterious consequences, causing DNA damage, injury and cell death. Oxidative stress caused by overproduction of ROS occurs in various tissues under diabetic conditions, and involved in the development of diabetes and its complications (Giacco and Brownlee 2010).

Palmitate is a potent inducer of ROS in pancreatic β cells (Sato et al. 2014). Indeed, the elevation of circulating free fatty acids (FFAs) including palmitate contributes to the pathogenesis of DM, and high concentrations of FFAs may impair insulin action and β-cell dysfunction (Giacca et al. 2011; Sato et al. 2014).

Taurine (2-aminoethanesulfonic acid) is a non-proteinogenic sulfur-containing amino acid and has been reported to have a cytoprotective effects (El-Sayed et al. 2011). Recently, the beneficial effects of taurine have emerged for the management of DM (Clark et al. 2017; Ito et al. 2012; Liu et al. 2017).

Loliolus beka is marine squid, whose meat is taurine-rich and widely consumed in Mediterranean, South American, and Asian countries. In a previous study, extract of L. beka meat (LBM) prepared at a moderate temperature evidenced a potent cellular protective effect, however, the taurine content was only about 7% when prepared in this manner (Han et al. 2017). The purpose of the present study was to investigate the effect of LBM extract prepared at high temperature, which is normally the case when it is prepared as food, on protecting against palmitate induced β-cell damage, which mimics exposure to high levels of fatty acids seen in DM.

2 Materials and Methods

2.1 Preparation of Hot Water Extract of Loliolus beka Meat (LBM)

Loliolus beka were purchased at a market, rinsed with freshwater to remove salt, epiphytes and sand. To prepare a hot water extract, 20 g of LBM was solubilized with 1 L of distilled water, for 4 h under continuous shaking at 100 °C, and then the extract was filtered through a Whatman No. 6 filter paper and freeze-dried. The extract was stored at −70 °C until further use and dissolved in cell culture media for cell treatment.

2.2 Cell Culture

A rat pancreatic β-cell line, Ins-1 cells, was cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and 55 μM β-mercaptoethanol, and was maintained in a humidified incubator with 5% CO_2.

2.3 Amino Acid Composition Analysis of LBM Extract

Amino acid composition of LBM extract was analyzed using an amino acid analyzer (S433-H, Sykam GmbH, Germany). LBM extract (50 mg) was hydrolyzed using 2 mL of 6.0 M HCl in a sealed vacuum ampule at 110 °C for 24 h. HCl was removed by rotary evaporator and the final volume was adjusted to 10 mL with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated and detected using a cation separation column (LCA K06/Na, 4.6 × 150 mm) with a flow rate of 0.45 mL/min (buffer) and 0.25 mL/min (reagent) at wavelengths of 440 and 570 nm. For the determination of free amino acids, 2 g of LBM extract was homogenized at 12,000 rpm twice for 2 min with 75% ethanol, followed by centrifuging at 2000 × g for 30 min. The supernatant solvents were removed by using a rotary evaporator, and redissolved in 8.0 mL of distilled water containing 5-sulfosalicylic acid (0.2 g) at 4 °C for 1 h. Then, the mixture was centrifuged at 2000 × g for 30 min, and 2 mL of the supernatant was transferred to a new tube containing 1 mL of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were determined.

2.4 Assessment of Cell Viability

Cell viability was estimated using a cell counting kit (D-PlusTM CCK; Dongin LS, Korea) that measures mitochondrial dehydrogenase activity. For the D-PlusTM CCK assay, Ins-1 cells (1×10^4 cells/well) were seeded onto 96-well plates. After 16 h, the cells were treated with LBM extract and/or palmitate (Sigma, St. Louis, MO). We treated the cells with different concentrations of palmitate (0.1, 0.2, 0.4, and 0.8 mM) for cell viability for 24 h and LBM extract (125, 250, 500, and 1000 μg/ml) for 24 h for toxicity. To study the protective effects of LBM extract, cells pretreated with vehicle (0.2% BSA) or 500 μg/ml LBM extract for 1 h, and subsequently incubated with or without 0.2 mM palmitate for 24 h at 37 °C. The D-PlusTM CCK solution was then added to the wells to a total reaction volume of 110 μl. After 2 h of incubation, the absorbance was measured at a wavelength of 450 nm. The optical density of the formazan generated in the control cells was considered to represent 100% viability.

2.5 Preparation of Colloidal $Fe(DETC)_2$

Sodium diethyldithiocarbamate (DETC, 4.5 mg, Sigma, St.Louis, MO) and $FeSO_4$ $7H_2O$ (2.8 mg, Daejung, Korea) were dissolved in two separate volumes (10 ml) of deoxygenated Krebs solution. Equal volumes of these parent solutions were rapidly mixed to form 0.5 mM $Fe(DETC)_2$ colloidal solution. No aggregate formation was observed at least during 30 min. The $Fe(DETC)_2$ colloidal solution was used immediately after the preparation.

2.6 Estimation of Intracellular Nitric Oxide, Alkyl Radical and Hydroxyl Radical Spectra

Ins-1 cells (0.5×10^5 cells/well) were seeded onto 48-well plates. The cells were treated with vehicle (0.2% BSA) or 500 μg/ml LBM, and 1 h later, 0.2 mM palmitate was added and the cells were incubated for 24 h. The cells were dissociated with trypsin and resuspended in PBS. Intracellular nitric oxide, alkyl and hydroxyl radicals were detected by the electron spin resonance spectroscopy. For nitric oxide, the dissociated cells were mixed with 200 μM colloid $Fe(DETC)^2$, incubated for 20 min at 37 °C in a water bath, and then transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3290 G, modulation frequency 100 kHz, modulation amplitude 5 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C. For alkyl radicals, the dissociated cells were mixed with 10 mM

4-α-(4-pyridyl N-oxide)-N-tert-butylnitrone (Sigma, St. Louis, MO), incubated for 30 min at 37 °C in a water bath, and then transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C. For, hydroxyl radicals, the dissociated cells were mixed with 50 mM α-phenyl-N-tert-butylnitrone incubated for 30 min at 37 °C in a water bath, and then transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3470 G, modulation frequency 100 kHz, modulation amplitude 0.5 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C.

2.7 Assessment of DNA Strand Breaks

The alkaline comet assay for assessment of DNA strand breaks was conducted according to the method of Samarakoon et al. (2013). Ins-1 cells (0.5×10^5 cells/well) were seeded onto 48-well plates. The cells were treated with vehicle (0.2%BSA) or 500 μg/ml LBM, and 1 h later, 0.2 mM palmitate was added and the cells were incubated for 24 h. The cells were dissociated with trypsin and the cell was mixed with 75 μL of 0.5% low melting agarose, and added to slides pre-coated with 1% normal melting agarose. After solidification of the agarose, the slides were covered with another 100 μL of 0.5% low melting agarose, and then immersed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid, 10 mM Tris, and 1% sodium lauroylsarcosine, 1% Triton X-100 and 10% dimethyl sulfoxide) for 30 min at 4 °C. Slides were subjected to electrophoresis, stained DNA (20 μg/mL ethidium bromide) was observed by fluorescence microscopy (BX-FLA; Olympus Optical Co. Ltd., Tokyo, Japan) and the image was analyzed using a Komet 5.0 imager (Liverpool, UK).

2.8 Measurement of Insulin Secretion

Ins-1 cells (1×10^5 cells/well) were plated on 24-well plates for insulin secretion measurements as previously described (Oh et al. 2011). Briefly, the cells were incubated with KRB buffer with 3 or 20 mM glucose for 2 h at 37 °C. The supernatants were collected, and released insulin was measured using an ELISA kit according to the manufacturer's protocol (ALPCO, Salem, NH). Insulin content was normalized to protein content, which was determined by a DC™ protein assay kit (Bio-rad, Hercules, CA).

2.9 Statistical Analysis

All measurements were carried out in triplicate and all values are represented as the mean ± S.E. The results were subjected to an analysis of variance (ANOVA) with the One-way test to analyze the differences.

3 Results

3.1 Hot Water Extract of LBM Prevents Palmitate-Induced Cytotoxicity in Ins1-Cells

First, to determine whether LBM extract has taurine, we analyzed the amino acid content. Taurine content was 38.22% of the total amino acids, which was the most prevalent amino acids (Table 1). Taurine is known as a cytoprotective agent in a

Table 1 Free amino acid composition (%) of LBM hot water extract

Amino acid name	% Amino acid
Taurine	38.22
Phosphoserine	0.29
Urea	10.75
Aspartic acid	0.98
Threonine	1.44
Serine	1.75
Glutamic acid	4.57
Proline	0.81
Glycine	0.86
Alanine	4.27
Citrulline	1.23
Valine	1.51
Methionine	1.36
Isoleucine	1.40
Leucine	3.28
Tyrosine	1.80
Phenylalanine	1.41
β-Alanine	1.81
γ-Amin-n-butyric acid	0.01
Histidine	0.52
Ornitine	0.94
Lysine	2.60
Arginine	17.58
Total	**100.00**

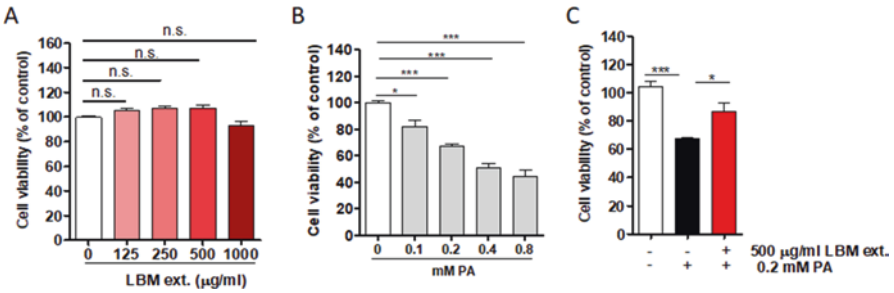

Fig. 1 Hot water extract of LBM prevents palmitate-induced cytotoxicity in Ins-1 cells. (**a**). Ins-1 cells were incubated with the indicated concentrations of LBM extract (ext.) for 24 h. (**b**). Ins-1 cells were incubated with the indicated concentrations of palmitate (PA) for 24 h. (**c**). Ins-1 cells were incubated with 500 μg/ml LBM ext. for 1 h and then further incubated with or without 0.2 mM PA for 24 h. CCK-8 assays were subsequently performed. Experiments were performed in triplicate. $*p < 0.05$, $***p < 0.001$, n.s. no significance

variety of tissues (Ito et al. 2012). To examine whether LBM or palmitate show cytotoxicity, Ins-1 cells were treated with various does of these compounds.

LBM extract alone did not show any cytotoxicity in Ins-1 cells in the concentration range tested (125–1000 μg/ml) (Fig. 1a). A significantly lower cell viability was observed in Ins-1 cells treated with palmitate in a dose-dependent manner (Fig. 1b). To examine whether LBM extract has a protective effect on palmitate-induced cytotoxicity, Ins-1 cells were preincubated with LBM extract for 1 h and then further incubated with palmitate for 24 h. Pretreatment with LBM extract increased the cell viability in the presence of 0.2 mM palmitate compared with cells treated with palmitate alone (Fig. 1c), indicating that the taurine-rich-containing LBM extract possesses a cytoprotective effect against palmitate-induced damage in Ins-1 cells.

3.2 Hot Water Extract of LBM Scavenges Palmitate-Induced Nitric Oxide, Alky, and Hydroxyl Radical in Ins-1 Cells

Taurine is a potent free radical scavenger (Sree and Sethupathy 2014) and palmitate induces reactive oxygen species such as free radicals (Sato et al. 2014). Therefore, to investigate whether LBM extract has scavenging effects on the palmitate-induced radicals production in Ins-1 cells, we measured nitric oxide, alky, and hydroxyl radicals in LBM extract-treated Ins-1 cells in the presence of palmitate. The intracellular nitric oxide (Fig. 2), alky (Fig. 3), and hydroxyl (Fig. 4) radicals spectra were elevated over controls by palmitate treatment, whereas LBM extract pretreatment of cells reduced this elevation. LBM extract alone did not affect radicals spectrum (data not shown). These results indicate that LBM extract prevents palmitate-induced radicals overproduction.

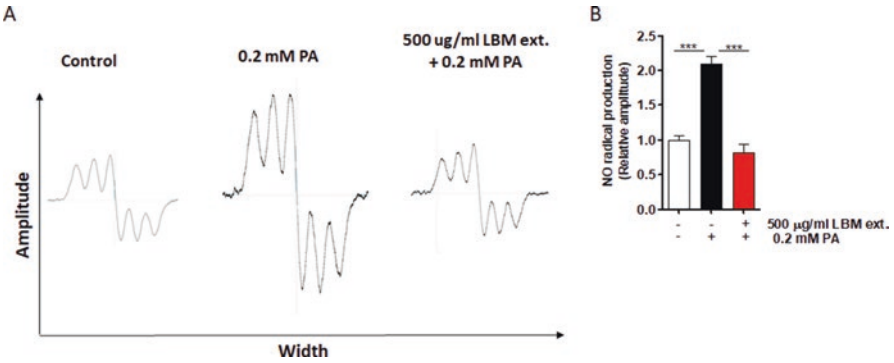

Fig. 2 Hot water extract of LBM scavenges palmitate-induced nitric oxide radicals in Ins-1 cells. Ins-1 cells were incubated with 500 μg/ml LBM extract for 1 h and then further incubated with or without 0.2 mM palmitate (PA) for 24 h. Thereafter, the cells were trypsinized and electron spin resonance assays for nitric oxide radical were subsequently performed. (**a**). Representative spectrum of nitric oxide radicals. (**b**). Mean values of relative amplitude. Experiments were performed in triplicate. ∗∗∗$p < 0.001$

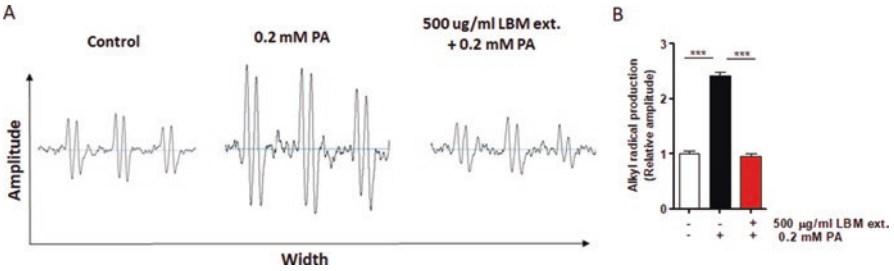

Fig. 3 Hot water extract of LBM scavenges palmitate-induced alky radicals in Ins-1 cells. Ins-1 cells were incubated with 500 μg/ml LBM extract for 1 h and then further incubated with or without 0.2 mM palmitate (PA) for 24 h. Thereafter, the cells were trypsinized and electron spin resonance assays for alky radicals were subsequently performed. (**a**). Representative spectrum of alkyl radicals. (**b**). Mean values of relative amplitude. Experiments were performed in triplicate. ∗∗∗$p < 0.001$

3.3 Hot Water Extract of LBM Prevents Palmitate-Induced DNA Damage in Ins1-Cells

Taurine attenuates the damage caused by excessive oxygen free radicals (Sree and Sethupathy 2014) and palmitate induced DNA damage (Giroud et al. 2009). We determined whether LBM extract can attenuate palmitate-induced DNA damage. As expected, the DNA tail (meaning that DNA fragmentation occurred) was significantly increased by palmitate treatment, whereas pretreatment with LBM extract significantly reduced the palmitate-induced DNA tail (Fig. 5), suggesting that the LBM extract attenuates palmitate-induced DNA damage.

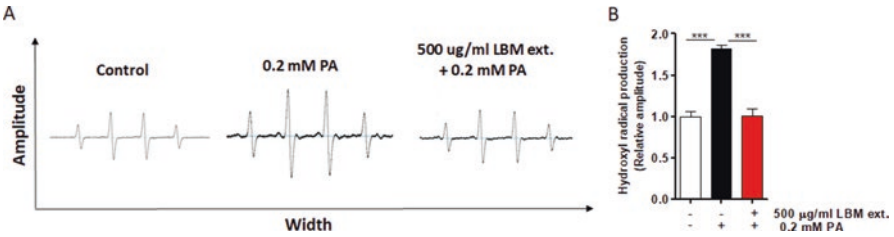

Fig. 4 Hot water extract of LBM scavenges palmitate-induced hydroxyl radicals in Ins-1 cells. Ins-1 cells were incubated with 500 µg/ml LBM extract for 1 h and then further incubated with or without 0.2 mM palmitate (PA) for 24 h. Thereafter, the cells were trypsinized and electron spin resonance assays for hydroxyl radicals were subsequently performed. (**a**). Representative spectrum of Hydroxyl radicals. (**b**). Mean values of relative amplitude. Experiments were performed in triplicate. ***p < 0.001

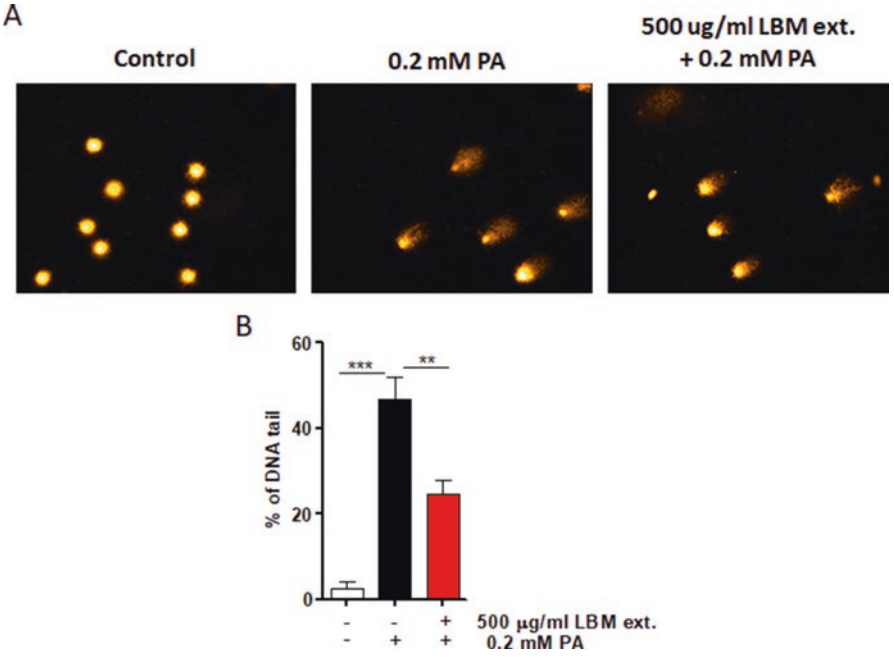

Fig. 5 Hot water extract of LBM protects palmitate-induced DNA damage in Ins1-cells. Ins-1 cells were incubated with 500 µg/ml LBM extract (ext.) for 1 h and then further incubated with or without 0.2 mM palmitate (PA) for 24 h. Thereafter, the cells were harvested in low melting point agarose. (**a**). Comet assays were subsequently performed and (**b**). quantified. Experiments were performed in triplicate. **p < 0.01, ***p < 0.001

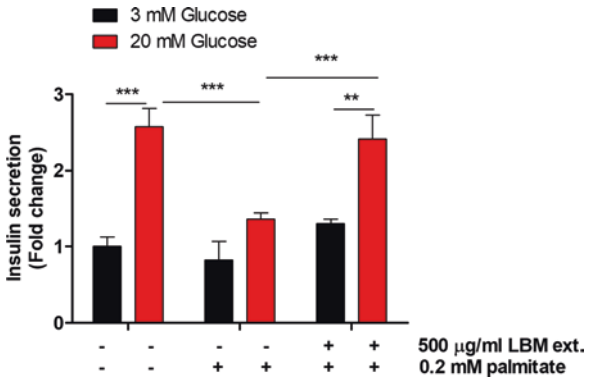

Fig. 6 Hot water extract of LBM protects against palmitate-induced dysfunction in Ins-1 cells. Ins-1 cells were incubated with 500 μg/ml LBM extract (ext.) in 5 mM glucose media for 1 h and then further incubated with or without 0.2 mM palmitate for 24 h. Thereafter, the cells were starved in 0.2 mM glucose-containing KRB buffer for 2 h. Insulin release was measured after 2 h of incubation in either 3 mM or 20 mM glucose. ELISA assays for insulin were subsequently performed. Experiments were performed in triplicate. $**p < 0.01$, $***p < 0.001$

3.4 Hot Water Extract of LBM Protects against Palmitate-Induced Dysfunction in Ins-1 Cells

To investigate whether LBM extract has protective effects on palmitate-induced β-cell dysfunction, we measured insulin secretion in LBM extract-treated Ins-1 cells in the presence of palmitate. Although palmitate had no effect on basal insulin secretion (3 mM glucose), insulin secretion stimulated by high glucose concentration (20 mM) was inhibited by treatment with palmitate. When Ins-1 cells were preincubated with LBM extract prior to palmitate treatment, the suppressed insulin secretion was restored to normal levels (Fig. 6), suggesting that the LBM extract showed the protective effects on inhibition of insulin secretion in the presence of palmitate in Ins-1 cells.

4 Discussion

Increased FFAs, alone or with hyperglycemia, have been shown to trigger the loss of β-cells in both type 1 and 2 diabetes (Leonardi et al. 2003; Shi et al. 2014). In addition, lipotoxicity induced by long-term elevated FFAs, especially saturated FFAs such as palmitate, leads to the β-cell dysfunction as a result of DNA damage (Lupi et al. 2002; Maedler et al. 2001).

The initial trigger whereby excess FFAs alter cellular function is the imbalance between NO and reactive oxygen species (ROS), leading to cell dysfunction (Creager et al. 2003). The toxicity of NO increases when it reacts with the superoxide radical, a kind of ROS, forming the highly reactive peroxynitrite anion ($ONOO\cdot-$) (Amaeze et al. 2011). Therefore, regulation of NO and ROS balance is important for the prevention of cell damage. As well, the highly reactive hydroxyl radical reacts with the heterocyclic DNA bases, consequently, leading to a toxic adduct radicals. There is mounting evidence for an important role of free radical-induced DNA damage in the etiology of numerous diseases including diabetes (Asmat et al. 2016).

Taurine has a beneficial effect on islet dysfunction induced by FFAs (Oprescu et al. 2007). These beneficial effects of taurine appear to be mostly based on its antioxidant activity (Xu et al. 2008), as well as on various protective effects against high glucose exposure in cultured beta cells and oxidative stress (Messina and Dawson 2000). However, it is not known whether hot water extract of taurine-rich food such as LBM might ameliorate diabetic related disease.

In agreement with previous studies (Hu et al. 2017; Luo et al. 2017), our results show that exposure to palmitate induced significant cell death with DNA damage in Ins-1 cells. In addition, palmitate treatment induced overproduction of free radicals in Ins-1 cells.

Taurine-rich-containing extract of LBM scavenged palmitate-induced production of radicals and significantly attenuated palmitate-induced cytotoxicity and DNA damage in Ins-1 cells, suggesting that treatment with taurine-rich-containing extract of LBM suppressed palmitate-induced oxidative stress. Further understanding of the mechanisms of free radical-induced DNA damage and repair by LBM extract will be of outmost importance for diabetes prevention and treatment.

5 Conclusion

In conclusion, our study shows that LBM extract can effectively protect insulin-secreting β-cells from toxicity induced by palmitate. Moreover, LBM extract improves insulin secretion in Ins-1 cells damaged by palmitate treatment. These results suggest that LBM extract may be an ingredient for functional foods for DM patients and a source of pharmaceutical agents for treating DM.

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The Potential Effects of Taurine in Mitigation of Radiation Nephropathy

Ning Ma, Toshihiro Kato, Tamami Isogai, Yeunhwa Gu, and Takenori Yamashita

Abstract Taurine (2-aminoethanesulfonic acid) is a sulfur-containing organic acid possessing several important effects, including antioxidant and anti-inflammatory ones. Exposure to ionizing radiation generates free radicals and reactive oxygen species (ROS) in irradiated cells, and free radical generation leads to oxidative stress. It is known that radiation nephropathy includes an inflammation-based process in which ROS and cytokines are responsible. Different doses of explored radiation can cause apoptosis, inflammation and a profound oxidative stress in kidneys. Oxidative stress is involved in renal injury after exposure to both ionizing radiation and inflammation. In this review, we describe the protective effect of taurine against several kidney diseases and the potential effects of taurine in the mitigation of radiation nephropathy. We also report that X-irradiation decreased the expression of taurine and TauT in the kidney. Taurine administration suppressed the decrease in the expression of taurine and TauT in the kidney after radiation exposure. Taurine might contribute to the mitigation of kidney injury induced by radiation.

Keywords Kidney · Radiation · Mitigation · Taurine transporter · Reactive oxygen species (ROS)

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Abbreviations

ROS reactive oxygen species
Tau taurine
TauT taurine transporter

1 Introduction

Exposure to ionizing radiation generates free radicals and ROS in irradiated cells, and free radical generation leads to oxidative stress. It has been reported that radiation exposure can induce kidney radiation-nephropathy at different doses of irradiation. It is known that radiation nephropathy is caused by chronic increases in ROS, and result in inflammatory reactions and fibrosis in the kidney (Zhao et al. 2007). Radiation kidney damage is involved transforming growth factor (TGF)-β, angiotensin II, ROS production and oxidative stress.

Taurine has protective effects on several kidney damages which were caused by cisplatin, 5-fluorouracil and diabetic. Taurine (2-amino-ethanesulfonic acid) is a sulfur-containing organic acid possessing several important effects, including antioxidant and anti-inflammatory functions. In this review, we describe the protective effect of taurine against several kidney diseases and the potential of taurine in mitigation of radiation nephropathy, including our findings.

2 Radiation-Induced Organ Damages

In recent years, incidence of cancer is increased in human beings. Treatment modalities of cancer are known such as surgery, chemotherapy and radiation therapy. Radiation therapy is an important component of cancer treatment (Baskar et al. 2012). It is known that ionizing radiation generates ROS and free radicals in cells. ROS and free radicals cause radiation-related injuries in tissues. The radiation therapy necessitates exposure of normal tissues and may induce side effects (Dörr 2010). It is known that exposure of normal tissues to radiation induces both early and late side effects. The early effect of radiation induces the loss of villus epithelial cells or crypt stem cells and lymphocytopenia, neutropenia and thrombocytopenia (Rosen et al. 2015; Dainiak 2002). The late effect of radiation induces resulting in chronic inflammation, organ dysfunction and fibrosis (Zhao et al. 2007). It is known that radiation nephropathy includes inflammatory reactions and fibrosis (Cohen and Robbins 2003). Those late effects are caused by chronic increases in ROS (Zhao et al. 2007). Exposure to ionizing radiation induces the functional changes in

mitochondria that are associated radiation-induced late effects (Datta et al. 2012). The functional changes in mitochondria after ionizing radiation exposure cause by increasing ROS production, and mitochondrial dysfunction causes persistent oxidative stress that may contribute to promotion of radiation-induced genomic instability (Yoshida et al. 2012; Leach et al. 2001; Kim et al. 2006). Chronic increases of ROS can lead to inflammation and oxidative stress in the kidneys (Zhao and Robbins 2009). Therefore, suppressing the ROS production might contribute to the mitigation of radiation nephropathy.

3 Protective Effect of Taurine Against Several Kidney Diseases

3.1 Cisplatin-Induced Nephrotoxicity

Cisplatin is one of the most effective chemotherapeutic agents used in the treatment for many solid tumors. Nabeel et al. (2018) have reported that both cisplatin and exposure to ionizing radiation induced kidney damage, and significantly decreased the expression of anti-apoptotic protein (Bcl-2) in kidneys. Cisplatin induces nephrotoxicity through increasing ROS formation, DNA oxidation and tumor necrosis factor-α (TNF-α). Moreover, caspase-3, p53 and factor-kappa B (NF-κB) were upregulated in the cisplatin induced kidney damage (Chtourou et al. 2016). In the cisplatin-induced acute nephrotoxicity, increased oxidative DNA damage and p53, decreased taurine transporter (TauT) were found in the kidneys. Moreover, taurine inhibited p53 activation and improved antioxidant activity in the cisplatin-induced nephrotoxicity (Tsunekawa et al. 2017).

3.2 5-Fluorouracil-Induced Nephrotoxicity

5-Fluorouracil (5-FU)-based chemotherapy is the most widely prescribed treatment for gastrointestinal solid tumors, but there are several drawbacks such as toxicities in kidney. 5-Fluorouracil increases creatinine, blood urea nitrogen and malondialdehyde levels in kidney tissues, while it reduces activities of catalase and superoxide dismutase. The mechanism of 5-Fluorouracil induced kidney toxicity is activating apoptotic pathway by up regulated caspase-3, p53 and Bax, and down regulated Bcl-2 (Rashid et al. 2014). Yousef and Aboelwafa (2017) tested protective effect of taurine against 5-Fluorouracil-induced nephrotoxicity in rats, and reported that taurine improved the progression of 5-fluorouracil-induced nephrotoxicity through antioxidant effects.

3.3 Taurine in Diabetic Kidney Injury

The diabetes-induced complications are one of the major causes of morbidity and mortality for patients worldwide, and diabetic nephropathy is the common microvascular complication of diabetes (Yang et al. 2018). In diabetes, high extracellular levels of glucose lead to intracellular accumulation of sorbitol, and is most likely to cause depletion of intracellular compounds including taurine (Li et al. 2005). A decrease of taurine level in diabetic patients may be involved in the diabetic complications (Ito et al. 2012). Das and Sil (2012) reported that Taurine ameliorate alloxan induced oxidative stress and intrinsic apoptotic pathway in the hepatic tissue of diabetic rats after taurine administration for 3 weeks (1% w/v in drinking water). Taurine administration also decreased the levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β). Moreover, taurine reduced kidney oxidative stress, improved kidney function and protected kidney tissue from apoptosis *via* the regulation of caspase-9/3 proteins and Bcl-2 family. Pandya et al. (2017) reported that taurine protected kidney tissue against diabetes-induced nephrotoxicity. Trachtman et al. (1995) have shown that administration of taurine to rats with STZ-diabetes ameliorates diabetic nephropathy. Taurine reduces kidney oxidant injury by decreasing lipid peroxidation within the kidney. Koh et al. (2014) have shown that taurine improves the progression of diabetic nephropathy through anti-fibrotic and antioxidant effects in Otsuka Long-Evans Tokushima Fatty (OLETF) rat.

4 Mitigation Effect of Taurine Against Radiation Nephropathy

It is known that radiation nephropathy includes inflammatory reactions and fibrosis (Cohen and Robbins 2003). TGF-β, angiotensin II, ROS production and oxidative stress are involved kidney damage, including radiation nephropathy (Yoshida et al. 2012; Sánchez-López et al. 2009; Kim et al. 2006; Robbins et al. 2002; Leach et al. 2001). TGF-β is considered as the key regulator of kidney fibrosis (Kaneto et al. 1993; Quesada et al. 2018). Many agents that mitigates radiation fibrosis directly or indirectly inhibit the TGF-β signaling pathway (Citrin et al. 2010). Robb et al. (2010) demonstrated that taurine administration prevented serum TGF-β1 levels upregulation after radiation exposure.

Inhibition of angiotensin-converting enzyme or blockade of angiotensin II receptors can prevent the structural and functional changes that occur after kidney irradiation (Lenarczyk et al. 2009; Cohen et al. 2002). Robbins et al. (2002) reported that angiotensin II receptor blocker markedly abrogates the severity of radiation nephropathy in experimental models (Robbins et al. 2002). It is reported that taurine reduces the actions of angiotensin II on angiotensin II signaling, Ca^{2+} transport and protein synthesis (Schaffer et al. 2000). ROS production and oxidative stress induces radiation nephropathy. Although taurine is incapable of directly scavenging the

classic ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, numerous studies suggest that taurine is an effective inhibitor of ROS generation (Cetiner et al. 2005; Hansen 2001). Taurine is also a regulator of mitochondrial protein synthesis that protects the mitochondria against excessive superoxide generation (Jong et al. 2012). Schaffer et al. (2009) reported that taurine may inhibit the ROS production *via* regulation of mitochondria. This effect is associated with various pathways in radiation nephropathy.

Research on the protective effects of taurine against biological damage induced by whole body irradiation has been carried out since the 1960s (Sugahara et al. 1969). Administration of taurine increased the survival of mice after whole body irradiation (Abe et al. 1968). Taurine is taken up by tissues *via* TauT (Kwon and Handler 1995). We reported that the expression of TauT in epithelial cells of intestine was reduced after high-dose radiation exposure, and resulted in taurine depletion after irradiation (Yamashita et al. 2017). Exposure to ionizing radiation causes several injuries depending on the dose of radiation received. It is known that exposure to ionizing radiation at high dose (15–20 Gy) could cause kidney damage (Kucuktulu. 2012). Moulder and Cohen (2014) reported that exposure to radiation at low dose (4–5 Gy) could cause significant kidney damage as well.

We tested the taurine administration on kidney injuries of mice after 4.5 Gy whole body X-irradiation. The distribution of taurine and TauT in the kidney of mice after radiation exposure is shown in Figs. 1 and 2. X-irradiation decreased the expression of taurine and TauT in the kidney, and taurine administration suppressed the decrease in the expression of taurine and TauT in the kidney after radiation

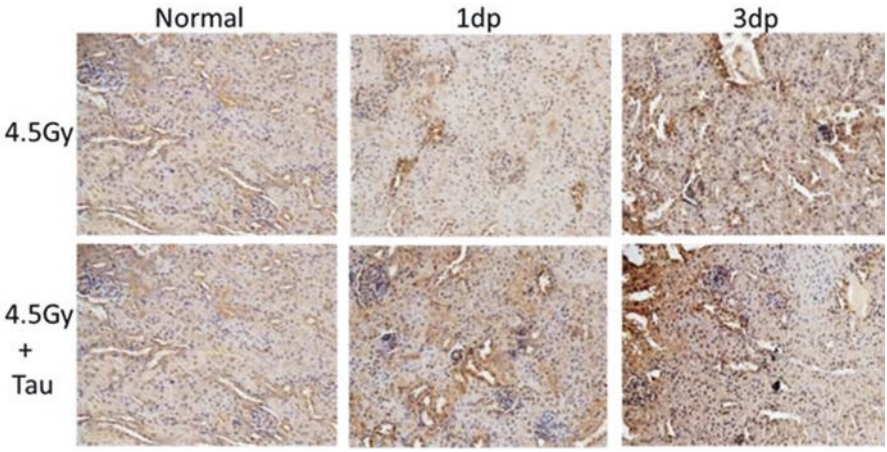

Fig. 1 Taurine distribution in the mice kidney after 4.5 Gy whole body X-irradiation. 1500 mg/kg b.w. per day of taurine was given orally by dissolving it in the drinking water to 4.5 Gy + Tau group. Taurine was administered 30 min after irradiation. The expression of taurine in the kidney of 4.5 Gy group decreased more than that of normal kidney at day 1 (1dp) and day 3 (3dp) after irradiation. The groups of taurine administration suppressed the decrease in the expression of taurine in the kidney of mouse after radiation exposure. (Magnification 200×)

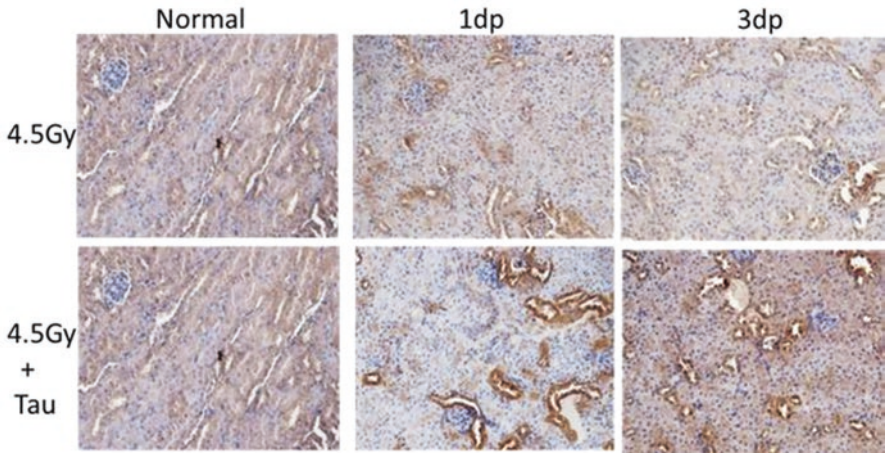

Fig. 2 TauT distribution in the mice of kidney after 4.5 Gy whole body X-irradiation. Figure showed representative TauT & hematoxylin staining of kidney sections. 1500 mg/kg b.w. per day of taurine was given orally by dissolving it in the drinking water to 4.5 Gy + Tau group. Taurine was administered 30 min after irradiation. The expression of TauT in the kidney of 4.5 Gy group decreased more than that of normal kidney day 1 (1dp) and day 3 (3dp) after irradiation. The groups of taurine administration suppressed the decrease of TauT expression in the kidney of mice after radiation exposure. (Magnification 200×)

exposure. A taurine depletion causes a decreased taurine-modified tRNA, which might impair electron transport capacity (Kirino et al. 2004). Further, taurine depletion in kidney may be linked to the functional changes in mitochondria. Therefore, taurine administration after irradiation might contribute to the mitigation of radiation nephropathy. This study suggests that taurine administration after radiation exposure compensate for taurine losses from cells *via* TauT. Taurine administration might contribute to mitigation of radiation-induced kidney injury by suppressing mitochondrial dysfunction and ROS. The result of this study also suggests that the increased expression of TauT after taurine administration plays a role in the mitigation of radiation-induced kidney injury.

5 Conclusion

ROS production, oxidative stress, TGF-β and angiotensin II are involved radiation nephropathy. Taurine may reduce the effect of angiotensin II and inhibit the TGF-β signaling pathway. Moreover, taurine may inhibit the ROS production *via* regulation of mitochondria function (Schaffer et al. 2000, 2009). We found that X-irradiation decreased the expression of taurine and TauT in the kidney, and taurine administration suppressed the decrease of taurine and TauT expression in the kidney after radiation exposure. Thus, taurine might contribute to mitigation of

radiation induced kidney injury. As described in this review, taurine may play a critical role in the mitigation of radiation-induced kidney injury. Taurine supplementation would be expected to provide a novel therapeutic strategy for patients who have been exposed to high or low doses of ionizing radiation.

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Taurine Increases Spermatozoa Quality and Function in Asthenospermia Rats Impaired by Ornidazole

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Abstract Asthenospermia has been considered as one of the crucial causes of male infertility, which was closely related to epididymal dysfunction. Lots of documents have revealed that taurine palys an important role in male reproduction, including antioxidation, membrane stabilization, stimulation of sexual hormone secretion and elevation of sperm quality. The objective of this study was to expose the effect of taurine on spermatozoa quality and function in ornidazole-induced asthenospermia rats. We found that taurine treatment could obviously recover the decline of cauda epididymal sperm count, viability and motility, and the elevation of sperm abnormality in asthenospermia animals. Spermatozoa acrosin, LDH-X, SDH and CCO activities of model rats also were notably increased by taurine administration. The present data indicated that taurine could raise spermatozoa quality and function by elevating mitochondrial energy metabolism. Notably, taurine supplementation markedly raised serum GnRH, LH and T levels in asthenospermia rays, suggesting taurine rescued asthenosperm by means of stimulating hypothalamic-pituitary-testicular axis secretion. We also found that concentrations of asthenospermia epididymal carnitine, SA, α-Glu and ACP, and mRNA expression levels of MMP7 and IDO2 were significantly rised by taurine administration, indicating taurine may protect epididymal epithelium structure, improve secretion activity, and maintain intraluminal microenvironment homeostasis. Finally, the present results showed taurine effectively increased cauda epididymal SOD, GSH and γ-GT levels in model rats, reduced ROS and MDA production, suggesting epididymal antioxidant ability of asthenospermia rats could be elevated by taurine treatment. To sum up, our results indicated that taurine can promote spermatozoa quality and function in

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ornidazole-induced asthenospermia rats by facilitating epididymal epithelium secretion and luminal microenvironment homeostasis.

Keywords Taurine · Spermatozoa quality and function · Sexual hormone · Epididymal function · Ornidazole-induced asthenospermia rats

Abbreviations

ACP	acid phosphatase
CCO	cytochrome c oxidase
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GSH	reduced glutathione hormone
IDO2	indoleamine 2, 3-dioxygenase
LDH-X	lactate dehydrogenase X
LH	luteinizing hormone
MDA	malondialdehyde
MMP7	matrix metalloproteinase 7
ROS	reactive oxygen species
SA	sialic acid
SDH	sorbitol dehydrogenase
SOD	superoxide dismutase
T	testosterone
α-Glu	α-glucosidase
γ-GT	γ-glutamyl-transferase

1 Introduction

It has been reported that infertility affects approximately one-seventh couples who prepare to conceive, and the male infertility factors account for around 50% in all infertility cases, which means that approximately 7% of all men have fertility problems (Forti and Krausz 1998). The most frequent etiologies of male infertility are oligospermia which means low sperm concentration, and asthenospermia that means reduced morphologically normal sperm and motile perm (Curi et al. 2003). Oligospermia and asthenospermia frequently occur in combination known as oligoasthenozoospermia (Irvine 1998). While a large number of studies have demonstrated that the pathogenic causes of oligospermia, asthenospermia and oligoasthenozoospermia are various, including male reproductive endocrine system dysfunctions, chromosome abnormalities, environmental factors, drugs,

infection- and immune- related factors (Krausz 2011), the mechanism have not been defined. Oxidative stress has recently been recognized as playing important pathophysiological roles in oligoasthenozoospermia (Agarwal et al. 2014a), the effect of oxidative stress on endocrine dysfunction of testis and epididymis may exacerbate low semen quality (Zhang et al. 2014). It has been detected that the level of seminal plasma reactive oxygen species (ROS, markers of oxidative stress) elevated (Iwasaki and Gagnon 1992), and ROS-mediated sperm damage increased in infertile patients (Aitken 1995; Agarwal et al. 1994). Further studies have shown that, as lacking of intracellular antioxidants, sperm were highly susceptible to oxidative attack, and easy to happen plasma membrane lipid peroxidation, which resulted in damage of sperm motility and viability (Gharagozloo and Aitken 2011). Therefore, researchers make extensive efforts to find antioxidants used to intervene and therapy male infertility, and a number of antioxidants, such as vitamin C/D and zinc, have been identified and used in clinical practice, although its exact efficacy and mechanism were not yet proven (Ajina et al. 2017; Kobori et al. 2014).

Taurine (2-aminoethane sulfonic acid), a conditionally essential amino acid, plays a wide range of biological functions in various organs of human and animals. It has been revealed that taurine is aboundant in testis, epididymis, seminal fluid and spermatozoon (Holmes et al. 1992; Hinton 1990), and can be synthesized in male reproductive organs (Yang et al. 2010b), suggesting that taurine might involve in testicular spermatogenesis, epididymal spermioteleosis and normal sperm function. Furthermore, recent studies have demonstrated that taurine may participate in spermatogenesis via increasing germ cells' antioxidant ability (Higuchi et al. 2012). In addition, it has been proved that taurine may stimulate sperm motility, capacitation, and acrosome actions in mammals (Meizel et al. 1980), sustain hamster sperm motility and fertility by inhibiting Na^+, K^+-ATPase activity (Engström et al. 1985). In the following Studies of Alvare and Storey demonstrated that loss of forward motility of rabbit epididymal sperm can be inhibited with taurine treatment by means of decreasing lipid peroxidation (Alvarez and Storey 1983). Our previous studies also have confirmed that taurine can stimulate male reproductive hormone secretion, elevate sperm quality in aged rats by inhibiting oxidative stress and apoptosis (Yang et al. 2010a, 2015), and can rescue steroidogenesis and spermatogenesis in diabetic rats though increasing reproductive system endocrine activity (Liu et al. 2017a). Further, there were substantial researches showed that taurine can elevate cytoprotection against oxidative stress by means of inhibiting mitochondria producing excessive ROS, reducing the lipid peroxidation, which suggested that taurine may act as ROS scavenger (Murakami et al. 2018; Alam et al. 2011; Shimada et al. 2015). But until now, there are little information on the effects of taurine on oligoasthenozoospermia. The aim of this study was to investigate the potential beneficial influence of taurine on rats with asthenospermia.

2 Materials and Methods

2.1 Experimental Animals and Treatments

A total of forty healthy adult male Sprague-Dawley rats (180–200 g) were purchased from the experimental animal center of Chinese Medical University, and were housed at the barrier environment facilities of Shenyang Agricultural University which was maintained under a 12 h light/12 h dark cycle, a temperature of 22 ± 2 °C and 40–70% relative humidity. After 1 week of adaptation, all animals were randomly divided into 4 groups, ten in each group. The control group (Con) rats were intragastrically treated with vehicle (0.5% carboxymethylcellulose solution) once a day and drank tap water (Zhang et al. 2009), the model group (M) rats were intragastrically treated with ornidazole suspension(200 mg/kg/d) and drank tap water, the taurine preventive group (M + Tau) rats were intragastricly treated with ornidazole suspension (400 mg/kg/d) and drank 2% taurine water, and the taurine group (Tau) rats were intragastricly administrated with vehicle and drank 2% taurine water. Rats were supplied with a commercial basal diet and water ad libitum. After being treatment for 20 days, the rats were anesthetized sacrificed to collect blood and epididymis. Blood samples were used for serum biochemical assay. The left cauda epididymis were prepared for sperm quality evaluation and sperm function biochemical assay. The part of right cauda epididymis were homogenized in cold PBS buffer and used for epididymal biochemical analyzation, the other part of right cauda epididymis were stored at −80 °C for total RNA extraction and real-time PCR. All the experimental protocols were approved by Shenyang Agricultural University Ethical Committee, and animals were deeply concerned with animal welfare.

2.2 Sperm Quality and Function Markers Analysis

Caudal epididymides were carefully separated from the testis and minced in 1 ml of the 37 °C PBS buffer at 10 min. The sperm suspension was detected sperm count, sperm viability, sperm motility and sperm abnormality according to the previous methods (Linder et al. 1995). Meanwhile, the activities of acrosin, lactate dehydrogenase X (LDH-X), sorbitol dehydrogenase (SDH) and cytochrome c oxidase (CCO) were measured by ELISA as per the manufacturer's (Nanjing Jiancheng Bioengineering Institute (NJJCBIO), China) protocols.

2.3 Serum Reproductive Hormone Assay

The levels of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) were determined by the means of ELISA kits of Beijing north Institute of biological technology, China.

2.4 Epididymal Biochemical Detection

Epididymal reactive oxygen species(ROS), superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione hormone (GSH), γ-glutamyl-transferase (γ-GT), carnitine, Ca^{2+}, sialic acid (SA), α-glucosidase (α-Glu) and acid phosphatase (ACP) levels were analyzed according to the protocols of respective reagent kit (NJJCBIO, China).

2.5 Epididymal MMP7 and IDO2 mRNA Real-Time RT-PCR Analyses

Total RNA was extracted from the epididymis, and was quantified by UV spectrophotometry. cDNA was reverse transcripted using AMV First Strand cDNA Synthesis Kit (Sangon, China), and specifc amplified as a template on a Bio-Rad iQTM5 system using SYBR Green PCR Master Mix (ABI, USA). The primers were designed for the objective genes: matrix metalloproteinase 7 (MMP7) forward primer: 5'-GCCAGGGAACACTCTAGGTCATGC-3′, reverse primer: 5'-TGCGTCCTCACCATCAGTCCAGTA-3′; indoleamine 2, 3-dioxygenase (IDO2) forward primer: 5′- CACAGAGCCTCTGAAGTACTC -3′, reverse primer: 5′- CTAAGCACCAGGACACAGGA'; and β-actin forward primer: 5'-TCGTGCGTGACATTAAAGAG-3′, reverse primer: 5'-ATTGCCGATAGTGATGACCT-3′. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of β-actin mRNA which served as an internal control in the same sample (Schmittgen and Livak 2008). Results were expressed as relative fold change according to the control group.

2.6 Statistical Analysis

The data were presented as mean ± SE. One-way ANOVA and Duncan's multiple range test were analyzed the differences among all groups by SPSS 16.0 software. Differences at p values less than 0.05 were considered statistically significant.

3 Results

3.1 Sperm Quality and Function Markers

Figure 1 showed the effect of taurine on sperm quality and function markers in ornidazole-induced asthenospermia rats. The results illustrated that the sperm viability, motility and abnormality in asthenospermia model rats were obviously affected compared with the control ($P < 0.05$), among which, sperm viability and motility were significantly decreased ($P < 0.05$), while the sperm abnormality was remarkably increased ($P < 0.05$). Taurine treatment could significantly restore the index associated with sperm quality of ornidazole-treated model rats nearly to the normal levels ($P > 0.05$). Although, sperm motility in taurine group was notably higher than the control group ($P < 0.05$), there were no statistical differences in 3 other sperm quality parameters ($P > 0.05$). There were no difference in acrosin and LDH-X levels ($P > 0.05$) between the wild type and model rats, while taurine administration in normal rats could significantly elevate acrosin activity ($P < 0.05$). The activities of SDH and CCO were statistically decreased in ornidazole-treated rats compared with the control rats ($P < 0.05$), but taurine treatment could obviously inhibit the decline of the two parameters ($P < 0.05$).

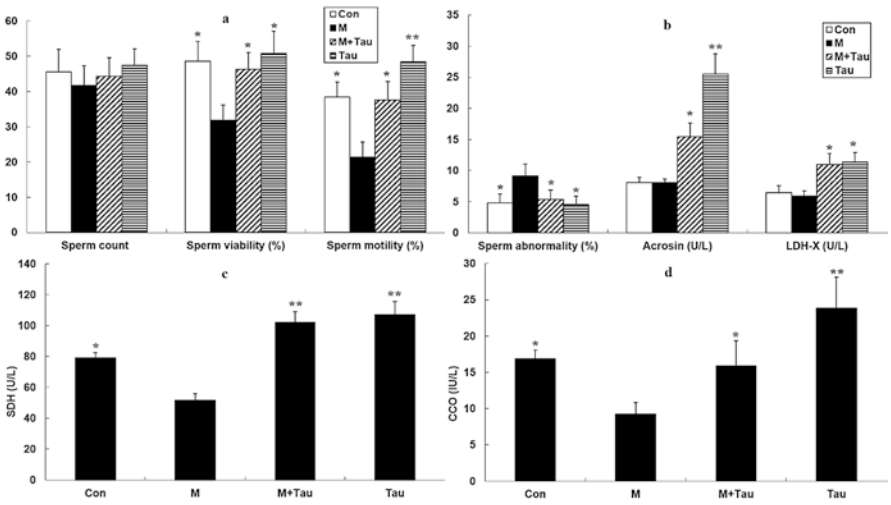

Fig. 1 Taurine increased spermatozoa quality and function markers levels in asthenospermia rats. The values were presented as means ± SE (n = 8). ∗P < 0.05 vs M, ∗∗P < 0.01 vs M

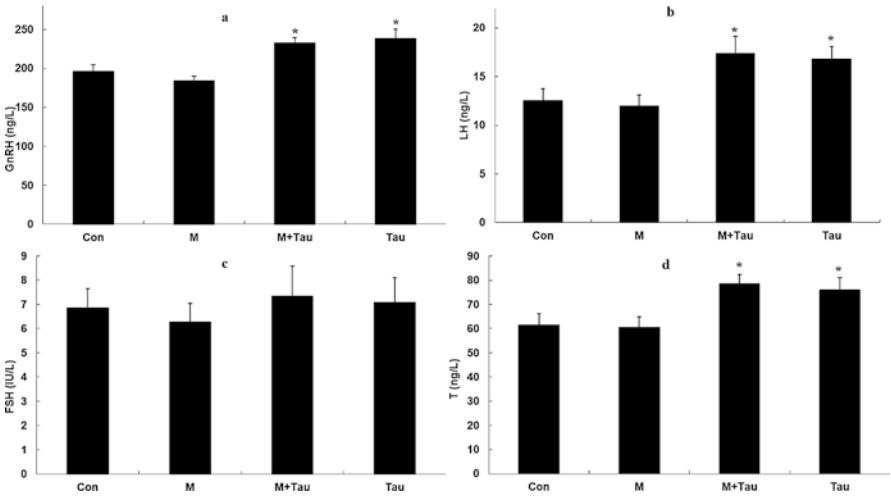

Fig. 2 Taurine raised serum sexual hormone levels in asthenospermia rats. The values were expressed as the means ± SE (n = 8). ∗P < 0.05 vs M, ∗∗P < 0.01 vs M

3.2 Levels of Serum Sex Hormones

As Fig. 2 illustrated, compared to control rats, there were no statistical difference in serum GnRH, LH, FSH and T levels of ornidazole-treated rats ($P < 0.05$), whereas, taurine administration could significantly raise the levels of serum GnRH, LH and T in normal and asthenospermia model rats ($P < 0.05$). In addition, there were no significant differences with regard to serum FSH level between groups ($P > 0.05$).

3.3 Epididymal Secretions and Biochemical Index

Figure 3 showed that the levels of carnitine, ACP, α-Glu, SA, MMP7 and IDO2 mRNA expression were obviously descendent in cauda epididymis of ornidazole-induced asthenospermia rats compared with the control rats ($P < 0.05$). Conversely, taurine supplementation could notably ameliorate the decline of all the cauda epididymal biochemical markers ($P < 0.05$). the greatest elevation were seen in the taurine group ($P < 0.05$). It was worth noting that epididymal carnitine, SA and MMP7 mRNA expression levels were outstandingly elevated in wild-type rats by taurine administration ($P < 0.05$).

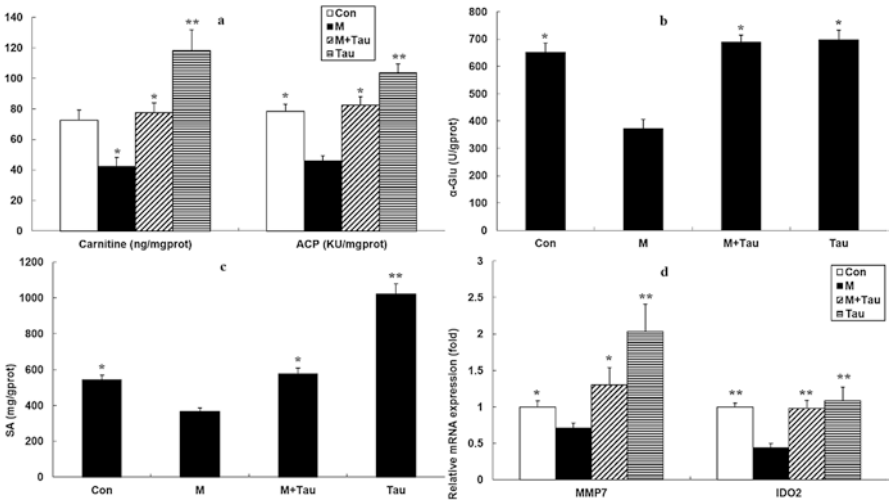

Fig. 3 Taurine stimulated cauda epididymal secretion and functional markers expression in ornidazole-treated rats. The values were presented as the means ± SE (n = 8). ∗P < 0.05 vs M, ∗∗P < 0.01 vs M

3.4 Levels of Epididymal Antioxidant Index

As shown in Fig. 4, the levels of epididymal ROS and MDA were statistically higher in asthenospermia rats than the control rats (P < 0.05), meanwhile the levels of SOD, GSH, γ-GT, and carnitine were obviously lower than control rats (P < 0.05). Taurine-addition showed the positive effect on epididymal secretions index in ornidazole-treated animals, the ROS and MDA levels were obviously reduced (P < 0.05), while the SOD, GSH, γ-GT, and carnitine concentrations were remarkably raised (P < 0.05). There were no statistically differences in Ca2+ concentration among groups (P > 0.05).

4 Discussion

Volumes of data have demonstrated that asthenospermia is one of the main causes that resulted in male infertility, and which may be attributed to epididymal dysfunction normally involved in oxidative stress (Curi et al. 2003; Purvis et al. 1991; Agarwal et al. 2014a). It has been identified that taurine, an antioxidant and one of the most aboundant free amino acids in epididymis and semen, could be biosynthesized in the epididymis (Li et al. 2006), and taurine supplementation could increase reproductive hormone secretion and spermatogenesis (Yang et al. 2015), protect spermatozoon plasmalemma (JG and BT 1983), stimulate sperm motility and fertility (Engström et al. 1985), raise sperm quality (Yang et al. 2010a). All these

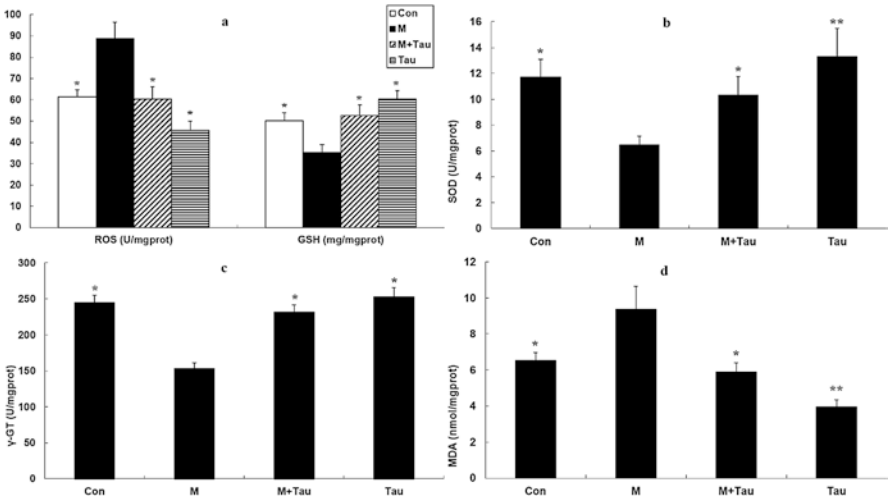

Fig. 4 Taurine promoted cauda epididymal antioxidant ability of asthenospermia animals. The values were presented as the means ± SE (n = 8). ∗P < 0.05 vs M, ∗∗P < 0.01 vs M

previous results indicated that taurine may have beneficial effects on asthenospermia. To verify the ameliorative effect of taurine on asthenospermia rats impaired by ornidazole, we first detected the changes of seminal quality and function marker enzymes activity. As an antimicrobial drug, ornidazole has been reported that it could reduce sperm quality, result in male infertility, and could be used for establishing asthenospermia model in rats (Mcclain and Downing 1988; Pang et al. 2005). In the present study, we found that epididymal sperm count, viability and motility were significantly decreased in ornidazole treated rats, sperm abnormality was obviously increased, which was in concordance with previous report (Zhang et al. 2009). We also found that spermic SDH and CCO levels were significantly decreased in asthenospermia rats, while taurine treatment has converse results. It was worth noting that taurine addition could remarkably elevate the activities of spermic function marker enzymes (acrosin, LDH-X, SDH and CCO) in model and normal rats. Acrosin, mainly located in sperm inner acrosomal membrane, has been identified it could facilitate sperm penetration through the oocyte vestments, and low acrosin activity may result in male infertility (Abdulaziz et al. 1996). LDH-X, SDH and CCO, key enzymes involved in sperm energy metabolism, have been used as sperm function marker enzymes that are closely related to spermatozoa metabolism, development, capacitation and fertilization (Miki 2007). It has been identified that taurine, as one of the important free amino acids in mitochondria, participates in the mitochondrial energy metabolism and function maintenance (Sun et al. 2011; Suzuki et al. 2011). Previous studies have found that the mechanism of ornidazole-induced asthenospermia may be contributed to its' inhibition on sperm energy metabolism that is essential for spermatozoa motility and maturation (Oberländer et al. 1996). The present results suggested that taurine could increase sperm quality

and function in asthenospermia rats by promoting sperm mitochondrial function implicated in energy metabolism.

After formation in the testes, spermatozoa are transported to the epididymis for maturation and then storage in cauda epididymidis. Normal epididymal structure and functions are essential for spermatozoa maturation that was modulated by androgen (Robaire and Hamzeh 2011). It has been demonstrated that taurine supplementation could stimulate sexual hormone secretion by acting on hypothalamic-pituitary-testicular axis (Yang et al. 2010b). To validate whether taurine ameliorate sperm quality and function in asthenospermia rats by increasing androgen secretion, we analyzed the effects of taurine addition on the levels of serum hypothalamic-pituitary-testicular axis hormone. Results showed that there were no statistical difference in serum GnRH, LH, FSH and T concentration between wild type and model animals. The result are consistent with studies of McClain and Downing who certified that ornidazole could induce asthenospermia by initiating epididymal oxidative stress and interfering metabolism other than affecting testicular function (Mcclain and Downing 1988). The present data also showed that serum GnRH, LH and T levels were obviously increased by taurine treatment in normal and model rats, the results was also in accordance with previous data (Liu et al. 2017a), in spite of the difference of the experimental animals and design. Our findings indicated that taurine could raise epididymal spermioteleosis and function in asthenospermia rats partly by stimulating hypothalamic-pituitary-testicular axis endocrine activity.

The epididymis plays crucial role in spermioteleosis including spermatozoa acquisition of motility and fertilizing ability, which is closely related to epididymal epithelial cells secretory activity and luminal microenvironment. To decipher the effects of taurine on the epididymal function in asthenospermia rats, the levels of epididymal secretion originates and function marker index were determined in the present study. We found that taurine treatment could effectively ameliorate ornidazole-induced decline of carnitine, SA, α-Glu, ACP, MMP7 and IDO2 mRNA expression levels in cauda epididymis. Ruiz-Pesini et al. (2001) have demonstrated that seminal carnitine concentration was associated with sperm cells mitochondrial functionality and seminal quality (Ruiz-Pesini et al. 2001). Meanwhile, androgen has been documented to stimulate carnitine secretion in the epididymis (Jeulin et al. 1994). The reason of cauda epididymal carnitine concentration elevation may be attributed to taurine's stimulation on T secretion. SA, α-Glu and ACP, secreted by epididymal epithelial cells, has been proved to take part in sperm maturation, and was well known as marker factors of epididymal functionality. Data of previous studies have shown that epididymal MMP7 and IOD2 mRNA expression levels notably decreased in injured epididymis, suggested that the two factors may be used as biomarkers of epididymal epithelium (Yan et al. 2009; Yoshida et al. 1980). The present data indicated that taurine may play an important roles in epididymal epithelium histology, secretion activity and intraluminal homeostasis.

Many studies have validated that epididymal oxidative stress should be regarded as one of the important causes of male infertility (Agarwal et al. 2014b). Taurine has

been identified as a natural antioxidant by neutralizing and detoxifying free radicals and lipid (Cozzi et al. 2010), and by inhibiting oxidative stress induced cytomembrane permeability (Timbrell et al. 1995). To investigate whether taurine elevate epididymal function in ornidazole-induced asthenospermia rats by means of inhibiting epididymal lipid peroxidation. The present findings showed that the levels of ROS and MDA in cauda epididymis markedly raised by oridazole-induced asthenospermia rats, while SOD, GSH and γ-GT levels significantly reduced. However, all the index could be rescued by taurine treated. It has been found that ornidazole could specifically stimulate epididymal oxidative stress action, destroy spermatozoa structure, inhibit sperm maturation (Liu et al. 2017b). The production of free radicals mainly ROS would increase when ornidazole acted on epididymis, then excessive ROS stimulate consecutive reactions producing more ROS, and induce lipid peroxidation resulting in lipid peroxide such as MDA, which furthermore induces cellular damage. In addition, epididymal SOD, GSH andγ-GT would eliminate oxidative components to protect cellular integrity. A number of studies have shown that serum and tissue antioxidant biomarkers including SOD, GSH and catalase were increased by taurine supplementation, while levels of ROS and MDA decreased (Dogru-Abbasoglu et al. 2001; Balkan et al. 2002; Koyama et al. 1992; Adedara et al. 2017), these findings are in agreement with the present data, despite that animals treatment and taurine addition concentration was different. The present data suggested that taurine could increase cauda epididymal antioxidant ability in oridazole-induced asthenospermia rats.

5 Conclusion

The results indicated that taurine can increase spermatozoa quality and function in ornidazole-induced asthenospermia rats by ameliorating spermatozoa mitochondrial energy metabolism, enhancing epididymal secretion function and maintaining luminal microenvironment that may be implicated in taurine's androgen stimulated and antioxidant activity.

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Author Contributions JY and JH conceived the study and participated in its design and coordination and helped to draft the manuscript. TM performed the sexual function tests, participated in the study design and drafted the manuscript. SL and GW performed the hormone study. QY and YZ participated in the detection of NOS/NO and performed the statistical analysis. All authors read and approved the final manuscript.

Conflict of Interest All authors declare no conflicts of interest, financial or otherwise.

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Part IV
Taurine and Heart Health

Taurine-Conjugated Metabolites in Hearts

Takashi Ito, Shigeru Murakami, and Stephen W. Schaffer

Abstract Mammalian tissues, especially the heart, contain high concentrations of taurine, a beta-amino acid that possesses a variety of physiological functions. While it is well known that taurine reacts with several metabolites, such as bile acids and fatty acids, taurine-conjugated metabolites in the heart have not been specifically studied. Recently, we performed Liquid chromatography-mass spectrometry- (LC-MS-) based metabolome analysis, comparing metabolome profiles of hearts from taurine transporter knockout (TauTKO) mice and wild-type mice to identify differences in taurine-conjugated metabolite content of the two phenotypes. Comparison of the metabolite profiles revealed taurine-containing dipeptides, such as glutamyl-taurine, which are present in wild-type but not in TauTKO hearts. These data suggest that taurine functions not only as a free osmolyte but also as a conjugated metabolite within the heart.

Keywords Taurine transporter-knockout mouse · Taurine-depleted cardiomyopathy · Metabolomics

Abbreviations

TauT	taurine transporter
TauTKO	taurine transporter knockout

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1 Introduction

Taurine is abundant in mammalian tissue, especially in the heart (about 20 μmol/g tissue). The size of the intracellular taurine pool is maintained through both the diet and biosynthesis in the liver. Taurine possesses various cellular actions, such as the regulation of intracellular osmolality, calcium handling, modulation of neurotransmission, etc. (Schaffer et al. 2000, 2014a, b; Ito et al. 2014). Taurine is an effective pharmaceutical agent against cardiovascular and hepatic diseases (Ito et al. 2014). On the other hand, severe taurine deficiency develops in cats and foxes fed a taurine deficient diet, as those species cannot readily synthesize taurine. Because taurine is a conditionally essential nutrient, cats fed a taurine deficient diet develop several tissue disorders, including dilated cardiomyopathy, blindness etc. (Pion et al. 1987; Moise et al. 1991). Moreover, knockout of the taurine transporter (TauT) leads to severe taurine depletion in the heart, resulting in the development of cardiomyopathy characterized by cardiac atrophy, ventricular wall thinning and the induction of heart failure marker genes (Ito et al. 2008). While many actions of taurine may contribute to the maintenance of normal cardiac function, the mechanism(s) have not been fully elucidated.

2 Various Taurine-Conjugated Metabolites (Fig. 1)

2.1 Bile Acids

Taurine can form a conjugate with several intrinsic metabolites. In mammals, bile acids conjugate with both taurine and glycine. Taurine-conjugated bile acids, such as taurocholic acid, are produced in the liver by bile acid amino acid transferase (BAAT) (O'Byrne et al. 2003). In addition, taurine-conjugation also plays a role in the metabolism of xenobiotics, which contain carboxyl groups, such as fenofibrate

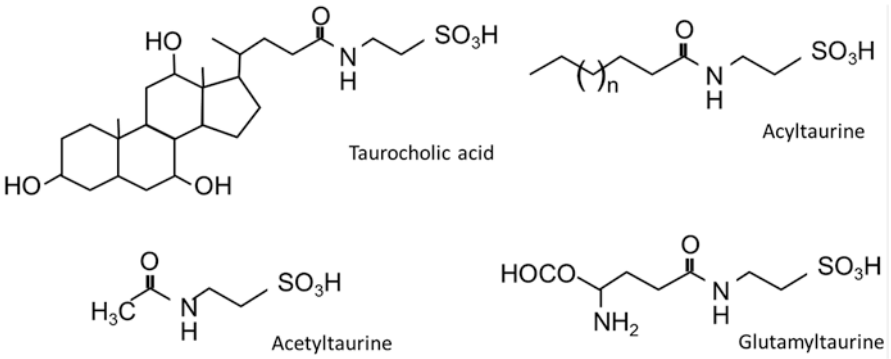

Fig. 1 Various taurine-conjugated metabolites

and ibuprofen (Shirley et al. 1994; Liu et al. 2009). Both free and conjugated bile acids act as ligands for farnesoid X receptor (FXR), a nuclear receptor that plays a crucial role in cholesterol metabolism (Parks et al. 1999). Moreover, bile acids activate TGR5, a G-protein-coupled receptor (Kawamata et al. 2003). TGR5 activation by bile acids stimulates adenylate cyclase, increasing cyclic AMP and activating cell signaling pathways. Since TGR5 is distributed in various tissues, it may assume a variety of physiological actions (Häussinger and Keitel 2017). Tauroursodeoxycholic acid (TUDCA), the taurineated-form of ursodeoxycholic acid, acts as a chemical chaperone in cells to reduce the levels of unfolded protein, thereby diminishing endoplasmic reticulum stress (Xie et al. 2002). It has been reported that TUDCA treatment is beneficial for several diseases, including heart failure (Rani et al. 2017).

2.2 Gangliosides

Taurine conjugated ganglioside (GM2) has been identified in the brain of a patient suffering from Tay-Sachs disease, which is a lysosomal glycosphingolipid storage disease characterized by cerebral GM2 accumulation (Li et al. 2003). The carboxyl group of *N*-acetylneuraminic acid in GM2 is amidated by taurine in the Tay-Sachs brain. The conjugation of taurine with GM2 presumably increases water solubility, as unmodified GM2 is very insoluble in water. Whereas the role of tauro-GM2 is not clear, the conjugation reaction may be involved in brain detoxification through the alleviation of GM2 toxicity (Li et al. 2008).

2.3 Fatty Acids

Fatty acids also conjugate with taurine to produce N-acyltaurines (Saghatelian et al. 2004). Long-chain (C16-C24) saturated, monounsaturated and polyunsaturated species of N-acyltaurine have been reported (Long et al. 2011). N-acyltaurine is widely distributed in several tissues, including the heart, brain, liver, adipose tissue etc. (Long et al. 2011). High levels of N-acyltaurine are detected in the brain of fatty acid amide hydratase (FAAH) knockout mice, largely because acyltaurine is hydrolyzed by FAAH to form taurine and fatty acids. The conjugation of acyl-CoA with taurine is catalyzed by acyl-CoA:amino acid N-acyltransferase (ACNAT1), a peroxisomal acyltransferase that is highly expressed in liver, kidney and the adrenal gland, but not in other tissues (Reilly et al. 2007). N-acyltaurines activate the members of transient receptor potential (TRP) family of calcium channels, including TRPV1 and TRPV4 (Saghatelian et al. 2006). N-acyltaurines accumulates in the islets of Langerhans of type 2 diabetic mice (Aichler et al. 2017). Interestingly, N-acyltaurines enhance insulin secretion from pancreatic b-cells through TRP channel activation (Waluk et al. 2013).

2.4 Acetic Acid

N-acetyltaurine has been identified as a metabolite of ethanol in the mouse (Shi et al. 2012). Acetate, but not acetyl-CoA, is a substrate for the reaction. N-acetyltaurine is synthesized in skeletal muscle cells, as well as hepatic cells, and increases in blood and skeletal muscle after exercise (Miyazaki et al. 2015). The physiological role of N-acetyltaurine is presently not known.

2.5 Amino Acids

Gamma-glutamyltaurine (also known as glutaurine), a taurine-dipeptide, has been found in the parathyroid gland and the brain (Bittner et al. 2005). Gamma-glutamyl transferase is responsible for the biosynthesis of glutamyltaurine. The dipeptide possesses various neural effects, such as anti-conflict and anti-epileptic activity. It also mediates a positive inotropic effect in isolated locust heart.

2.6 tRNAs

In addition to small molecules, taurine also forms a conjugate with transfer RNA (tRNA). Taurine-conjugated uridines, 5-taurinomethyluridine and 5-taurinomethyl-2-thiouridine, are present in mitochondrial tRNAs, $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$, respectively (Suzuki et al. 2002). Since the taurine-conjugated uridines are located in the wobble position of the tRNA, they play a crucial role in the binding of the anticodon to the codon of mitochondrial mRNA (Kirino et al. 2004; Schaffer et al. 2014b). The lack of taurine-conjugation of mitochondrial tRNA are observed in patients suffering from mitochondrial encephalomyopathic diseases, such as MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonus epilepsy associated with ragged-red fibers). The lack of taurine-conjugation in mitochondrial tRNA diminishes the translation of certain mitochondria encoded proteins, in particular that of ND6 (Schaffer et al. 2014b; Jong et al. 2017). This defect causes a reduction in complex I of the respiratory chain, leading to oxidative stress and impaired energy metabolism (Schaffer et al. 2016; Jong et al. 2017; Fakruddin et al. 2018). Because energy deficiency and oxidative stress both lead to impaired contractile function, impaired formation of taurine conjugated tRNA likely contributes to the development of cardiomyopathy. Also contributing to the development of cardiomyopathy is oxidative stress-mediated apoptosis (Jong et al. 2017). However, taurine also forms conjugates with other intermediates, the product of which could also influence the development of cardiomyopathy. The aim of the present study is to identify some of the other taurine containing compounds.

3 Taurine-Conjugated Metabolites in the Heart

Saghatelian et al. first identified N-acyltaurines by untargeted metabolome analysis using Liquid chromatography-Mass spectrometry (Saghatelian et al. 2004). Untargeted metabolome analysis recognizes novel metabolites by comparing metabolome profiles of 2 groups, such as WT and knockout mice. In the study of Saghatelian et al. unknown metabolites which increased in the brain of FAAH knockout mice, were chosen for subsequent analysis; among the novel metabolites identified were the N-acyltaurines.

In our study, untargeted metabolome analysis was performed on tissues isolated from normal wild-type and TauTKO mice, the latter which exhibits severe taurine depletion, with a ~99% decrement in the heart (Ito et al. 2008, 2018). We expected that LC-MS-based metabolome analysis would detect taurine-conjugated metabolites in the heart of wild-type mouse that were absent or reduced in the taurine-depleted TauTKO heart (Fig. 2).

By using the TauTKO mouse model, we performed LC-MS-based metabolome analysis. The metabolome profile was analyzed using LTQ Orbitrap XL (Thermo Fischer Scientific, USA) in the positive ion mode. Data analysis was performed by Powerget software (Kazusa DNA Institute) (Sakurai et al. 2014). The peak intensity for taurine was 186-fold higher in the WT heart than in the TauTKO heart, which is consistent with the previous report as measured by the pre-column derivatization HPLC method. We further analyzed the peaks that differed in the samples obtained from wild-type and TauTKO mice. One of the taurine containing peaks in the WT samples was glutamyltaurine (C7H14N2O6S1). We also identified three additional taurine containing peaks that were present in the WT but not in the TauTKO tissue samples. These peaks are likely aspartyltaurine (C6H12N2O6S1), leucyltaurine (C8H18N2O4S1) and isoleucyltaurine (C8H18N2O4S1). The importance of these intermediates toward normal function of the heart remains to be determined.

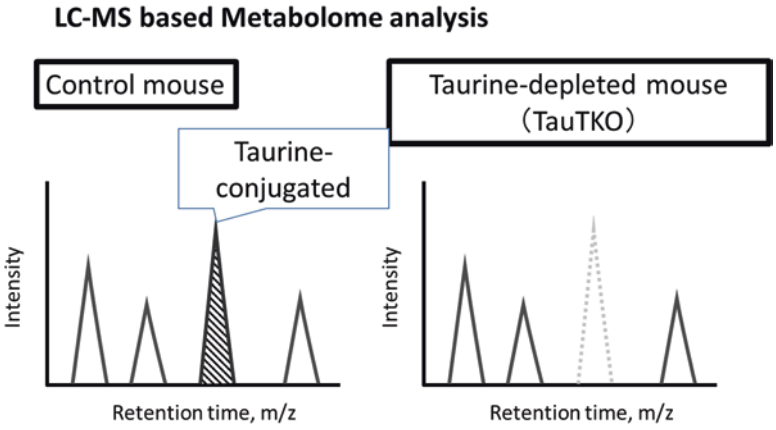

Fig. 2 Strategy to identify taurine-conjugated metabolites in control mice by LC-MS analysis

As is well known, taurine plays various functions in the heart, such as osmoregulation, protein stabilization and the regulation of calcium handling and mitochondrial function. In addition to the well-established actions of taurine itself, taurine dipeptides or other intermediates may modulate cardiac action. Indeed, the physiological actions of several dipeptides, such as carnosine (beta-alanyl-L-histidine) and anserine (beta-alanyl-N-methyl histidine), have been established. Further study is required to clarify the cardiac action of the novel taurine dipeptides.

4 Ethical Approval

All applicable international, national and institutional guidelines for the care and use of animal were followed.

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Part V
Taurine and Anti-cancer

Anti-Cancer Mechanisms of Taurine in Human Nasopharyngeal Carcinoma Cells

Feng He, Ning Ma, Kaoru Midorikawa, Yusuke Hiraku, Shinji Oikawa, Yingxi Mo, Zhe Zhang, Kazuhiko Takeuchi, and Mariko Murata

Abstract Taurine displays anti-tumor activity in some kinds of human cancers. However, the underlying mechanisms are poorly understood. Epstein-Barr virus-related nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck cancer in Southeast Asia with the highest incidence in South China. We examined an apoptosis-inducing effect of taurine against NPC cells (HK1 and HK1-EBV) to clarify the mechanisms of anti-tumor effects of taurine by immunocytochemical methods. We observed that taurine induced cleavage of caspase-9/3 in a concentration-dependent manner, suggesting the involvement of mitochondrial apoptotic signals. Both PTEN and p53 activation were detected in a dose-dependent

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manner after taurine treatment in NPC cells. In conclusion, taurine may play an anti-tumor role by activating tumor suppressor PTEN and p53.

Keywords Taurine · Nasopharyngeal carcinoma · PTEN · p53

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a natural amino acid, expressed widely in mammalian tissues (Schaffer and Kim 2018). Several studies have demonstrated that taurine possesses anti-tumor properties through inhibiting cell proliferation and inducing apoptosis in certain cancers by upregulating pro-apoptotic and downregulating anti-apoptotic proteins (Choi et al. 2015; Ibrahim et al. 2018; Tang et al. 2015; Tu et al. 2018; Zhang et al. 2014).

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck malignancy, with the incidence rising to 15–50 cases per 100,000 in the southern regions of China (McDermott et al. 2001) while the frequency in Caucasians in other countries is less than one case per 100,000 person-years (Yao et al. 2017). The etiology of NPC is multifactorial. Latent Epstein–Barr virus (EBV) infection, environmental carcinogens, and genetic factors play important roles in the development of NPC (Tsao et al. 2014). There is an urgent need to develop novel preventive and therapeutic strategies.

Our recent study has demonstrated that taurine can inhibit the proliferation and induce apoptosis in NPC cell lines (HK1 and HK1-EBV) in a concentration-dependent manner, as detected by MTT assay and flow cytometer (He et al. 2018). More importantly, taurine exerts a significant suppression of cell proliferation in NPC cell lines without toxic effects on normal nasopharyngeal epithelial cells under 32 mM of taurine for 48 h. Therefore, we decided upon the conditions of taurine treatment (0–32 mM for 48 h) for the current experiments.

In this study, we investigated the effects of taurine on apoptosis in NPC cells by detecting cleaved caspase 9/3 by immunocytochemistry. Tumor suppressor proteins, such as phosphatase and tensin homolog (PTEN) and p53, are important in promoting apoptosis. To clarify the anti-cancer mechanism, we examined the protein levels of PTEN and p53 in taurine-treated cells.

2 Methods

2.1 Cell Lines

Human NPC cell lines (HK1 and HK1-EBV) were a kind gift from Professor Sai-Wah Tsao (Hong Kong University) (Li et al. 2006; Lo et al. 2006; Tsang et al. 2010). Cells were maintained at 37 °C in a 5% CO_2 incubator. NPC cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml

streptomycin (Gibco). Taurine (purity ≥98.5%) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All cell lines were treated with taurine at various concentrations (0–32 mM, dissolved in medium) for 48 h.

2.2 Immunocytochemistry (ICC)

Cells were fixed on slides with 4% (v/v) formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature after the treatment of taurine and washed three times with PBS. The cells were treated with 1% (v/v) Triton X-100 for 10 min, and then incubated with 5% (w/v) skim milk for 60 min at room temperature. ICC was performed by incubation with rabbit monoclonal cleaved caspase-9 (1:100, Cell Signaling Technology Inc., 20750S, Dancers, MA, USA), cleaved caspase-3 (1:100, Cell Signaling Technology Inc., 9661S), anti-PTEN (1:100, Cell Signaling Technology Inc., 9188S), or mouse monoclonal anti-p53 (1:100, Santa Cruz Biotechnology, sc-47,698, San Diego, CA, USA) overnight at room temperature. Then, they were incubated with fluorescent secondary antibodies Alexa Fluor 594–labeled goat anti-rabbit IgG or Alexa Fluor 594-labeled goat anti-mouse IgG (1:400, Thermo Fisher Scientific, Massachusetts, USA) at room temperature for 2 h. Nuclei were stained with DAPI (Southern Biotech, Birmingham, USA), and the stained cells were examined under a fluorescence microscope (BX53, Olympus, Tokyo, Japan). The numbers of positively-staining cells were analyzed using the ImageJ software (ver. 1.48).

2.3 Statistic Analysis

Values were presented as means ± SD. Differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc analysis using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). A p-value <0.05 was considered to be statistically significant.

3 Results

3.1 Taurine Increases Cleaved Caspase-9/3 in HK1 and HK1-EBV Cells

To analyze taurine-induced apoptosis, caspase activation was assessed by ICC. Treatment of the cells with taurine showed a significant increase in the levels of cleaved caspase-9 (Fig. 1a–d) and caspase-3 (Fig. 1e–h), which suggested that taurine may enhance the apoptosis-inducing effect through the mitochondrial pathway.

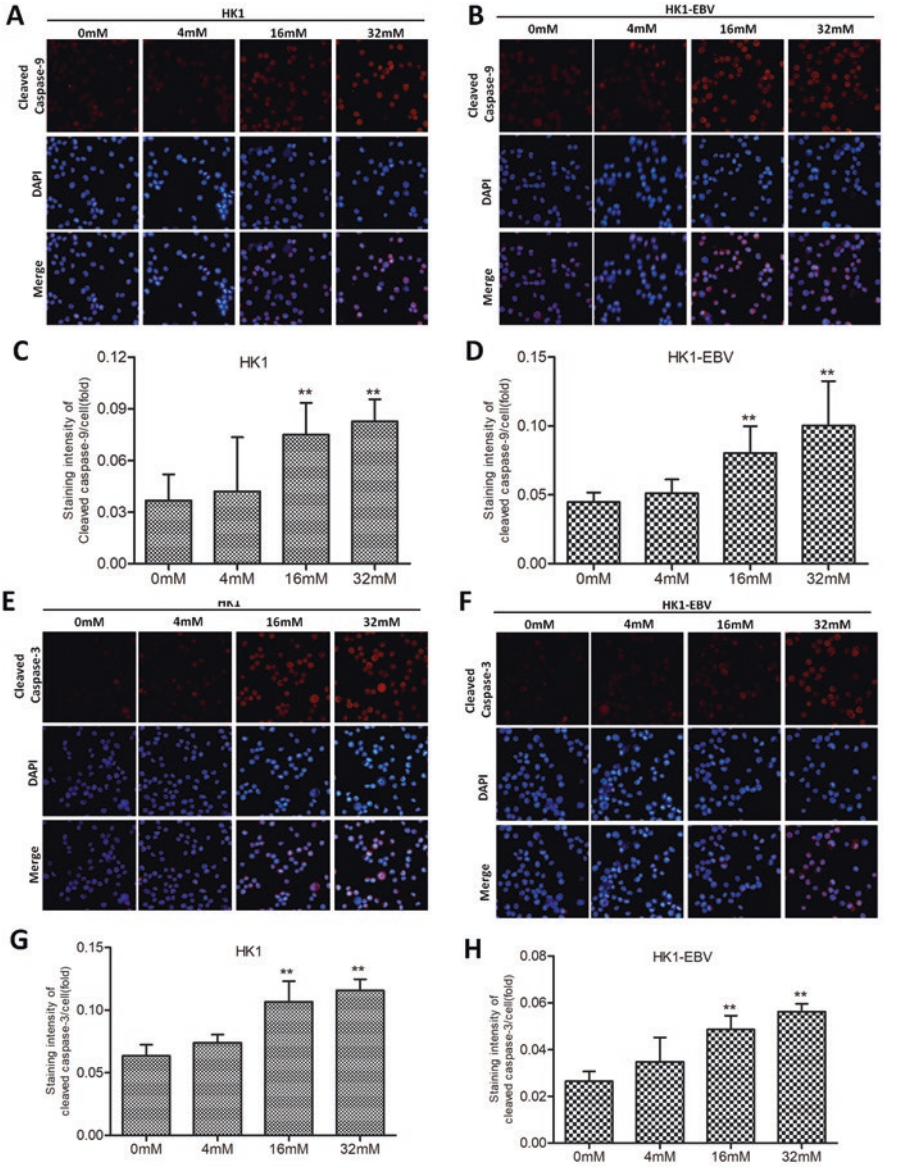

Fig. 1 Taurine yields cleaved caspase-9/3 in HK1 and HK1-EBV cells. Cells were treated with taurine for 48 h and photographed to observe cleaved caspase-9 (**a**, **b**) and cleaved caspase-3 (**e**, **f**). Data represent quantitative image analysis for cleaved caspase-9 (**c**, **d**), and caspase-3 (**g**, **h**). Intensity of staining and total number of DAPI-stained cell nuclei was analyzed by ImageJ software. $**p < 0.01$ compared to taurine 0 mM group

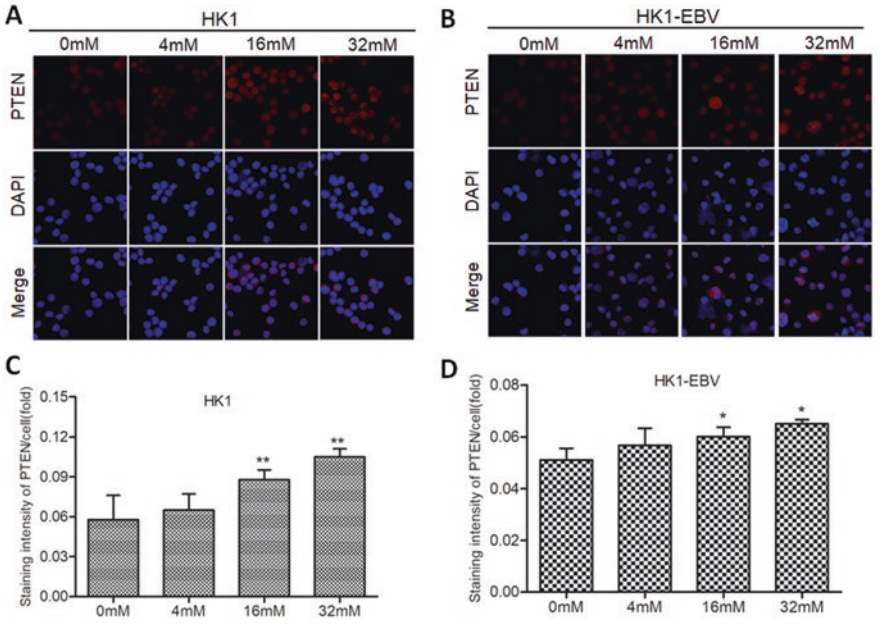

Fig. 2 Taurine activates PTEN in HK1 and HK1-EBV cells. Cells were treated with taurine for 48 h and (**a**, **b**) photographed to observe PTEN (red), DAPI (blue) staining using a microscope. (**c**, **d**) Data represents quantitative image analysis for taurine-induced PTEN activation. Intensity of staining and total number of DAPI-stained cell nuclei was analyzed by Image J software. $*p < 0.05$, $**p < 0.01$ compared to taurine 0 mM group

3.2 Taurine Activates PTEN in HK1 and HK1-EBV Cells

To identify whether PTEN is involved in the anti-tumor effect on NPC, the levels of PTEN were assessed in HK1 and HK1-EBV cells incubated with different concentrations of taurine for 48 h by ICC (Fig. 2). Taurine dose-dependently increased the levels of PTEN compared with the control group. These results suggest that taurine may show an anti-tumor ability on NPC cells through PTEN activation.

3.3 Taurine Activates p53 in HK1 and HK1-EBV Cells

Studies have shown that p53 causes apoptosis in response to DNA damage, which can be induced by anti-cancer drugs or radiation. p53 plays a critical role in activating apoptosis via direct activation of transcription of proapoptotic genes, such as Bcl-xL (Dudgeon et al. 2006). We found that p53 was upregulated with increasing concentrations of taurine (Fig. 3).

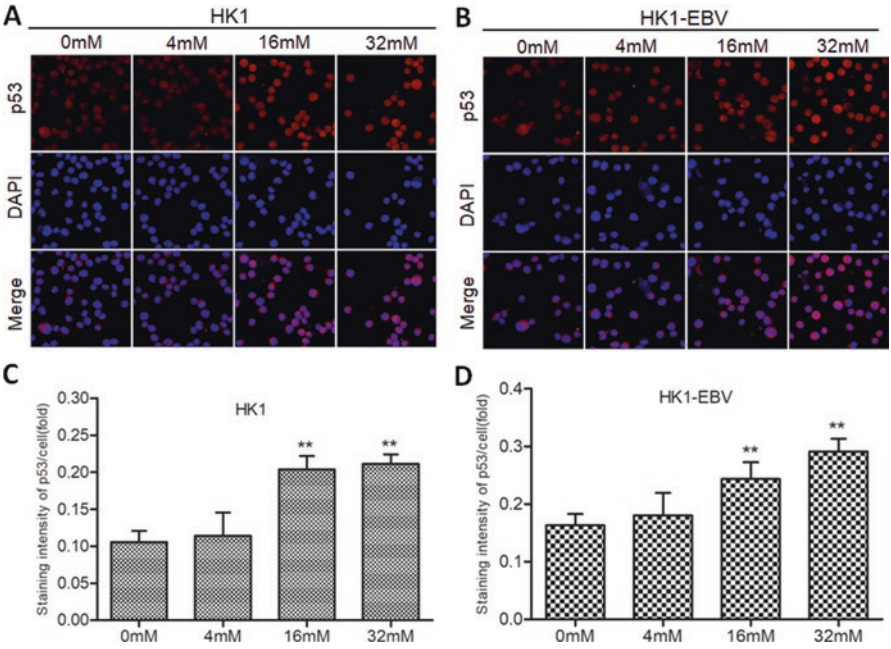

Fig. 3 Taurine activates p53 in HK1 and HK1-EBV cells. Cells were treated with taurine for 48 h (**a**, **b**) and photographed to observe p53 (red), DAPI (blue) staining using a microscope. (**c**, **d**) Data represent quantitative image analysis for taurine-induced p53 activation. Intensity of staining and total number of DAPI-stained cell nuclei was analyzed by Image J software. $**p < 0.01$ compared to taurine 0 mM group

4 Discussion

Apoptosis is an important homeostatic mechanism balancing cell division and cell death, which is a highly attractive and widely studied area to search for more effective agents for treatment of human cancers (Cheng et al. 2005). A wide variety of in vivo and in vitro studies published in recent years suggested that many chemotherapeutic agents could induce apoptosis in different cancer cells (Lian et al. 2018). Apoptosis is mainly divided into two pathways, one is the death receptor-dependent (external) pathway and the other is the mitochondrial-dependent (inner) pathway (Fulda and Debatin 2006). Additionally, a group of cell death enzymes, cysteine aspartases (caspase), which play an important role in apoptosis, has been intensively studied. Caspase-3 is involved in the terminal phase of apoptosis (Cryns and Yuan 1998), and caspase-9 is a key player of apoptosis in the mitochondrial pathway (Li et al. 2017). Our results demonstrated that taurine increased the levels of cleaved caspase-9 and cleaved caspase-3 in a dose-dependent manner (Fig. 1), suggesting that the taurine can induce apoptosis through a mitochondrial pathway. This result is consistent with the results in colon cancer cell lines (Zhang et al. 2014).

PTEN, identified in 1997 in chromosome 10q23, is a well-known tumor suppressor gene. PTEN is inactivated in patients with advanced cancers, and has gradually become a hot issue in cancer research in the past decades (Lee et al. 2018; Que et al. 2018). The main function of PTEN is negative regulation of PI3K/Akt/mTOR pathway, which plays an important role in the regulation of many important signaling pathways, including cell proliferation and metastasis (Chen et al. 2016; Jiang and Liu 2009). We evaluated PTEN expression after taurine treatment and found that PTEN was upregulated in a dose-dependent manner (Fig. 2). PTEN suppresses Akt activation, which inhibits pro-apoptotic molecules. Increases in PTEN leads to apoptosis. Taurine may induce apoptosis via the PTEN/Akt pathway.

p53 also known as TP53, is one of the most widely studied tumor suppressor genes and plays an important role in cell cycle regulation (Poon 2014). Inactivation or a mutation in p53 can damage its DNA-binding properties and transcription factor function, thereby driving tumorigenesis (Wang et al. 2017). Inhibition of the p53 pathway can accelerate cancer progression and cause resistance to chemotherapy and radiotherapy (Malkin et al. 1992; Wan et al. 2018). p53 generates multiple pro-apoptotic signals in its network, via regulation of target genes, such as PTEN (Freeman and Espinosa 2013). It is suggested that the accumulation of PTEN in the nucleus leads to p53 stabilization and p53-mediated apoptosis of liver cancer cells (Zhang et al. 2016). In this study, we demonstrated that taurine promoted an upregulation of both PTEN and p53 in a concentration-dependent manner (Figs. 2 and 3, respectively). Taurine may have an impact on the p53 network of pro-apoptotic signaling, including the PTEN/Akt pathway.

The results of the present study have provided novel evidence demonstrating that taurine can upregulate the expressions of PTEN and p53. Although further study is needed to clarify the activation pathway of PTEN and p53 by taurine, this study indicates that taurine may exert an anti-tumor effect by upregulating the expression of PTEN and p53 in NPC.

5 Conclusion

In summary, our current work demonstrates that taurine can induce apoptosis in nasopharyngeal carcinoma cells via a mitochondrial pathway through activation of PTEN and p53. These results may provide a new therapeutic strategy for patients with nasopharyngeal carcinoma.

Acknowledgements This work was supported in part by JSPS KAKENHI Grant Numbers JP16H05255 (MM).

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Targeting Taurine Transporter (TauT) for Cancer Immunotherapy of p53 Mutation Mediated Cancers – Molecular Basis and Preclinical Implication

Xiaobin Han

Abstract Taurine transporter (TauT) has been identified as a target gene of p53 tumor suppressor. TauT is also found to be overexpressed in variety type of human cancers, such as leukemia. This study showed that expression of TauT was upregulated by c-Myc and c-Jun oncogenes. To explore whether blocking of TauT inhibits tumor development, the RNA interference (RNAi) and immune targeting approaches were tested in tumor cells in vitro and in p53 mutant mice in vivo. Knockdown of TauT expression by RNAi resulted in cell cycle G2 arrest and suppressed human breast cancer MCF-7 cells proliferation determined by colonies production and cell migration assays. Knockdown of TauT also rendered MCF-7 cells more susceptible to chemotherapeutic drug-induced apoptosis. An antibody specifically against TauT blocked taurine uptake and induced cell cycle G2 arrest leading to cell death of variety type of tumor cells without affecting the viability of normal mammalian cells. TauT peptide vaccination significantly increased median lifespan (1.5-fold) of the p53 null mice and rescued p53+/− mice by extending the median lifespan from 315 days to 621 days. Furthermore, single dose treatment of tumor-bearing (thymic lymphoma) p53 null mice with TauT peptide reduced tumor size by about 50% and significantly prolonged survival of these mice from average 7 days (after observing the thymic lymphoma) to 21 days. This finding demonstrates that a novel TauT peptide vaccine can delay, inhibit, and/or treat p53 mutation related spontaneous tumorigenesis in vivo. Therefore, TauT peptide may be used as a universal cancer vaccine to prevent and/or treat patients with p53 mutation-mediated cancers.

Keywords TauT · TauT peptide · Cancer immunotherapy · p53

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Abbreviations

p53	p53 tumor suppressor gene
RNAi	RNA interference
TauT	taurine transporter

1 Introduction

Cancer affects millions of people in the world each year. About 50% of these cancers are curable with surgery, radiation therapy, and chemotherapy. Despite significant technical advances in these three types of treatments, each year more than 500,000 people will die of cancer in the United States alone reported by National Institute of Health (NIH) 2016.

The goal of cancer treatment is to develop modalities that specifically target tumor cells, thereby avoiding unnecessary side effects to normal tissue. Immunotherapy has the potential to provide an alternative systemic treatment for most types of cancer. The advantage of immunotherapy over radiation and chemotherapy is that it can act specifically against the tumor without causing normal tissue damage.

One form of immunotherapy, vaccines, is particularly attractive because they can also provide for active immunization, which allows for amplification of the immune response (Sagiv-Barfi et al. 2018). In addition, vaccines can generate a memory immune response. The possibility that altered features of a tumor cell are recognized by the immune system as non-self and may induce protective immunity is the basis for attempts to develop cancer vaccines. Whether or not this is a viable strategy depends on how the features of a transformed cell are altered. Appreciation of the central role of mutation in tumor transformation gave rise to the hypothesis that tumor antigens arise as a result of random mutation in genetically unstable cells. Although random mutations might prove immunogenic, it would be predicted that these would induce specific immunity unique for each tumor. Vaccines are given to protect against certain diseases, such as cervical (HPV vaccines) (Wittet and Tsu 2008), prostate (Provenge) (Plosker 2011) and bladder cancers (BCG vaccine) (Witjes 2007), and infections by boosting your immune system.

There is a need for new, selective and universal anticancer agents that differentiate between malignant and nonmalignant cells based on specific cell surface proteins that provide essential environment to support cancer cell proliferation. The benefits of such agents would include a higher therapeutic index and lower toxicity than conventional therapies.

Taurine transporter (TauT) has been identified as a target gene of p53 tumor suppressor and Wilms' tumor suppressor gene, as well as c-Jun and c-Myc oncogenes (Han et al. 2006), respectively. TauT is also found to be overexpressed in variety type of human cancers, such as leukemia (Learn et al. 1990). In this study, we tested

role of TauT in tumor cells development by targeting TauT using RNAi, specific TauT antibody, and TauT peptide vaccine both in vitro and in vivo models.

2 Methods and Materials

2.1 Cell Culture and Experiments

Human embryonic kidney (293), LLC-PK1, MDCK, Jurkat, marine fibroblast (10)1, MCF-7 cells were cultured according to American Type Culture Collection guidelines. HL-60 and primary thymocytes were cultured in the RPMI-1640 medium containing 10% fetal bovine serum. Briefly, cells were grown as confluent monolayers in 10-cm diameter tissue culture plates in media specific for each cell line with 10% fetal calf serum at 37 °C in the presence of 5% CO2 in a humidified incubator. Cells were plated 18 h before transfection and fed with fresh medium 4 h before transfection. Taurine transport, transient transfection, electrophoretic mobility shift assay, western blot analysis, and RNAi were performed as described previously (Han et al. 1996, 1997a, b, 1999, 2002; Han and Chesney 2013).

2.2 Animal Study

All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The University of Tennessee Health Science Center's Animal Care and Use Committee approved all animal studies (Protocol number: 12–088.0). p53 knockout mice were obtained from Dr. Gerard Zambetti of St. Jude Children' Research Hospital. Primers 5′-AGCGTG-GTGGTACCTTATGAGC-3′ (p53-x6), 5′-GGATGGTGGTATACTCAGAG-CC-3 (p53-x7), and 5′-GCTATCAGGACATAGCGTTGGC-3′ (Neo19) were used for mice genotyping. TauT peptide vaccine was prepared by coupling TauT peptide to KLH with a kit (Imject SuperCarrier EDC system for Peptides; Pierce) (US patent, pending). To test the anti-cancer effect of TauT-peptide vaccine, p53 knockout (KO) mice were immunized with 100 μg/kg and boosted with an equal amount of antigen 2 weeks later and the tumor development and survival of the mice were followed up accordingly. To develop and test TauT-Ab, animals were immunized with 100 μg/kg TauT-peptide and boosted with an equal amount of antigen 2 weeks later. Then animals were bled on Days 21 and 36 to measure the antibody response. Antisera were collected on Day 49 and purified by affinity column chromatography (Pierce). Antisera were stored at −80 °C.

2.3 Statistical Analysis

All experiments were performed in triplicate. Luciferase assays are expressed in units of relative light output. The data represent the mean ± S.E. of three or four experiments. For all animal experiments, the data represent the mean ± standard error of at least four mice. We evaluated differences between two groups by unpaired *t*-test and multiple groups by one-way analysis of variance ANOVA). All computations were performed using GraphPad Prism 5 (GraphPad Software Inc. La Jolla, CA, USA). Significance was defined as $P < 0.05$.

3 Results

3.1 TauT is a Target Gene of Tumor Suppressor and Oncogenes

The promoter of TauT gene has been cloned and studied extensively. TauT promoter contains multiple sites for transcription factors, including p53, c-Myc, c-Myb, c-Jun, WT1, and a tonicity response site-TonE (Fig. 1a) (Han et al. 2000a, b, 2002; Han and Chesney 2003; Ito et al. 2004). p53 binds to TauT promoter (Fig. 1b) and downregulates TauT expression (Fig. 1c), while expression of TauT is upregulated by c-Myc, c-Myb, c-Jun, WT1, and hypertonic condition (Fig. 1d–j).

3.2 TauT Plays a Role in Cell Cycle Regulation

Knockdown of TauT by RNAi decreases TauT expression (Fig. 2a and b) and results in reduced cell proliferation (Fig. 2c), fewer colonies formation (Fig. 2d and e), slower cell migration (Fig. 2f), and cell cycle G2 arrest (Fig. 2g and h) compared to control cells.

3.3 Inhibition of TauT Sensitizes Tumor Cells to Chemotherapeutic Drugs Induced Apoptosis

Control (MCF-7) and TauT-knockdown (MCF-7/C3-3) cells were treated with tamoxifen or doxorubicin for indicated time. We showed that drug treated MCF-7/C3-3 cells have fewer number of live cells compared to parental MCF-7 cells (Fig. 3a and b). Knockdown of TauT rendered MCF-7 cells more susceptible to drug-induced apoptosis determined by DNA fragmentation (Fig. 3c and d) and flow cytometry analysis (Fig. 3e and f).

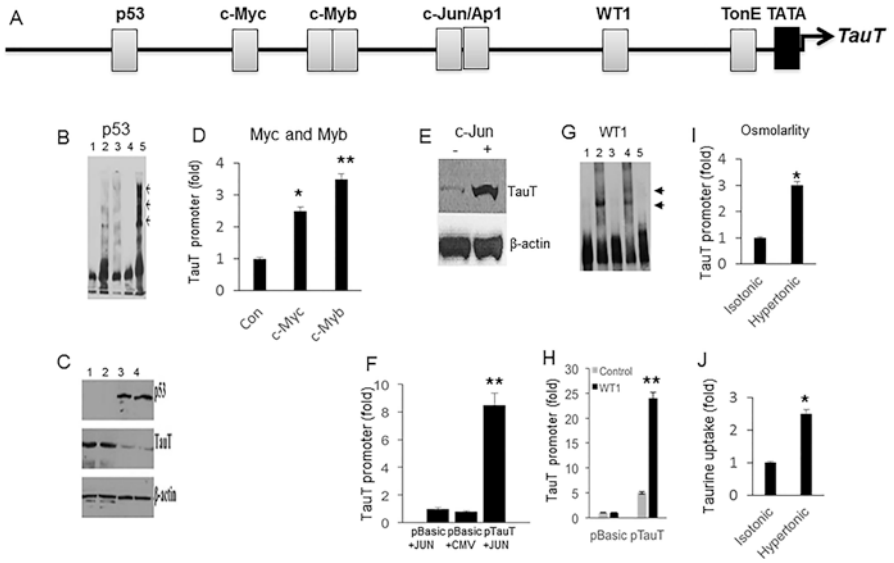

Fig. 1 Regulation of TauT by p53, c-Myc, c-Myb, c-Jun, WT1, and osmolarity. (**a**) Scheme of TauT promoter. (**b**) p53 binds to TauT promoter region determined by gel shift assay. (**c**) Overexpression of p53 downregulates TauT expression. (**d**) Upregulation of TauT promoter activity by c-Myc and c-Myb. (**e** and **f**) Upregulation of TauT by c-Jun. (**g** and **h**) WT1 binds to TauT promoter and upregulates TauT promoter activity. (**i** and **j**) Regulation of TauT by osmolarity. N = 3 for each experiment. $*p < 0.05$, or $**p < 0.01$ vs controls

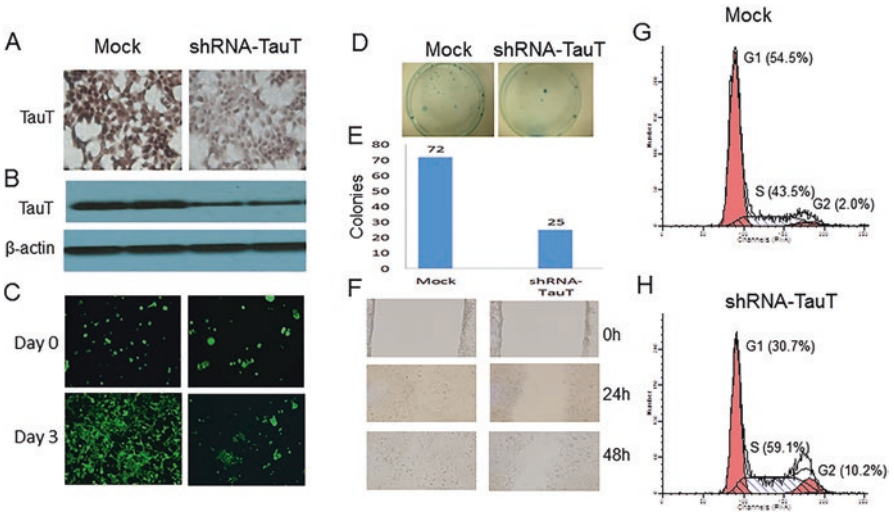

Fig. 2 Knockdown of TauT causes cell cycle G2 arrest in MCF-7 cells. (**a** and **b**) Knockdown of TauT expression by shRNA results in reduced cell proliferation (**c**), fewer colonies formation (**d** and **e**), impaired cell migration (**f**), and cell cycle G2 arrest (**g** and **h**)

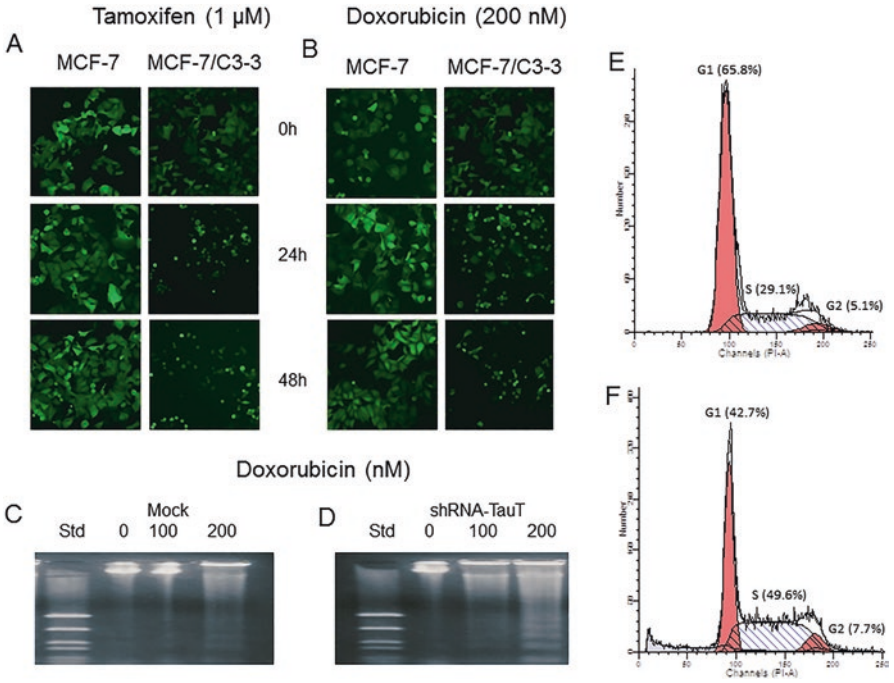

Fig. 3 Knockdown of TauT renders MCF-7 cells susceptible to chemotherapies drugs. Cells were culture to 80% of confluent and tamoxifen or doxorubicin was added to the medium for indicated time. Knockdown of TauT results in MCF-7 more sensitive to tamoxifen (**a**) and doxorubicin (**b**) treatment leading to cell undergo apoptosis (**c** and **d**), cell cycle G2 arrest (**e** and **f**)

3.4 TauT Blocking Antibody Induces Apoptosis in TauT-Overexpression Cells

Cells were treated with equal amount (10 μg/ml) of pre-immune IgG and T-antibody for indicated time. Untreated cells were used as control. Cells were counted and cell viability was calculated basis on the percentage of life cells out of totally counted cells. T-antibody inhibited taurine uptake by TauT-expressing oocytes and LLC-PK1 cell (Fig. 4a) without affecting the cell viability of normal cells including LLC-PK1, 293, (10)1 and thymocytes (Fig. 4b). In contrast, T-antibody induced cell death of HL-60, MCF-7, Jurkat, and TauT-overexpressing LLC-PK1 cells in time-dependent manners (Fig. 4c–f). T-antibody also caused cell cycle G2 arrest in tumor cells (Fig. 5), consistent with the finding that inhibition of TauT expression by RNAi results in cycle G2 arrest described above (Fig. 2f).

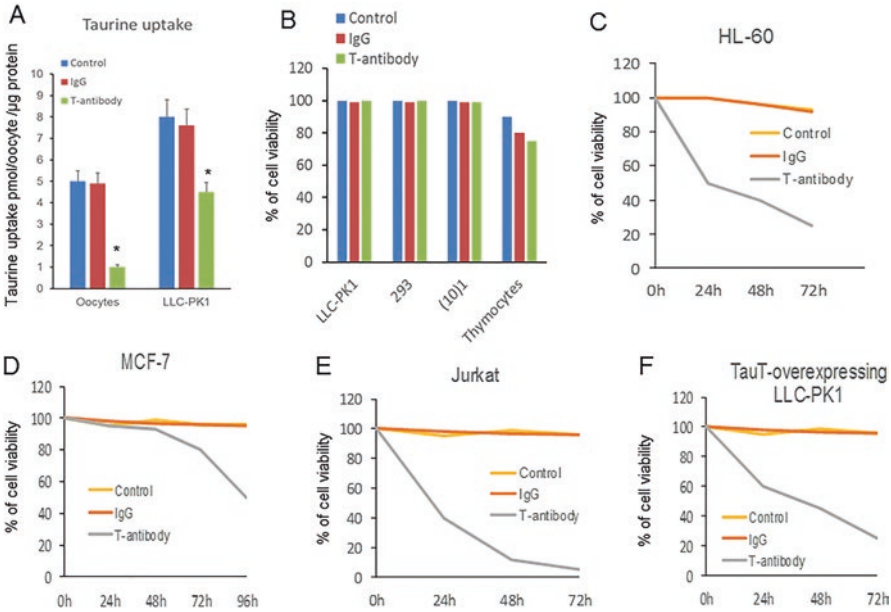

Fig. 4 TauT antibody inhibits taurine uptake and induces apoptosis in TauT-overexpression cells. Cells were culture to 80% of confluent, then TauT antibody (T-antibody, 5 μg/ml) and control IgG (5 μg/ml) were added to the medium and culture for indicated time. T-antibody blocked taurine uptake by TauT-expressing oocytes and LLC-PK1 cell (**a**). T-antibody had no effect on the cell viability of normal cells (**b**), and reduced cell viability of tumor cells (**c–e**) and TauT overexpression LLC-PK1 cells (**f**)

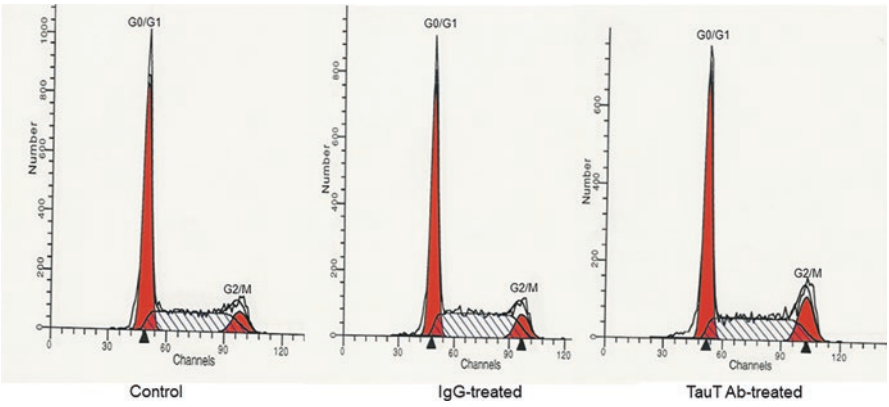

Fig. 5 TauT Ab (T-antibody) induces cell cycle G2 arrest in human Jurkat cells

3.5 *TauT Peptide Vaccination Prolongs Lifespan of p53 Mutant Mice*

To evaluate the efficacy of TauT-peptide as a cancer vaccine, p53−/− and p53+/− (C57BL/6 J background) were injected with either TauT-peptide (100 μg/kg) or vehicle only at the age of 4 weeks and boosted 2 weeks later (n = 15–20 for each group). All mice were observed daily for signs of morbidity and the development of tumors. Tumor (mainly thymic lymphoma) was observed as early as at the age of 75 days in p53−/− control mice (Fig. 6a). The median lifespan for p53−/− control mice was 138 days (Fig. 6b), which consistent with others studies (Hursting et al. 1994). The p53−/− mice vaccinated with TauT-peptide vaccine showed 100% response to the vaccine. TauT-peptide vaccination delayed tumor develops in all vaccinated p53−/− mice, which started to develop tumor (thymic lymphoma) at the age of 189 days (Fig. 6a). TauT-peptide vaccination significantly increased (1.5 fold) median lifespan of the p53−/− mice from 138 days (control) to 207 days (TauT-peptide vaccinated) (Fig. 6b).

Notably, we showed that single injection of TauT-peptide can extend the survival of tumor-bearing (thymic lymphoma) p53−/− mice (n = 4) from average of 7 days to 21 days (Fig. 6c, left) and reduce tumor size by about 50% (Fig. 6C, right). Consistently, the p53+/− control mice started to develop tumor (mainly osteosarcoma) at the age of 186 day (Fig. 6d). The median lifespan for p53+/− control mice was 315 days (Fig. 6e), which consistent with others studies (Komarova et al. 2012).

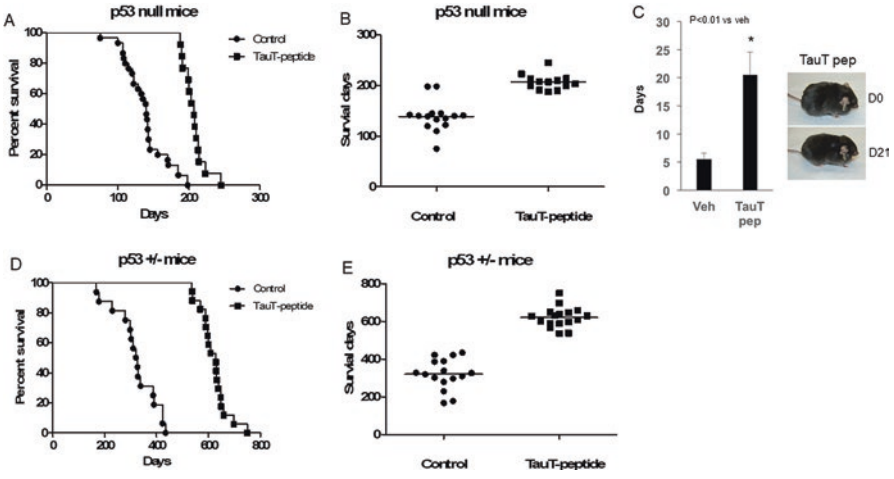

Fig. 6 TauT peptide cancer vaccine prolongs lifespan of p53 mutant mice. (**a** and **b**) Survival curve of p53 null mice vaccinated with TauT peptide compared to control (n = 15–20) ($p < 0.001$ vs control). (**c**) Single TauT peptide treatment significantly extended survival of tumor-bearing p53 null mice from average 7 days to 21 days (n = 4) ($p < 0.01$ vs control). (**d** and **e**) Lifespan of $p53^{+/-}$ mice was significantly prolonged after TauT peptide vaccination compared to control (n = 16) ($p < 0.001$ vs control)

The p53+/− mice vaccinated with TauT-peptide vaccine showed 100% response to the vaccine. TauT- peptide vaccination delayed tumor development in all vaccinated p53+/− mice, which delayed the development of tumor (mainly osteosarcoma) and significantly increased the survival of p53+/− mice (Fig. 6d). TauT-peptide vaccination significantly increased median lifespan of the p53+/− mice from 315 days (control) to 621 days (TauT-peptide vaccinated) (Fig. 6e).

4 Discussion

Tumor-specific and tumor-associated antigens are often derived from proteins directly involved in transformation of a normal cell to a tumor cell due to mutation of up-stream tumor suppressors and/or gain-of-function of oncogenes, which regulate cell cycle control or apoptosis. Additionally, the proteins that is the downstream target of the oncogenes directly causative for a transformation may be upregulated and thus be indirectly tumor-associated. Such indirectly tumor-associated antigens may also be targets of a vaccination approach. TauT is a downstream target gene of p53 tumor suppressor, WT1, and c-Jun oncogene (Han et al. 2002; Han and Chesney 2003, 2013), and TauT is overexpressed in many type of cancer cells. Yasunaga et al. have reported that SLC6A6 (TauT) plays an important role in the survival and maintenance of the cancer stem cell (CSC) population and its capacity for tumor initiation, starvation tolerance and MDR (Yasunaga and Matsumura 2014). Additionally, overexpression of TauT protects against cisplatin induced cell injury (Han et al. 1997a, b; Han and Chesney 2009).

Consistent with our previous report (Han and Chesney 2013), in this study we showed that knockdown of TauT decreased the growth rate, cell migration, colony formation, and caused cell cycle G2 arrest in MCF-7 human breast cancer cells. These findings suggest that TauT may play a role in tumor development through mediating cell cycle regulation during cell proliferation. Mammalian cells undergo dramatic cell volume change during mitosis (Boucrot and Kirchhausen 2008), and significant cell volume increase during cell division that could reach up to 30% for certain cells (Zlotek-Zlotkiewicz, et al. 2015). We have observed that TauT-deficient cells have a small cell size and decreased cell mobility (unpublished data), suggesting that TauT may play an important role during mitosis through regulation of cell volume by transportation of taurine (Hoffman et al. 2000; Olson et al. 2003; Lambert 2004).

Furthermore, we demonstrated that knockdown of TauT rendered MCF-7 cells more sensitive to tamoxifen and doxorubicin induced apoptosis. Interestingly, a TauT blocking antibody can block taurine uptake and selectively induce cell death in tumor cells and TauT overexpression non-tumor cells. Consistent with the observation that knockdown of TauT by RNAi results in cell cycle arrest, TauT antibody also causes cell cycle G2 arrest in human tumor cells, indicating that TauT may be an ideal target for cancer immunotherapy. Finally, we vaccinated p53 null and p53

heterogynous mice with the TauT peptide twice and found that TauT peptide vaccination significantly increased (1.5–3.0 fold) the lifespan of in both p53 null and p53 heterogynous mice compared to controls. Spontaneous tumorigenesis occurs and malignant lymphoma is the predominant tumor type in p53 mutant mouse models (Donehower et al. 1992; Harvey et al. 1993). In p53−/− mice, which predominantly develop thymic T-cell lymphomas within 6 months (Donehower et al. 1992; Harvey et al. 1993). Notably, a single dose of TauT peptide treatment significantly prolonged survival of tumor bearing (thymic lymphoma) p53 null mice from average 7 days after developing thymic lymphoma to 21 days. This finding demonstrates that a novel TauT peptide vaccine can delay, inhibit and/or treat p53 mutation related spontaneous tumorigenesis in vivo.

5 Conclusion

Our study first demonstrates that a novel TauT peptide vaccine can delay, inhibit, and/or treat p53 mutation related spontaneous tumorigenesis in vivo. Therefore, TauT peptide may be used as a universal cancer vaccine to prevent and/or treat patients with p53 mutation-mediated cancers.

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Tau-TCHF Inhibits Spleenic Apoptosis via PI3K-Akt Signaling Pathway in Chickens

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Abstract Taurine plays an important role in improving immunity, regulating cell proliferation and differentiation, apoptosis and so on. Traditional Chinese herb formula (TCHF) is a wealth of medicine materials for diseases control. There are many studies on Chinese herb formula in inducing cell apoptosis, differentiation and improving animal immunity. The factors in phosphatidylinositol 3-kinase/Protein Kinase (PI3K-Akt) signaling pathway are central regulators of normal cells, which integrates extra-cellular signals into cells and activates affects cell activities including cell proliferation, differentiation and apoptosis. We find the key factors (PIK3CA, PDPK1, AKT1, MDM2, ITGA2B, ITGB1, FAK and p53) in PI3K-Akt signaling pathway by RNA-Seq analysis in our previous research. The overall goal of this study to investigate the influence of taurine TCHF (Tau-TCHF) on cell proliferation, differentiation and apoptosis by estimating the factors above. The layers were fed with normal diet plus 1% of Tau-TCHF and the control group with normal diet to 42 days old. The spleen tissue samples from individual layers were used to analyze the influence of Tau-TCHF on the factors PIK3CA, PDPK1, AKT1, MDM2, ITGA2B, ITGB1, FAK and p53 in PI3K-Akt signaling pathway. The levels of transcription and protein expression of various factors were assessed by quantitative PCR (qPCR) and Western Blot. The results showed that the transcription levels of *itgb1, fak, pik3ca, akt1 and mdm2* on 42-day-old chicken spleen tissues were increased significantly in Tau-TCHF group comparing with control group ($P < 0.01$); the transcription levels of *itga2b, pdpk1* and *p53* were no significant difference ($P > 0.05$). The protein levels of PDPK1 and AKT (Ser437) were increased significantly ($P < 0.05$), but ITGA2B, ITGB1, FAK, PIK3CA, AKT1, MDM2 and p53 had no significant difference ($P > 0.05$). The results suggest that Tau-TCHF may influence proliferation and differentiation of chickens spleen via regulating PI3K-Akt

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signaling pathway. And Tau-TCHF may be provided as feed additives in improving the immunity of animals. AKT (Ser473) and PDPK1 may be considered as further targets to study mechanism of Tau-TCHF on anti-apoptosis via PI3K-Akt signaling pathway.

Keywords Chickens · Tau-TCHF · Apoptosis · PI3K-Akt signaling pathway

1 Introduction

Taurine (Tau) is a sulfur-containing non-protein amino acid found in almost all tissues of animals. Tau exists in free form in vivo and does not participate in the biosynthesis of proteins in vivo. It is involved in regulating a series of normal physiological activities such as cell proliferation, differentiation, and apoptosis and so on. It is mainly obtained directly from food, and can also be produced endogenously in the liver through cysteine oxidation and transamination. Taurine has been proved to have a variety of physiological functions, such as anti-oxidation, anti-inflammatory response, participation in cellular immunity (Ripps and Shen 2012; Stipanuk and Ueki 2011; Stipanuk 2004). A number of studies have found that taurine inhibits apoptosis in different organs (Aly and Khafagy 2014; Aydin et al. 2016; Li et al. 2017; Yang et al. 2015; Wu et al. 2018). Research on Chinese herb is deepening; studies have shown that Chinese herbs play an important role in cell proliferation and differentiation by influencing tumor-related factors (Chen et al. 2017; Yang et al. 2018).

The PI3K-Akt signaling pathway is a central regulation of normal cells in different physiological activities. It integrates extra-cellular signals into cells and is activated by cell surface receptors. After being activated, AKT is recruited into plasma membrane to activate the phosphorylation of AKT on plasma membrane. Activated AKT inhibits apoptosis and promotes cell proliferation by phosphorylation various substrates (Katso et al. 2001; Anderson and Jackson 2003; Chen and Xu 2018). In recent years, the PI3K-Akt signaling pathway has become a hot topic. It is reported that the factors in PI3K-Akt signaling pathway play an important role in cell proliferation, differentiation, and development (Parsons 2004; Samuels et al. 2004). With the further research, the Tau-TCHF will have far-reaching application prospects as feed additive resource in enhancing animal immunity and inhibiting cell apoptosis.

2 Materials and Methods

2.1 Animal Experiments and Sample Collection

A total of 60 healthy Hy-line Brown layers (day 1) were randomly allocated to two groups, with each group containing 30 chickens. The control group chickens were fed with normal diet and the Tau-TCHF group with normal diet plus 1% of

Tau-TCHF, and all chickens were given feed and water free throughout the experimental time period. All experiments were performed in accordance with the animal Guideline of Shenyang Agricultural University (SYAU) and the protocols were approved by the Ethical Committee of SYAU. The spleen tissues were collected and stored at −80 °C on 42 day age of chickens.

2.2 Total RNA Extraction and Expression Level Estimation of All Factors Using Quantitative PCR

Total RNA of the spleen tissues were extracted by RNAiso Plus (Takara Bio, China). cDNA was synthesized by using AMV First Strand cDNA Synthesis Kit (Sangon, China). Quantitative PCR (qPCR) was performed by using TransStart TIP Green qPCR SuperMix (Transgen BioTech, China) on Quant Studio 3 Real-Time PCR-system (Thermo Fisher, USA). All 20 μl reactions consisted of 1 μl template cDNA 10 μl, 2 × TransStart TIP Green qPCR SuperMix, 1 μl (10 μM) of each primer. PCR was performed in triplication for every chicken samples. The primers were designed for the objective genes: *itga2b* forward primer: 5′-AGATGGGCAACCCCATGAAG-3′ and reverse primer: -5′-CCGTTCTGACTCCTGAGCTG-3′, *Itgb1* forward 5′-CCAAGTGGGATACGGGTGAA-3′ and reverse 5′-TACGCTGCATACAGTGTCGT-3′, *fak* forward primer 5′-TACTGCCGACTGGTGAATGG-3′ and reverse primer 5′-CATTGTTGGCCAGCTTTGGT-3′, *pik3ca* forward primer 5′-AGGGTGCTAAAGAGGAGCACT-3′ and reverse primer 5′-TCCATGGGGTACTGCCCAAA-3′, *pdpk1* forward primer 5′-CAGCCACCTGTATGATGCTGTT-3′ and reverse primer 5′-TGCCACAGGTGGAAATGACAG-3′, A*kt1* forward primer 5′-CTCCCGCAGCCATAAGTAGC-3′ and 5′-CAGGCTGGTGGATCAGTCAA-3′, *mdm2* forward primer 5′-AGTCGGACAGCATCTCGTTG-3′ and reverse primer 5′-GGCATCAAGATCCGGAATGG-3′, *p53* forward primer 5′-GAAGGAGATCAATGAGGCGCT-3′ and reverse primer 5′-GTGGTCAGTCCGAGCCTTTT-3′, *β-actin* forward primer 5′-TGTTGACAATGGCTCCGGTA-3′ and reverse primer 5′-AACCATCACACCCTGATGTCTG-3′.

2.3 Western Blot

The proteins of the chicken spleen tissues were extracted by Cell lysis buffer for Western and IP (Beyotime, China), and the concentration of the proteins was determined by BCA assay kit (Beijing Dingguo Changsheng Biotechnology Co.

Ltd,China).The Proteins were first extracted by sodium dodeceyl sulfate polyacrylamide gel electrophresis (SDS-PAGE) and then electrotransferred to polyvinylidene fluoride (PVDF) membrane(Millipore, France). After blocking in 5% nonfat milk at room temperature for 2 h, the membrane was incubated with appropriate primary antibodies at 4 °C overnight. Antibodies utilized were p53 and GAPDH (Santa Cruz Biotechnology,USA), AKT1, AKT (Ser437) and ITGB1 (Selleck Chemicals, USA), PDPK1, PIK3CA, ITGA2B and MDM2 (Abcam China), FAK, PDPK1, PIK3CA, ITGA2B, MDM2 (ZSGB BIO, China). Then membranes were incubated with the secondary antibody (Goat anti-rabbit IgG, Abcam China). Micro Chemi 4.2 (DNR Bio Imaging Systems, ISREAL) was used to measure quantified densitometric analysis.

2.4 Statistics Analysis

All statistical data were analyzed by SPSS 18.0 and GraphPad Prism 5 software. All results are presented as the mean ± standard deviation from PCR and Western Blot with triplication, and analyzed by 1-way ANOVA and Duncan's multiple range tests with $p < 0.05$ as the significant difference.

3 Results

3.1 Transcription Level of the Factors in PI3K-Akt Signaling Pathway

Transcription levels of the factors in PI3K-Akt signaling pathway were analyzed in all chicken tissue samples. To compare with the control group, the transcription level of the factors *itgb1, fak, pik3ca*, *akt1* and *mdm2* in the Tau-TCHF group were significantly increased than those in the control group ($P < 0.01$). There was no significant difference in *itga2b, pdpk1* and *p53* ($P > 0.05$) (Fig. 1).

3.2 The Protein Level of the Factors in PI3K-Akt Signaling Pathway in Chicken Spleen Tissues

In order to understand inhibition of Tau-TCHF in cell apoptosis, all chicken tissue samples were analyzed for protein expression level of the factors in PI3K-Akt signaling pathway. PDPK1 and AKT (Ser473) in Tau-TCHF group were significantly increased than those in the control group ($P < 0.05$), but there was no significant difference in ITGA2B, ITGB1, FAK, PIK3CA, AKT1, MDM2 and p53 ($P > 0.05$) (Fig. 2).

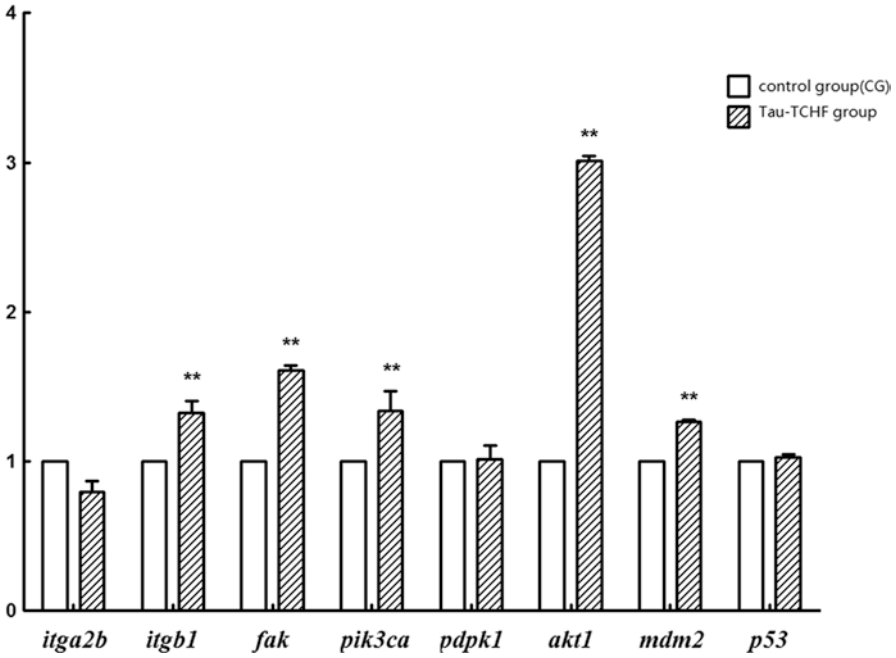

Fig. 1 Effect of Tau-TCHF on transcription level of the factors in PI3K-Akt signaling pathway
The values were presented as the means ± SE (n = 3). ∗P < 0.05 vs. CG, ∗∗P < 0.01 vs. CG

4 Discussions

Tauine has been widely researched as a growth-promoting additive or as an antioxidant in animals because of its multiple functions. However, few studies have explored its effects on avian immunity as feed additives. Taurine is also a cytoprotective nutrient which is important for the reduction of risk for apoptosis (Chian et al. 2017). In order to understand inhibition of Tau-TCHF on apoptosis, the factors in PI3K-Akt signaling pathway of chicken spleenic tissues were studied to illustrate the effect on cell proliferation and differentiation. ITGA2B and ITGB1 are members of the integrin family. ITGA2B is related to regulation of integrin activity, and ITGB1 can mediate cell-cell interaction (Tomar et al. 2018; Zhang et al. 2018; Yu et al. 2017). When integrin subunits bind to ligands in other extracellular matrices, FAK is activated and further binds to many intracellular signaling proteins such as PI3K, causing a series of intracellular signal transductions (Sun et al. 2014; Han et al. 2011). Therefore, PI3K is activated by binding of phosphorylated FAK to its SH2 domain (Waldschmidt et al. 2017). Activated PI3K recruits AKT into cell membrane, in which phosphorylation of AKT occurs on the membrane by PDPK1. Further, AKT enhances MDM2-mediated alteration of p53 function. And p53 plays a key role in DNA damage-induced apoptosis (Fig. 3).

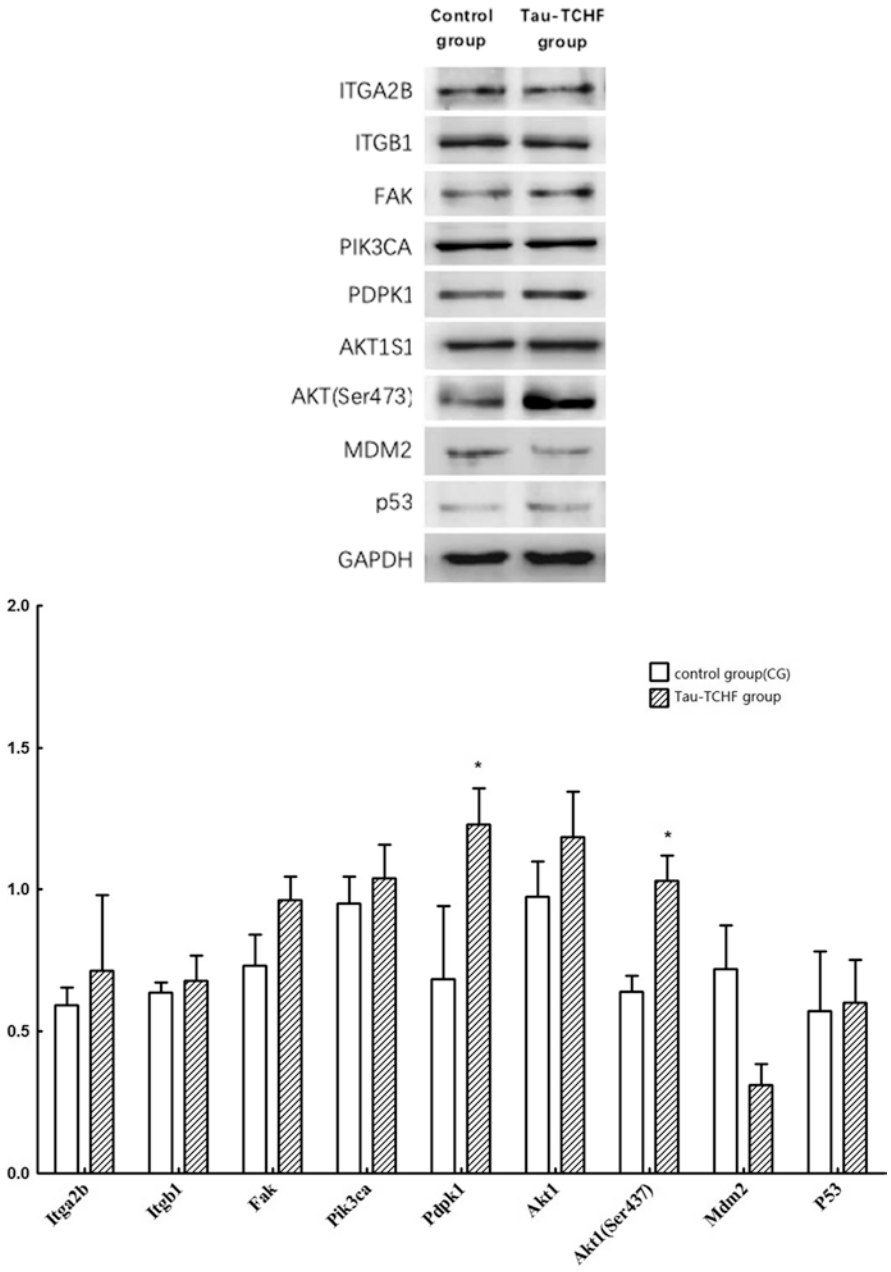

Fig. 2 Effect of Tau-TCHF on Protein level of the factors in PI3K-Akt signaling pathway
The values were presented as the means ± SE (n = 3). ∗P < 0.05 vs. CG, ∗∗P < 0.01 vs. CG

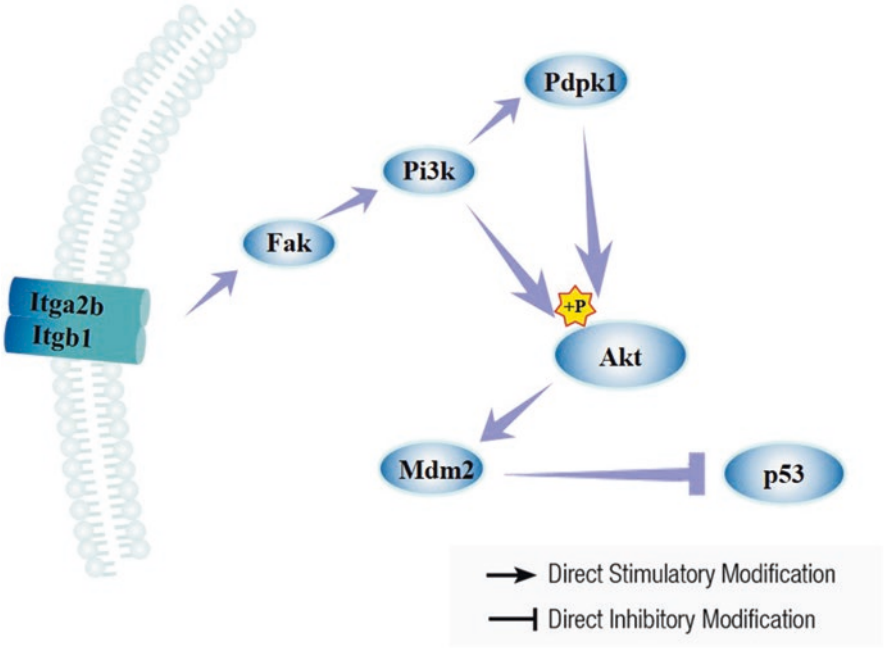

Fig. 3 Diagram of related factors in PI3K-Akt signaling pathway

In this study, AKT1 gene expression level and both AKT (Ser473) and PDPK1 protein levels are up-regulated in chicken spleen tissue samples. AKT activates inhibits cell apoptosis and promotes proliferation by phosphorylation of various substrates (Chen XL and Xu ZS 2018), which further indicates that AKT is a negative regulator of p53 protein and plays an important role in regulating cell life activity. This aspect of the research suggested that Tau-TCHF may have some effects on improving immunity through PI3K-Akt signaling pathway in chicken spleen tissue and be provided as feed additives in improving the immunity of animals. AKT (Ser473) and PDPK1 may be considered further targets to study mechanism of Tau-TCHF on anti-apoptosis via PI3K-Akt signaling pathway.

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Part VI
Taurine and Anti-oxidation and Anti-microbial

A Hepatoprotective Effect of a Hot Water Extract from *Loliolus beka* Gray Meat Against H_2O_2-Induced Oxidative Damage in Hepatocytes

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Abstract Here, we investigated the hepatoprotective effect of a hot water extract from *Loliolus beka* gray meat (LBMH) containing plentiful taurine in H_2O_2-induced oxidative stress in hepatocytes. LBMH potently scavenged the 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl

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(DPPH) radicals and exhibited the good reducing power and the oxygen radical absorbance capacity (ORAC) value. Also, LBMH improved the cell viability against H_2O_2-induced hepatic damage in cultured hepatocytes by reducing intracellular reactive oxygen species (ROS) production. In addition, LBMH inhibited apoptosis via a reduction in sub-G_1 cell population, as well as inhibition of apoptotic body formation from H_2O_2-induced oxidative damage in hepatocytes. Moreover, LBMH regulated the expression levels of Bax, a pro-apoptotic molecule and Bcl-2, an anti-apoptotic molecule in H_2O_2-treated hepatocytes. Additionally, pre-treatment with LBMH increased the expression of heme oxygenase 1 (HO-1), which is a hepatoprotective enzyme, by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) in H_2O_2-treated hepatocytes. Taken together, LBMH may be useful as a food ingredient for treatment of liver disease by regulating the Nrf2/HO-1 signal pathway.

Keywords *Loliolus beka* gray meat · Nrf2/HO-1 signal pathway · Antioxidant activity · ROS · Apoptosis

1 Introduction

Reactive oxygen species (ROS), one of the free radicals, play an important part in many diseases such as Alzheimer's, arthritis, ischemic reperfusion, gastric ulcers and hepatotoxicity (Waris and Ahsan 2006). Normally, ROS including free radicals such as superoxide anion radical (O^{2-}) and hydroxyl radical (OH^-) and non-radical species including hydrogen peroxide (H_2O_2), a singlet oxygen ($^1O^2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) is continuously formed in various aerobic organisms (Poprac et al. 2017). These radicals are unstable and leads to cell or tissue injury via reacting rapidly with other groups (Je et al. 2005).

Apoptosis, a major form of cell death, plays an essential part in several unique features, including membrane blebbing, DNA cleavage (DNA fragmentation) and nuclear condensation phenomenon (Cohen et al. 1992). Normal apoptosis plays a critical role in a wide variety of physiologic processes during fetal development and in adult tissues (Levine and Klionsky 2004). However, abnormal apoptosis is a major factor in many human diseases including neurodegenerative diseases, cancer, and hepatic damage via generating nuclear condensation and fragment in single cells (Kannan and Jain 2000). In addition, cytotoxic mediators, pro-apoptosis molecules, for example Bax, cleaved caspase and p53, can be induced by the formation of ROS in Chang liver cells. Moreover, cell damage in liver, can lead to an intracellular oxidant stress (Jaeschke 2000). In addition, several *in vivo* experiments have demonstrated that apoptosis occurs in oxidative stress-induced liver injury (Jenner 2003). With these points, the inhibition of ROS production and/or apoptosis is an important key for the improvement of cell and tissue damages. Many synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), t-butylhydroquinone (TBHQ) and propyl gallate (PG), may be used to retard oxidative stress in many

fields (Rajapakse et al. 2005). However, the use of synthetic antioxidants has been restricted due to the potential health damage caused by inappropriate use (Rajapakse et al. 2005). Therefore, the search for natural antioxidants, as alternatives to synthetic ones, is of great interest among researchers. Also, recent studies have reported about the nuclear factor erythroid 2-related factor 2 (Nrf2) as important proteins that mediate antioxidant mechanism by inducing the up-regulation of HO-1 protein (Chang et al. 2010). With these reports, the inhibition of oxidative stress and apoptosis is an important factor for the improvement of damages in cells and tissues that can be caused by various oxidant systems.

Cephalopoda is included in the molluscan class and is a various protein source containing plentiful bioactive substances (Lindgren et al. 2004, Siahpoosh and Alikhani 2016). Especially, most cephalopod species can release ink, which has an anticancer effect, improvement of bone marrow activity, and induction of cell proliferation factors (Siahpoosh and Alikhani 2016). However, until now, there are no scientific reports on the biological activity of Cephalopoda meat against apoptosis in liver cells.

Therefore, the principal objective of this study was to explore in more detail the mechanisms underlying the regulation of apoptosis and antioxidant enzymes by *Loliolus beka gray* meat (LBM), leading to hepatoprotective effects in H_2O_2-treated hepatocytes.

2 Materials & Methods

2.1 Materials

All testing reagents including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), fluorescein sodium salt, 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA) and 3-(4–5-dimethyl-2yl)-2–5-diphynyltetrasolium bromide (MTT) were from Sigma Chemical Co. (St, Louis, Mo, USA). The hepatocytes line (CCL-13) was obtained from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Paisley, UK). Antibodies for Bcl-2, p53, Bax, Nrf2, HO-1, secondary antibodies were purchased from Cell Signaling Technology Inc. and β-actin was purchased from Sigma. Other chemical and reagent were of the highest grade available commercially.

2.2 Preparation of LBMH

Loliolus beka (LB) was obtained from Yeosu, South Korea. It washed to remove impurities, and the meat was separated from LB (LBM). Hot water extract of LBM (LBMH) was prepared by following methods. LBM (50 g) and 1 L of distilled water was mixed for 4 h at 100 °C. Then, the extract was centrifuged and freeze-dried. The freeze-dried LBMH was used for this study.

2.3 Determination of the Free Amino Acid Composition

The free amino acid composition of LBMH was applied to the amino acid analyzer (S433-H, Sykam GmbH, Germany) and measured by the identical method indicated by Han et al. (2017).

2.4 Antioxidant Capacities of LBMH

To evaluate antioxidant capacities of LBMH, the effect of LBMH on ABTS and DPPH radicals, hydrogen peroxide, ORAC value, and reducing power were checked according to the identical methods of Han et al. (2017).

2.5 Cell Viability

Chang liver cells were incubated at 37 °C in a humidified atmosphere (5% CO_2), and cultured in DMEM containing 10% heat-inactivated FBS, streptomycin (100 μg/ml) and penicillin (100 unit/ml). Cell viability of LBWH was determined by MTT assay. The Chang liver cells (1 × 104 cells/wells) were divided in equal amounts and treated with various doses of LBMH (125–500 μg/ml) in a 96 well plate for 24 h. After incubation, 15 μl of MTT stock solution (5 mg/ml) was applied to each cell for 4 h. The absorbance of formazan crystals dissolved in 150 μl of dimethylformamide (DMSO) was measured at 540 nm using microplate reader.

To evaluate the cytoprotective effect of LBMH on H_2O_2-induced oxidative stress in Chang liver cells, the cells were incubated with a non-cytotoxic dose of LBMH (125, 250 and 500 μg/ml) at 37 °C for 2 h. Additionally, the cells were exposed to 1 mM H_2O_2 to generate oxidative stress, followed by incubation for 24 h. After completion of the incubation, the MTT assay was performed as described in the above methods.

2.6 DCFH-DA Assay

To examine effect of LBMH on intracellular ROS generation, DCFH-DA assay was performed. Hepatocytes (1.6×10^4 cells/wells) were pre-treated with different doses of LBMH (125, 250 and 500 μg/ml) before 1 h of H_2O_2 (1 mM) stimulation. After 1 h, the cells were stained with DCFH-DA (0.5 mg/ml in 100% ethanol) and the fluorescent was examined by recording values at excitation 485 nm/emission 528 nm. The absorbance of only H_2O_2-treated cells was considered as 100% and then the results were compared to the other groups.

2.7 Nuclear Staining with Hoechst 33342

Changes in nuclear morphology of hepatocytes such as apoptotic body formation following a bout of H_2O_2-induced oxidative stress was measured with a cell-permeable DNA dye, Hoechst 33342 (Sigma, USA, Missouri). Normally, the cells damaged by the exposure of oxidative stress include the characteristic formations, such as nuclear condensation of chromatin and/or nuclear fragmentation (Lovrić et al. 2005). First, hepatocytes (6×10^5 cells/wells) were pre-treated with LBMH (125, 250 and 500 μg/ml), and then 1 mM H_2O_2 was added. After 12 h, Hoechst 33342 was added to the cultured cells and plates were incubated for additional 30 min at 37 °C in the dark. The stained cells were observed with a fluorescence microscope (Olympus, Japan, Shinjuku).

2.8 Flow Cytometry Analysis

Cell cycle analysis was conducted to confirm the population of apoptotic sub-G_1 hypodiploid cells (Durelli et al. 2009). The hepatocytes (6×10^5 cells/dish) were treated with various concentrations of LBMH (125, 250 and 500 μg/ml) and then the cells were stimulated with H_2O_2 (1 mM) for 12 h. The cells were stained using PI solution (50 μg/ml) (Sigma, Missorui, USA) and RNase A (0.2 μg/ml) (Promega, Wisconsin, USA). Cell cycle was analyzed using CytoFLEX (Beckman coulter, California, USA). The effect of LBMH on the cell cycle was determined by changes in the percentage of the cell population at a specific cell cycle phase, as shown by histogram.

2.9 Western Blot

Hepatocytes (5×10^5 cells/wells) pre-treated with LBMH (125, 250 and 500 μg/ml) were exposed to 1 mM H_2O_2. After 12 and 24 h, the cells were lysed by RIPA buffer (Thermo, Rockford, IL) and the proteins were obtained. The obtained protein (40 μg) was used for western blot analysis. The primary antibodies (1:1000) were for Bcl-2, p53, Bax, Nrf2, HO-1 and β-actin (1:3000). The secondary antibodies were HRP-conjugated-anti-mouse IgG and anti-rabbit IgG (Cell Signaling Technology Inc.). The densitometric data analysis was examined using NIH Image J software (US National Institutes of Health, Bethesda, MD).

2.10 Effect of LBMH on Blockage of Nrf2/HO-1 Signaling in H_2O_2-Stimulated Hepatocytes

To confirm whether the hepatoprotective effect of LBMH is related to the Nrf2/HO-1 signaling in H_2O_2-treated hepatocytes, MTT and DCFH-DA assay were performed under the block of Nrf2/HO-1 signaling. The cells were treated with 5 μM ZnPP (HO-1 inhibitor) for 1 h and then LBMH for additional 1 h. The cells were stimulated with 1 mM H_2O_2, followed by incubation for 1 h and 24 h at 37 °C. The MTT and DCFH-DA assays were performed as described in the above method.

2.11 Statistical Analysis

Data are expressed as means ± SE in triplicate, and all statistical comparisons were made by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value <0.05 was statistically significant.

3 Results

3.1 Free Amino Acid Composition of LBMH

In Table 1, we observed LBMH contains a plentiful supply of free amino acid, such as taurine, glutamic acid, alanine and leucine. Among them, taurine content is the dominant amino acid, representing 22.97% of the free amino acid pool. With these results, LBMH consisted of a plentiful taurine could possess a high nutritional value.

Table 1 The free amino acid contents (%) of hot water extract prepared from *Loliolus beka* gray meat (LBMH)

	LBMH	
Amino acid	mg/100 g	% amino acid
Phosphoserine	14.08	0.36
Taurine	904.82	22.97
Phosphoethanolamine	9.01	0.23
Urea	N.D.	N.D.
Aspartic acid	12.61	0.32
Hydroxyproline	N.D.	N.D.
Threonine	17.40	0.44
Serine	17.78	0.45
Asparagine	N.D.	N.D.
Glutamic acid	402.20	10.21
Sarcocine	N.D.	N.D.
α-aminoadipic acid	N.D.	N.D.
Proline	227.32	5.77
Glycine	134.47	3.41
Alanine	343.82	8.73
Citrulline	98.92	2.51
α-aminobutyric acid	7.51	0.19
Valine	112.61	2.86
Cystine	14.97	0.38
Methionine	174.76	4.44
Cysthathionine	8.96	0.23
Isoleucine	132.48	3.36
Leucine	352.11	8.94
Tyrosine	151.51	3.85
Phenylalanine	159.77	4.06
β-alanine	0.96	0.02
β-aminoisobutyric acid	14.79	0.38
γ-amino-n-butyric acid	4.37	0.11
Histidine	50.39	1.28
3-methylhistidine	N.D.	N.D.
1-methylhistidine	N.D.	N.D.
Carnosine	N.D	N.D.
Anserine	N.D.	N.D.
Tryptopan	48.37	1.23
Hydroxylysine	16.78	0.43
Ornitine	85.61	2.17
Lysine	326.64	8.29
Ethanolamine	N.D.	N.D.
Arginine	93.60	2.38
Total	3938.57	100.00

3.2 *Determination of Antioxidant Activities*

Antioxidant activities of LBMH were confirmed by measurement the ABTS, DPPH radicals and hydrogen peroxide scavenging activities, ORAC value and reducing power. As shown in Fig. 1a–c, LBMH highly exhibited the ABTS and DPPH radicals, and hydrogen peroxide scavenging activities. In addition, Fig. 1d indicated that LBMH (50 μg/ml) had the ORAC value of 544.11 μM TE/mg sample. Furthermore, LBMH led to the superior reducing power activity at used all concentrations (Fig. 1e).

3.3 *Intracellular Antioxidant Activities of LBMH in H_2O_2-Stimulated Hepatocytes*

Effect of LBMH against H_2O_2-induced cytotoxicity was measured by MTT assay. LBMH has no significant cytotoxicity at the used all concentrations (Fig. 2a). Also, Fig. 2b exhibited only 1 mM H_2O_2 stimulation markedly decreased cell viability, compared to the non-treated control cells (21.90%). Interestingly, they were improved by the pre-treatment of LBMH. These results indicated that LBMH exhibits its significant cytoprotective activity in hepatocytes.

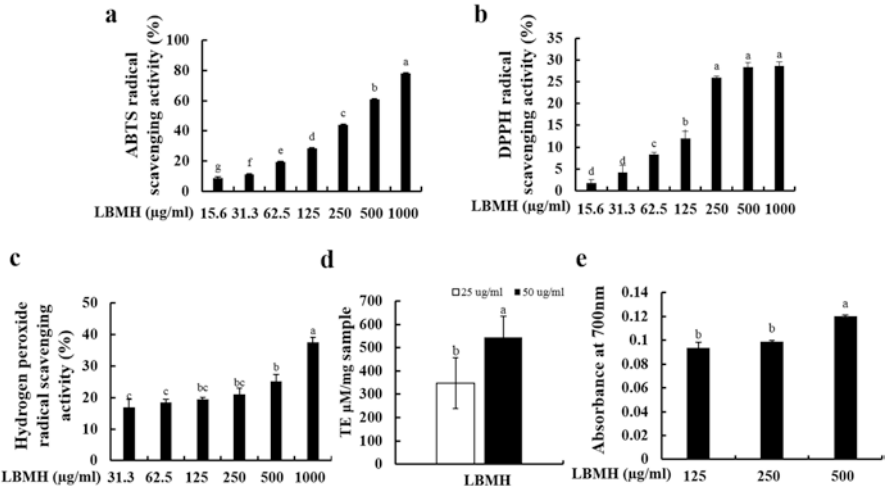

Fig. 1 Antioxidant effects (ABTS (**a**), DPPH (**b**), hydrogen peroxide radical scavenging activity (**c**), ORAC value (**d**) and reducing power scavenging activity (**e**)) of LBMH. Values represent means ± SE of three determinations. [a–g]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as means ± SE (n = 3)

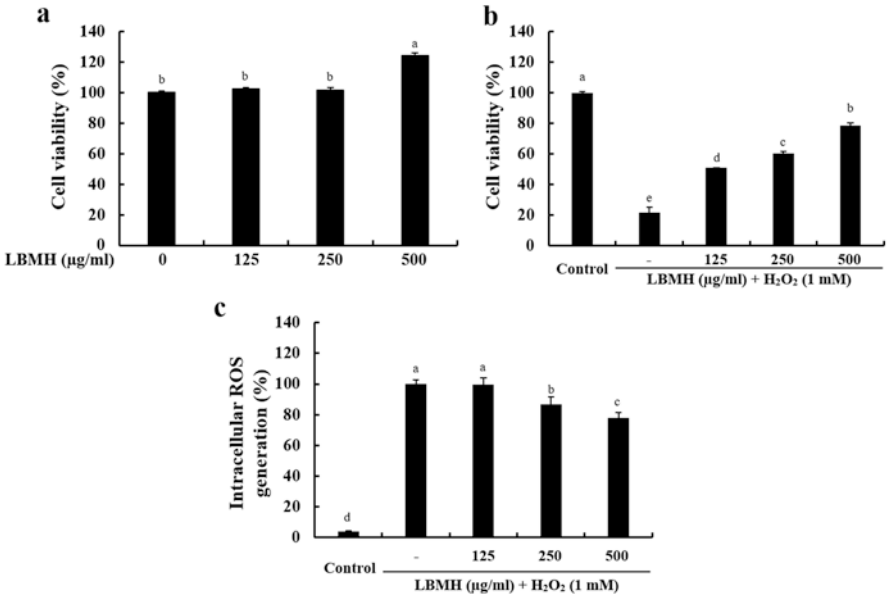

Fig. 2 Effects of LBMH on cell viability in the absence/presence of H_2O_2 (**a**, **b**) and intracellular ROS generation (**c**) induced by H_2O_2 in hepatocytes. [a–e]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as means ± SE (n = 3)

3.4 *Intracellular ROS Scavenging Activities of LBMH in H_2O_2-Stimulated Hepatocytes*

To measure effect of LBMH on intracellular ROS generation, we performed the DCFH-DA assay. Figure 2c exhibited the intracellular ROS generation was markedly increased by stimulation of H_2O_2. However, co-treatment with LBMH significantly reduced H_2O_2-induced intracellular ROS generation (77.71% at 500 μg/ml).

3.5 *Effects of LBMH Against the Apoptotic Body Formation and Sub-G_1 Hypodiploid Cells Caused by H_2O_2 Stimulation*

Normally, Hoechst 33342 dye is widely used to detect apoptosis (Kim et al. 2006). As shown in Fig. 3a, no apoptotic bodies were observed in the non-treated control cells, however, the stimulation with H_2O_2 (1 mM) caused the significant increase in the number of apoptotic bodies. Whereas the cells treated with LBMH exhibited the marked reduction in the number of apoptotic bodies. Moreover, PI analysis exhibited H_2O_2 increased the sub-G_1 DNA population content to 31.13% (Fig. 3b, c). However, the pre-treatment of LBMH improved the increment of sub-G_1 DNA contents in H_2O_2-stimulated hepatocytes. These results indicate that LBMH can protect cells as reducing apoptosis phenomenon.

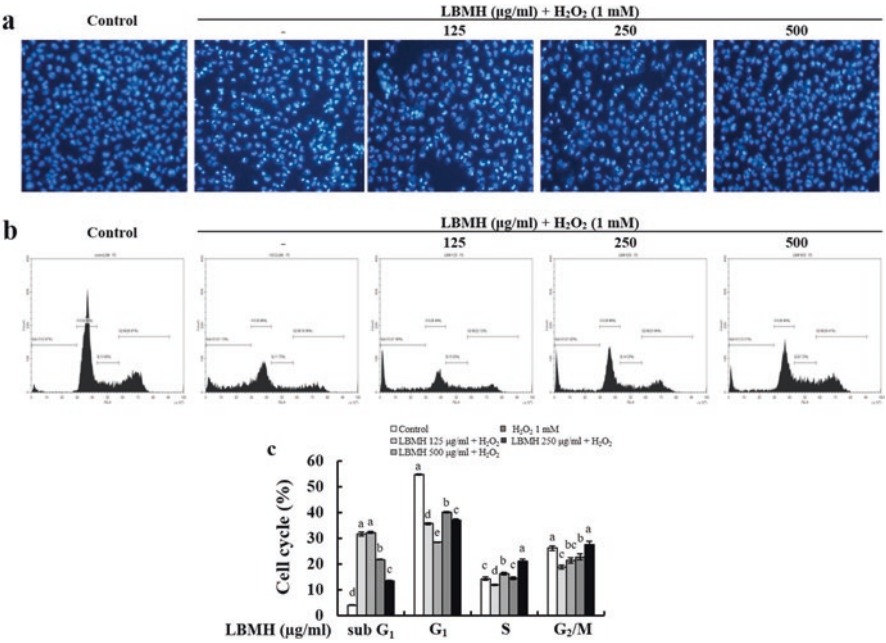

Fig. 3 The effect of LBMH on apoptotic body formation (**a**), cell cycle pattern (**b**) and graph of cell cycle percentage (**c**) induced by H_2O_2 in hepatocytes. [a–e]The bars with different letters are significantly different ($p < 0.05$)

3.6 Western Blot

To analyze whether LBMH inhibits apoptosis by modulating the mitochondrial signaling in H_2O_2-treated hepatocytes, western blot assay was performed. We identified that the stimulation of H_2O_2 led to the increased expression levels of Bax and p53 as well as the reduced expression level of Bcl-2 in H_2O_2-treated hepatocytes. Interestingly, they positively modulated by the pre-treatment of LBMH (Fig. 4). These results indicate that LBMH suppresses apoptosis by altering the content of apoptosis regulators.

3.7 Effect of LBMH on Nrf2/HO-1 Signaling in H_2O_2-Stimulated Hepatocytes

To evaluate whether the hepatoprotective effect of LBMH is related to the antioxidant defense system in H_2O_2-stimulated hepatocytes, we analyzed Nrf2/HO-1 signaling by western blot analysis. Figure 5 showed that the pre-treatment with LBMH activated Nrf2/HO-1 signaling as increasing the expression levels of nucleic Nrf2

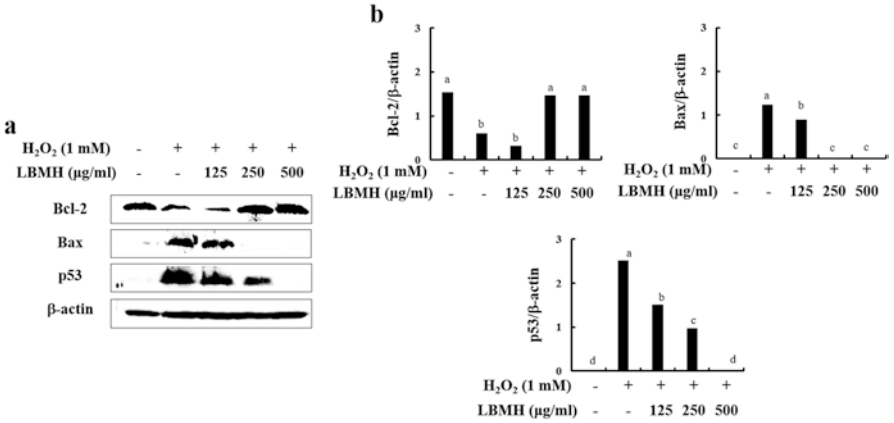

Fig. 4 Protective effect of LBMH against H_2O_2-induced apoptosis in cultured hepatocytes. Total cell lysates were prepared and subjected to western blot analysis to monitor the expression levels of protein regulators of apoptosis, namely Bcl-2, Bax and p53 (**a**). Densitometry analysis of protein expression levels (**b**). Values represent means ± SE of three determinations. [a–d]The bars with different letters are significantly different ($p < 0.05$)

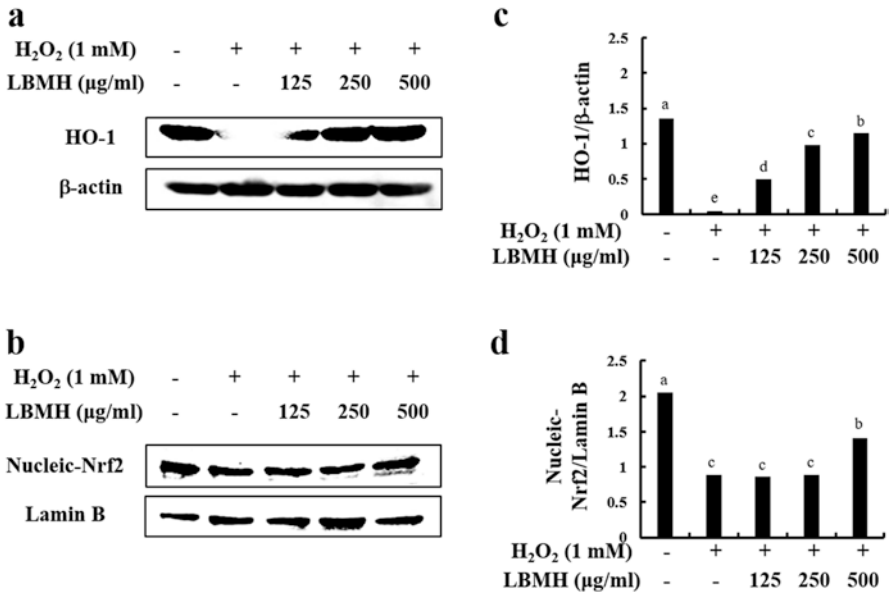

Fig. 5 Effect of LBMW on the expression of HO-1 (**a**) and Nuclear-Nrf2 (**b**) and densitometry (**c, d**) of H_2O_2-treated cultured hepatocytes. Quantitative representations of western blot analysis. Values represent means ± SE of three determinations. [a–e]The bars with different letters are significantly different ($p < 0.05$)

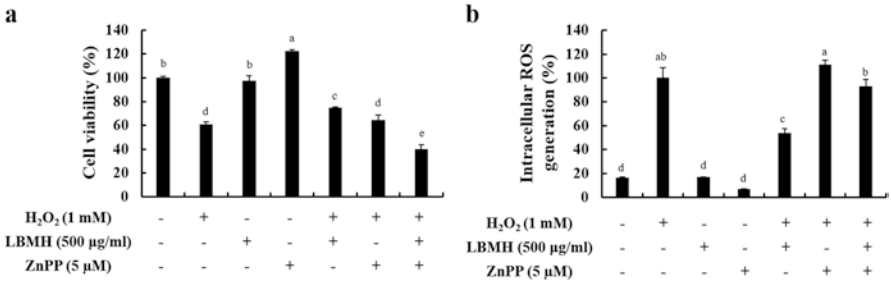

Fig. 6 Effect HO-1 blockage on LBMH' cell viability (**a**) and intracellular ROS generation (**b**) of H_2O_2-stimulated hepatocytes. Cell viability was assayed by MTT assay and intracellular ROS generation was assessed using the oxidation sensitive dye, DCFH-DA. [a–e]The bars with different letters are significantly different ($p < 0.05$)

and cytoplasmic HO-1. These results demonstrate that the hepatoprotective effect of LBMW is supported by the Nrf2/HO-1 signaling pathway. Indeed, in Fig. 6, we discovered the interesting result that the capacity of LBMH on the improved cell viability and the reduced intracellular ROS generation are abolished by the blockage of HO-1 following as the treatment of ZnPP, a HO-1 inhibitor. With these results, we indicate that LBMH exerts hepatoprotective activity as inhibiting H_2O_2-induced apoptosis through the activation of HO-1/Nrf2 signaling.

4 Discussion

Marine organisms usually contain plentiful amounts of taurine. *Loliolus beka* gray, a cephalopod, which mainly inhabits Korea and China serves as a processed food and contains abundant taurine (Um et al. 2017; Han et al. 2017). Taurine (2-aminoethanesulfonic acid) is a sulfur amino acid that is abundant in seafood but not in land animals. In a recent study, taurine exhibited various pharmacological and physiological effects, such as anti-diabetes, anti-inflammatory and antioxidant effects (Um et al. 2017). Interestingly, the cells and tissue can be damaged by oxidative stress in taurine deficiency. Also, taurine deficiency potentiates cell damage-mediated apoptosis in the previous study (Jong et al. 2012). Until now, no study has reported on the antioxidant potential of an extract of a marine organism, particularly those that contain large amounts of taurine. In particular, the potential of LBMH as an antioxidant with hepatoprotective activity against H_2O_2-induced oxidative damage has not been validated. Therefore, in this study, we evaluated the hepatoprotective activity of LBMH, which contains abundant amounts of taurine, against H_2O_2-induced oxidative stress of hepatocytes.

Normally, the ABTS radical scavenging assay is a method of antioxidant evaluation to measure the activity of lipophilic and hydrophilic compounds (You et al. 2009). The DPPH radical is a stable radical that accepts electrons or hydrogen radi-

cals to become a stable diamagnetic molecule, which is often used as a substrate to evaluate antioxidant activity (Choi et al. 2007). The reducing capacity, hydrogen peroxide scavenging and ORAC value of the compound provide the beneficail information on the potential use of the tested substance as a commercial antioxidant. As these studies, we also confirmed free radical sacavenging activity of LBMH *in vitro*. Our results showed that LBMH containing the large amouts of taurine led to the potent scavenging activity against ABTS, DPPH and hydroxyl radicals and high reducing power as well as ORAC values. According to the previous study, the antioxidant activity of taurine can affect to a biological antioxidant, including an ability to scavenge hydroxyl radicals and hydrogen peroxide, thereby decreasing cellular cytotoxicity and connective tissue damage (Lovrić et al. 2005). Based on the work of Wu et al. (2003), free amino acids and low molecular weight peptides obtained from fish have shown superior antioxidant activity against DPPH radical, it was similar to ours. Thus, the antioxidant activities of LBMH are thought to be caused by taurine, which is a single amino acid in contrast to that of a low molecular weight peptide (Wu et al. 2003).

Previous study showed that high concentrations of ROS to induce cell damage, including cell death and metabolic alterations (Lobo et al. 2010). Our data showed that H_2O_2 treatment induced death of hepatocytes however pretreatment with LBMH improved cell viability by decreasing ROS production. The data of Roy and Sil (2012) also showed that taurine is an effective antioxidant, protecting against cell death through reductions in intracellular ROS production. According to other investigators, taurine attenuates oxidative cell damage through the actions of the antioxidant enzymes, including SOD, GPX and CAT (Das et al. 2008; Han et al. 2017). Hence, taurine shows excellent protective activity against H_2O_2-induced oxidative stress. Therefore, our data supports that LBMH-mediated cell protection is due to the actions of taurine. Apoptosis caused by oxidative stress involves biochemical features of the apoptotic body formation process, including physiological, biochemical or noxious stimuli (Poprac et al. 2017). The biochemical and morphological characteristics of apoptosis have been extensively described elsewhere. In brief, these characteristics include cell shrinkage, chromatin condensation, apoptotic body formation and DNA degradation (Bortner et al. 1995). The fragmented cells (apoptotic cells) can be separated by the presence of chromatin condensation within the nucleus, bright blue and smaller nuclear bodies surrounded by cytoplasmic membrane (Kim et al. 2013). Moreover, apoptosis is followed by the activation and/or suppression of several proteins, such as Bcl-2, Bax, cleaved caspases and p53 (Kim et al. 2013). So, our data showed the inhibition of ROS important for the proection of cells and tissues against oxidative stress. Interestingly, our data showed the treatment with LBMH significantly decreased the number of the apoptotic body despite the presence of H_2O_2-induced oxidative stress. It also decreased the proportion of cells with sub-G_1 DNA content by modulating regulators of apoptosis (Bcl-2, Bax and p53). Wu et al. (2018) results supported our results, namely, high amounts of taurine prevent oxidative stress-induced apoptosis in hepatocytes by reducing ROS production (Wu et al. 2018). In addition, another study indicated that taurine treatment significantly reversed disturbances mediated by changes in Bax and Bcl-2

expression in mice liver (Li et al. 2017). Thus, our resuts demonstrated the imrovement of apoptosis by LBMH treatment, by mitochondrial pathways.

In further study, we checked whether HO-1 altered the hepatoprotective effect of LBMH. Recently, several studies have suggested that Nrf2 is an important regulator of antioxidative defense responses (Yang et al. 2009). The activation of Nrf2 is normally reduced in the cytosol by specific binding to Keap1. However, upon activation by other mediators, inhibition of Nrf2 protein is associated with its translocation into the nucleus, thereby suppressing HO-1 protein (Yang et al. 2009). Our results revealed that the pre-treatment with LBMH exerts protective effects via the modulation of H_2O_2-induced oxidative stress in hepatocytes. Interestingly, we also discovered the hepatoprotective capacity LBMH can be affected by the activation of HO-1/Nrf2 pathway.

5 Conclusion

Taken together, these results suggest that LBMH consisted of a large amount of taurine leads to the hepatoprotective effects on H_2O_2-induced oxidative stress and apoptosis by inhibiting ROS generation and modulating the Nrf2/HO-1 signaling.

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An Aqueous Extract from *Batillus Cornutus* Meat Protects Against H_2O_2-Mediated Cellular Damage via Up-Regulation of Nrf2/HO-1 Signal Pathway in Chang Cells

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Abstract In this study, we evaluated the protective effects of an aqueous extract from *Batillus cornutus* meat (BM) against cellular oxidative damage caused by hydrogen peroxide (H_2O_2) in human hepatocyte, Chang cells. First, we prepared an aqueous extract of BM meat (BMW) showing the highest taurine content among free amino acid contents. BMW led to high antioxidant activity showing 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, good reducing power and an oxygen radical absorbance capacity (ORAC) value.

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Also, BMW improved cell viability that was diminished by H_2O_2 exposure, as it reduced the generation of intracellular reactive oxygen species (ROS) in Chang cells. In addition, BMW up-regulated the production of antioxidant enzymes, such as catalase and superoxide dismutase (SOD), compared to H_2O_2-treated Chang cells lacking BMW. Moreover, BMW induced the expressions of nuclear Nrf2 and cytosolic HO-1 in H_2O_2-treated Chang cells. Interestingly, the treatment of ZnPP, HO-1 inhibitor, abolished the improvement in cell viability and intracellular ROS generation mediated by BMW treatment. In conclusion, this study suggests that BMW protects hepatocytes against H_2O_2-mediated cellular oxidative damage via up-regulation of the Nrf2/HO-1 signal pathway.

Keywords *Batillus cornutus* meat · Taurine · Antioxidant effect · Cellular oxidative stress · Protective effect · Nrf2/HO-1 signal pathway

1 Introduction

Batillus cornutus (*B. cornutus*) or the spiny top shell has economic value because it is one of the commonly consumed sea snail species in East Asian countries, such as Japan, Korea, and China. Their natural spawning grounds are Okinawa, Honshu and Kyushu islands in Japan. Due to increased consumption, large numbers of juveniles are produced and released into the open sea, where they grow and proliferate (Donaghy et al. 2010). Recently exploration of nutraceutical and functional properties of seafood has assumed enhanced interest, as concerns about diet and health have arisen. Many studies have been carried out to explore the functional properties of seafood-derived peptides and amino acids (Nikoo and Benjakul 2015; Kim and Wijesekara 2010). Based on emerging evidence, peptides of fish and shellfish contain antioxidants, anti-microbials, antihypertensives, anti-coagulants, anti-diabetic agents, immuno-stimulatory factors, anti-cancer compounds, calcium-binding substances and hypocholesteremic properties (Harnedy and Fitzgerald 2012). While numerous bioactive peptides and amino acids are reported from fish species, marine organisms such as crustacean and mollusks remain under-explored.

Oxidative stress is a primary factor causing a number of non-communicable chronic disease conditions including arteriosclerosis, diabetes, heart disease, stroke, and cancer. Increased generation of reactive oxygen species (ROS), such as hydroxyl (OH-) radicals, superoxide anion (O2-), hydrogen peroxide (H_2O_2), combined with the depletion of the endogenous antioxidant defenses controlled by enzymatic and

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non-enzymatic mediators, is a major contributor to the pathogenesis of chronic diseases (Schieber and Chandel 2014). Hence antioxidants should benefit the aforementioned disease conditions, helping to maintain physiological wellbeing. Although many synthetic antioxidants are incorporated into food products, such as butylated hydroxyanisole, butylated hydroxytoluene, tert-butylhydroquinone, and propyl gallate, prolonged consumption may cause detrimental side effects. Hence natural antioxidants have gained attention in terms of their health benefits with little or no side-effects (Shahidi and Zhong 2005).

Still, the exact antioxidant mechanism of amino acids and peptides are not fully understood. However, recent evidence suggests that amino acids and peptides can act as transition metal chelators, as well as radical scavengers (Sarmadi and Isamil 2010). Furthermore, some amino acids and peptides induce the synthesis of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as Phase II enzymes, including NAD(P)H quinone dehydrogenase 1 (NQO1) and heme oxygenase-1 (HO-1) through nuclear factor erythroid 2-realted factor2 (Nrf-2) activation (Kim and Jang 2014). Seafood derived peptides with antioxidant activity could possess potential as biocompatible alternatives to synthetic antioxidants and would help to uphold physiological wellbeing. The aim of the present study was to explore the antioxidant and nutraceutical benefits of an aqueous extract obtained from *B. cornutus* meat against oxidative stress conditions induced by H_2O_2.

2 Materials & Methods

2.1 Materials

Ferrozine, 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, Aldrich, USA. Chang cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbeco's modified Eagle's medium (DMEM), FBS, and antibiotics (penicillin and streptomycin) were purchased from GIBCO INC., USA. Antibodies were purchased from Santa Cruz Biotechnology, USA.

2.2 Preparation of BM Aqueous Extraction Technique (BMW)

B. cornutus were washed using tap water to remove salt and debris. The muscular part was collected, and the shells were discarded. Then the samples were freeze-dried, powdered and extracted using water at room temperature for 24 h with continuous agitation. The water extract of *B. cornutus* (BMW) was obtained by centrifugation followed by vacuum filtration. The extract was dried by lyophilization.

2.3 Evaluating Proximate Compositions and Amino Acid Compositions of BMW

The protein content in BMW was estimated by Thermo Scientific Pierce BCA protein assay kit (Thermo Scientific, Rockford, USA). The polysaccharide composition and the polyphenol composition was estimated by the phenol-sulfuric acid method (Dubois et al. 1956) and by the folin-ciocalteu method (Chandler and Dodds 1983) respectively. The amino acid compositions of the BMW was analyzed by amino acid analyzer (S433-H, Sykam GmbH, Germany).

2.4 ABTS Radical Scavenging Assay

ABTS radical scavenging activity of BMW was determined according to the method of Han (2017). The working solution was prepared by diluting samples with distilled water to an absorbance of 1.50 ± 0.05 at 414 nm. A stock solution containing 7 mM ABTS in 2.4 mM potassium persulfate was mixed with each 50 μl of BMW and allowed to stand for 10 min at R.T. The absorbance was measured at 414 nm using microplate reader. ABTS radical scavenging activity of BMW was calculated from the following equations.

$$\text{ABTS radical scavenging activity}(\%) = \left[\left(\text{Abs.control} - \text{Abs.sample}\right) / \text{Abs.control}\right] \times 100$$

Abs.control: Control absorbance at 414 nm
Abs.sample: Sample absorbance at 414 nm

2.5 ORAC Assay

To perform the Oxygen radical absorbance capacity (ORAC) assay, the method described by Zulueta was used with slight modifications. Briefly, phospate buffer (75 mM, pH 7.0) was used to prepare all of the solutions of BMW. BMW (50 μg/ml, 50 μl) was mixed with fluorescein (78 nM, 50 μl). This was incubated for 15 min at 37 °C. After incubation AAPH (221 mM, 25 μl) was added and fluorescence readings were recorded at 5 min intervals for 1 h duration (excitation wavelength: 485 nm, emission wavelength: 582 nm). A standard curve was plotted using trolox (range; 0–20 μM), the results were calculated using the area under the curve (AUC).

2.6 Reducing Power

A method described by Oyaizu (1986) with slight modifications was used to evaluate reducing power. For this a specific concentration of BMW (0.25, 0.5 and 1 mg/ml) was mixed with sodium phosphate buffer (0.1 M, pH 6.6, 300 µl) and potassium ferricyanide (1%, 500 µl) at 50 °C for 20 min. Then TCA (10%, 500 µl) was added and the mixture was centrifuged (1036 × g for 10 min). The resulting supernatant (100 µl) was diluted in half with distilled water and 20 µl of $FeCl_3$ (0.1%) was added to each sample. The absorbance readings were collected using 20 µl of the final solution at 700 nm wavelength (Oyaizu 1986).

2.7 Determination of Cell Viability and Intracellular ROS Scavenging Activity

Chang cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin mixture at 37 °C under a humidified atmosphere supplemented with 5% CO_2. The cells were maintained by periodical subculture. Cells that have attained exponential growth were seeded for the experiments at a concentration of 1×10^5 cells/ml. For cytotoxicity evaluation, different concentrations of BMW were treated to 24 h pre-cultured cells and incubated for 24 h. Intracellular ROS scavenging effects were evaluated in H_2O_2 (750 µM) treated cells after treating different concentrations of BMW with a 24 h incubation period. The cell viability was measured by MTT assay (Wang et al. 2017).

Intracellular ROS formation was measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA). Chang cells were seeded in 96 well plate (1×10^4 cells/wells). They were incubated for 18 h and treated with different concentrations of BMW. After 1 h incubation, 1 mM H_2O_2 was added and incubated for an additional 24 h, after which DCF-DA was added to each well (500 µM). The fluorescence intensity was measured after 5 min, under the conditions of excitation 485 nm/emission 528 nm. Intensities are compared that of the controls.

2.8 Western Blot

The levels of molecular mediators related to the antioxidant effects were analyzed by western blot (Fernando et al. 2018). Cells were seeded at a concentration of 2×10^5 cells/ml and incubated with different BMW concentrations and stimulated with H_2O_2 for 24 h. After the 24 h incubation period, the cells were collected, lysed, and the proteins were collected by centrifugation (13,000 × g). Then the protein

levels were standardized to 50 μg and loaded on 12% SDS-polyacrylamide gels. The protein bands were resolved by electrophoresis. The bands were transferred to nitrocellulose membranes and incubated with primary (4 °C for 12 h) and then with secondary (R.T for 2 h) antibodies.

2.9 Statistical Analysis

All data are expressed as means ± standard error based on at least triplicate determinations. All statistical comparisons were made by means of one-way analysis of variance followed by Duncan's test using PASW Statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

3 Results

3.1 Proximate Compositions and Amino Acid Compositions of BMW

As indicated in Table 1, the proximate compositions of BM indicated comparatively higher levels of proteins followed by lipids. In the result of amino acid compositions analysis, we identified that BMW contained the higher levels of taurine followed by alanine and then proline (Table 2).

3.2 Antioxidant Activities of BMW

The antioxidant activity of BMW was initially analyzed by several different chemical assays. ABTS radical scavenging and reducing power are two of the most popular antioxidant assays. The ORAC assay is a standard analytical procedure for evaluating antioxidant activity of food products. As shown in Fig. 1a, the ABTS radical scavenging activity increased with increasing sample concentrations of BMW. The IC50 concentration for ABTS radical scavenging activity was 0.69 ± 0.00. Shown in Fig. 1b are the results of the reducing power assay, revealing that BMW elevates antioxidant activity in dose-dependent manner. The antioxidant capacity reached 234 μM TE/mg at 50 μg/ml sample concentration.

Table 1 Proximate compositions (%) of BM

Samples	Protein	Lipid	Moisture
BM	40.65 ± 1.70	15.68 ± 0.53	2.04 ± 0.01

Values are the means ± S.E of three determination

Table 2 The free amino acid contents (%) of BMW

	BMW	
Amino acid	mg/100 g	% amino acid
Phosphoserine	N. D	N. D
Taurine	258.178	24.74
Phosphoethanolamine	N. D	N. D
Urea	N. D	N. D
Aspartic acid	22.948	2.20
Hydroxyproline	N. D	N. D
Threonine	40.539	3.89
Serine	21.029	2.02
Asparagine	4.526	0.43
Glutamic acid	49.698	4.76
Sarcocine	N. D	N. D
α-Aminoadipic acid	N. D	N. D
Proline	72.334	6.93
Glycine	30.406	2.91
Alanine	134.161	12.86
Citrulline	N. D	N. D
α-Aminobutyric acid	4.67	0.45
Valine	43.279	4.15
Cystine	N. D	N. D
Methionine	31.895	3.06
Isoleucine	27.733	2.66
Leucine	55.319	5.30
Tyrosine	26.637	2.55
Phenylalanine	24.095	2.31
β-Alanine	1.848	0.18
β-Aminoisobutyric acid	5.358	0.51
γ-Amino-n-butyric acid	0.816	0.08
Histidine	27.502	2.64
3-Methylhistidine	2.779	0.27
1-Methylhistidine	0.963	0.09
Carnosine	0.907	0.95
Anserine	N. D	N. D
Tryptopan	N. D	N. D
Hydroxylysine	N. D	N. D
Ornitine	N. D	N. D
Lysine	108.068	10.36
Ethanolamine	N. D	N. D
Arginine	38.714	3.71
Total	1043.40	100.00

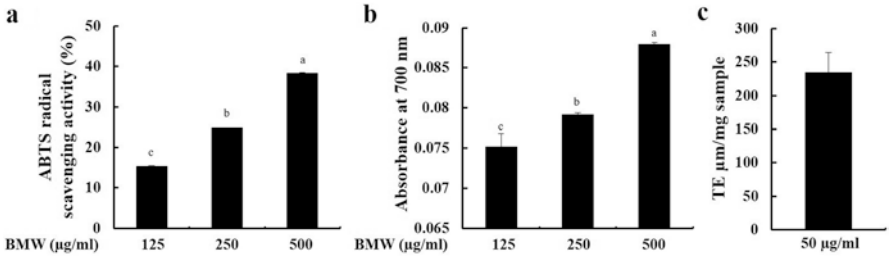

Fig. 1 ABTS (**a**), reducing power (**b**) and ORAC value (**c**) of BMW. Values means ± S.E of three determinations. [a–c]The bars with different letters are significantly different ($p < 0.05$)

3.3 Cytotoxicity and Intracellular ROS Scavenging Effect of BMW

To investigate the cytotoxicity of BMW on Chang cells, we performed MTT assay. Interestingly BMW did not show the cytotoxicity, compared to the control (Fig. 2a). As indicated in Fig. 2b, H_2O_2 treatment caused a rapid reduction in cell viability wheras BMW treatment diminished H_2O_2-induced toxicity, resulting in the elevated cell viability. Figure 2c also exhibited that the intracellular ROS level was promptly increased upon H_2O_2 treatment. Subsequent treatment with increasing concentrations of BMW significantly reduced intracellular ROS levels in a dose-dependent manner.

3.4 Effects on Antioxidant Enzyme Activities

Catalase (CAT) and superoxide dismutase (SOD) are two of the key intracellular antioxidant enzymes which help to regulate antioxidant defense. As shown in Fig. 3a, H_2O_2 treatment decreased the activity of CAT and SOD compared to the control. However, the enzyme activity increased dose-dependently with increasing concentrations of BMW.

3.5 Effects of BMW on HO-1 and Nrf2 Protein Expression Levels

HO-1 is another important member of the anti-oxidant defense system in cells. As shown in Fig. 4a, H_2O_2 treatment decreased the levels of the antioxidant enzyme, HO-1, whereas BMW treatment significantly increased it in a dose-dependent manner. Nrf2 is a key molecule that mediates the production of antioxidant enzymes. As evidenced in Fig. 4b, Nrf2 levels in the nucleus decrease with H_2O_2 treatment and increase upon BMW treatment.

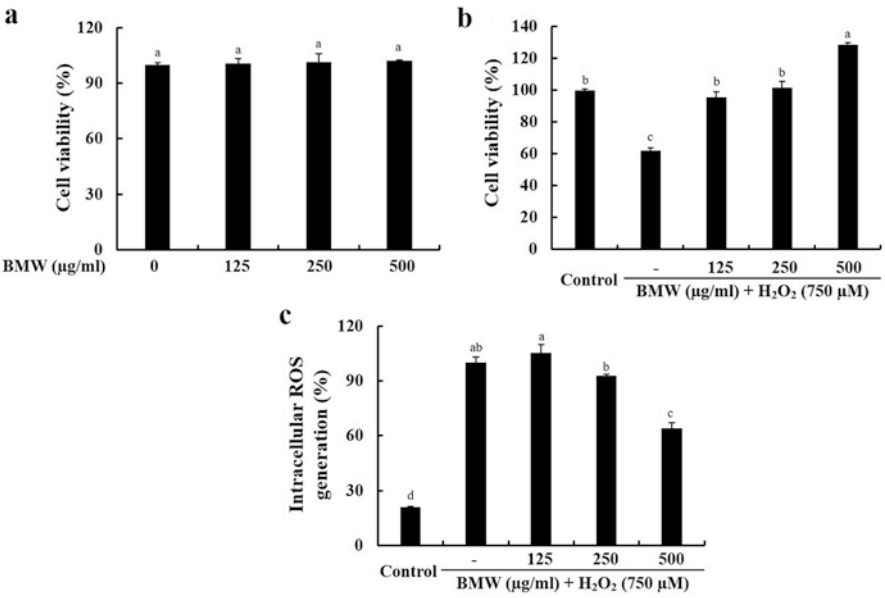

Fig. 2 Effects of BMW on cell viability with/without H_2O_2 stimulation (**a** and **b**) and ROS generation (**c**) in Chang cells. Chang cells were pre-treated with various doses of BMW (125, 250 and 500 μg/ml). Values represent means ± S.E of three determinations. [a–e]The bars with different letters are significantly different ($p < 0.05$)

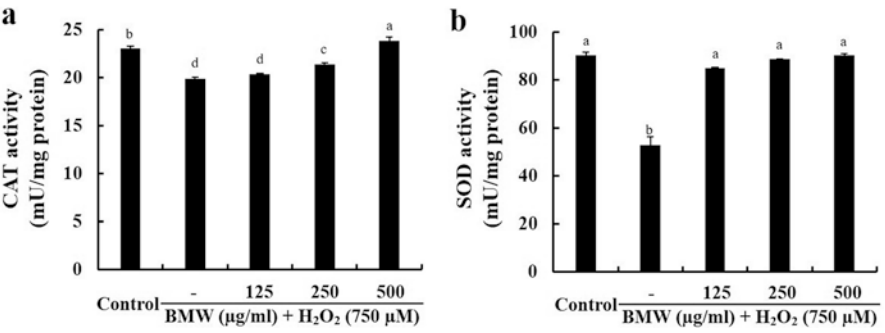

Fig. 3 Effect of BMW on antioxidant enzyme activities in H_2O_2-treated Chang cells. (**a**) CAT activity, (**b**) SOD activity. Values represent means ± S.E of three determinations. [a-d]The bars with different letters are significantly different ($p < 0.05$)

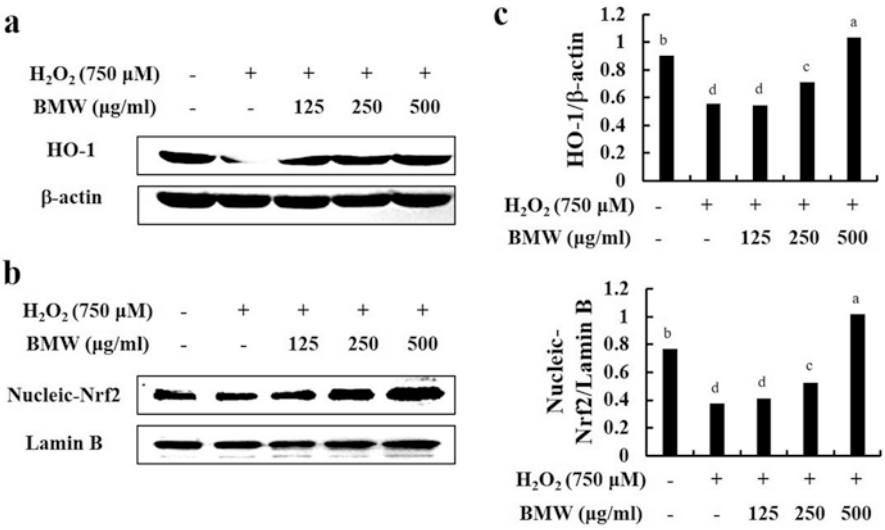

Fig. 4 Effects of BMW on the expression of HO-1 (**a**) and Nrf2 (**b**) in cultured Chang cells. Cells were incubated with various concentrations of BMW (125, 250 and 500 μg/ml) to evaluate the effect of the extract on HO-1 and Nrf2 protein expression and densitometry analysis (**c**). Values means ± S.E of three determinations. [a–e]The bars with different letters are significantly different ($p < 0.05$)

3.6 Effects of BMW on Cell Viability, ROS Production and HO-1 Content

The HO-1 inhibitor, ZnPP, was used to clarify the effect of HO-1 on cell viability and ROS production. As indicated in Fig. 5a, cell viability promptly decreased in H_2O_2 treated, HO-1 inhibited cells while ROS levels increased (Fig. 5b). For cells co-treated with H_2O_2 and BMW, a minor increase in cell viability was seen and ROS generation was reduced compared to H_2O_2 (only) treated cells. Because HO-1 is inhibited by ZnPP, BMW did not cause a significant alteration in ROS levels or cell viability in the H_2O_2-treated cells. The results suggest that the effects of BMW on intracellular ROS levels are altered by changes in HO-1 activity.

4 Discussion

Oxidative stress is considered as a central contributor to the pathogenesis of a number of detrimental disease conditions. The implementation of antioxidants has widely expanded during the past few years to counteract these issues. The liver is considered as the major organ involved in detoxification and elimination of potentially harmful substances. ROS, including hydrogen peroxide, are involved in

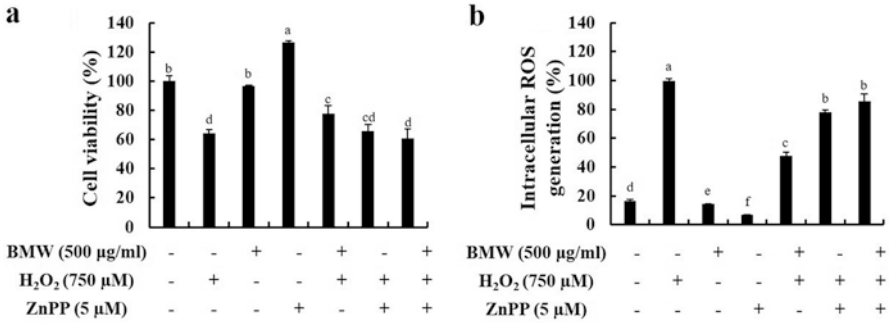

Fig. 5 Effects of BMW and ZnPP on cell viability (**a**) and ROS production (**b**) in H_2O_2-treated Chang cells. Values means ± S.E of three determinations. [a–f]The bars with different letters are significantly different ($p < 0.05$)

damaging liver cells. Hydrogen peroxide is an unstable metabolite which is responsible for generating hydroxyl radicals and singlet oxygen. It potentially initiates lipid peroxidation and damages essential macromolecules (Heo et al. 2005). Hence, the measurement of H_2O_2 scavenging activity is a useful method for determining the utility of antioxidants and food products. Recently, an increasing number of studies has been undertaken to evaluate the nutraceutical and functional properties of edible marine food products. These include fish, invertebrates (mollusks, echinoderms), and seaweed. Apart from seaweed, one of the major constituents of marine animals is their protein. A large number of studies have demonstrated the functional properties of their proteins, peptides and amino acids (Ngo et al. 2012). Some of these functional properties are antioxidant, antimicrobial, anti-hypertensive, opioid agonistic, prebiotic, immunomodulatory, mineral binding, hypocholesterolemic activity, and anti-thrombotic effects. Previous studies have shown that peptides from various marine organisms, such as fish, blue mussel, conger eel, microalgae, and squid, could act as potent antioxidants.

Based on the results, the major constituent of the *B. cornutus* meat extract is protein. The analysis of the free amino acid composition indicated that the extract is rich in taurine, followed by alanine and then proline. Based on several recent investigations, taurine is identified as a compound capable of inducing antioxidant properties. Its activity largely depends on promoting intracellular defense by activating a number of key antioxidant enzymes including HO-1, GSH, SOD, CAT, and GPx by mediating the Nrf2/HO-1 antioxidant pathway (El-Maraghi et al. 2018).

The antioxidant activity determined by chemical assays indicate that BMW is capable of scavenging free radicals and it possesses relatively good antioxidant properties. The increase of radical scavenging potential with increasing contractions of BMW suggests that increasing consumption of *B. cornutus* meat extract may have a positive impact on human health. The cytotoxicity of the samples indicates a somewhat unlikely pattern in which 125 µg/ml BMW showed 74.09% of cell viability while the cell viability of 500 µg/ml concentration was almost at the level of the

control. These results suggest that BMW at 500 μg/ml could be used to carry out further experiments. Since *B. cornutus* is an edible material, further experiments could be carried out regardless of its observed reductions in cell viability at low sample concentrations. The intracellular ROS levels were evaluated by using the DCFH-DA assay. Substances that could lower intracellular ROS levels could act as cytoprotective agents that protect cells against ROS-induced cellular damage. BMW reduced intracellular ROS levels in Chang cells which were stimulated by H_2O_2 in a dose-dependent manner. The corresponding cell viability, which is a measure of the cytoprotective effect, was analyzed by MTT assay. Accordingly, viability increased with increasing sample concentrations in a dose-dependent manner. Interestingly the cell viability at 500 μg/ml sample concentration reached a level higher than the level of the control suggesting that BMW is effective as a cytoprotective agent against H_2O_2-induced cytotoxicity.

Identifying the molecular mechanism of intracellular antioxidant activity is of utmost importance in understanding the applicability of this material for a wide range of applications. Evaluations were carried out to determine the levels of antioxidant enzymes, including CAT, SOD, and HO-1. Based on the results, treatment of BMW could attenuate the decrease in the antioxidant enzymes by ROS. Interestingly the levels of both SOD and CAT became equal to the levels of the control at a sample concentration of 500 μg/ml. HO-1 is a major antioxidant enzyme which increases heme (a pro-oxidant which mediates redox balance in cells) catabolism (Fernando et al. 2016). As evident from the results, BMW increases HO-1 levels in H_2O_2-treated cells in a dose-dependent manner. Based on densitometry analysis, the HO-1 level rose to levels exceeding the control at 500 μg/ml sample concentration, which correlates with the observed increase in cytoprotective effects.

The transcription factor "Nrf2" increases the expression levels of several antioxidant enzymes. Nrf2 bound to the inhibitor protein Keap1 resides in the cytosol. Stress conditions activate the cellular defense mechanisms regulated by Nrf2 which its translocation into the nucleus upon detachment from Keap1, resulting in the transcription of genes encoding antioxidant enzymes (Loboda et al. 2016). The results indicate inhibition of Nrf2 levels upon H_2O_2 treatment, but the levels were increased with increasing BMW concentration. These results relate to the observed increase in HO-1 levels in H_2O_2-treated Chang cells. Further experiments were carried out to clarify the effects of HO-1. The use of the HO-1 inhibitor ZnPP allowed the identification of the effect of HO-1 upon the antioxidant activity of BMW. Comparing the intracellular ROS levels in Chang cells treated with H_2O_2 and ZnPP with that of H_2O_2, ZnPP, and BMW, no significant difference was observed in ROS levels. In both instances, the incorporation of ZnPP inhibits HO-1 expression. However, in the absence of the inhibitor, BMW treatment causes a significant lowering of ROS levels in H_2O_2-treated Chang cells. These results suggest that HO-1 is a major factor responsible for the regulation of intracellular ROS levels upon BMW treatment.

5 Conclusion

This is the first study to report the antioxidant activity of the *B. cornutus* meat extract. BMW effectively attenuates H_2O_2-induced oxidative stress in Chang cells by promoting HO-1 mediated antioxidant defense mechanisms. Hence BMW might serve as a potent antioxidant that could counter oxidative stress conditions. It could have potential applications in the functional food industry. With further evaluations, the bioactive principals responsible for the observed effects could be isolated and applied to numerous other studies, such as drug discovery.

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An Aqueous Extract of *Octopus ocellatus* Meat Protects Hepatocytes Against H_2O_2-Induced Oxidative Stress via the Regulation of Bcl-2/Bax Signaling

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Abstract *Octopus ocellatus* meat (OM) is well known as a plentiful protein source. In this study, we evaluated the hepatoprotective effect of an aqueous extract of OM (OMA) against H_2O_2-triggered oxidative stress in human hepatocytes. First of all, taurine rich OMA showed a good ORAC value and reducing power and it was

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similar with that of ascorbic acid, which is known as a strong antioxidant. Also, OMA significantly improved H_2O_2-decreased cell viability by reducing the generation of intracellular reactive oxygen species (ROS) in hepatocytes. Interestingly, the stimulation of H_2O_2-induced the formations of apoptotic bodies and sub-G_1 DNA content, whereas they were inhibited by the treatment with OMA. Furthermore, OMA regulated the protein expression levels of apoptotic molecules, such as Bax and Bcl-2. Taken together, this study suggests that OMA, which contains an abundant amount of taurine, protects hepatocytes from H_2O_2-triggered oxidative stress and might be a functional food material with hepatoprotective effects.

Keywords Taurine · *Octopus ocellatus* meat · Aqueous extract · Protective effect

1 Introduction

Oxidative stress causes many changes in the body; there are many causes for oxidative stress, but reactive oxygen species (ROS) is the main cause. ROS is constantly formed in the human body. Free-radical mechanisms have been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases (Aruoma 1998; Han et al. 2017). Among biologically active ROS are superoxide radical (O_2^-), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2). Especially, H_2O_2 is a reactive oxygen species that is used experimentally to damage liver (Jaeschke et al. 2012). This is because it reacts with several elements in the body and produces a lot of other ROS.

Health problems caused by oxidative stress include liver injury. Oxidative stress is thought to play a major role in the pathogenesis of liver diseases, as their metabolites can induce hepatocellular injury and fibrogenesis (Roskams et al. 2003). Liver injury is one of the conditions responsible for complications, such as cirrhosis and liver cancer (Farrell and Larter 2006). These liver injuries include drug liver injury, alcoholic liver injury and drug-hepatitis (Davern et al. 2011). Recently, the incidence of non-alcoholic liver disease has been on the rise due to changes in lifecycle and eating habits (Chalasani et al. 2012). Oxidative stress induces the primary metabolic abnormalities that lead to hepatic steatosis and are also involved in lipotoxicity caused by changes in nutrition and alterations in hepatic lipid metabolism (Videla et al. 2006). In addition, oxidative stress induced non-alcoholic disease is an important clinical entity because it may progress to fibrosis or cirrhosis, with a fatal outcome (Malaguarnera et al. 2005). Thus, reducing oxidative stress can prevent liver damage (Fukuda et al. 2007).

Apoptosis, programmed cell death, can be naturally produced by excessive oxidative stress in multicellular organisms (Chen et al. 2011). However, many researchers have reported that in apoptosis the integrity of the cell membrane is maintained but it leads to cell swelling and cell lysis (Kannan and Jain 2000). Apoptosis can be triggered by a lot of factors, such as receptor-mediated signals (Bcl-2 and Bax), withdrawal of growth factors, and damage to DNA (Buttke and Sandstrom 1994).

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid and a free amino acid (Sturman and Hayes 1980). Taurine has a number of features and functions, including conjugation with bile acid, reduction of blood cholesterol and triglyceride levels, promotion of neuron cell differentiation and growth, antioxidant effects, maintenance of cell membrane stability, retinal development, energy generation, depressant effects, regulation of calcium level, muscle contraction and relaxation, bone formation, anti-inflammatory effects, anti-cancer and anti-atherogenic effects, and osmotic pressure control (Yoon et al. 2015). However, the exact physiological functions of taurine are not clear. *Octopus ocellatus* (*O. ocellatus)* meat (OM) is a material known to be rich in protein, but also taurine (Lee et al. 2017). And it is known to lower cholesterol and prevent cancer (Yamazaki et al. 2002).

In this study, we observe protective effects of a hydrolysate from OM against hydrogen peroxide-induced oxidative stress.

2 Materials & Methods

2.1 Materials

All testing reagents 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), fluorescein sodium salt, 10% TCA, 3-(4–5-dimethyl-2yl)-2–5-diphynyltetrasolium bromide (MTT) fluorescein sodium salt, 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St, Louis, Mo, USA). And Chang liver cell line (CCL-13) was obtained from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Paisley, UK). Other chemical and reagent were of the highest grade available commercially.

2.2 Preparation of OM Aqueous Extracts (OMA)

To prepare the aqueous extract from OM, OM (20 g) was dissolved in distilled water (1 L) for 24 h with R.T. After centrifugation for 15 min at 4000 rpm, the supernatant was filtered, freeze-dried and stored at −20 °C for further use. The aqueous extract of OM was named as OMA.

2.3 Determination of Free Amino Acid Composition

Free amino acid component in OMW was measured by using an amino acid analyzer (S433-H, Sykam GmbH, Germany) with the previous indicated method (Han et al. 2017).

2.4 Determination of Antioxidant Activities

ORAC Assay
The ORAC assay was performed by a modified method described by Zulueta et al. (2009). Briefly, 50 µl of the OMA were mixed with 50 µl of 78 mM fluorescein and incubated for 15 min at 37 °C. Then, 25 µl of 221 nM AAPH was rapidly added to the mixture and the fluorescence was recorded at each 5 min for 2 h (excitation wavelength 485 nm, emission wavelength 538 nm). Finally, the ORAC values were calculated using the regression equation between trolox concentration and the net area under the curve (AUC) and were expressed as µM trolox equivalent (TE)/mg sample.

Reducing Power
The reducing power was performed using the method described by Oyaizu (1986). OMA (125, 250 and 500 µg/ml) were mixed with 300 µl of 0.1 M sodium phosphate buffer (pH 6.6) and 500 µl of potassium ferricyanide (1%, w/v). The mixture was kept at 50 °C for 20 min, and then 500 µl of 10% TCA was added, the mixture was centrifuged at 3000 rpm for 10 min. Finally, 100 µl of each supernatant solutions were mixed with 100 µl of distilled water and 20 µl of $FeCl_3$ (0.1%, w/v), and the absorbance were determined at 700 nm.

2.5 Antioxidant Activities of OMA in Chang Liver Cells

2.5.1 Cell Culture

Chang liver cells were incubated at 37 °C in a humidified atmosphere (5% CO_2), and cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 µg/ml) and penicillin (100 unit/ml).

2.5.2 Effects of OMA Against Cell Viability in H_2O_2-Induced Oxidative Stress

The cell viability of OMA was determined using the MTT assay by Han et al. (2017). Chang liver cells were seeded in a 96-well plate at a density of 1×10^4 cells/wells. After 24 h, the Chang liver cells were pretreated with different concentrations of OMA for 1 h, and then washed with phosphate buffered saline (PBS). The cells were then exposed to 750 µM H_2O_2 to induce oxidative stress, followed by incubation for 24 h at 37 °C. After 24 h incubation, the MTT assay was performed to determine the cell viability. Absorbance was measured at 540 nm using a microplate reader (SpectraMax® M2/M2e, CA, USA).

2.5.3 Effects of OMA on Intracellular ROS Scavenging Activities

Chang liver cells were seeded in a 96-well plate at a density of 1.6×10^4 cells/wells. After 24 h, Chang liver cells were treated with OMA. Again after 1 h, H_2O_2 was added at a concentration of 750 μM and then the cells were incubated for an additional 1 h at 37 °C under a humidified atmosphere. The fluorescence emission of 2′,7′-dichlorofluorescin due to oxidation was measured at Excitation 485 nm/Emission 528 nm. The percentage of fluorescence intensity (ROS generation) of the samples were compared with that of the control cells (without OMA), which were arbitrarily assigned a value of 100%.

Effects of OMA on Apoptotic Body Formation

The nuclear morphology of Chang liver cells against H_2O_2-induced oxidative stress was evaluated using cell-permeable DNA dye, Hoechst 33342 staining (Sigma, USA, Missouri). Hoechst 33342 was an obvious choice as the DNA stain because it was known to produce stoichiometric DNA staining against various oxidative stress (Shapiro 1981). First, cells were seeded in 6-well plates at the density of 6×10^5 cells/wells. After 18 h incubation, cells were pre-treated with OMA (125, 250 and 500 μg/ml), and then 750 μM H_2O_2 was added. Further incubated for 12 h, Hoechst 33342, a DNA specific fluorescent dye was treated the cultured cells at a final concentration of 2 μg/ml and was incubated for additional 30 min at 37 °C in the dark. Finally, the formation of apoptotic body was observed by using a fluorescence microscope (Olympus, Shinjuku, Japan).

Effects of OMA on the Population of Sub-G_1 DNA Content

To analysis cell cycle, we performed flow cytometry analysis. First, Chang liver cells were seeded in 6 cm dish at a concentration of 6×10^5 cells/dish. After 18 h, OMA (125, 250 and 500 μg/ml) was treated into the cells. The cells were washed with PBS (1 X) and fixed by 70% ethanol at 4 °C. Next, the cells were incubated with 500 μl of 2 mM PBS-EDTA containing PI (50 μg/ml) (Sigma, Missouri, USA) and RNase A (0.2 μg/ml) for 30 min in the dark. Flow cytometric analysis was conducted with CytoFLEX (Beckman coulter, California, USA).

Effects of OMA on the Expressions of Apoptosis Molecules

Chang liver cells (5×10^5 cells/wells) were treated with H_2O_2 (750 μM) and OMA (125, 250 and 500 μg/ml) for 24 h. After the incubation, the cells were lysed in a RIPA buffer (Thermo, Rockford, IL) and the obtained lysates were used as the cellular proteins. The 40 μg of cellular proteins were electrophoresed in SDS-polyacrylamide gels (12%) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA, Massachusetts). The membrane was incubated with 5% skim milk in tris buffered saline (TBS) containing Tween-20 (TBS-T) for 2 h, and then the membrane was incubated overnight at 4 °C with specific primary antibodies as Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.), Bax (1:1000 dilution, Cell Signaling Technology Inc.), p53 (1:1000 dilution, Cell Signaling Technology Inc.) and β-actin (1:3000 dilution, Sigma) in 5% skim milk. After the incubation, the HRP-conjugated secondary antibodies (anti-mouse IgG and

anti-rabbit IgG, 1:5000, Cell Signaling Technology Inc.) were added into the membrane at R.T. for 90 min. The bands were detected by using an enhanced Super Signal West Femto Maximum Sensitivity Substrate (Thermo, Canada, Burlington) reagents and analyzed using NIH Image J software (US National Institutes of Health, Bethesda, MD).

2.6 Statistical Analysis

Data were analyzed using the SPSS package (Version 21). Values were expressed as means ± standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A *p*-value less than 0.05 was considered significant.

3 Results

3.1 Amino Acid Composition of OMA

Table 1 indicated that the amino acid composition of OMA had the various amino acid composition such as taurine, leucine, lysine and phenylalanine, which accounted for 29.66%, 9.90%, 9.01% and 5.50% of the total amino acid pool, respectively. Especially, taurine was the main amino acid in OMA.

3.2 Antioxidant Activities of OMA

In order to determine the antioxidant activity of OMA, we first performed the ORAC and the reducing power assays. The ORAC assay provides the information regarding the sample's ability to scavenge peroxyl radicals through a hydrogen atom transfer mechanism (Prior et al. 2003). As shown in Fig. 1a, OMA exhibited the superior ORAC value of 858 μmol TE/mg.

The reducing power assay measures the degree to which Perl's prussian blue, which is generated by reducing the Fe^{3+} present in the potassium ferricyanide to Fe^{2+} at 700 nm (Zulueta et al. 2009). Our result showed that OMA led to the reducing power in a dose dependent manner (Fig. 1b). From these results, we indicate that OMA has the *in vitro* antioxidant activity.

Table 1 Free amino acid of OMA

	OMA	
Amino acid	mg/100 g	% amino acid
Phosphoserine	34.02	0.23
Taurine	4351.14	29.66
Phosphoethanolamine	66.03	0.45
Urea	N.D.	N.D.
Aspartic acid	240.88	1.64
Hydroxyproline	N.D.	N.D.
Threonine	115.93	0.79
Serine	128.08	0.87
Asparagine	N.D.	N.D.
Glutamic acid	601.32	4.10
Sarcocine	N.D.	N.D.
α-aminoadipic acid	N.D.	N.D.
Proline	206.11	1.40
Glycine	343.07	2.34
Alanine	647.68	4.41
Citrulline	418.25	2.85
α-aminobutyric acid	17.16	0.12
Valine	689.49	4.70
Cystine	N.D.	N.D.
Methionine	779.45	5.31
Cysthathionine	N.D.	N.D.
Isoleucine	653.07	4.45
Leucine	1451.80	9.90
Tyrosine	731.53	4.99
Phenylalanine	807.21	5.50
β-alanine	171.85	1.17
β-aminoisobutyric acid	N.D.	N.D.
γ-amino-n-butyric acid	N.D.	N.D.
Histidine	48.68	0.33
3-methylhistidine	N.D.	N.D.
1-methylhistidine	N.D.	N.D.
Carnosine	N.D	N.D.
Anserine	16.53	0.11
Tryptopan	0.00	0.00
Hydroxylysine	39.08	0.27
Ornitine	417.58	2.85
Lysine	1322.20	9.01
Ethanolamine	5.73	0.04
Arginine	366.59	2.50
Total	14670.47	100.00

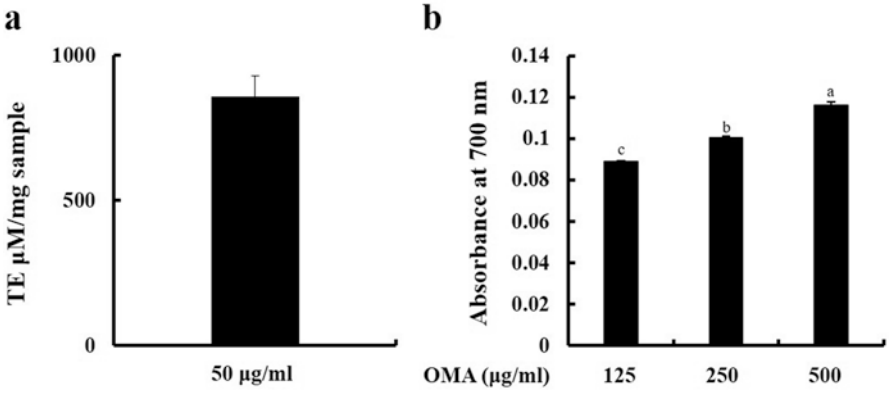

Fig. 1 ORAC assay (**a**) and reducing power (**b**) of aqueous extract of OM (OMA). [a–c]The bars with different letters are significantly different ($p < 0.05$)

3.3 *Effects of OMA on Cell Viability and Intracellular ROS Generation in H_2O_2-Exposed Hepatocytes*

As shown in Fig. 2a, OMA has no cytotoxic effects at the used all concentrations, compared to the non-treated control cells. Figure 2b also exhibited that the exposure with 750 μM H_2O_2 significantly decreased the hepatic cell viability (57.11%), comparing to the non-treated control cells (100%). Interestingly, it was recovered by the application of OMA (500 μg/ml) (Fig. 2b). With these results, we indicate that OMA protect the cells against H_2O_2-induced hepatotoxicity.

Oxidative stress, defined as the imbalance between ROS and antioxidants, contributes to the development of aging and various diseases in humans. Therefore, antioxidant therapy is vital in scavenging free radicals (Finkel and Holbrook 2000). The capacity of OMA as a ROS scavenger was determined by using the DCFH-DA assay. Figure 2c exhibited that the intracellular ROS production was increased in H_2O_2-exposed Chang liver cells compared to non-treated cells. However, it was significantly improved by the application of OMA at 500 μg/ml, although the lower concentrations of OMA did not affect to them.

3.4 *Effects of OMA on Cellular Damages in H_2O_2-Exposed Hepatocytes*

Hoechst 33342 is usually used for the staining of DNA nuclear fragments, such as apoptotic monocytes (Allen et al. 2001). We investigated the ability of OMA to protect cells against H_2O_2-induced apoptosis using Hoechst 33342 and PI staining. As shown in Fig. 3a, the exposure to H_2O_2 increased the formation of apoptotic bodies compared to that of non-treated cells. However, the application of OMA reduced the formation of H_2O_2-induced apoptotic bodies in Chang liver cells.

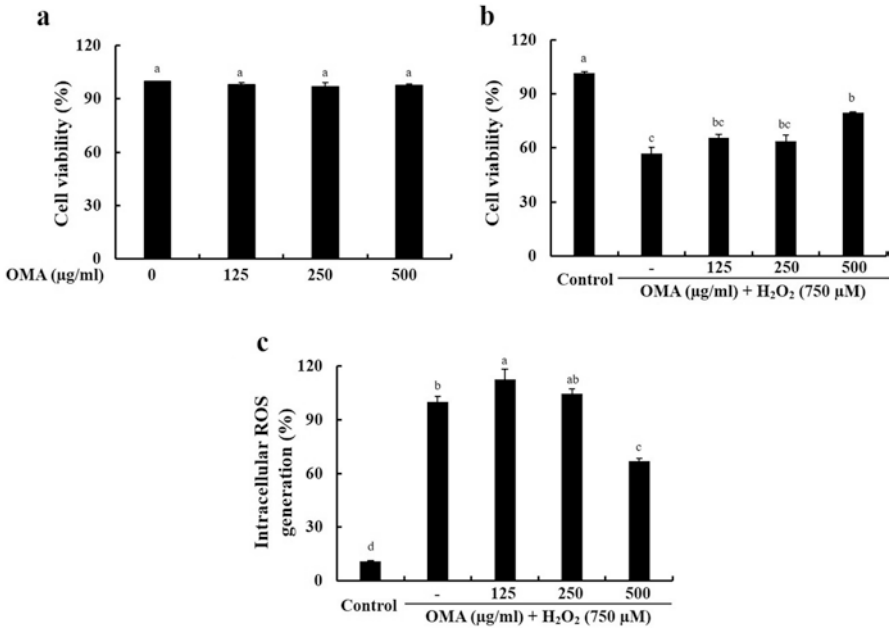

Fig. 2 Effects of OMA on cell viability (**a**, **b**) and intracellular ROS (**c**) generation in H_2O_2-treated Chang liver cells. Chang liver cells were pretreated with various dose of OMA (125, 250 and 500 μg/ml). [a–d]The bars with different letters are significantly different ($p < 0.05$)

In a subsequent study, we examined sub-G_1 DNA content in the H_2O_2-treated cells by PI staining. PI stains nuclear DNA following plasma membrane permeabilization, which usually occurs during cell death (Lecoeur et al. 2002). As a result, the stimulation of H_2O_2 increased sub-G_1 DNA content in Chang liver cells, whereas it was abolished by the application of OMA (Fig. 3b).

3.5 Effect of OMA on Apoptosis-Related Molecules in H_2O_2-Exposed Hepatocytes

To establish the protective mechanism of OMA against DNA damages induced by the treatment of H_2O_2 in Chang liver cells, we performed western blot analysis. As shown in Fig. 4, the exposure to H_2O_2 decreased the expression level of Bcl-2, an anti-apoptotic molecule compared to that of the non-treated control cells, whereas increased those of Bax and p53, pro-apoptotic molecules. Interestingly, OMA modulated the apoptosis via the up-regulation of Bcl-2 expression level and the down-regulation of Bax and p53. This result suggests that OMA protects the cells against DNA damages induced by the treatment of H_2O_2 via the modulation of apoptosis in Chang liver cells.

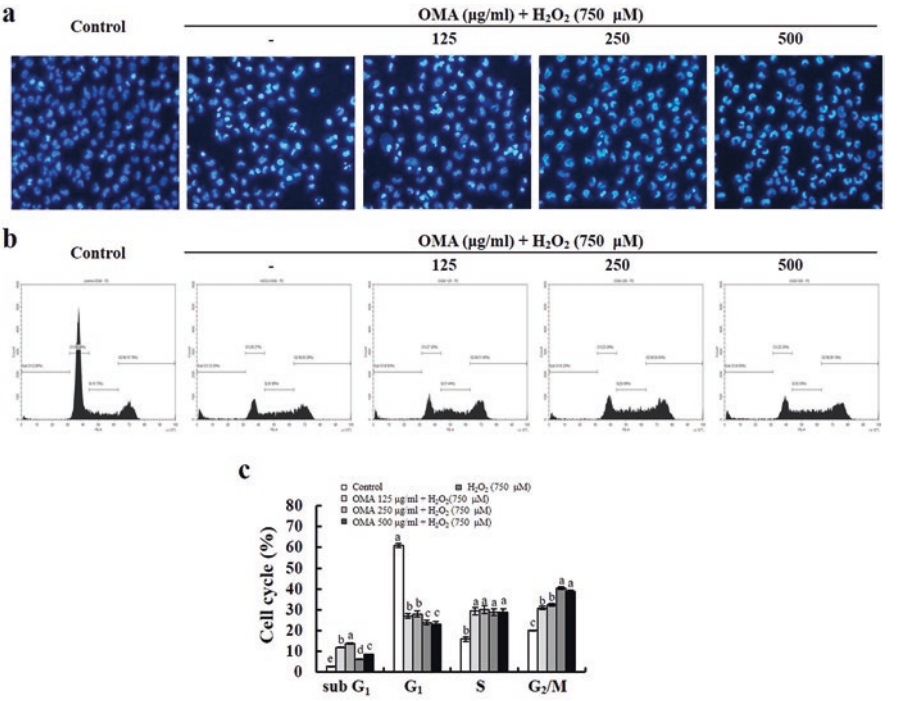

Fig. 3 Effects of OMA on apoptotic body formation (**a**), cell cycle pattern (**b**) and percentage (**c**) of Chang liver cells undergoing apoptosis following exposure to H_2O_2. Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining while the cell cycle was observed from PI staining using flow cytometry. [a–e]The bars with different letters are significantly different ($p < 0.05$)

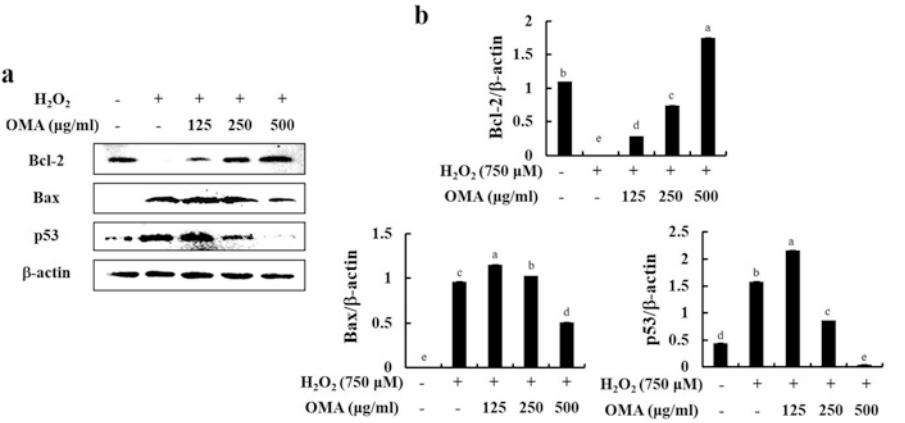

Fig. 4 Protective effect of OMA against H_2O_2-induced apoptosis in cultured Chang liver cells. The expression levels of protein regulators of apoptosis, namely Bcl-2, Bax and p53 (**a**). Densitometry analysis of protein expression levels (**b**). Values means ± SE of three determinations. Bars with different letters are significantly different ($p < 0.05$)

4 Discussion

In this study, we investigated the hepatoprotective activity of OMA against H_2O_2-induced oxidative stress in Chang liver cells. Normally, *O. ocellatus* contains a lot of essential amino acids, such as valine, isoleucine, methionine and threonine, and the semi-essential amino acid, taurine. These essential amino acids have potential biological activities such as anti-oxidant, anti-inflammatory and anti-cancer activities (Yoon et al. 2015). Our data also showed that *O. ocellatus* contained plentiful amounts of taurine (29.66%) compared to that of the other amino acids (Um et al. 2017).

According to Sila and Bougatef (2016), fish-derived bioactive peptides with antioxidative properties may be a potential substitute for synthetic antioxidants, a concept that relies on human nutrition and functional foods. Based on these results, we used the ORAC assay and the reducing power assay to confirm the antioxidant activity of OMA samples. The ORAC assay is a method using oxygen radical absorption capacity, and the reducing power assay is an antioxidant activity measurement method using the reduction force of Fe^{2+} ions (Ou et al. 2002). Interestingly, our result showed that OMA containing high amounts of taurine had potent scavenging activity in the ORAC and reducing power assays. Thus our results indicate that OMA has potential biological activity as a natural antioxidant. Next, we checked the hepatoprotective effects of OMA in Chang liver cells. Normally, high concentrations of ROS induce oxidative stress, which cause liver damage (Weber et al. 2003). These free radicals may attack intracellular nucleic acids, proteins, and lipids, ultimately leading to hepatocyte oxidative damage and death (Weber et al. 2003). Based on *in vitro* result, we wanted to identify the direct antioxidant activity mechanism of the OMA sample. In another study, Miranda demonstrated that mate tea was able to decrease H_2O_2-induced DNA breakage after intervention only in liver cells (Miranda et al. 2008). We used H_2O_2 as oxidative stress stimulator, and stimulated Chang liver cells. As a result, treatment with H_2O_2 significantly decreased cell viability, whereas, pre-treatment with OMA attenuated the degree of H_2O_2-decreased cell viability up to 79.54% by suppressing the production of intracellular ROS. According to the study of Ghosh et al. (2009), arsenic reduced cell viability, but taurine improved cell viability through reductions in ROS. This result is similar to that of our own. So taurine is expected to exert antioxidant activity, as that shown for OMA. According to Chen result (Chen et al. 2017), oxidative stress induced the apoptosis and modulated apoptosis molecules such as Bcl-2 and cleaved caspase-3 in hepatocytes. In further study, we have checked whether the OMA sample affects oxidative stress-induced apoptosis action. As oxidative stress-induced apoptosis progresses, it changes levels of protein such as Bcl-2, Bax and p53 in cells. At this time, the amount of Bcl-2 is decreased because it is anti-apoptotic protein, and the amount of Bax and p53 are increased because it is pro-apoptotic protein (Thomas et al. 1996). Moreover, the Bcl-2 family modulates the mitochondrial pathway of apoptosis by regulating mitochondrial outer membrane permeabilization (MOMP) (Brunelle and Letai 2009). Briefly, after the cell stimulation, the BH3-only protein is activated and binds to anti-apoptotic proteins including Bcl-2 and Bcl-xL (Zong et al. 2001). Bax,

a pro-apoptotic, promotes mitochondrial outer membrane permeabilization (MOMP) that leads to the release of apoptogenic factors from the mitochondria (García-Sáez et al. 2005). When MOMP occurs, cytochrome c is released from the mitochondria and apoptosis is formed and induced (Lavrik 2010). The result of western blot analysis exhibited that OMA modulated the expression of apoptosis molecules including Bcl-2, Bax and p53 against H_2O_2–induced apoptosis. In other results, taurine treated cardiomyocytes, the number of apoptotic cells was significantly low (12.9%) indicating that taurine protected cardiomyocytes from as induced apoptosis (Ghosh et al. 2009). Recent studies have reported taurine content has the various biological effects such as osmoregulation in brain cells, anti-inflammation, and antioxidant effect in diabetes (Pasantes-Morales and Schousboe 1997; Marcinkiewicz and Kontny 2014; Schaffer et al. 2009). Based on the result, the antioxidant capacity of taurine content in OMA has affected reduction of apoptosis.

Thus, we confirmed that OMA containing rich full taurine has a protective ability against liver damage derived from oxidative stress through Bcl-2/Bax signaling regulation.

5 Conclusion

Taken together, these results suggest that OMA containing rich taurine has a beneficial effect on H_2O_2-induced oxidative stress through Bcl-2/Bax signaling regulation.

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Antioxidant Activities of Viviparus Contectus Extract Against Tert-Butylhydroperoxide-Induced Oxidative Stress

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Abstract In this study, the antioxidant properties of Viviparus contectus (V. contectus) extract were evaluated for various radical scavenging activities, ferric reducing antioxidant power (FRAP), ABTS radical scavenging activity and oxygen radical absorbance capacity (ORAC). In addition, inhibition effect of the V. contectus extract against DNA scission induced by hydroxyl radical was measured. We also studied the protective effect of V. contectus extract against oxidative damage through measurements of intracellular reactive oxygen species (ROS) in Chang cells and zebrafish embryo. We found that V. contectus extract contains strong radical scavenging activities and antioxidant properties, which prevent tert-butylhydroperoxide (t-BHP)-induced oxidative stress, enhance cell viability, reduce ROS production, inhibit oxidative damage and improve mitochondrial function in Chang cells. Also, we determined that the V. contectus extract reduced ROS production mediated by t-BHP induced oxidative stress on zebrafish embryo.

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1 Introduction

Viviparus contectus (V. contectus, pond snail) is a species of large, freshwater snail with an operculum and a gill, an aquatic gastropod mollusk in the family Viviparidae, the river snails. V. contectus is a common group of vivipard gastropods and is widespread in many countries. Lu et al. (2014) reported that Viviparidae are widely distributed around the globe, but there are many types of Viviparidae and considerable gaps in the taxonomic record.

V. contectus resides in pools, lakes, streams and other water bodies and feeds on organic particles and microbes (Chiu et al. 2002). It is used as a food supplement in China, as it contains high amounts of protein, essential amino acids, taurine, calcium, iron and zinc (Cao and Yao 2005). It has been processed into many kinds of foods, appearing as sauces, in cans or freshly cooked. In addition, V. contectus has been used as a traditional medicine for the treatment of liver diseases and alcohol poisoning in China and Korea. Recent studies have demonstrated that extracts of C. chinensis meat, which is from the same family of Viviparidae, exert various pharmacological effects, such as anticancer activity, cardioprotective actions against injury and hepatoprotective effects in vivo (Fu and Zhang 2010; Cui and Zhao 1989; Jiang et al. 2013). However, physiological studies of the V. contectus extract have not been sufficiently performed.

Reactive oxygen species (ROS) play a positive role, such as energy production, phagocytosis, regulation of cell growth, and intracellular signaling (Meng et al. 2017). On the other hand, certain ROS such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) mediate cell death via oxidation of DNA, proteins, lipids, and almost any other cellular constituent (Ye et al. 2015). ROS and free radicals are continuously produced during normal physiological events, and eliminated by antioxidant defense mechanisms (Zhou et al. 2014). Normally, cells have several antioxidant defense mechanisms that prevent the destructive effects of ROS. These defense mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, and of small molecules such as glutathione and vitamins C and E (Kirkman et al. 1999; Flohé 1988). There is a balance between the generation of ROS and the antioxidant system in organisms is very important. Therefore, many researchers have been interested in antioxidants that can prevent oxidative damage.

In recent years, there has been considerable interest in finding natural antioxidants for use in food or pharmaceutical materials.

The objectives of this study were to determine the in vitro antioxidant activities of V. contectus extract and to evaluate its protective effect against oxidative stress in Chang cells and zebrafish embryo.

2 Materials and Methods

2.1 Reagent

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), FeCl3, 2, 4, 6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu reagent (FCR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt(ABTS), Fluorescein sodium salt, N-Acetyl-L-cysteine (NAC), potassium persulfate and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest commercially available grade.

2.2 Extract Preparation

After removing the shell of the V. contectus, 10 times its weight in distilled water was boiled, after which the suspension was boiled for 90 min. Then it was filtered using Whatman No. 41 paper. Finally, the filtrate was evaporated in a rotary evaporator and lyophilized. The V. contectus extract (VCE) was stored at −20 °C until use.

2.3 Amino Acid Composition

The amino acid composition was analyzed using following steps. First, samples were mixed with 10 mL of 6 N HCl. After that, N_2 gas was used to purge the samples in the test tube and then the samples were hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and amino acid composition was determined by measuring the absorbance at 440 and 570 nm.

2.4 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined according to previous studies (Guo et al. 2018) with slight modification. 50 μL of DPPH ethanol solution (2.5 mM) was mixed with 50 μL of sample ethanol solution at varying concentrations (500, 250, 125, 62.5 and 31.25 μg/mL). Absorbance was measured at 515 nm after keeping in dark for 30 min at room temperature.

The percentage of DPPH scavenging was calculated by the formula:

$$\text{DPPH scavenging activity}(\%) = \left[\left(\text{control absorbance} - \text{sample absorbance}\right)\right] / \text{Control absorbance}] \times 100.$$

Where control absorbance (DPPH solution without sample), sample absorbance (DPPH solution plus test sample), and blank absorbance (sample without DPPH solution).

2.5 Ferric Reducing Antioxidant Power(FRAP) Assay

To measure the antioxidant capacity of the V. contectus extract, the FRAP assay was performed according to the method of Benzie and Strain (1996) with slight modifications. Extracted samples (200 μL) were mixed with 3.0 mL FRAP reagent in test tubes and then vortexed. Blank samples were prepared for both methanol and deionized water extraction. Both samples and blanks were incubated in water bath for 30 min at 37 °C and the absorbance of the samples was determined against a blank at 593 nm. A series of stock solutions at 125, 250, 500 and 1000 μM were prepared using aqueous solution of $FeSO_4$ for a standard curve. The values obtained were expressed as mM of ferrous equivalent $FeSO_4$ per mg of freeze dried sample.

2.6 ABTS Radical Scavenging Activity

The total antioxidant activity of the V. contectus extracts was measured using the ABTS· + radical cation decolorization assay (Tachakittirungrod et al. 2007). 7.4 mM ABTS was mixed with 2.6 mM potassium persulfate in the same volume. The mixture was then stored in the dark at room temperature (RT) for 12–14 h before use. After radical generation, the ABTS radical solution was diluted with deionized water until its absorbance was 0.70 ± 0.02 at 734 nm. To determine the radical scavenging activity, 0.9 mL of ABTS radical solution was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm. The antioxidant activity of the VCE were expressed as trolox equivalents antioxidant capacity (TEAC), or mM of trolox equivalent per mg of extract (mM Trolox eq./mg extract).

2.7 Oxygen Radical scavenging (ORAC) Activity

The ORAC assay was performed using a modified method of Ou et al. (2002). Samples and Trolox solutions were prepared using 75 mM phosphate buffer (pH 7.4). Fifty microliters of blank, Trolox standard or extract were mixed with

50 µL of fluorescein (78 nM) solution and incubated for 15 min at 37 °C. Upon addition of 25 µL of 221 mM AAPH, fluorescence intensity was measured every 5 min time for about 120 min (excitation wavelength 485 nm, emission wavelength 535 nm) using a spectrofluorometer (SpectraMax M2/M2e, CA, USA). The final ORAC values of the samples were calculated using the net area under the decay curves (AUC) and were expressed as µmol Trolox equivalent per milligram extract (µmol TE/mg extract).

$$\mathrm{ORAC}\left(\mu\mathrm{M\,TE}\right) = \mathrm{Ctrolox}\cdot\left(\mathrm{AUCsample}\quad\mathrm{AUCblank}\right)$$
$$\mathrm{Csample}\cdot\left(\mathrm{AUCtrolox}\quad\mathrm{AUCblank}\right)$$
$$\mathrm{AUC} = 1 + \mathrm{f5}/\mathrm{f0} + \mathrm{f10}/\mathrm{f0} + \ldots + \mathrm{fn} + 5/\mathrm{f0}$$

2.8 Inhibition Against Hydroxyl Radical-Induced DNA Scission

Inhibitory activity of APE against DNA scission mediated by hydroxyl radical was determined according to the method described by Ambigaipalan and Shahidi (2015) with slight modification. In an E-tube, samples (0.1, 0.5, 1.0 mg/mL, 2 µL), DW (3 µL), supercoiled pBR 322 DNA (0.5 µg/mL, 1 µL), FeSO4 (0.04 mM, 3 µL), and H2O2 (30%, 4 µL) were added. A control (DNA with radicals) and a blank (DNA only) were also prepared. After incubation at 37 °C for 1 h, 2 µL of the loading dye was added. The mixture was loaded onto 0.8% agarose gel. Electrophoresis was performed at 100 V for 30 min in TAE buffer using a mini gel electrophoretic unit (Optima Inc., Tokyo, Japan). DNA bands were visualized under UV light using a gel imaging system (Davinch-GelTM, Younghwa Ltd., Seoul, Korea).

2.9 Cell Culture

The murine melanoma Chang cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/mL) under 5% CO_2 in a humidified incubator at 37 °C.

2.10 MTT Assay

The MTT assay was performed to evaluate the cytotoxicity of VCE. Briefly, Chang cells (7 × 103 cells/well) were seeded in 48-well culture plates. Cells were treated with various concentrations of VCE (25, 50, 100 and 200 µg/mL) and incubated at

37 °C for 24 h. Next, MTT solution was added to each well, and the cells were incubated again at 37 °C for 4 h. The medium was discarded and the intracellular formazan product was dissolved in 200 μL DMSO for 10 min with shaking. Absorbance was measured at 540 nm using a microplate reader (Thermo Scientific Multiskan GO, Thermo ScientificTM, Waltham, MA, USA), and cell viability was expressed as a percentage of the control. After pretreatment of the VCE (25, 50, 100 and 200 μg/mL) for 1 h, oxidative stress (t-BHP, 100 μM) was induced and the protective effect was evaluated by the MTT method.

2.11 Measurement of Intracellular ROS Production

The amount of intracellular ROS was assessed using the oxidant sensitive dye DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate) as a substrate, as described previously (Kim et al. 2012). Chang cells were seeded in a 96 well black plate at a concentration of 2 × 104 cells/well. Cells were treated with different concentrations of VCE (25, 50, 100 and 200 μg/mL) and incubated for 1 h, then t-BHP (200 μM) was added and the reaction was allowed to proceed for 30 min. Control cells and pre-treatment cells were incubated for 30 min with DCFH-DA (10 μg/mL) at 37 °C in the dark. Nonfluorescent DCFH-DA dye, which can penetrate cells and undergo hydrolysis by intracellular esterase to form brightly fluorescent 2′,7-dichlorofluorescein (DCF), the latter which becomes trapped inside cells. The formation of DCF as a result of DCFH-DA oxidation in the presence of ROS was read after 30 min at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrofluorometer (SpectraMax M2/M2e; Molecular Devices).

2.12 Origin and Maintenance of Zebrafish

Adult zebrafish were purchased from a Seoul Aquarium (Korea) and 10 fish were kept in a 3 L acrylic tank under the following conditions: 28.5 °C, 14/10 h light/dark cycle. The fish were fed live brine shrimp (Artemia salina) at least three times per day up to 6 days in a week. Embryos were obtained from natural pawning and induced by switching on the light in the morning. The collection of embryos was completed within 30 min.

2.13 Estimation of Oxidative Stress-Induced Intracellular ROS

Generation of ROS in zebrafish embryos was induced using oxidative stress with t-BHP. Production of intracellular ROS in zebrafish embryos was detected using an oxidation-sensitive fluorescent probe dye, DCFH-DA. At 3–4 h, embryos were

treated with the sample and 1 h later, 1 mM t-BHP was added. After the treatment with t-BHP for 24 h, the embryo medium was changed and the embryos developed up to 2 dpf. The embryos were transferred into 6-well plates and treated with DCFH-DA (20 μg/mL). Then, the plates were incubated for individual reaction times in the dark at 28.5 °C. After the incubation, the embryos were rinsed in fresh embryo media and the stained embryos were observed using a fluorescence microscope (Zeiss AX10, Carl Zeiss, Göttingen, Germany).

2.14 Statistical Analysis

All results were represented as means ± standard deviation (SD). Experimental data were assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test using Graph Pad Prism software version 5.00 (Graph Pad Software Inc., San Diego, CA). $p < 0.05$ was considered as statistical significance.

3 Results

3.1 Amino Acid Composition of the V. contectus *Extract*

The free amino acid and total amino acid composition of VCE is shown in Table 1. The proximate composition of VCE consisted of crude protein 16.8%, ash 0.6%, crude fat 1.5%, and carbohydrate 5.4%. The amount of the total amino acid pool was 4.80 mg/g, and the primary amino acids were glutamic acid (24.07%), lysine (8.51%), aspartic acid (8.13%), arginine (8.07%) and alanine (6.52%), respectively. The content of the total free amino acid pool was 6.05 mg/g, and the primary free amino acids in VCE were glutamic acid (14.95%), arginine (14.57%), alanine (13.87%), leucine (6.06%).

3.2 Radical Scavenging Activity

DPPH is a stable free radical that is widely used in a method to evaluate antioxidant activity. The degree of discoloration reveals the scavenging potential of the antioxidant extract, which is due to its hydrogen donating ability (Brand-Williams et al. 1995). As seen Fig. 1, VCE possesses DPPH radical scavenging activity in a concentration-dependent manner. At a concentration of 1000 μg/mL, 500 μg/mL, 250 μg/mL and 125 μg/mL of VCE, the DPPH radical scavenging activities were found to be 84.50%, 79.64%, 38.55% and 12.37%, respectively. Oyaizu (1986) reported that iron chelators upon interacting with DPPH can neutralize free radicals by transferring electrons. Therefore, these results indicate that VCE scavenge free radicals, perhaps by transferring electrons to it.

Table 1 Free amino acids and total amino acid composition of *V. contectus* extract

Total amino acids			Free amino acids		
Amino acid	mg/g	%	Amino acid	mg/g	%
Asp	0.39	8.13	Tau	0.06	0.95
Thr	0.20	4.12	Asp	0.14	2.30
Ser	0.24	5.00	Thr	0.19	3.08
Glu	1.16	24.07	Ser	0.35	5.74
Gly	0.28	5.83	Glu	0.90	14.96
Ala	0.31	6.53	Gly	0.16	2.71
Cys	0.05	1.13	Ala	0.84	13.87
Val	0.17	3.50	Cit	0.03	0.51
Met	0.06	1.29	Val	0.30	4.88
Ile	0.11	2.27	Met	0.09	1.43
Leu	0.23	4.81	Ile	0.17	2.81
Tyr	0.10	2.11	Leu	0.37	6.07
Phe	0.14	2.87	Tyr	0.14	2.25
Lys	0.41	8.51	Phe	0.19	3.22
NH3	0.21	4.37	b-Ala	0.01	0.16
His	0.09	1.95	g-ABA	0.04	0.59
Arg	0.39	8.07	Hylys	0.05	0.78
Hypro	0.05	1.04	Orn	0.11	1.88
Pro	0.21	4.40	Lys	0.31	5.12
Total	4.80	100.00	His	0.17	2.79
			Car	0.31	5.13
			Arg	0.88	14.58
			Pro	0.25	4.17
			Total	6.05	100.00

3.3 ABTS Radical Scavenging Activity

Generation of the ABTS radical cation forms is applied to the measurement of the total antioxidant activity of pure substances, solutions, aqueous mixtures and beverages. (Miller 1996). The scavenging activity of VCE on ABTS radicals generated by potassium persulfate was compared with a standard amount of Trolox. The results, expressed as TEAC, showed a higher radical scavenging activity with VCE (TEAC value, 1.146 ± 0.002) than BHT (TEAC value, 0.654 ± 0.004) (Table 2). These results showed that VCE possesses strong ABTS radical scavenging activity.

3.4 FRAP Assay

The FRAP assay is one of the most widely cited assays for total antioxidant capacity. The antioxidant capacities of VCE are given in Table 2. The results showed that VCE (2.774 ± 0.016 mM $FeSO_4$ eq./mg extract) has 2 times higher FRAP antioxidant activity than that of BHT (1.363 ± 0.12 mM $FeSO_4$ eq./mg extract, used as a positive control).

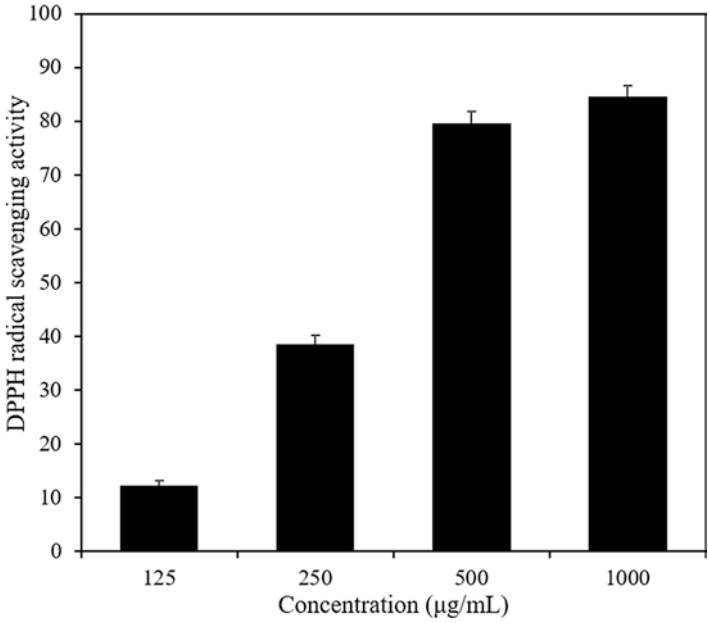

Fig. 1 DPPH radical scavenging activity of VCE. Each value is the average of three experiments and is expressed as mean ± SD

Table 2 Values for ABTS radical scavenging, FRAP activity and ORAC of *V. contectus* extract

Samples	TEAC (mM Trolox eq./mg extract)	FRAP (mM $FeSO_4$ eq./mg extract)	ORAC (µM Trolox eq./mg extract)
VCE	1.146 ± 0.002	2.774 ± 0.016	97.99 ± 0.29
BHT	1.151 ± 0.012	1.274 ± 0.152	54.02 ± 1.22

Values represent means ± SD ($n = 3$)
TEAC trolox equivalent antioxidant capacity, *FRAP* ferric reducing antioxidant power, *ORAC* oxygen radical absorbance capacity

3.5 ORAC Assay

The total antioxidant capacity values of VCE with the ORAC assay were 97.99 ± 0.29 µM TE/mg extract (Table 2). VCE exhibits stronger antioxidant capacity than that of BHT (54.02 ± 1.22 µM TE/mg) as positive control. Some researchers have reported that a protein fraction consisting of amino acids, such as tyrosine, tryptophan, histidine, lysine, and methionine, exerts a strong antioxidant effect (Chen et al. 2003; Rival et al. 2001).

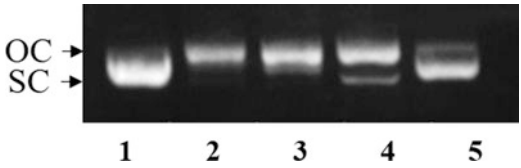

Fig. 2 Agarose gel electrophoretic patterns of plasmid DNA breaks by OH generated from a Fenton reaction in the presence of VCE. pBR 322 DNA (1 μg) was incubated at 37 °C for 1 h in 0.04 mM $FeSO_4$ and 30% H_2O_2 with the following additive combinations: Lane 1, no addition (plasmid DNA control); Lane 2, $FeSO_4$ and H_2O_2 (DNA damage control); and Lanes 3–5, $FeSO_4$ and H_2O_2 in the presence of VCE with concentrations of 0.1, 0.5, and 1.0 mg/mL, respectively

3.6 DNA Strand Break Assay

In this study, Hydrogen peroxide and Fe^{2+} were used to generate hydroxyl radical. Hydroxyl radicals were used to determine inhibition of DNA cleavage by APE. As shown in Fig. 2, supercoiled DNA of the damaged group was converted to the open circular DNA configuration by hydroxyl radicals. APE was able to significantly inhibit DNA scission compared to the damaged group. Some studies have reported that inhibition of DNA scission by antioxidants might be due to a combination of radical scavenging and Fe^{2+} chelation (Hussein 2011; Gülçin 2010). Therefore, inhibition of APE against DNA scission induced by hydroxyl radical might also be due to a combination of radical scavenging, Fe^{2+} chelating, and H_2O_2 scavenging.

3.7 Cell Viability

To examine the cytotoxicity of VCE, Chang cells were cultured for 24 h in VCE and cell viability was determined using the MTT assay (Fig. 3). Compared with the cell viability of the control (as 100%), treatment with different VCE concentrations (25–200 μg/mL) showed no cytotoxicity and cell viability was significantly increased ($p < 0.05$).

Next, we examined cell viability of VCE with t-BHP induced oxidative stress. Similar results were observed with oxidative stress, pre-treatment of VCE for 1 h, cell viability was increased dose-dependently (Fig. 4). Therefore, VCE protects against t-BHP induced oxidative stress in Chang cells.

3.8 Intercellular ROS Production

In general, AAPH, H_2O_2, and t-BHP are the most commonly used oxygen radical generators in cells and can penetrate cells through the cell membrane, leading to the generation of other radicals (mainly superoxide anion radicals and hydroxyl radicals) (Wang et al. 2016). The resulting excess of ROS then attack biological

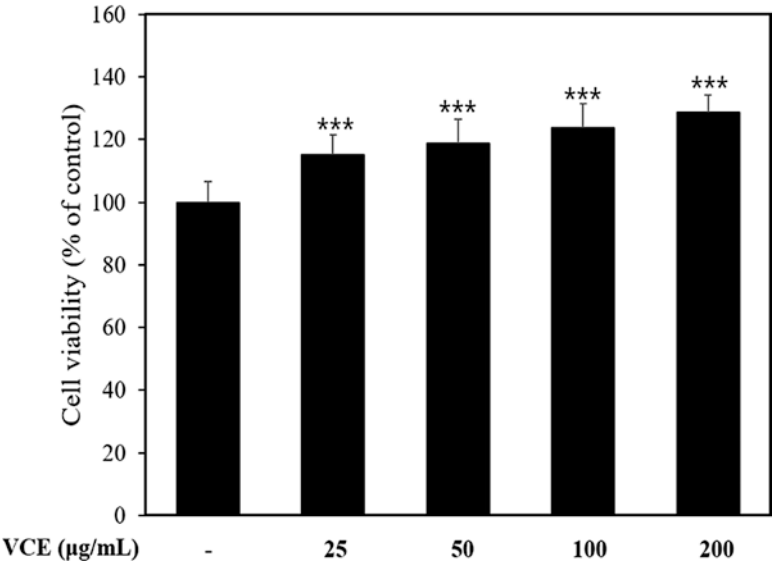

Fig. 3 Effect of VCE on the viability of Chang cells using the MTT assay. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's test $*p < 0.05$ compared to control

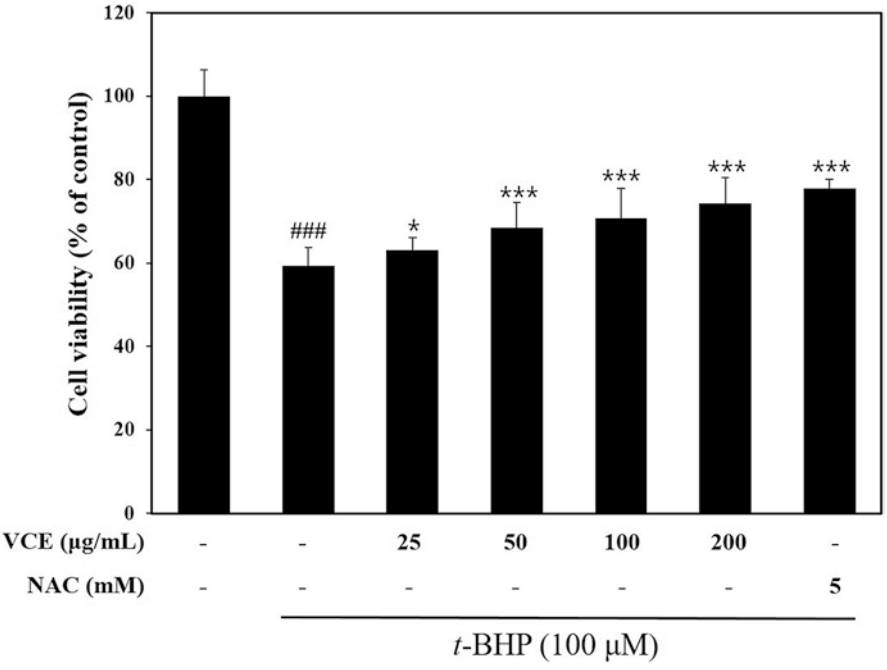

Fig. 4 Effect of pre-treatment of VCE on oxidative stress. Results are expressed as means ± SD from three independent experiments. One-way ANOVA was used for comparison of multiple group means followed by Tukey's test $*p < 0.05$ compared to control

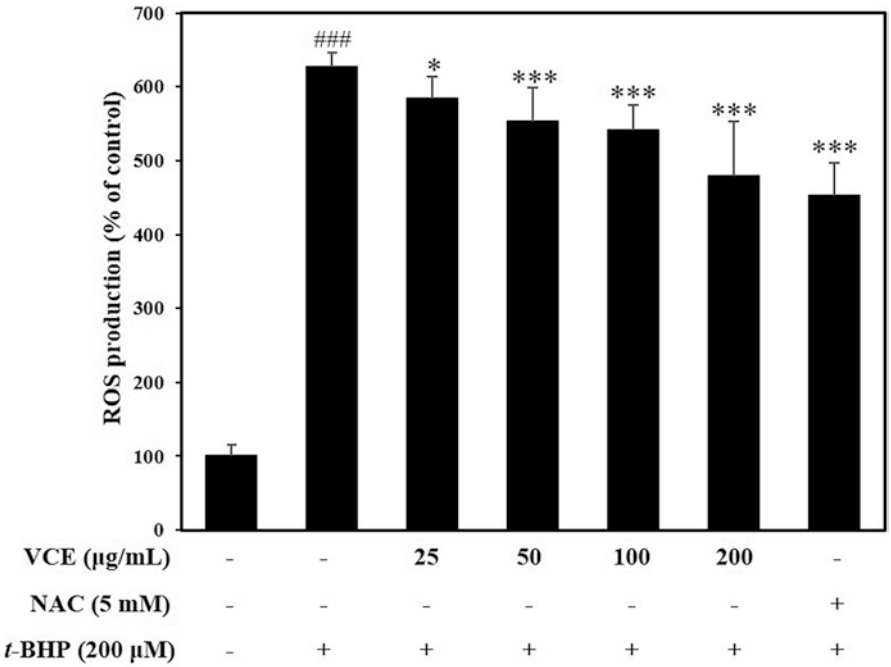

Fig. 5 Intracellular ROS production changes in intracellular ROS levels generated using *t*-BHP treatments in Chang cells. Chang cells were treated with different concentrations of VCE (25, 50, 100, and 200 μg/mL) for 1 h prior to *t*-BHP (200 μM) treatment for 30 min. Data were reported as means ± SD (n = 4) using a one-way ANOVA followed by Tukey's Test: $^{###}p < 0.001$ vs. control and $**p < 0.01$, $***p < 0.001$ vs. *t*-BHP

molecules leading to cell or tissue damage (Wijeratne et al. 2005). Therefore, in the present study, an oxidative stress model of Chang cells induced by t-BHP was used to evaluate the intracellular ROS scavenging capacity of VCE. The intracellular ROS level and ROS scavenging capacities of VCE in oxidized cells induced by t-BHP (200 μM) are shown in Fig. 5. The t-BHP group has the highest intracellular ROS level, being 6.2-fold higher than that of control group ($p < 0.05$). Interestingly, compared to the damaged group, the intracellular ROS level of all the groups that had been pretreated with the VCE (25–200 μg/mL), decreased significantly in a dose-dependent manner ($p < 0.05$). It is therefore suggested that the VCE exhibited excellent intracellular ROS scavenging capacity in oxidized Chang cells induced by t-BHP. These data indicate that the protective effect of VCE against t-BHP-induced cytotoxicity damage is mainly due to its ability to reduce ROS production.

3.9 ROS Production in Zebrafish

Oxidative stress-induced ROS production was detected in zebrafish embryo using a free permeable radical sensor (DCFH-DA). This non-fluorescent form of fluorescein is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon cleavage of the acetate group through oxidation. t-BHP induced oxidative stress increased ROS levels, but the VCE group and NAC groups decreased ROS compared to that of the t-BHP group (Fig. 6).

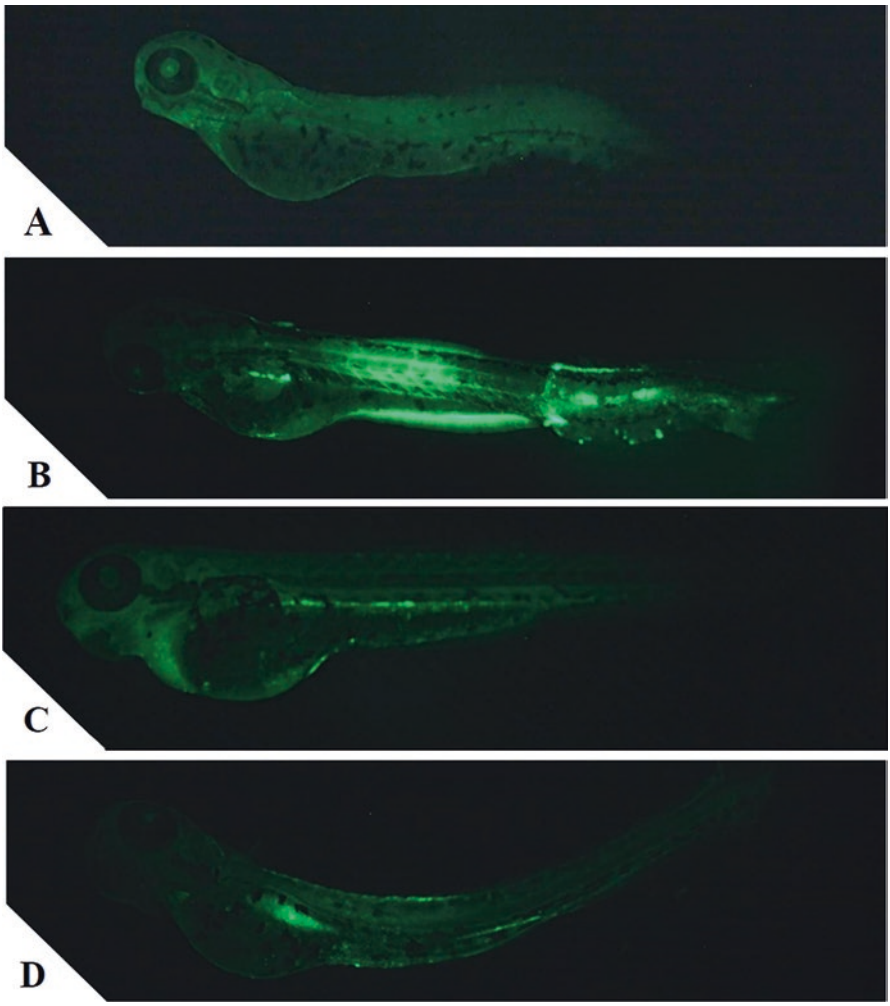

Fig. 6 ROS detection in zebrafish embryos. The embryos were incubated with pre-treatment of VCE for 1 h followed by a 24 h treatment with *t*-BHP, photographs were taken by using a fluorescence microscope. (**a**) control, (**b**) *t*-BHP (1 mM), (**c**) *t*-BHP (1 mM) + VCE (200 μg/mL), (**d**) *t*-BHP (1 mM) + NAC (0.1 mM)

4 Discussion

Reactive oxygen species (ROS) are known to be produced as a result of normal cellular metabolism and environmental factors, such as air pollutants or tobacco smoke (Birben et al. 2012). The imbalance between ROS levels and the antioxidant defense system causes oxidative stress (Patlevič et al. 2016). ROS can damage cell structures, such as carbohydrates, nucleic acids, lipids, and proteins, and alter their function (Wu et al. 2013). Many researchers have shown that oxidative stress is associated with a wide range of diseases, including cancer, atherosclerosis, neurological disorders, diabetes, hypertension, ischemia/perfusion, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and asthma (Jenner 2003; Toshniwal and Zarling 1992; Kerr et al. 1999; Kašparova et al. 2005). Therefore, reducing excess ROS generation as a way of preventing the disease is very important. Antioxidants are capable of preventing oxidative damage. Antioxidants from natural sources are considered to be good food and food supplements.

In this study, we evaluated the antioxidant effect of the V. contectus extract using various radical scavenging activity and protective effect against oxidative stress in Chang cells. The V. contectus extract contains strong radical scavenging activity. Under normal metabolic conditions, DNA in each cell of our body is exposed to adequate oxidative damage. However, oxidative stress causes DNA damage. Therefore, we measured the protective effect of VCE on DNA damage by oxidative stress.

As shown in Fig. 2, supercoiled DNA in the control was fully converted into open circular DNA by hydroxyl radicals, however the linear form was not observed. The result showed that VCE inhibits DNA scission resulting from H_2O_2 damage. According to Zhong and Shahidi (2012), inhibition against DNA scission of EGCG and its derivatives might be due to a combination of radical scavenging and Fe^{2+} chelation. Therefore, inhibition against DNA scission induced by hydroxyl radical might also be due to the cumulative effect of radical scavenging, Fe^{2+} chelating ability and H_2O_2 scavenging ability of VCE.

The amount of intracellular ROS was significantly decreased in a dose-dependent manner by the V. contectus extract in Chang cells. Also, the V. contectus extract exhibited ROS scavenging activity in zebrafish embryo. The protective effect of the V. contectus extract against t-BHP-induced oxidative stress could be attributed to it's antioxidant activity and the reduction in ROS production. These results suggest that the V. contectus extract attenuates t-BHP-induced oxidative stress by decreasing ROS production in Chang cells and the zebrafish embryo model.

Conflict of Interest The authors declare that there are no conflicts of interest.

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Antioxidant and Protective Effects of Atrina Pectinata Extract

Yon-Suk Kim, Eun-Kyung Kim, Xin Dong, Woen-Bin Shin, Jin-Su Park, Su-Jin Kim, Eun-Ae Go, Hee-Guk Byun, and Pyo-Jam Park

Abstract Atrina pectinata (A. pectinata), called pen shell, is an edible shellfish that adheres to the seabed pointed downward and has a triangular shell reaching about 40 cm in length.

In this study, we examined the antioxidant effect of an A. pectinata extract exhibiting various radical scavenging activities. These scavenging activities were evaluated using electron spin resonance. Anti-oxidant activities were also determined using the ferric reducing antioxidant power (FRAP) and the ABTS radical scavenging assays. Lipid peroxidation inhibitory activity was confirmed using ferric thiocyanate and thiobarbituric acid assays. Furthermore, the protective effect of the A. pectinata extract against t-BHP-induced oxidative stress on Chang cells were evaluated using MTT assay and the measurement of reactive oxygen species (ROS). These results showed that the A. pectinata extract have strong radical scavenging activities, and exerts protective effect against oxidative stress through reducing intracellular ROS content of Chang cells.

Keywords Antioxidant · Protective effects · Atrina pectinata

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1 Introduction

Shellfish are rich in various nutrients required by humans. In general, shellfish contain appreciable quantities of digestible proteins, bioactive peptides, essential amino acids, long-chain polyunsaturated fatty acids, astaxanthin and other carotenoids, vitamin B12 and other vitamins, minerals, including copper, zinc, inorganic phosphate, sodium, potassium, selenium, iodine, and also other nutrients, which offer a variety of health benefits to the consumer (Venugopal and Gopakumar 2017). Shellfish is a major component of global seafood production. Among various kinds of shellfish, we have focused on Atrina pectinata (A. pectinata).

A. pectinata is a large wedge or fan-shaped suspension-feeding bivalve (Okutani 1997) that belongs to the family Pinnidae. Its habitat ranges from muddy-to-sandy sediments to tidal flats or shallow subtidal environments up to 20 m deep (Yurimoto et al. 2003). A. pectinata, a popular food source due to its great taste, is important in a number of Asia-Pacific countries, including Korea, and to fisheries (FAO 1998). However, A. pectinata has been studied only for its structural and mechanical characterics, but there are not many studies on tits antioxidant activities.

Reactive oxygen species (ROS) are produced as a result of normal cellular metabolism by living organisms. Moderate concentrations of ROS serve various physiological functions, but at high concentrations, they produce adverse effects, modifying lipids, proteins, and DNA (Valko et al. 2006; Wang et al. 1996). A shift in the balance between the oxidants and antioxidants that favors the oxidants is termed “oxidative stress” (Birben et al. 2012). Meanwhile, oxidative stress causes profound alterations in various biological structures, including cellular membranes, lipids, proteins and nucleic acids (Zitka et al. 2012; Luo et al. 2018). Therefore, oxidative stress plays a major role in the pathology of various diseases including atherosclerosis, hypertension, ischemia/perfusion, hypoxia and cancer (Trujillo et al. 2013; Dhalla et al. 2000; Kasparova et al. 2005; Kerr et al. 1999).

Antioxidants are important substances which possess the ability to protect cells from damage caused by free radical induced oxidative stress. Recently, there is a growing interest in antioxidants, especially with regard to the natural antioxidants (Capecka et al. 2005).

The aim of the present study is conducted to evaluate the antioxidant potential and protective effects of an A. pectinata extract. The antioxidant capability of A. pectinata in vitro was analyzed by DPPH, FRAP, ABTS and ORAC assays. The protective effect of A. pectinata against oxidative stress was measured by the MTT assay and the measurement of intracellular ROS production by Chang cells.

2 Materials and Methods

2.1 Reagent

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium (FL), 2,2-azobis (2-methylpropionamide)-dihydrochloride (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4,6-tripyridyl-S-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2′,7′-dichlorofluorescein diacetate (DCF-DA), NAC was purchased from Invitrogen (Eugene, OR, USA). All other reagents were of the highest commercially available grade.

2.2 Extract Preparation

After removal of the shell, A. pectinata was washed with flowing water to remove the salt. Distilled water was added to A. pectinata (at 10 times weight/weight), after which it was boiled for 2 h. The boiled water extract was filtered with filter paper and the filtrate was evaporated in a rotary evaporator and lyophilized. The A. pectinata extract was stored at −20 °C until use.

2.3 Amino Acid Composition

The amino acid composition of the extract was analyzed using following steps. First, samples were mixed with 10 mL of 6 N HCl. After that, N_2 gas was used to purge the sample in a test tube and then the sample was hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and the amino acid composition was determined by measuring the absorbance at 440 and 570 nm.

2.4 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined according to previous studies (Guo et al. 2018) with slight modification. 50 μL of DPPH ethanol solution (2.5 mM) was mixed with 50 μL of sample, with the ethanol solution containing varying

concentrations (500, 250, 125, 62.5 and 31.25 μg/mL). Absorbance was measured at 515 nm after keeping samples in the dark for 30 min at room temperature.

The percentage of DPPH scavenging was calculated by the formula:

$$\text{DPPH scavenging activity}(\%) = \left[\left(\text{control absorbance} - \text{sample absorbance}\right)\right] / \text{Control absorbance}] \times 100.$$

where control absorbance represents (DPPH solution without sample), sample absorbance represents (DPPH solution plus test sample), and blank absorbance represents (sample without DPPH solution).

2.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant capacity of A. pectinata extract was measured using the FRAP method described by Benzie and Strain (1996) with little modifications. In these experiments, a 3.0 mL aliquot of FRAP reagent, a mixture of 0.3 M acetate buffer containing 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride (10:1:1 v/v/v), were mixed with 1.0 mL of A. pectinata extract. To determine the antioxidant capacity of the samples, the absorbance values were compared with those obtained from the standard curves using FeSO4 (0–10 mM). The antioxidant capacity values were expressed as mM of FeSO4 equivalent per mg of extract (mM $FeSO_4$ eq./mg extract).

2.6 ABTS Radical Scavenging Activity

The total antioxidant activity of the A. pectinata extract was measured using the ABTS· + radical cation decolorization assay. ABTS radical cation was produced by reacting 7.4 mM ABTS and 2.6 mM potassium sulfate overnight in the dark. After radical generation, the product was diluted with water until its absorbance was 1.50 ± 0.02 at 734 nm.

To determine radical scavenging activity, 0.9 mL of ABTS reagent was mixed with 0.1 mL of sample or BHT (as a positive control) and the absorbance was measured at 734 nm after reaction at RT. The antioxidant activities of the A. pectinata extract was expressed as trolox equivalents antioxidant capacity (TEAC), or mM of trolox equivalent per mg of extract (mM Trolox eq./mg extract).

2.7 ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay is a widely used procedure for measuring total antioxidant capacity of biological samples, supplements and food samples (Prior et al. 2003; Wu et al. 2004). The modified ORAC assay was conducted according to Dávalos et al. (2004). AAPH, Trolox, FL and sample solutions were prepared with 75 mM phosphate buffer (pH 7.4). The final reaction mixture contained 20 μL of sample, 120 μL of FL solution, and 60 μL of AAPH solution, and the final concentration of FL and AAPH in the reaction mixture was 70 nM and 12 mM, respectively. 20 μL of sample and 120 μL of FL were added in a well of a black 96-well microplate and pre-incubated at 37 °C for 15 min. To the reaction mixture 60 μL of AAPH solution was added and the mixture was shaken for 30 s. The reaction was conducted at 37 °C. The fluorescence of the reaction mixture was recorded using a spectrofluorometer (Spectramax M2/M2e, Molecular Devices, Silicon Valley, CA, USA) every 5 min for 120 min. The excitation and emission wavelengths were 485 and 520 nm, respectively.

2.8 Cell Culture

The Chang cell line was obtained from Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/mL) in an incubator with 5% CO_2 at 37 °C.

2.9 MTT Assay

Cell viability was assessed using an MTT assay. Briefly, Chang cells (7 × 103 cells/well) were seeded in 48-well culture plates. Cells were treated APE for 24 h and the MTT solution was added into each well; the cells were further incubated for 4 h. The medium was discarded and the intracellular formazan product was dissolved with 200 μL DMSO for 10 min. The absorbance was measured at 540 nm using a microplate reader (Thermo electron corp, Waltham, MA. USA). Cell viability was expressed as a percentage of the control.

2.10 Reactive Oxygen Species (ROS) Assay

The cells were seeded in a 96-well black plate at a concentration of 2 × 104 cells/well. After 24 h, cells were pre-treated with APE or NAC (positive control) for 1 h, then 200 μM t-BHP was added to each well and incubated for 30 min. The cells were then incubated with DCFH-DA (5 μg/mL) for 30 min at 37 °C in the dark. Non-fluorescent DCFH-DA dye can freely permeate into cells, to be hydrolyzed by intracellular esterase to 2′7-dichlorofluorescein, which are trapped inside the cells. The cells were washed with cold PBS twice, intracellular ROS was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrofluorometer (SpectraMax M2/M2e, CA, USA).

2.11 Measurement of Mitochondrial Membrane Potential (MMP)

Rhodamine 123 accumulates in membranes in a manner which is dependent on membrane polarization and rhodamine fluorescence can also be used as a measure of membrane polarization in live cell assays both within mitochondria (Chen 1988). Depolarization of MMP results in the loss of Rhodamine123 from the mitochondria and a decrease in intracellular fluorescence (Satoh et al. 1997). The cells were pre-treated with APE or NAC (positive control) for 1 h and then treated with t-BHP for 24 h, rhodamine123 was added to become a final concentration of 10 μM and incubated for 30 min at 37 °C. Harvested cells were analyzed for MMP by flow cytometry (FACSCalibur, Becton & Dickinson Co., Franklin Lakes, NJ, USA).

2.12 Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Experimental data were assessed with one-way analysis of variance (ANOVA) followed by Tukey's test and Dunnett's test. Graph Pad Prism software version 5.00 (Graph Pad Software Inc., San Diego, CA) was used for analyses. $p < 0.05$ was considered as statistical significance.

3 Results

3.1 Amino Acid Composition

A. pectinate contained carbohydrate (0.8 g), fat (1.1 g), protein (10.3 g), ash (1.3 g), and moisture (86.6 g) per 100 g edible portion. The amino acid composition of the APE is reported in Table 1. The amount of total amino acids was 6.02 mg/g, and the major amino acids were glutamic acid (23.74%), lysine (8.44%), aspartic acid (7.99%), arginine (8.06%) and alanine (6.49%). The amount of free amino acid was 5.91 mg/g, and the major amino acids were glutamic acid (15.47%), arginine (14.88%) and alanine (13.63%).

Table 1 Total and free amino acid composition of *A. pectinata* extract

Total amino acids			Free amino acids		
Amino acid	mg/g	%	Amino acid	mg/g	%
Asp	0.48	7.99	Tau	0.04	0.76
Thr	0.24	4.07	Asp	0.15	2.62
Ser	0.30	4.94	Thr	0.18	3.13
Glu	1.43	23.74	Ser	0.34	5.78
Gly	0.37	6.21	Glu	0.91	15.47
Ala	0.39	6.49	Gly	0.16	2.71
Cys	0.07	1.16	Ala	0.81	13.63
Val	0.21	3.48	Cit	0.03	0.56
Met	0.08	1.33	Val	0.29	4.84
Ile	0.14	2.27	Met	0.08	1.32
Leu	0.29	4.85	Ile	0.17	2.80
Tyr	0.13	2.14	Leu	0.35	5.96
Phe	0.17	2.85	Tyr	0.13	2.18
Lys	0.51	8.44	Phe	0.18	3.04
NH3	0.29	4.87	b-Ala	0.01	0.14
His	0.12	1.91	g-ABA	0.04	0.61
Arg	0.49	8.06	Orn	0.12	2.01
Hypro	0.05	0.75	Lys	0.31	5.29
Pro	0.27	4.42	His	0.16	2.63
Total	6.02	100.00	Car	0.31	5.20
			Arg	0.88	14.88
			Pro	0.25	4.31
			Total	5.91	100.00

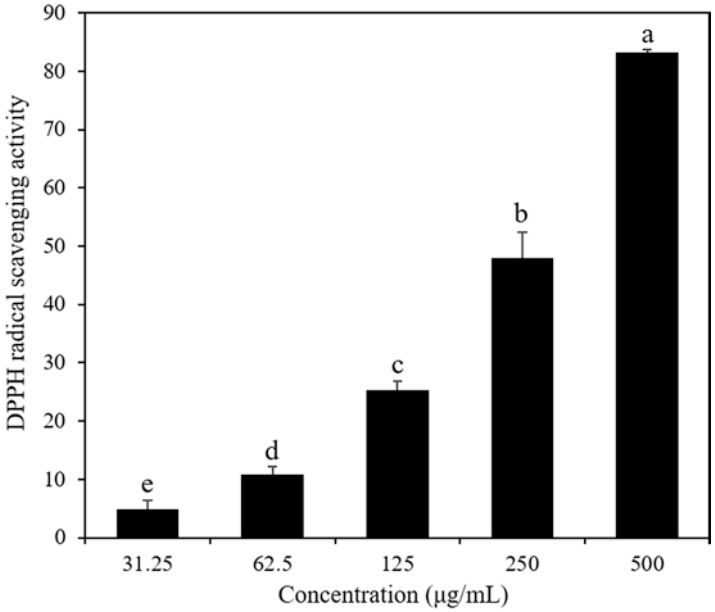

Fig. 1 Free radical scavenging activity of the *A. pectinate* extract using DPPH radical. Measurements without letter in common are significantly different according to the Tukey's test ($p < 0.05$)

3.2 DPPH Radical Scavenging Activity

The antioxidant activities in vitro of APE were evaluated for DPPH radical scavenging activity, ferric-reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). Figure 1 shows the reaction of APE with DPPH. There is a change in color from purple to yellow as the sample concentration increases. Our results show that APE scavenges the DPPH radical in a dose-dependent manner. APE showed 83.16 ± 0.53, 48.01 ± 4.45, 25.34 ± 1.53, 10.82 ± 1.36 and 4.82 ± 1.63% of DPPH radical scavenging activity at 500, 250, 125, 62.5 and 31.25 μg/mL, respectively.

3.3 Anti-oxidant Activity Using FRAP and ABTS Assay

The antioxidant activities were measured using commonly used methods of scavenging, including the ABTS radical, and ferric reducing antioxidant power (FRAP) assays. The antioxidant activity of APE using FRAP and TEAC values is shown in Table 2. The TEAC value of APE was 1.146 ± 0.003, which is similar to the TEAC value of BHT. The FRAP value of APE (2.736 ± 0.020) is larger than that of BHT (1.274 ± 0.152). These results show that APE has potent antioxidant activity.

Table 2 Values for ABTS radical scavenging, FRAP activity and ORAC of the *A. pectinata* extract

Samples	TEAC (mM Trolox eq./mg extract)	FRAP (mM $FeSO_4$ eq./mg extract)	ORAC (μM Trolox eq./mg extract)
APE	1.146 ± 0.003	2.736 ± 0.020	97.81 ± 0.28
BHT	1.151 ± 0.012	1.274 ± 0.152	54.02 ± 1.22

3.4 ORAC Assay

ORAC values are based on the standard curve of Trolox, derived from water-soluble vitamin E. The ORAC assay has been widely used to assess the antioxidant capacity of food, dietary supplements, wines, juices, and biological samples, such as plasma and urine (Prior et al. 2003; Wu et al. 2004). The ORAC assay utilizes an AAPH-derived peroxyl radical, which mimics lipid peroxyl radicals involved in a lipid peroxidation chain reaction in vivo (Tai et al. 2011). Inhibition of peroxyl radical-induced oxidation of a fluorescent probe, fluorescein, by antioxidants is serially monitored (Prior et al. 2005). APE showed stronger antioxidant activity than that of BHT in the ORAC assay.

3.5 Effect of A. pectinata *Extract on Cell Viability*

Cell viability was assessed using the MTT assay which relies on mitochondrial metabolic capacity of viable cells. The results show that APE has no cytotoxicity at 200 μg/mL. After treatment with APE for 24 h, viability of Chang cells was increased (Fig. 2).

Furthermore, the protective effect of APE was evaluated against oxidative stress induced by t-BHP (100 μM) in Chang cells. The cell viability of Chang cells decreased to 59.37% after exposure to t-BHP (100 μM) for 24 h. However, we found that cell viability increased to 68.39 ± 8.31%, 69.64 ± 3.10%, 71.22 ± 4.81% and 70.62 ± 5.58% in cells pretreated with APE at concentrations of 25, 50, 100 and 200 μg/mL, respectively (Fig. 3). The MTT assay results revealed that APE and the positive control (NAC, 5 mM) displayed protective effects against t-BHP-induced oxidative stress in Chang cells.

3.6 Intercellular ROS Production

In order to investigate the ability of APE to reduce intracellular oxidative stress, cells were pre-treated with APE (25, 50, 100 and 200 μg/mL) and the level of intracellular ROS was determined. t-BHP (200 μM) increased ROS production in Chang cells. An antioxidant, N-acetyl-cysteine (NAC) suppressed intracellular ROS production. The cells treated with APE suppressed ROS production caused by exposure of the cells to t-BHP. These results suggest that there is a cytoprotective effect through ROS inhibition (Fig. 4).

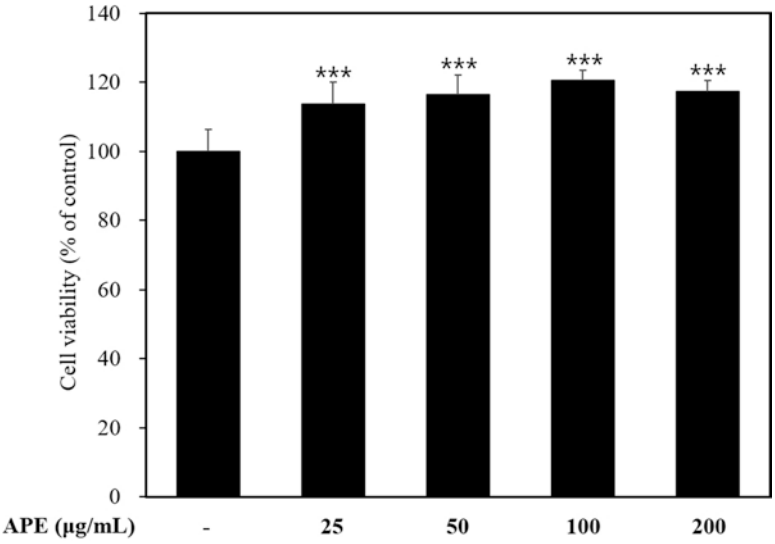

Fig. 2 Effect of the *A. pectinata* extract on viability of Chang cells using MTT assay. Results are expressed as means ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's test $*p < 0.05$ compared to control

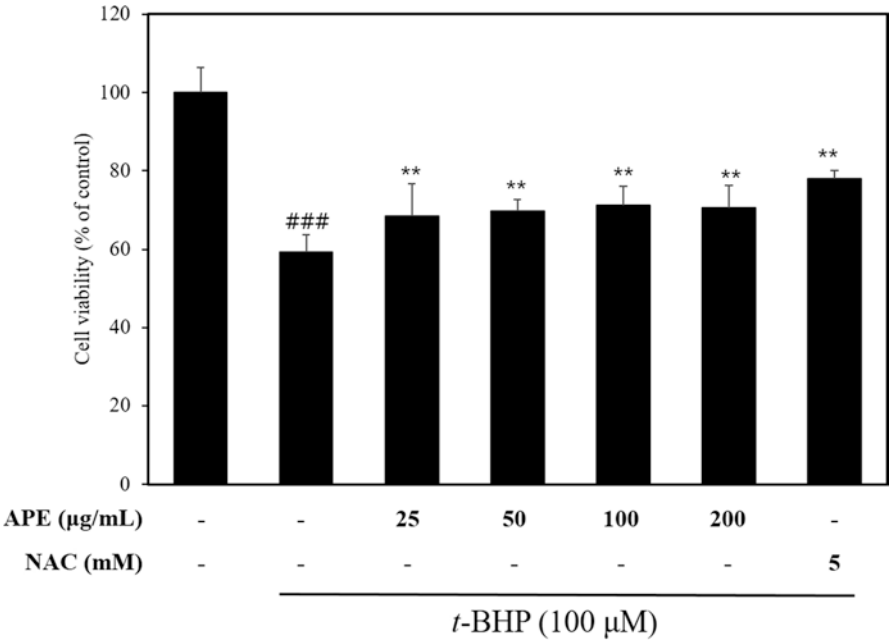

Fig. 3 Protective effect of APE on Chang cells using the MTT assay. Results are expressed as means ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Tukey's test $*p < 0.05$ compared to the control

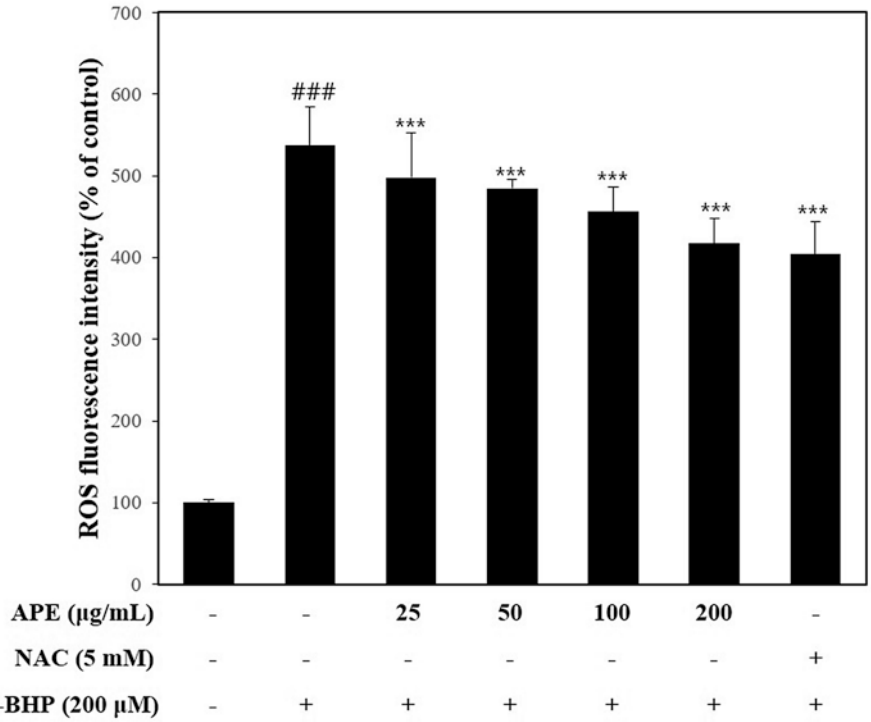

Fig. 4 Changes in intracellular ROS levels generated using *t*-BHP treatments in Chang cells. Chang cells were treated with different concentrations of APE (25, 50, 100, and 200 μg/mL) for 1 h prior to *t*-BHP treatment at 200 μM for 30 min. Data were reported as means±SD (n = 4) using a one-way ANOVA followed by Tukey's Test: $^{###}p < 0.01$ vs. control and $**p < 0.01$, $***p < 0.001$ vs. *t*-BHP

3.7 Measurement of MMP

We investigated whether APE protected mitochondria of Chang cells from depolarization caused by t-BHP. We determined the MMP by measuring the fluorescence of rhodamine123. After t-BHP (100 μM) treatment for 24 h, the percentage of cells exhibiting rhodamine123 fluorescence was 62.53%, and the fluorescence intensity of rhodamine123 decreased to 58.49% (50 μg/mL), 49.29% (100 μg/mL), and 45.43% (200 μg/mL) after pretreatment with APE (Fig. 3). These results indicate that APE prevented depolarization of MMP against oxidative stress (Fig. 5).

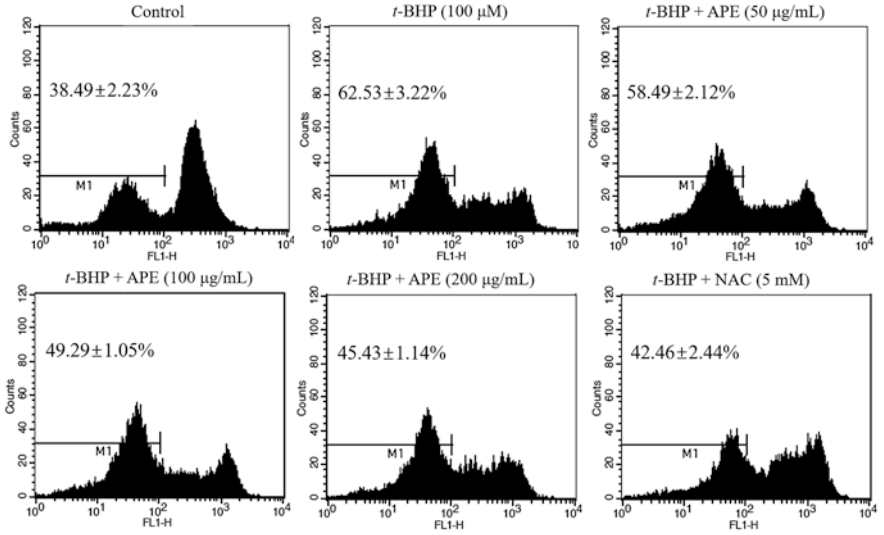

Fig. 5 Effect of APE on MMP of Chang cells. (**a**) control, (**b**) *t*-BHP (100 μM), (**c**) *t*-BHP (100 μM) + APE (50 μg/mL), (**d**) *t*-BHP (100 μM) + APE (100 μg/mL), (**e**) *t*-BHP (100 μM) + APE (200 μg/mL), (**f**) *t*-BHP (100 μM) + NAC (5 mM)

3.8 Morphological Changes

To confirm morphological changes, cells were observed using a phase-contrast microscope. t-BHP treatment group showed morphological changes of cells, rounded shape, reduced the cell size and density (Fig. 6). However, APE (200 μg/mL) and NAC (5 mM) pretreatment decreased the number of morphologically-altered cells and increased the number of normal cells.

4 Discussion

Taurine is an amino acid with antioxidant, anti-inflammatory and immunomodulatory properties (Stapleton et al. 1998), and it reduces oxidant-induced damage in a variety of experimental models (Timbrell et al. 1995; Gordon et al. 1998). Venkatachalam et al. (2014) also reported that taurine protects lungs from ROS production-induced damage by augmenting the function of antioxidants. In this study, taurine content of the A. pectinata extract was measured by amino acid analysis.

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. ROS normally exist in all aerobic cells in balance with biochemical antioxidants. ROS include superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (•OH) (Waris and Ahsan 2006).

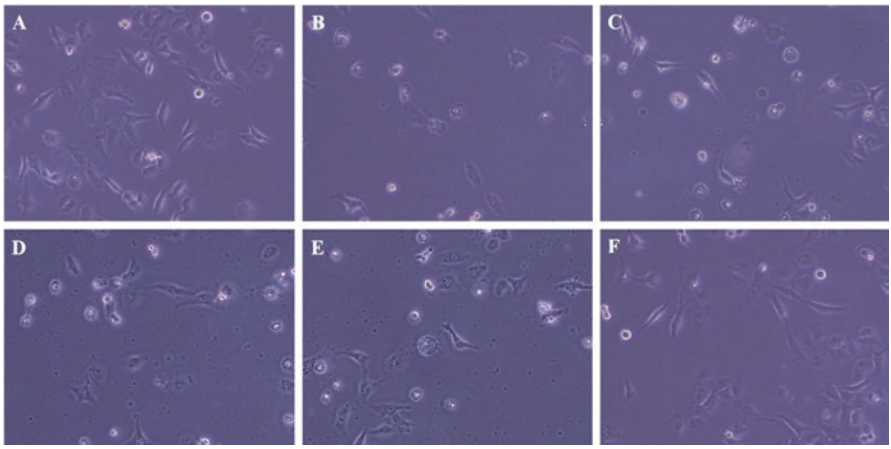

Fig. 6 Morphological change in Chang cells. The incubated cells were pre-treated with APE for 1 h and followed 24 h by treatment with *t*-BHP. Photographs were taken using an inverted microscope. (**a**) control, (**b**) *t*-BHP (100 μM), (**c**) *t*-BHP (100 μM) + APE (50 μg/mL), (**d**) *t*-BHP (100 μM) + APE (100 μg/mL), (**e**) *t*-BHP (100 μM) + APE (200 μg/mL), (**f**) *t*-BHP (100 μM)) + NAC (5 mM)

ROS can induce cell death by damaging DNA, oxidizing membrane lipids, and/or directly activating the expression of genes and proteins responsible for apoptosis (Redza-Dutordoir and Averill-Bates 2016). However, living cells cannot avoid ROS because they are produced continuously through biochemical reactions and external factors (Loft and Poulsen 1998). Multiple cellular defense mechanisms exist to quench free radicals and prevent intracellular damage by reducing the harmful effects of ROS (Rahman 2007). Therefore, reducing ROS production is critical for cell survival. It is therefore important to find external antioxidant sources to prevent oxidative damage.

In present study, the antioxidant activity of APE was determined by assaying various radical scavenging activity. The results of the FRAP and ORAC assays and antioxidant capacity of APE was two times higher than those of BHT. In addition, APE inhibited DNA scission induced by hydroxyl radical. APE decreased intracellular ROS production against t-BHP-induced oxidative stress in Chang cells. Taken together, it is considered that the strong radical scavenging activity of APE inhibits the production of intracellular ROS.

Further studies are ongoing to isolate and identify the active antioxidant compound of APE. Also, in vivo studies are needed to better understand the mechanism of action.

Conflict of Interest The authors declare that there are no conflicts of interest.

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Antioxidant Effects of an Alcalase Hydrolysate from *Batillus cornutus* Meat

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Abstract *Batillus cornutus* (*B. cornutus*) is one of the gastropoda, which are distributed along the coast of China, Japan and South Korea and northeast area. In this study, we first identified the antioxidant effects of a *B. cornutus* meat (BM) enzymatic hydrolysate in H_2O_2-treated Vero cells. First of all, we prepared an Alcalase hydrolysate from BM (BMA) and revealed a high taurine content. Also, taurine rich BMA dose-dependently increased 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, reducing power and the higher oxygen radical absorbance capacity (ORAC) value. In addition, BMA significantly increased

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the cell viability via the down-regulation of intracellular reactive oxygen species (ROS) production, as well as the decreased formation of apoptotic bodies and sub-G_1 DNA population in H_2O_2-treated Vero cells. Furthermore, BMA increased the expression of the anti-apoptotic molecule, Bcl-2, and decreased the expressions of Bax, p53 and cleaved PARP, all of which are pro-apoptotic molecules, in H_2O_2-treated Vero cells. Based on these results, this study suggests that BMA may be used as a potential protector on damage caused by oxidative stress.

Keywords *Batillus cornutus* meat · Taurine · Antioxidant effect · Vero cell

1 Introduction

ROS is an entire class of highly reactive molecules derived from the metabolism of oxygen and often generated products of biological reactions or by exogenous factors (Lucas-Abellán et al. 2011). ROS is used for short-lived diffusible entities, such as alkoxyl, hydroxyl or peroxyl radicals, and for some radical species of medium lifetime, such as nitroxyl radical and superoxide. Also non-radical ROS contains hydrogen peroxide, organic hydroperoxides and hypochlorous acid (Gill and Tuteja 2010). Over production of ROS resulting in oxidative stress may involve a large variety of mechanisms, including mitochondrial respiration, inflammation and the mitochondria injury (Mailloux and Harper 2011; Sun et al. 2011a). Especially, oxidative stress leads to cellular and DNA damage, which includes single- and double-strand breaks, oxidation of proteins and lipid peroxidation (Smith et al. 2013; Cichoż-Lach and Michalak 2014). These types of oxidative damage can be regulated by apoptotic modulators. Recently, many studies have shown that anti-apoptotic molecules, such as Bcl-xL and Bcl-2, play fundamental roles in apoptosis and protect T cells from apoptosis (Um 2016; Sharpe et al. 2004). Also, previous research has shown that up-regulation of Bcl-2 can protect Chang liver cells from apoptosis (Gokila Vani et al. 2013). Protein such as Bcl-2 has an influence on the release of cytochrome c from the mitochondria, thereby enhancing cell survival (Inayat-Hussain et al. 2010). As a result, Bcl-2 one of regulators of apoptosis, has been shown to confer proteins with an antioxidant function (Ola et al. 2011). Bax, which is pro-apoptotic, promotes mitochondrial outer membrane permeabilization (MOMP) that induces the release of apoptogenic factors from the mitochondria (Garcia-Saez et al. 2005). Also, it has been shown that Bax interacts with the membrane, causing permeabilization (Ichim et al. 2015). This fact indicated that increasement of Bax expression accelerated cell death (Mohan et al. 2012). Moreover, p53, one of pro-apoptotic proteins is involved in cell apoptosis through increased expression of Bax (Khutornenko et al. 2010). PARP is a pro-apoptotic protein that is promoted by caspase-3 (Ruemmele et al. 1999). Thus, ROS-induced oxidative stress causes a very wide spectrum of genetic, metabolic and cellular responses, including cell death, which are associated with diverse diseases, such as Alzheimer's disease

(Huang et al. 2016), Parkinson's disease (Pisoschi and Pop 2015), liver diseases (Cichoż-Lach and Michalak 2014), cancer (Birben et al. 2012), hepatitis and arthritis (Droge 2002; Wang and Weinman 2006). Therefore, the reduction and removal of ROS is an important key for the prevention and therapy of a various of diseases related oxidative stress.

Recent studies have indicated that various antioxidants inhibit or delay the oxidation of other molecules by reducing the initiation or propagation of oxidizing chain reactions (Velioglu et al. 1998). Normally, antioxidants are divided into natural antioxidants, such as tocopherols, flavonoids, alkaloids, polyphenols and ascorbic acid (Kumar 2011; Sun et al. 2011b), and synthetic antioxidants, such as hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Borsato et al. 2014). Until now, synthetic antioxidants have been used as antioxidants. However, the synthetic antioxidants have side effects, such as skin rash and cough (Kim and Wijesekara 2010). Therefore, many researchers have studied the naturally derived antioxidants that induce no deleterious side effects (Han et al. 2017) such as edible mushrooms (Cheun et al. 2003), cantaloupe (cucumis melo) (Ismail et al. 2010) and cherries (Karabegović et al. 2014). Interestingly, many recent studies have indicated that natural products, consisting of plentiful amounts of taurine, lead to antioxidant and protective effects against oxidative cellular and DNA damage (Um et al. 2017).

Batillus cornutus (*B. cornutus*) is one of the gastropoda that is found in the sea of the South Korea peninsula, some parts of the Chinese continent, Japan, and the Philippine islands (Saito and Aono 2014). However, until now, there is no report about its antioxidant activity in vitro and in the cell.

Therefore, in this study, we first evaluated the free amino acid composition of an *B. cornutus* meat (BM) enzymatic extract and its antioxidant activity against the adverse effects of H_2O_2 treatment.

2 Materials & Methods

2.1 Materials

All testing reagents including 1,1-diphenyl-2-picrylhydrazy (DPPH), bovine serum albumin (BSA), gallic acid, folin-ciocalteu reagent, Hydrogen peroxide, 2,2-azobis (2-methylpropion-amidine) dihydrochloride (AAPH), 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), fluorescein sodium salt, 3-(4–5-dimethyl-2yl)-2–5-diphynyltetrasolium bromide (MTT), 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Paisley, UK). Other chemicals and reagents were of the highest grade available commercially.

2.2 Proximate Composition of BM

The content of moisture, protein and lipid from BM were checked according to the Official Analytical Chemists (Association of Official Agricultural Chemists (AOAC) 1980).

2.3 Preparation of BMA

BM was obtained from Pohang, South Korea. To prepare an Alcalase hydrolysate of BM, BM (10 g) was mixed with 500 ml of distilled water and Alcalase enzyme 100 mg for 24 h at 50 °C. The enzyme mixture was inactivated, and then centrifuged. The filtered solution (BMA) was freeze-dried and stored at −20 °C until use.

2.4 Measurement of Free Amino Acid Composition

The free amino acid composition of BMA was checked by amino acid analyzer (S433-H, Sykam GmbH, Germany) based on the previously modified methods (Han et al. 2017).

2.5 Protein, Carbohydrate and Phenolic Contents of BMA

The protein content of BMA was based from the method of Lowry et al. (1951). Briefly, samples (1 ml) were hydrolyzed by adding 100 µl and heating the solution for 10 min at 100 °C and then 1 ml Lowry reagents was added to the mixture. Following addition of 0.1 ml folin-ciocalteu reagent, the reaction was allowed to proceed for 30 min in the dark. The absorbance was measured at 750 nm using a microplate reader (SpectraMax® M2/M2e, CA, USA). The carbohydrate content of BMA was measured using the method of Nielsen (2010). Briefly, after adding 1 ml sample and 80% phenol solution (25 µl in test tube), 2.5 ml sulfuric acid was mixed, and then the mixture was incubated for 30 min at R.T. The absorbance was measured at 480 nm using a microplate reader. The total phenolic content of BMA was determined by using the method of Singleton et al. (1999). The mixture of 500 µl sample and 95% ethanol 500 µl was blended with 2.5 ml distilled water, and 50% folin-ciocalteu reagent 250 µl was added. Following the addition 500 µl 5% Na_2CO_3, the reaction was allowed to proceed for 1 h. The absorbance was examined from microplate reader at absorbance of 725 nm. Respective calibration standard was confirmed as glucose, gallic acid and BSA.

2.6 ABTS Radical Scavenging Activity

The ABTS radical scavenging activity of BMA was determined according to the method of Han et al. (2017). The working solution was prepared by adding distilled water to achieve an absorbance of 1.50 ± 0.05 at 414 nm. 150 μl of used stock solution containing 7 mM ABTS in 2.4 mM potassium persulfate was mixed with each 50 μl of BMA and allowed to stand for 10 min at R.T. The absorbance was measured at 414 nm using a microplate reader. The ABTS radical scavenging activity of BMA was calculated with the following equation.

2.7 ORAC Assay

To evaluate effect of BMA on ORAC value, we performed ORAC assay with a modified method of Thaipong et al. (2006). All solutions were made in 75 mM phosphate buffer (pH 7.0). A 50 μl of 78 nM fluorescein was mixed with 50 μl of BMA (50 μg/ml) and incubated for 15 min at 37 °C. Then, 25 μl of 221 mM AAPH was added quickly, and the fluorescence was recorded every 5 min for 120 min (excitation wavelength 485 nm, emission wavelength 582 nm). The standard curve was measured using differed concentrations of trolox (0–40 μM). The net area under the curve (AUC) was determined and data expressed as μM trolox equivalent (TE)/mg sample.

2.8 Reducing Power

The reducing power was examined according to the method modified by Lue et al. (2010) and Han et al. (2017). BMA 200 μl, 0.1 M Na_2HPO_4 buffer (pH 6.6) 300 μl and $K_3[Fe(CN)_6]$ (1%, w/v) were added in e-tube. After 20 min incubation, e-tube was mixed 10% TCA (Trichloro acetic acid) 500 μl and was centrifuged at 3000 rpm for 10 min. The supernatant 500 μl, distilled water 500 μl and 0.1% $FeCl_3$ 100 μl was added in e-tube and the absorbance value was measured at 700 nm.

2.9 Cell Culture

Vero cells were obtained from ATCC (Manassas, VA, USA). Vero cells, known as an African green monkey kidney cell were incubated at 37 °C with 5% CO_2 in RPMI 1640 containing 10% heat-inactivated FBS, streptomycin (100 μg/ml) and penicillin (100 unit/ml).

2.10 Cell Viability

To determine the cytoprotective activity of BMA, the MTT assay was performed using the method of Hansen et al. (1989). After 16 ~ 18 h of Vero cell seeding with 1×10^4 cells/wells, the three concentrations of BMA were treated in wells for 1 h and additionally incubated with 500 μM H_2O_2. After 24 h, MTT reagent (5 mg/ml) was applied to the cells for 4 h and the formazan was resolved in dimethylformamide (DMSO) solution (pH 4.7, 10% sodium dodecyl sulfate (SDS) and 50% DMSO). The absorbance was measured at 570 nm using a microplate reader.

2.11 Determination of Intracellular ROS Generation

The intracellular ROS generation was determined 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay with the method of Um et al. (2017). Vero cells (1.6×10^4 cells/wells) were treated with various concentrations of BMA for 1 h and stimulated by H_2O_2 (500 μM) for additional 1 h at 37 °C. Finally, DCFH-DA (0.5 mg/ml) was added to the cells, and the absorbance was measured for 5 min at Excitation 485 nm/Emission 528 nm. The fluorescence percentage of intracellular ROS generation was calculated with the standard of 100% for the group treated with H_2O_2 without sample.

2.12 Nuclear Staining with Hoechst 33342

Chromosomal condensation and morphological change in the nucleus were investigated by Hoechst 33342 staining (Sigma, USA, Missouri). In brief, Vero cells (6×10^5 cells/wells) were treated with various concentrations (125, 250 and 500 μg/ml) of BMA for 1 h and then reacted with H_2O_2 (500 μM). After 12 h, the BMA treated cells were dyed using Hoechst 33342 of 2 μg/ml concertation for 0.5 h at 37 °C. Finally, the formation of apoptotic bodies was confirmed using a fluorescence microscope (Olympus, Shinjuku, Japan).

2.13 Flow Cytometry Analysis

To check effect of BMA on the proportion of apoptotic sub-G_1 hypodiploid cells, propidium iodide staining was performed (Yang et al. 2011). Vero cells (6×10^5 cells/wells) placed in 6 well plate and treated with BMA for 1 h and then reacted with H_2O_2 (500 μM). After 12 h, the cells were centrifuged to remove the supernatant and 70% ethanol 1 ml was added to fix the cell. After washing the fixed cells twice with PBS, and incubated in darkroom for 0.5 h in 2 mM PBS-EDTA

containing propidium iodide (PI) (50 μg/ml) (Sigma, USA, Missorui), and RNase A (0.2 μg/ml) (Promega, USA, Wisconsin). Flow cytometric analysis was conducted using a CytoFLEX (Beckman coulter, USA, California).

2.14 Western Blot

To examine the activity of BMA on the expression of apoptotic regulators in H_2O_2-treated Vero cells, Western blot analysis was executed (Han et al. 2017). Briefly, Vero cells (5×10^5 cells) were treated with BMA for 1 h and then reacted with H_2O_2 (500 μM) for additional 12 h. After 12 h, the cells were used for the extraction of cytosolic proteins by RIPA buffer (Thermo, Rockford, IL). Each protein (40 μg) was subjected to electrophoresis in 12% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA, Massachusetts). The membrane was blocked for 3 h at R.T in 5% skim milk in tris buffered saline (TBS) containing Tween-20 (TBS-T). Finally, the membrane was incubated with anti-Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.), anti-Bax (1:1000 dilution, Cell Signaling Technology Inc.), anti-cleaved PARP (1:1000 dilution, Cell Signaling Technology Inc.), anti-p53 (1:1000 dilution, Cell Signaling Technology Inc.) and anti-β-actin (1:3000 dilution, Sigma) in 5% skim milk. Following three washes with TBS-T, and incubation with appropriate HRP-conjugated anti-mouse IgG and anti-rabbit IgG (1:5000, Cell Signaling Technology Inc.) for 90 min at R.T, the bands were detected using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Canada, Burlington) reagents and their densitometric analysis was examined by NIH Image J software (US National Institutes of Health, Bethesda, MD).

2.15 Statistic Analysis

All results are expressed as the mean ± S.E in triplicate. Differences between means of each group were assessed by one-way analysis of variance followed by Duncan's test using PASW statistics 21.0 software (SPSS, Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

3 Results

3.1 Proximate Compositions of BM and BMA

The compositions of BM and BMA are shown in Table 1 and Table 2. Table 1 showed BM contained the plentiful protein composition of 40.65 ± 1.70% than that of moisture (2.04 ± 0.01%) and lipid (15.68 ± 0.53%). In Table 2, we identified the

Table 1 Proximate compositions (%) of BM

Sample	Protein	Lipid	Moisture
BM	40.65 ± 1.70	15.68 ± 0.53	2.04 ± 0.01

Table 2 The extraction yields and the content of protein, carbohydrate and phenol (%) of BMA

Sample	Extraction yields	Protein	Lipid	Moisture
BMA	87.79 ± 0.88	37.94 ± 0.17	27.30 ± 0.68	3.17 ± 0.03

extraction yield of BMA was 87.79 ± 0.88% and contained a large amount of protein and carbohydrate. But, it contained a small amount of polyphenol comparing to other constituents. Particularly, the BMA compositions was highest in protein content (37.94 ± 0.17%) followed by carbohydrate (27.30 ± 0.68%) and finally phenol (3.17 ± 0.03%).

3.2 Free Amino Acid Contents of BMA

The free amino acid content of BMA is indicated in Table 3. The most plentiful amino acid of BMA was taurine, which represented 29.74% of the total amino acid pool. Also, BMA is abundant in leucine, alanine and glutamic acid, which accounted for 8.81%, 7.88% and 5.37% of the amino acid pool, respectively.

3.3 Antioxidant Effect of BMA In Vitro

The antioxidant effect of BMA was evaluated by measuring ABTS radical scavenging, reducing power and ORAC value *in vitro*. As shown in Fig. 1a, BMA scavenged the ABTS radical in a dose-dependent manner. Also, BMA significantly increased reducing power activity (Fig. 1b). In Fig. 1c, BMA (50 μg/ml) exhibited an ORAC value of 667.97 μM TE/mg. From these results, we suggest that BMA contained the rich taurine has the high antioxidant activity *in vitro.*

3.4 Effect of BMA on Cell Viability in H_2O_2-Treated Vero Cells

To check the cytotoxic effect of BMA, we performed MTT assay in Vero cells. As indicated in Fig. 2a, BMA showed no cytotoxic effect at all concentrations examined, comparing with the control cells. Next, the effect of BMA on the viability of H_2O_2-treated Vero cells was evaluated using the MTT assay. The results revealed that treatment with 500 μM H_2O_2 reduced Vero cell viability to 67%, whereas it was

Table 3 Free amino acid contents (%) of BMA

	BMA	
Amino acid	mg/100 g	% amino acid
Phosphoserine	N.D.	N.D.
Taurine	361.432	29.74
Phosphoethanolamine	N.D.	N.D.
Urea	1.456	0.12
Aspartic acid	14.325	1.18
Hydroxyproline	N.D.	N.D.
Threonine	47.581	3.92
Serine	47.63	3.92
Asparagine	51.225	4.21
Glutamic acid	65.272	5.37
Sarcocine	N.D.	N.D.
α-aminoadipic acid	N.D.	N.D.
Proline	36.886	3.04
Glycine	54.926	4.52
Alanine	95.751	7.88
Citrulline	N.D.	N.D.
α-aminobutyric acid	N.D.	N.D.
Valine	67.722	5.57
Cystine	N.D.	N.D.
Methionine	39.147	3.22
Isoleucine	55.5	4.57
Leucine	107.112	8.81
Tyrosine	23.323	1.92
phenylalanine	50.441	4.15
β-alanine	28.945	2.38
β-aminoisobutyric acid	N.D.	N.D.
γ-amino-n-butyric acid	N.D.	N.D.
Histidine	11.22	0.92
3-methylhistidine	1.543	0.13
1-methylhistidine	N.D.	N.D.
Carnosine	N.D	N.D.
Anserine	N.D.	N.D.
Tryptopan	8.077	0.66
Hydroxylysine	N.D.	N.D.
Ornitine	N.D.	N.D.
Lysine	23.189	1.91
Ethanolamine	N.D.	N.D.
Arginine	22.642	1.86
Total	1,215.35	100.00

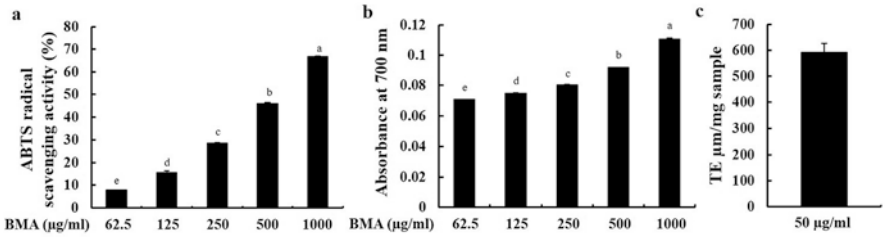

Fig. 1 Antioxidant effects of BMA *in vitro*. The ABTS radical scavenging effect (**a**), Reducing power (**b**), ORAC value (**c**). Data represented as means ± S.E. [a–e]The values with different subscripts indicate significant difference within each sample ($p < 0.05$)

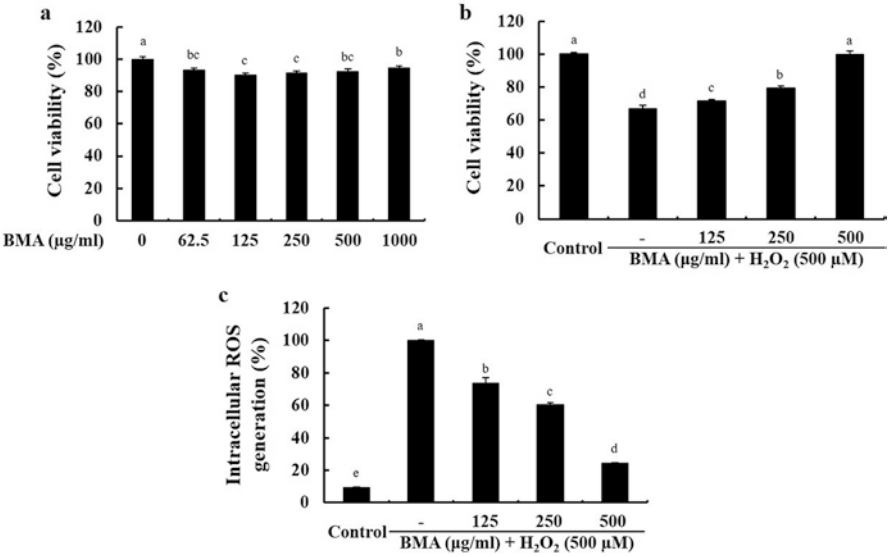

Fig. 2 Effects of BMA on cell viability (**a**, **b**) and intracellular ROS production (**c**) in Vero cells. Cell viability was determined in only BMA-treated Vero cells using the MTT assay (**a**). Cell viability was assessed in Vero cell co-treated with H_2O_2 and BMA using the MTT assay (**b**). Intracellular ROS production was checked in Vero cell co-treated with H_2O_2 and BMA using the DCFH-DA assay (**c**). Data represented as means ± S.E. [a–e]The values with different subscripts indicate significant difference within each sample ($p < 0.05$)

significantly improved by pretreatment of BMA with the increment of concentrations. In particular, the cell viabilities reduced by H_2O_2 exposure were rescued by the application of BMA (500 μg/ml) up to about 99.67% (Fig. 2b). These results indicate that BMA protects against damage caused by H_2O_2-treatment of the Vero cells. Next, we measured the effect of BMA on intracellular ROS generation caused by exposure of Vero cells to H_2O_2 containing medium. In the DCFH-DA assay, DCFH-DA is hydrolyzed to non-fluorescent DCFH by an intracellular esterase, and

by oxidation of DCFH to form fluorescent DCF in the presence of ROS (Park et al. 2016). Figure 2c exhibited after the exposure of H_2O_2, the intracellular ROS formation was markedly over-produced in Vero cells, whereas it was significantly inhibited by the treatment of BMA with the increment of dose. Therefore, this result indicates that BMA has the cytoprotective effect against H_2O_2-mediated oxidative stress.

3.5 Protective Effects of the BMA Against H_2O_2-Induced Apoptosis in Vero Cells

The protective effect of BMA against H_2O_2-mediated apoptosis was investigated by nuclear staining with Hoechst 33342. As shown in Fig. 3a, the negative control possessed intact nuclei, whereas the H_2O_2-treated group contained apoptotic bodies, including significant nuclear fragmentation, indicating apoptosis. But, pretreatment with BMA attenuated the formation of apoptotic bodies caused by exposure to H_2O_2. Figure 3b showed that the exposure to H_2O_2 led to the abnormal increment of apoptotic sub-G_1 DNA contents in Vero cells, compared to the non-treated control cells. Interestingly, pretreatment with three concentrations (125, 250 and 500 μg/ml) of BMA reduced apoptotic sub-G_1 DNA content of H_2O_2-treated Vero cells in a dose-dependent manner. From these results, our study suggest that BMA protects cells via the down-regulation of H_2O_2-mediated oxidative stress by reducing the production of apoptotic bodies and sub-G_1 DNA.

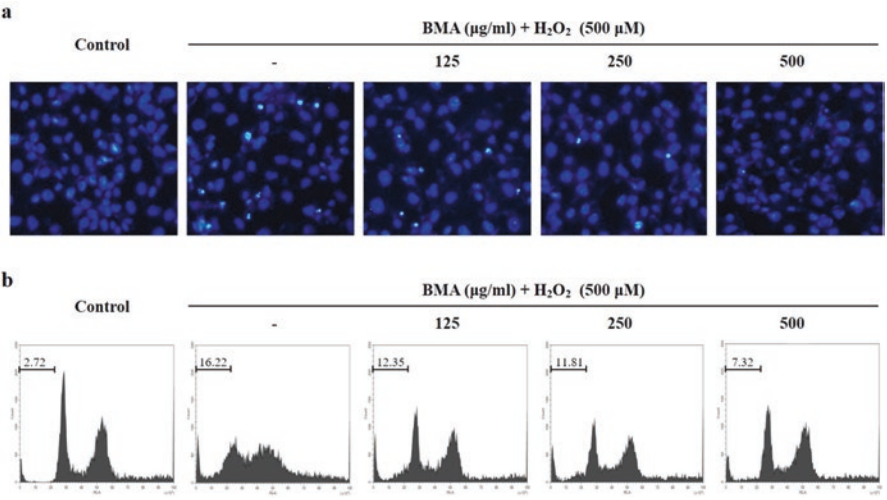

Fig. 3 Effects of BMA on apoptotic body formation (**a**) and apoptotic sub-G_1 DNA content (**b**) induced by H_2O_2 in Vero cells. Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and the cell cycle was observed by PI staining using flow cytometry

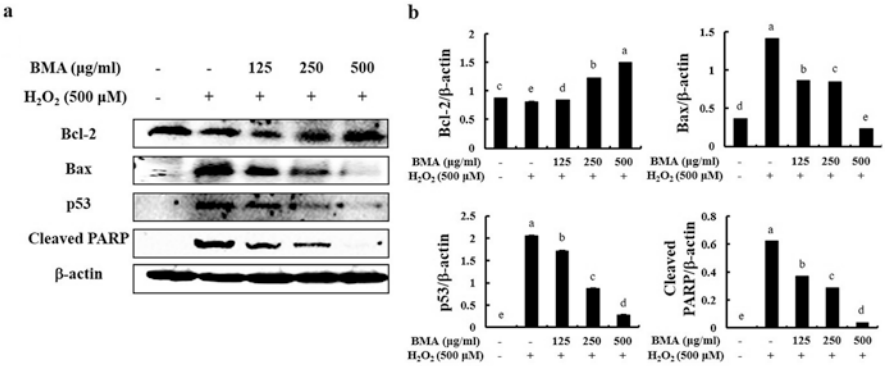

Fig. 4 Effect of BMA on the expression of apoptotic regulators in H_2O_2-treated Vero cells. Total cell lysates were prepared and subjected to western blot analysis to monitor the levels of apoptotic proteins, namely Bcl-2, Bax, p53, and cleaved PARP (**a**). Densitometry analysis of protein levels (**b**). Values means ± S.E of three determinations. [a–e]The bars with different letters are significantly different ($p < 0.05$)

3.6 Effect of BMA on the Expression of Apoptosis-Related Molecules in H_2O_2-Treated Vero Cells

To establish the effect of BMA, the cellular levels of the anti-apoptosis molecule, Bcl-2, and pro-apoptosis molecules, Bax, p53 and cleaved PARP, were determined by western blot analysis. As shown in Fig. 4, exposure to H_2O_2 slightly decreased the level of Bcl-2 compared to that seen in the non-treated control cells. However, BMA mediated an up-regulation in Bcl-2. Also, BMA significantly down-regulated the expression of pro-apoptotic molecules, including Bax, p53 and cleaved PARP in cells exposed to H_2O_2. This result suggests that BMA protects the cells against H_2O_2-mediated oxidative stress by modulating the expression of apoptotic regulators.

4 Discussion

Taurine (2-aminoethanesulfonic acid) is found in all animal cells (Oliveira et al. 2010) and is plentiful in humans (Nguyen et al. 2013). In a previous study, many researchers indicated that taurine exerts beneficial effects in various biological and physiological functions (Zeng et al. 2010). *B. cornutus* is one of the gastropoda found in Asia, including South Korea, Japan and China. Also, we confirmed that BMA is rich in taurine, alanine and leucine. Especially, among the free amino acids, taurine exerts beneficial effects, such as antiatherosclerotic, hepatic injury (Balkan et al. 2001), membrane stabilization effect and antioxidant effect (Shiny et al. 2005). However, there are no report on the antioxidant effect of *B. cornutus*. Here, we investigated the antioxidant effect of BMA against H_2O_2-induced oxidative stress in Vero cells.

Various previous studies confirmed that enzyme extraction technique has many kinds of advantages such as the useful acquisition of biologically active compounds, the higher extraction yields, and the water soluble extraction technique and has been used for the extraction from a great variety of natural products (Um et al. 2017). Here, BMA was prepared from *B. cornutus* meat by the enzymatic extraction with Alcalase, a protease and evaluated its antioxidant effect by measuring ABTS radical scavenging, reducing power and ORAC values. ABTS radical scavenging assay is radical cation decolorization test and it is widely used as evaluation of antioxidant activity (Miliauskas et al. 2004). Reducing power is one of the useful measurements of antioxidant activity and its principle is the conversion of potassium ferricyanide (Fe^{3+} form) to potassium ferrocyanide (Fe^{2+} form), the latter which forms a ferric ferrous complex in the presence of ferric chloride (Jayanthi and Lalitha 2011). ORAC assay is a method of measuring the reduction of protein fluorescence as a result of oxidative damage caused by peroxyl radical (Zulueta et al. 2009). Previous our studies have reported the taurine rich extracts derived from the marine resources had the beneficial capacities as the antioxidants (Um et al. 2017; Han et al. 2017). In this study, we identified that BMA led to the excellent antioxidant activity by scavenging ABTS radicals, improving reducing power and increasing the ORAC value. With these points, we can indicate that BMA can be used as a potential natural antioxidant.

Normally, oxidative stress induced by overproduction of ROS can cause DNA damages including apoptotic body formation and sub-G_1 DNA content increment in cells and implicate cellular injury during hypoxia and re-oxygenation (Markesbery 1997; Li and Jackson 2002). It also can generate severe damage to proteins and lipids (Curtin et al. 2002; Simon et al. 2000), cause cell death, and finally play a role in various diseases such as Alzheimer's disease (Huang et al. 2016), kidney disease and diabetes (Forbes et al. 2008). Based on these results, the capacity of ROS scavenging can affect to protect cells and animals against oxidative stress-caused various damages. Interestingly, our data exhibited that the exposure of H_2O_2 significantly decreased cell viability as increasing the intracellular ROS generation, whereas it was improved by the treatment of BMA in Vero cells. Moreover, DNA damages such as apoptotic body formation and sub-G_1 DNA content increment caused by the generation of intracellular ROS were abolished by the treatment of BMA. Previous reports suggested that taurine decreased the intracellular ROS production and DNA damages including the reduced number of apoptotic bodies and a high content of apoptotic sub-G_1 DNA by increasing of the antioxidant activity (Das and Sil 2012; Chang et al. 2014; Hu et al. 2015). Also, taurine reduces oxidative stress in the mitochondria, stimulates mitochondrial anti-oxidative enzymes (Mn-SOD), regulates mitochondrial calcium homeostasis and preserves mitochondrial function (Xu et al. 2015). Our data also showed that BMA reduced the DNA damages such as apoptotic body formation and sub-G_1 DNA content increment in H_2O_2-treated Vero cells. From these results, we suggest that the rich taurine content of BMA led to the antioxidant effect and it contributed to the protection of cells as inhibiting DNA damages against oxidative stress caused by the increment of intracellular ROS generation.

The Bcl-2 family of proteins regulates the mitochondrial pathway of apoptosis by regulating MOMP (Brunelle and Letai 2009). Briefly, when cells are exogenously stimulated, the BH3-only protein is activated and binds to anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Zong et al. 2001). After binding, pro-apoptotic such as Bax is activated and oligomerized to form pores in the outer mitochondrial membrane and cause MOMP (Siu et al. 2008). When MOMP occurs, cytochrome c is released from the mitochondria and induced apoptosis (Lavrik 2010). DNA damage and oxidative stress increases the expression levels of pro-apoptotic molecules, Bax, p53 and cleaved PARP, but reduces that of anti-apoptotic molecule, Bcl-2 (Xu 2003; Um et al. 2017; Han et al. 2017). Recent report indicated that taurine significantly decreased the expression of the pro-apoptotic protein, Bax, whereas increased the expression of the anti-apoptotic protein, Bcl-2, in oxidative stress-exposed cells (Chang et al. 2014). Recent studies have reported that taurine exerts anti-apoptotic activity, however, it is unclear if its anti-apoptotic and antioxidant activities are directly related (Ricci et al. 2008). Moreover, mitochondrial ROS is the only cause of apoptosis. Our western blot analysis exhibited BMA consisted of the plentiful taurine content inhibited the apoptosis as up-regulating the expression level of anti-apoptotic molecule, Bcl-2 and down-regulating those of the pro-apoptotic molecules, Bax, p53 and cleaved PARP, in comparison with the H_2O_2-treated cells. These data indicate that BMA protects cells against cellular and DNA damage by inhibiting H_2O_2-induced apoptosis via the prevention of mitochondrial ROS formation.

5 Conclusion

Taken together, these results suggest that BMA consisted of large amounts of taurine, which protects cells against damage caused by ROS-induced oxidative stress and apoptosis.

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Cytoprotective Effects of an Aqueous Extracts from Atrina Pectinate Meat in H_2O_2-Induced Oxidative Stress in a Human Hepatocyte

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Abstract In the present study, we investigated the antioxidant activity of an aqueous extract from *Atrina pectinate* meat (APW) against H_2O_2-induced oxidative stress in a human hepatocyte. The extraction yield of APW was 30.01 ± 0.83% and which contained the highest taurine content among free amino acid contents. APW led to the high antioxidant activity showing 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) radical scavenging activity, good reducing power and oxygen radical absorbance capacity (ORAC) value. Also, the results showed that APW improved the cell viability decreased by H_2O_2 stimulation as well as the reduction of intracellular reactive oxygen species (ROS) generation in hepatocytes. Additionally, APW up-regulated the production of antioxidant mechanisms related

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enzymes such as catalase and superoxide dismutase (SOD), compared to the only H_2O_2-treated hepatocytes. Moreover, APW increased the expressions of nuclear Nrf2 and cytosolic HO-1 in H_2O_2-treated hepatocytes. Interestingly, the treatment of ZnPP, a HO-1 inhibitor abolished the cell viability and intracellular ROS generation induced by APW treatment. In conclusion, this study suggests that APW protects H_2O_2 induced oxidative stress via up-regulating of Nrf2/HO-1 signal pathway in hepatocytes.

Keywords *Atrina pectinate* · Anti-oxidant · Hepatocytes · Nrf2/HO-1 signal pathway

1 Introduction

Atrina pectinate (*A. pectinate*) is an aquatic mollusk, which has a compressed body enclosed a hinged shell (bivalve). *A. pectinate* belongs to the order- Mytiloida, family- Pinnidae. *A. pectinate* is a common bivalve in tropical marine eco-systems. *A. pectinate* usually grow in muddy or sandy sediment (Cao et al. 2018; Mohibbullah et al. 2018). Specifically, *A. pectinate* has a good demand as a seafood in Asian pacific countries including China, Japan, and Korea (Cao et al. 2018; Awaji et al. 2018). Moreover, in Korea *A. pectinate* is called as Khi-Jo-Gae and play an important role as a seafood type in Korea due to its commercial value and protein content. According to the previous reports, during 2017 the production of *A. pectinate* in the Korean peninsula was approximately around 3,743,000 tons and its market value was 7,983,103,600 $ (Mohibbullah et al. 2018).

Reactive oxygen species (ROS) like hydroxyl radical (OH^-), superoxide anion (O^{2-}), and hydrogen peroxide (H_2O_2) produced during the oxygen metabolism because of normal cell function. Additionally, exposure of cells to the external sources such as cigarette smoke fine-dust, and vehicle smoke lead to produce ROS in cellular systems (Park et al. 2018). To avoid damages caused by ROS, the cellular systems have antioxidant defense mechanisms such as Nrf2/HO-1 mediated antioxidant signal pathway. The activation of ROS defense mechanisms has the possibility to remove excess ROS into normal levels and protect cells and organs from ROS-induced damages (Niu et al. 2018). Excessive production of ROS in the cells have the potential to made an imbalance between oxidant production and antioxidant systems and ultimately causes oxidative stress in the cellular systems (Martindale and Holbrook 2002). With the evidences of previous studies, the expo-

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sure of cells to continuous and long-term oxidative stress contributed to the pathogenesis of oxidative stress related conditions such as, cancer, inflammation related disorders, cardiovascular diseases, neurodegeneration diseases (Huntington's disease, Alzheimer's disease, Parkinson's disease) and diabetes (Fernando et al. 2016). Commercial antioxidants such as 2,6,-di-t- butyl-4- methyl phenol (BHT) and 2-t-butyl-4-methoxyphenol (BHA) are commonly used as additive in the food industry to control oxidative stress (Soobrattee et al. 2005). However, the long term use of synthetic antioxidants found to induce side effects such as blood clotting and tumor progression. Therefore, it is an urgent requirement to develop effective antioxidants from natural sources with less side effects (Soobrattee et al. 2005; Kahl and Kappus 1993). Taken together, in the present study, we attempted to evaluate the antioxidant activity of an aqueous extract from *A. pectinate* meat (APW) against H_2O_2-induced oxidative stress in a human hepatocyte.

2 Materials and Methods

2.1 Chemicals and Regents

2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), RIPA buffer, zinc protoporphyrin IX (ZnPP), ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein sodium salt, 3-(4-5-dimethyl-2yl)-2-5- diphynyltetrasolium bromide (MTT), were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Nuclear protein extraction kit was purchased from, Thermo Scientific Inc., IL. Antibodies against heme oxygenase-1 (HO-1), nuclear factor E2-related factor 2 (Nrf2), β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Chang liver cell line was purchased from the Korean cell line bank. Penicillin/streptomycin and Dulbeco's modified Eagle's medium (DMEM) were purchased from GibcoBRL (Grand Island, NY, USA).

2.2 Collection and Extraction of Sample

To prepare the water extract from AP, AP (50 g) was mixed with 1 L of distilled water for 24 h at R.T. Then, the mixture was centrifuged and filtered through a Whatman No. 6 filter paper. The filtrate (APW) was freeze-dried and stored at −20°C until further use.

2.3 Evaluation of Free Amino Acid Content of Extracts

Amino acid composition of APW was analyzed with an amino acid analyzer (S433-H, Sykam GmbH, Germany). A 100 mg of APW was hydrolyzed using 2 ml of 6 M HCl in a sealed vacuum ampoule at 110 °C for 24 h. Hydrochloric acid was removed by rotary evaporator and brought to a final volume of 10 ml with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated and detected using a cation separation column (LCA K06/Na, 4.6 × 150 mm) with a flow rate of 0.45 ml/min (buffer) and 0.25 ml/min (reagent) at wavelengths of 440 and 570 nm. For the determination of free amino acids, 100 mg of APW was homogenized at 12,000 rpm twice for 2 min with 95% ethanol, followed by centrifuging at 2000 × g for 30 min. The supernatant solvents were removed by using a rotary evaporator, and re-dissolved in 8 ml of distilled water containing 5′-sulfosalicylic acid (200 mg) at 4 °C for 1 h. The mixture was then centrifuged at 2000 × g for 30 min, and the 2 ml of the supernatant was transferred to a tube containing 1 ml of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were proved using the same amino acid analyzer.

2.4 ABTS Radical Scavenging Assay

To generate the ABTS radical, 7 mM ABTS and 2.4 mM potassium persulfate was incubated for 16 h in the dark. And then, the solution was diluted with distilled water to an absorbance of 1.50 ± 0.05 at 414 nm. The ABTS solution was mixed with 50 μl of the APW (15.3–1000 μg/ml). The mixture was incubated at R.T for 10 min, and the absorbance was measured at 414 nm using microplate reader (SpectraMax® M2/M2e, CA, USA). Calculation of ABTS radical scavenging activity was as follow.

$$\text{ABTS radical scavenging activity}(\%) = \left[\left(\text{Abs.control} - \text{Abs.sample}\right) / \text{Abs.control}\right] \times 100$$

Abs.control: Control absorbance at 414 nm
Abs.sample: Sample absorbance at 414 nm

2.5 ORAC Assay

The Oxygen radical absorbance capacity (ORAC) assay was performed according to the method describe by Bentayeb et al. (2014) with slight modifications. First, fluorescein solution (0.8 ml) and different concentrations of APW (0.1 ml) mixed 8:1 ratio and then 0.6 ml of the AAPH solution was added to the previous mixture to start radical reaction (Bentayeb et al. 2014). The fluorescences were respectively recorded every 5 min for 60 min (excitation wavelength 485 nm, emission

wavelength 582 nm). Different concentrations of trolox (0–20 μM) were used in the preparation of a standard curve, and ORAC values of the APW were calculated by using the net area under the decay curves (AUC) and expressed as μM trolox equivalent (TE)/mg sample.

2.6 Reducing Power

The reducing power of APW was measured following the method described by Hyun et al. (2016). Briefly, APW (125–500 μg/ml) was mixed with 3 ml of reaction mixture which contained 28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulfuric acid. The reaction mixture was incubated 90 min at 95°C and the absorbance was measured at the 695 nm using a ELISA reader (Hyun et al. 2016).

2.7 Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging effect of APW was evaluated using previously optimised method. In Brief, 0.1 ml APW hydrolysates (125–500 μg/ml) was mixed with 0.1 ml of 0.1 M sodium phosphate buffer (pH 5.0) in a 96 well plate. After that, 20 μl of 20 mM H_2O_2 solution was added and incubated 5 min at 37°C. The reaction mixture was incubated for 10 min at same temperature with 30 μl of 1 U/ml peroxidase and 30 μl of 1.25 mM ABTS. Then, the reaction mixture was measured at 405 nm using a ELISA reader (Um et al. 2017).

2.8 Cell Culture and Evaluation of Cell Viability

Chang liver cell line was periodically sub-cultured in DMEM containing 10% FBS and 1% penicillin/streptomycine. MTT assay was used to evaluate the chang cell (with and without APW and/or H_2O_2) viability as a measurement of cytotoxicity (Sanjeewa et al. 2018).

2.9 Intracellular ROS Scavenging Assay

The cells were cultured in a black color 96 well plate at 1×10^4 cells/wells density. After 24 h, the cells were incubated with the APW (125–500 μg/ml) with or without the HO-1 inhibitors. After 1 h, the cells were stimulated with 1 mM H_2O_2 and incubated additional 1 h at 37 °C. The fluorescence emission of 2′7′-dichlorofluorescin

was measured at emission wave length 528 nm and excitation wave length at 485 nm. The percentage of fluorescence intensity of APW treated wells was calculated after compared with the intensity of non-treated wells (consider as 100% intensity).

2.10 Superoxide Dismutase (SOD) and Catalase (CAT) Activity

SOD and CAT activity was measured using a commercial colorimetric assay kit (Abcam, Cambridge, MA, USA) following the vender's instruction.

2.11 Western Blot Analysis of Antioxidant Protein Expression Levels

Cytosolic and nuclear proteins were prepared using RIPA buffer and nuclear extraction kit, respectively. The blotted proteins were incubated with anti-heme oxygenase-1 (HO-1, 1:2000), anti-βactin (1:2000), anti-Lamin B (1:2000), and anti-Nrf2 (1:2000). The bands were visualized using anti-rabbit horseradish peroxidase-conjugated IgG was used with enhanced chemiluminescence reagents (Thermo Scientific Inc.).

2.12 Statistical Analysis

All results were expressed as the mean standard deviation (SD) of three determinations (N = 3). The differences between means of each group were assessed using one-way analysis of variance followed by Duncan's test using SPSS 20.0 software (SPSS, Chicago, IL, USA).

3 Results

3.1 The Free Amino Acid Contents of APW

The free amino acid contents of APW were shown in the Table 1. According to the results, APW had the highest amount of Taurine (410.533 mg/100 g or 21.69%) and followed by β-alanine (19.10%), Leucine (15.41%), Glycine (11.41%), and Arginine (9.68%). This result indicates that APW contains the plentiful amount of Taurine.

Table 1 The free amino acid contents (%) of APW

Amino acid	APW	
	mg/100 g	% amino acid
Phosphoserine	N. D	N. D
Taurine	410.533	21.69
Phosphoethanolamine	N. D	N. D
Urea	152.508	8.05
Aspartic acid	2.358	0.12
Hydroxyproline	N. D	N. D
Threonine	12.3	0.64
Serine	6.775	0.35
Asparagine	1.521	0.08
Glutamic acid	2.519	0.13
Sarcocine	N. D	N. D
α-Aminoadipic acid	N. D	N. D
Proline	31.126	1.64
Glycine	215.975	11.41
Alanine	291.846	15.41
Citrulline	N. D	N. D
α-Aminobutyric acid	N. D	N. D
Valine	54.685	2.88
Cystine	N. D	N. D
Methionine	28.255	1.49
Isoleucine	33.181	1.75
Leucine	40.825	2.15
Tyrosine	16.115	0.85
Phenylalanine	28.186	1.48
β-Alanine	361.551	19.10
β-Aminoisobutyric acid	7.158	0.37
γ-Amino-n-butyric acid	2.768	0.14
Histidine	1.694	0.08
3-Methylhistidine	2.225	0.11
1-Methylhistidine	N. D	N. D
Carnosine	1.257	0.06
Anserine	N. D	N. D
Tryptopan	N. D	N. D
Hydroxylysine	N. D	N. D
Ornitine	N. D	N. D
Lysine	4.027	0.21
Ethanolamine	N. D	N. D
Arginine	183.277	9.68
Total	1892.67	100.00

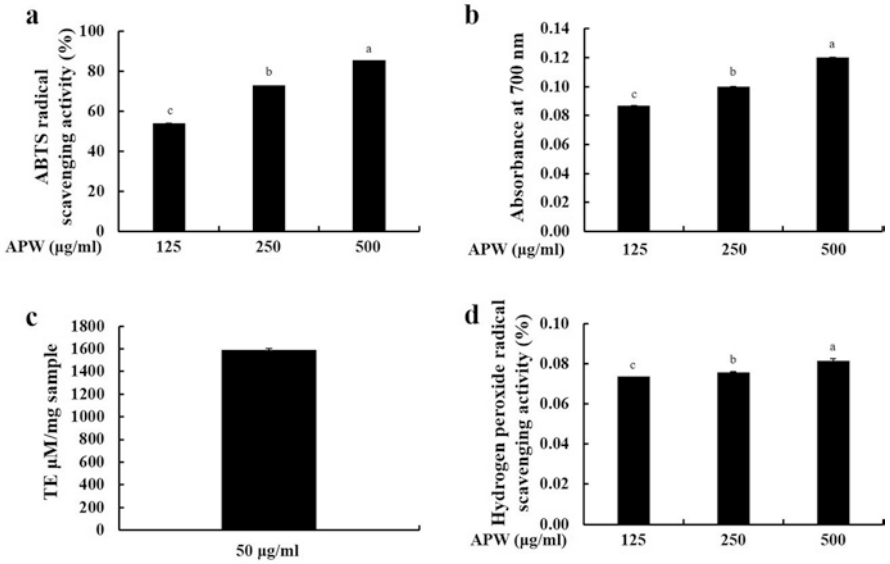

Fig. 1 Antioxidant effects (ABTS radical (**a**), reducing power (**b**), ORAC value (**c**) and hydrogen peroxide scavenging activity (**d**) of APW. [a–c]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE. (n = 3)

3.2 Radical Scavenging Effects of APW

ABTS radical scavenging activity of APW were increased in a dose-dependently at the concentrations between 125 and 500 μg/ml, (Fig. 1a). At the concentration of 500 μg/ml, APW scavenged more than 85% of ABTS radical. In the reducing power, evaluate the ability of testing compounds to convert Fe^{3+} in to Fe^{2+}. The formation of Fe^{2+} evaluate calorimetrically using the strength of Perl's Prussian blue in reaction mixture at 700 nm. According to the results APW had a strong reducing power at the tested concentrations (Fig. 1b). Figure 1c shows the ORAC of APW at 500 μg/ml. The ORAC of APW was 1584.84 TE μM/mg. In addition, APW dose-dependently inhibited the hydrogen peroxide radicals at the concentrations between 125 and 250 μg/ml.

3.3 **In vitro** *Antioxidant Activities of APW*

As shown in Fig. 2a, treatment of APW did not show any considerable cytotoxic effect in Chang liver cells. In addtion, APW improved the cell viability reduced by the exposure of H_2O_2 (Fig. 2b). As a next study, we attempted to evaluate the ROS scavenging activities of APW against H_2O_2-induced ROS production from Chang liver cells using DCF-DA assay (Fig. 2c). The result exhibited that the pre-treatment of APW dose-dependently reduced the ROS production from H_2O_2-induced Chang liver cells.

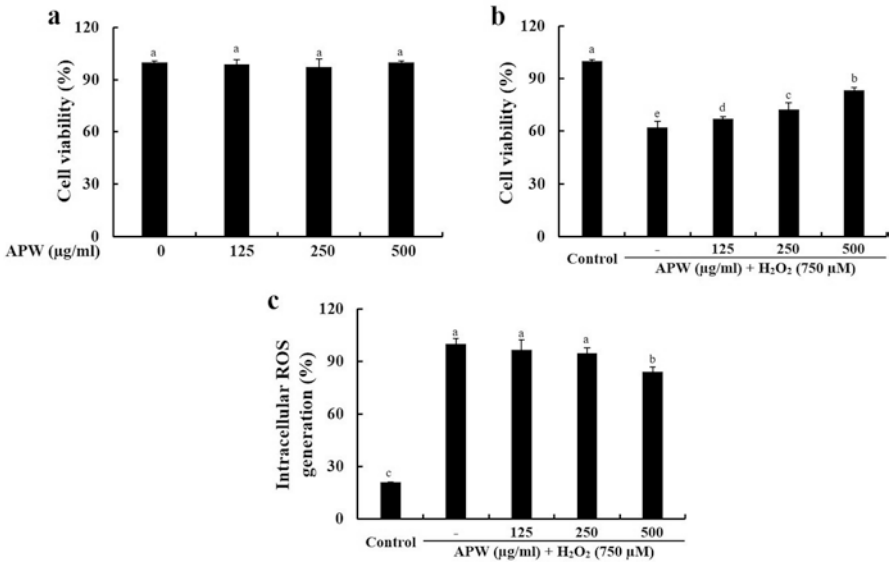

Fig. 2 Effects of APW on cell viability with/without H_2O_2 (**a** and **b**) and intracellular ROS production (**c**) induced by H_2O_2 in Chang liver cells. The cell viability was assayed by MTT assay and intracellular ROS generation was assessed using the oxidation sensitive dye, DCFH-DA. [a–e]The bars with different letters represent significant differences ($p < 0.05$). Values are expressed as mean ± SE (n = 3)

3.4 Antioxidant Enzyme Activities of APW against H_2O_2-Induced Chang Liver Cells

The levels of two defence antioxidant enzymes (SOD and CAT) in Chang liver cells were determined. The Fig. 3 shows the dose-dependent upregulation of CAT (Fig. 3a) and SOD (Fig. 3b) in H_2O_2-induced Chang liver cells. Both anti-oxidant enzymes were dose-dependently increased than that of the non-treated group under the tested conditions.

3.5 Western Blot Analysis of HO-1 and Nrf2 Protein Levels in H_2O_2-Induced Chang Liver Cells

Western blot analysis was used to evaluate the antioxidant action of APW against H_2O_2-induced Chang liver cells, and we confirmed the anti-oxidant signaling mechanisms through the protein expressions including HO-1 and Nrf2 (Fig. 4a and 4b). As shown in Fig. 4, exposure of H_2O_2 resulted in down-regulation of both HO-1 and Nrf2 in cytosol and nucleus, respectively. However, pre-treatment with APW increased the expression levels of antioxidant proteins in H_2O_2-exposed Chang liver cells.

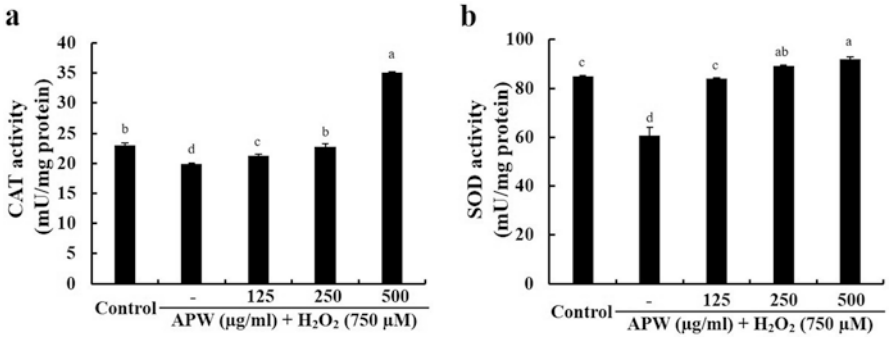

Fig. 3 Effect of APW on antioxidant enzyme activities in H_2O_2-treated Chang liver cells (**a**) CAT activity, (**b**) SOD activity. Values mean ± S.E of three determinations. Bars with different letters are significantly different ($p < 0.05$)

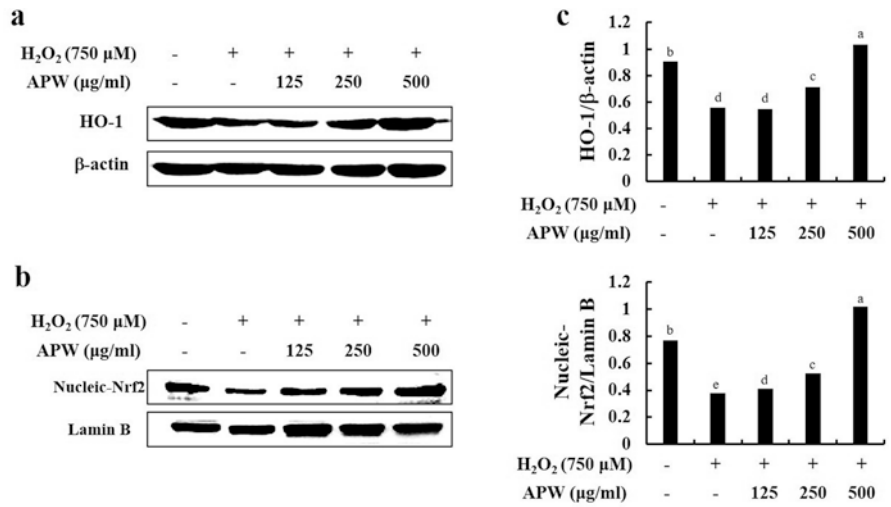

Fig. 4 Effect of APW on the expression of HO-1 (**a**) and Nucleic-Nrf2 (**b**) in cultured Chang liver cells. Cells were treated concentrations of APW for the check Nucleic-Nrf2 and HO-1 protein expressions (c) the ratio of HO-1/β-actin and Nrf-2/Lamin B. Quantitative representations of western blot analysis. Values mean ± SE of three determinations. [a–e]Bars with different letters are significantly different ($p < 0.05$)

3.6 Role of APW as HO-1 Stimulator

To determine the effect of HO-1 protein expression on the H_2O_2-exposed Chang liver cells, the cell viability and intracellular ROS production were measured by MTT assay and DCF-DA assay in ZnPP-treated cells, repectively. The pre-incubation of ZnPP (5 μM) significantly reduced the protective effect of APW via the decrease of cell viability and intracellular ROS production against H_2O_2-induced

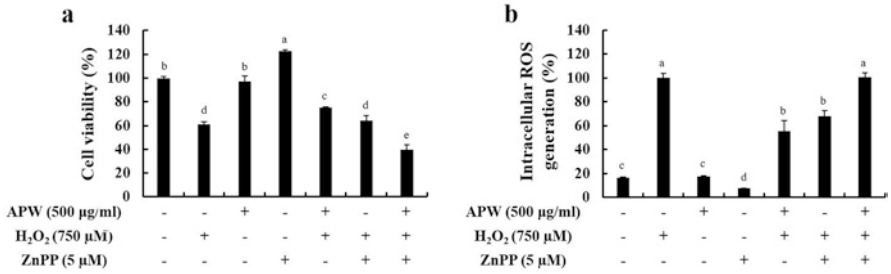

Fig. 5 Effect of APW with ZnPP (HO-1 inhibitor) on cell viability on H_2O_2 (**a**) and intracellular ROS production (**b**) induced by H_2O_2 in Chang liver cells. The cell viability was assayed by MTT assay and intracellular ROS generation was assessed using the oxidation sensitive dye, DCFH-DA. [a-e]Bars with different letters are significantly different ($p < 0.05$)

cell death (Fig. 5). These results suggest that the up-regulation of HO-1 by APW protects the cells against H_2O_2-induced cell death and ROS production.

4 Discussion

Taurine is a sulfur-containing β-amino acid (2-aminoethanesulfonic acid), which presents at large quantities in many cells of most animal species (Sturman 1993). When consider about humans, taurine is formed from methionine/cysteine metabolism via hypotaurine in hepatocytes. Moreover, cells like neutrophils contain large amounts of taurine due to taurine uptake from the blood, a source of endogenously from the urine in the kidney and through the absorption of food in the intestine (Klebanoff 2005; Lambert et al. 2015). According to the previous studies, taurine keeps homeostasis and has cyto-protection of cells in inflammation related to stress and oxidative stress (Marcinkiewicz and Kontny 2014). Usually, meat products and seafood are considering as main supplement of taurine, for human body (Um et al. 2017). *A. pectinate* has a large adductor muscle, and they have long been considering as a seafood species with high commercial value (Korea, China, and Japan) (Awaji et al. 2018). Even though, many seafood studies focused on taurine and its antioxidant properties, effect of *A. pectinate* as a taurine rich seafood or as source of antioxidant has not been validated yet. Taken together, in the present study, we attempted to evaluate the antioxidant properties of taurine rich aqueous extract from *A. pectinate* on H_2O_2-induced Chang liver cells. Previously, a number of studies confirmed that species belong to phylum mollusca are rich source of taurine and has a potential to developed in to functional ingredient due to their interesting bioactive properties (El-Sayed 2014; Lee et al. 2017). In the present study we have confirmed, aqueous extract separated from APW has large amount of taurine as well as strong antioxidant properties against H_2O_2-induced oxidative stress in Chang cells.

Moreover, APW demonstrated strong radical scavenging activities against hydrogen peroxide, ABTS radical, ORAC, and reducing power.

H_2O_2 is considered as a stable ROS species and freely diffusible within and between cells. Furthermore, when the cells exposed to H_2O_2, which induce the malfunctions of cells and even cell death via oxidizing DNA, lipids, and proteins. Moreover, the inability of the H_2O_2-exposed cells to deal with oxidative stress has been implicated pathogenesis of disease conditions (Jian et al. 2011). However, according to the results pre-incubation of APW together with H_2O_2 comparatively increased the viability and reduced the elevated ROS levels, compared to the only H_2O_2-exposed cells.

Nrf2, a cap'n'collar (CNC) transcription factor which contains basic leucine zipper and CNC domains. According to the previous studies, Nrf2 has a regulatory role in the expression of cytoprotective genes such as HO-1 and NQO-1 (Hayes and McMahon 2001). According the previous studies, Nrf2 does not have any antioxidant functions. When cells exposed to the oxidative stress, first inactivated cytoplasmic Nrf2 translocates into the nucleus and then induces the expression of its target antioxidant genes such as HO-1 (Nguyen et al. 2003). Thus, the activation and translocation of Nrf2 in cells provides a strong support to enhance antioxidative capacity of cells (Suzuki and Yamamoto 2015). In the present study, we compared the activation levels Nrf2 and its down-stream antioxidant protein HO-1 using western blot analysis. According to the results, pre-treatment of APW increased the expression levels of both tested proteins. This might be a one reason for increased CAT and SOD levels after treatment of APW. ZnPP is a specific HO-1 inhibitor, which can use to evaluate the effect of HO-1 in antioxidant studies (Lee et al. 2008). Recently, a number of studies reported ZnPP has a suppressive effect on Nrf2/HO-1 mediated antioxidant pathway (Jian et al. 2011). Our results also demonstrated that the addition of ZnPP reduced the protective effect of APW against H_2O_2-exposed hepatocytes. With these results, this study suggests the cytoprotective effect of APW in H_2O_2-exposed hepatocytes via Nrf2/HO-1 antioxidant pathway.

5 Conclusions

In the present study we reported that APW is a rich source of taurine and it has a potential to developed as an active ingredient in functional foods. Specifically, APW protects H_2O_2-exposed hepatocytes by triggering anti-oxidant mechanisms in the cells. The up-regulated expression of Nrf2/HO-1 signal cascade is the possible reason for cytoprotective effect observed in this study. The contents in this research suggests that might be useful to develop value added functional ingredient from *A. pectinate* might be a functional indegredient in the future.

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Effects of Taurine on Broiler Aortic Endothelial Cells Activity and Antioxidant Ability Impired by Heat Stress In Vitro

Da-lin Fang, Shumei Lin, Zhenyong Wang, Wei-fan Gao, Yiqing Cui, Rui-yuan Sun, Wen-hua Sun, Fan Huang, and Qiufeng Lv

Abstract In order to provide a theoretical basis for the amelioration of heat stress-related diseases in broilers by taurine supplementation, the effect of taurine on the viability and antioxidant ability of aortic endothelial cells in broilers under heat stress was investigated in the present study. In this experiment, 10d healthy broilers were sacrificed, then aortic tissue was used for aortic endothelial cells isolation and cultivation. Tissue patching was used to cultivate primary broiler aortic endothelial cells. The 3rd to 5th generations of cells were used and randomly divided into five groups, including the control group (C), the heat-stressed group (HS), the Tau(HS + LTau) group, the Tau(HS + MTau) group and the Tau(HS + HTau) group. Cells were cultivated for 24 h in a cell incubator (37 °C, 5%CO2). Then heat-stressed cells were placed in a 43 °C thermostatic water bath for 6 h, followed by incubation in the cell incubator under 37°Cfor 1 h. The results were as follows (1) Based on MTT colorimetry and AO/EB staining, the activity of aortic endothelial cells was decreased, but the rate of apoptosis was increased in the HS group. Compared with the HS group, the taurine groups showed significantly higher level in relative survival rates ($P < 0.05$), and significantly lower apoptosis rates ($P < 0.05$); (2) compared to control group, LDH activity and MDA content of endothelial cells in the HS group were significantly increased ($P < 0.01$), while the levels of T-SOD, GSH-Px and T-AOC were significantly decreased ($P < 0.01$). The LDH activity and MDA content of endothelial cells were significantly lower in Tau group than those

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of HS group ($P < 0.05$), while the T-SOD activity, GSH-Px activity and T-AOC of endothelial cells were significantly increased ($P < 0.05$) in the taurine group. The results show that HS decreases antioxidant capacity, which causes severe oxidative damage to the endothelial cells; while taurine administration prevents the decline in LDH activity and MDA content, and increases the activity of several antioxidant enzymes, including SOD, GSH-Px and T-AOC, which implies that taurine can improve the broiler aortic endothelial cells activity and antioxidant ability under heat stress.

Keywords Taurine · Cellular activity · Antioxidant ability · Heat stress · Broiler aortic endothelial cells

1 Introduction

Taurine, known as beta-aminoethanesulfonic acid, was originally isolated from bull bile acids. Taurine is colorless, tasteless, stable in nature and soluble in water (Xiaoqiong and Deyi 2011). Taurine is widely distributed in animal tissues and organs as a free amino acid, where it exerts many physiological functions.

Vascular endothelial cells (VEC) are single-layered flat cells that cover the blood vessels' surface and are arranged longitudinally. They provide a smooth inner surface for blood vessels, but are recognized for their ability to regulate function of adjacent cells. Due to their wide distribution, they are considered a tissue with extensive biological activity in the body. As one of the most obvious tissue cells in early traumatic injury, they contribute to inflammatory reactions and apoptosis (Zhengtao 2014).

Plumier et al. (1996) showed that heat stress destroys vascular integrity and permeability, and injures endothelial cells.

Death from heat stroke is mainly caused by massive hemorrhage, injury and thrombosis of the endothelial cells. These physiological changes are related to intense heat stress-induced cytotoxicity, inflammation and coagulation, leading to severe damage to endothelium cells and affecting normal function of multiple organ tissues.

Li et al. (2016) showed that heat stress can induce endothelial cells apoptosis of human umbilical veins, and that cells treated with intense heat can induce the production of ROS, indicating that apoptosis is an important pathological feature of heat stress.

Taurine has been the focus of many studies, largely because it exerts many physiological actions and pharmacological effects. However, few reports have focused on the cytoprotective mechanism of Tau against heat stress-induced endothelial cell damage. The present study examines the beneficial effects of different amounts of Tau against heat stress-induced injury of broilers' endothelial cells at 43 °C, focusing on taurine-mediated changes in apoptosis and the protective mechanism of Tau on aortic endothelial cell injury. The study should provide insight into treatment of

heat stress-induced diseases in broilers, with an emphasis on traditional measures for improvement of broiler performance and disease resistance.

2 Methods

2.1 Instrument

The CO_2 incubator was purchased from Hong Kong Heraeus Co., Ltd.; the fully automated cell counter was obtained from Shenzhen Daboju Technology Co., Ltd.

2.2 Main Reagents

DMEM/F12 medium and PBS solution were purchased from Hyclone, USA; rabbit anti-human factor VIII related antigen antibody, SABC immunohistochemistry kit, and DAB chromogenic kit were from Beijing Dred reagent company.

2.3 Culture of Broiler Aortic Endothelial Cells

2.3.1 Primary Culture of Broiler Aortic Endothelial Cells

Ten day-old healthy broilers were selected and killed by cutting the carotid artery. The chest and abdomen were disinfected with 75% alcohol and carefully cut with autoclaved ophthalmic scissors. After aseptic removal of the aorta, it was put into a small beaker containing 1% double antibody PBS. After that, the cell flask was placed in an incubator (at 37 ° C., with 5% CO 2) for 1–2 h to facilitate adherence to tissue blocks. DMEM/F12 medium containing 20% fetal bovine serum was added to 1–2 ml of freshly cultured cells. The tissue blocks were placed in the incubator. After 2 days, the endothelial cells had formed monolayers.

2.3.2 Subculture of Broiler Aortic Endothelial Cells

After primary endothelial cells had grown into monolayers, the original culture fluid was discarded. The cells were washed twice with PBS solution, and digested with 0.05% trypsin (containing 0.02% EDTA) at room temperature. After adding trypsin, cells were examined with an inverted microscope for evidence of changes in cell morphology. Culture medium containing 10% calf serum was added and the cells were incubated until a single layer was formed. Subsequent experiments were performed on cells in the logarithmic growth phase of 3–5 generations.

2.4 Identification of Porcine Aortic Endothelial Cells

Digested cells were transferred to a 6-well plate at a density of 2 × 10 5/ml. They were allowed to fuse into a single layer. They were rinsed 3 times with PBS before being treated with 4% paraformaldehyde at room temperature for 20 min. They were again rinsed with PBS 3 times and then treated with 0.05% Triton X-100 at room temperature for 20 min. Following further rinsing with PBS the cells were treat with %H2O2 at room temperature for 15 min, after which they were again rinsed with PBS. They were then exposed to 5% BSA at 37 °C for 20 min. to absorb the blocking solution. Primary antibody (1:50) was dilated with PBS; PBS was used in the negative control instead of the primary antibody. After a further 1–2 h incubation at 37 °C in the wet box, the cells were rinsed with PBS three times and then incubated with the second antibody (1:100) in the wet box at 37 °C for 30 min and then rinsed with PBS three times. The samples were incubated in SABC (1:100) at 37 °C wet box for 20 min.Following further rinsing with PBS, the color was allowed to develop for 5–8 min and then rinsed with distilled water. The cells were subjected to hematoxylin counterstain 15–30s and after rinsing with distilled water, the cells were placed on glass slides, and observed under a microscope.

2.5 Study Design and Sample Collection

Endothelial cells in the logarithmic growth phase of 3–5 generations were divided into control (C), heat stress (HS), and heat stress groups treated with low, medium and high levels of Tau (LTau, MTau). HTau). Among them, normal control cells were always cultured in a cell incubator (at 37 °C, with 5% CO2), and the cell culture medium concentration of Tau in each heat stress group was 0 mmol/L Tau (HS group), 20 mmol/L Tau (LTau group), 40 mmol/L (MTau group) and 80 mmol/L (HTau group) respectively.

Each group of cells was placed in a cell culture incubator for 24 h. Cells belonging to the heat stress groups were placed in a constant temperature water bath at 43 °C. After 6 h of heat stress, they were incubated in the incubator for 1 h, and the cells and culture fluids were collected for follow-up indicators (Jinshun et al. 2014). Refer to heat stress treatment methods (Wu et al. 2016).

2.6 Detection of Apoptotic Rate and Viability of Aortic Endothelial Cells in Heat-Stressed Broilers

The rate of apoptosis was measured according to the AO/EB staining method; cell viability was detected by MTT colorimetry.

$$\text{Formula : Rate of apoptosis}(\%) = \left(\text{Apoptotic cell number / Normal number of living cells + Apoptotic cell number + Necrocytosis}\right) * 100$$

2.7 Detection of Oxidative Damage Related to Aortic Endothelial Cells in Heat-Stressed Broilers

After heat stress treatment followed by rewarming for 1 h, the medium was collected for analysis of lactate dehydrogenase (LDH) activity. Endothelial cells werelysed and the samples were analyzed for malondialdehyde (MDA) content, total superoxide dismutase (T-SOD) activity, glutathione peroxidase in endothelial cells after heat stress (GSH-Px) activity and total antioxidant capacity (T-AOC) effects.

2.8 Statistical Analysis

SPSS 17.0 analysis software was used for statistical analysis. Differences between groups were analyzed by one-way ANOVA, and the LSD method was used for multiple comparisons. Data were expressed as means±standard deviation (x ± SD), and $P < 0.05$ was statistically significant.

3 Results

3.1 Primary Culture, Passage Culture and Identification of Broiler Aortic Endothelial Cells

After 24–48 h of culture of the primary broiler aortic aneurysm, cells began to migrate around the aortic mass and then began to adhere. After 48 h, they gradually proliferated, growing from the center of the tissue block to the periphery. Most cells assumed the shape of short spindles or polygons. They were flat, uniform in size. After 3–5 days, the tissue blocks were picked out and the cells fused into monolayers, which appeared as typical paving stone, as shown in Fig. 1a.

The cells were adherent for about 6–8 h after passage, and subcultured for 3–5 days to overgrown the bottom wall of the cell bottle by about 80–90%, as shown in Figs. 1b and c.

After passage to the seventh generation, the cells began deteriorate. Immunohistochemical staining of factor VIII-related antigen was positive, brown-yellow particles appeared in the cytoplasm, and the cultured cells were confirmed to be of aortic endothelial origin. According to statistics, the yield of endothelial cells was greater than 90%, as shown in Fig. 1d.

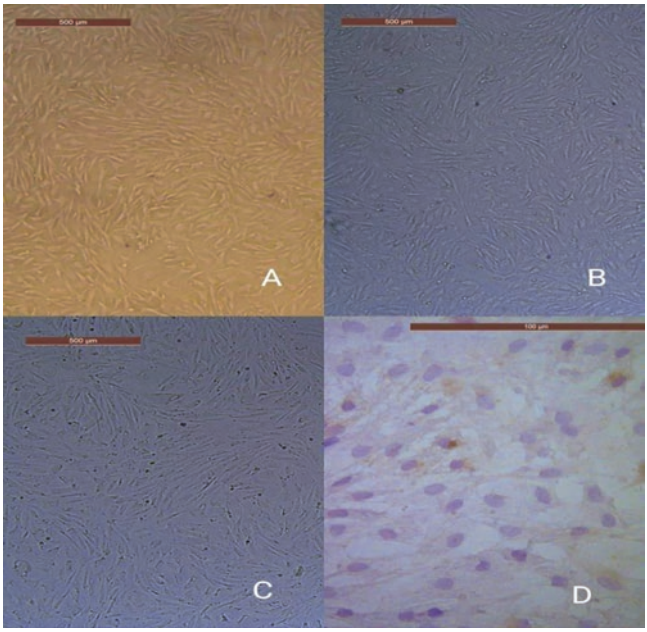

Fig. 1 The results of primary culture, subculture culture and identification of broiler aortic endothelial cells. (a) Primary cell cultures 48h(× 50); (b) Passaging cells cultures 3d(× 50); (c) Passaging cells cultures 5d(× 50); (d) Identification of endothelial cells(× 400)

3.2 Effects of Taurine on Apoptotic Rate of Aortic Endothelial Cells from Heat-Stressed Broilers

3.2.1 Effects of Tau by AO/EB on Apoptosis Rate of Aortic Endothelial Cells in HS Broilers

After AO/EB staining, normal broiler aortic endothelial cells were observed to be green by AO under an inverted fluorescence microscope, showing evenly distributed dye. Orange cells in green were early apoptotic; while agglomerate apoptotic bodies of orange-red cells of even color were terminally apoptotic.

As shown in Table 1 and Fig. 2, after heat stress treatment at 43 °C for 6 h, a large number of apoptotic cells were observed in HS group (0 mmol/L Tau), indicative of an apoptotic rate of 63.33%. The endothelial cell death rate due to heat stress was reduced in the three taurine treated groups, showing death rates of 28.67%, 29% and 54% at concentrations of 20 mmol/L, 40 mmol/L and 80 mmol/L, respectively ($P < 0.05$). Analysis of AO/EB measurements, 20 mmol/L and 40 mmol/L Tau exhibited the most protection against heat stress damage of endothelial cells.

Table 1 Effects of different concentrations of Taurine on rate of endothelial cells apoptosis after heat-stress

heat stress time(h)	Tau concentration(mmol/L)	Rate of apoptosis(%)
6	0	63.33 ± 3.33^{Bc}
6	20	28.67 ± 3.18^{Aa}
6	40	29.00 ± 3.61^{Aa}
6	80	54.00 ± 2.65^{Bb}

Note: The values in the same column contain different small letters are significantly different ($P < 0.05$); Capital letters are extremely significantly different ($P < 0.01$) The same below

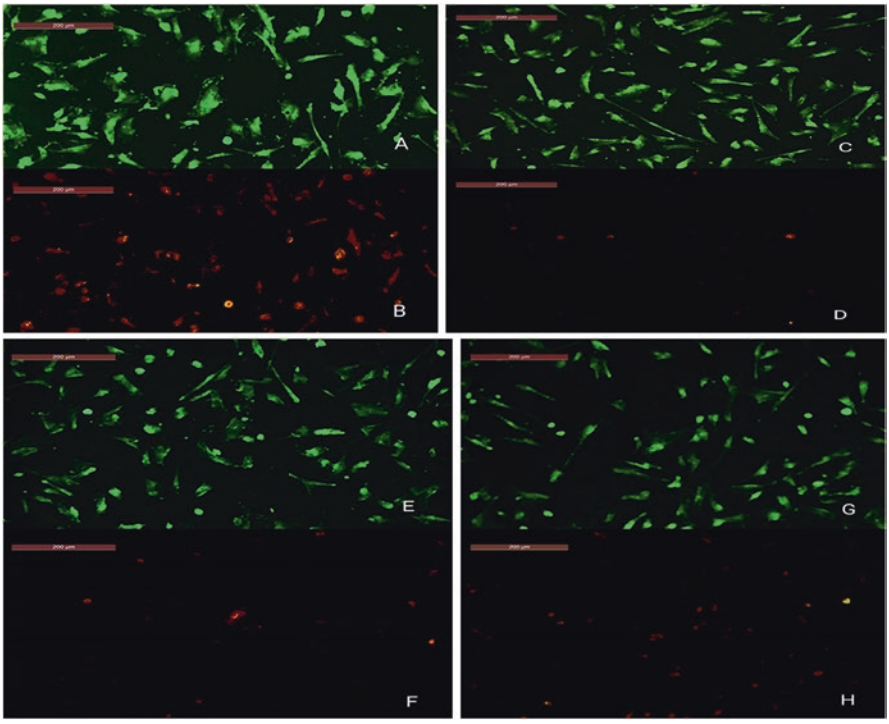

Fig. 2 Effects of different concentrations of Taurine on rate of endothelial cells apoptosis after heat-stress. **a** and **b**: Deying results of AO/EB of 0 mmol/L Tau(×200); **c** and **d**: Deying results of AO/EB of 20 mmol/L Tau(×200); **e** and **f**: Deying results of AO/EB of 40 mmol/L Tau(×200); **g** and **h**: Deying results of AO/EB of 80 mmol/L Tau(×200)

3.2.2 Effects of Tau on Viability of Aortic Endothelial Cells in Heat-Stressed Broiler Chickens Using the MTT Assay

As shown in Fig. 3, the relative survival rate of cells in the HS group (0 mmol/L Tau) was lower after heat stress at 43 ° C for 6 h. Compared to the HS group, the taurine treated groups (20 mmol/L, 40 mmol/L, and 80 mmol/L) exhibited higher survival rates, which increased by 34.6%, 35.3% and 17.9%, respectively.

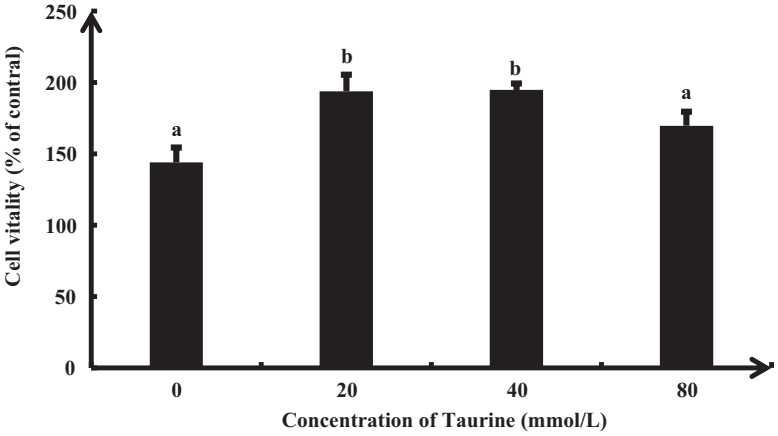

Fig. 3 Effects of different concentrations of Taurine on survival of endothelial cells after heat-stress
Note: Different small letters indicate significant difference ($P < 0.05$); Capital letters are extremely significantly different ($P < 0.01$) The same below

(($P < 0.05$)) Results of the MTT assay showed a similar pattern, with 20 mmol/L and 40 mmol/L Tau exhibiting more protection against HS than the highest taurine concentration.

3.3 Detection Results of Taurine on Oxidative Damage of Aortic Endothelial Cells in HS Broilers

3.3.1 Detection of Lactate Dehydrogenase (LDH) in Medium of HS Endothelial Cells by Tau

As shown Fig. 4, LDH activity in the medium of the HS group was significantly increased ($P < 0.01$) after HS, compared with that of the C group, indicating that heat stress caused endothelial cell membrane damage and the release of LDH from the cell. The amount of LDH activity in the cell medium was reduced when taurine was added to the incubation medium prior to the HS procedure, with values declining by 47.93%, 57.61% and 56.02% for L, M and H, respectively. ($P > 0.05$). There was no significant difference between the three groups of Tau.

The above results indicate that HS can cause endothelial cell damage, and that addition of Tau significantly reduced the cytotoxic effects induced by heat stress.

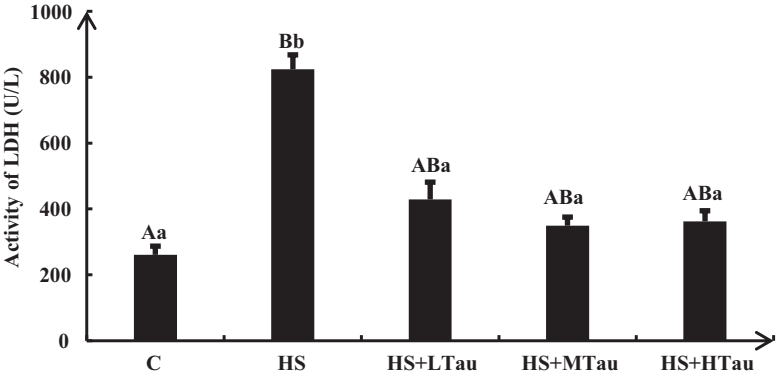

Fig. 4 Change in LDH activity in EC's culture medium

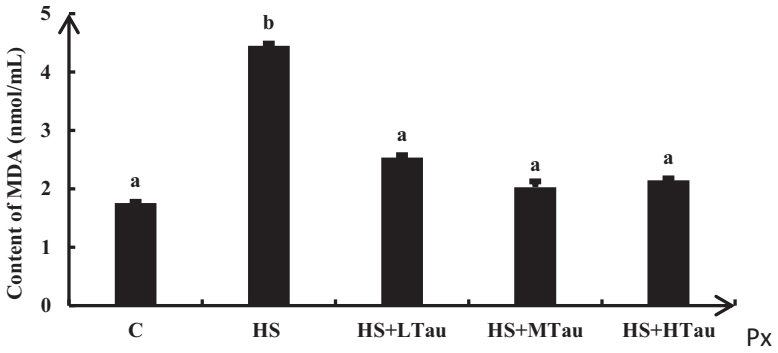

Fig. 5 Change of content of MDA in EC's culture supernatant

3.3.2 Effects of Taurine on Release of LDH from HS-Treated Endothelial Cells

As seen Fig. 5, HS treatment significantly increased by 152.84% the MDA content of the endothelial cells, indicating that HS caused lipid peroxidation in endothelial cells. The MDA content of endothelial cells of the Tau groups (L,M and H) was increased were significantly decreased ($P < 0.05$). There were no significant differences among the Tau groups ($P > 0.05$). The MDA results showed that Tau diminishes oxidative damage of aortic endothelial cells caused by HS.

3.3.3 Effects of Tau on Endothelial Cell Glutathione Peroxidase (GSH-Px) After HS

As seen Fig. 6 HS caused a 61.1% decrease in GSH-Px activity of endothelial cells. Taurine treatment attenuated the fall in GSH Px activity, Compared with the HS group, Tau increased the activity of GSH-Px by 44.74%, 118.66% and 123.32% for the L, M and H concentrations, respectively. The difference between the LTau group and HS group the was not significant ($P < 0.05$); The activity of GSH-Px in endothelial cells of MTau and HTau was significantly higher than that in the HS group ($P < 0.05$), indicating that addition of Tau enhances the antioxidant capacity of aortic endothelial cells.

3.3.4 Detection of Tau in Total Endothelial Superoxide Dismutase (T-SOD) in Culture Medium of HS-Treated Endothelial Cells

Figure 7 show that the T-SOD activity of endothelial cells was higher in the C than in the HS group ($P < 0.01$), indicating that HS caused endothelial cells to lose antioxidant capacity. The Tau groups restored T-SOD activity to the normal range Taurine blocks HS-mediated oxidative damage of aortic endothelial cells.

3.3.5 Effects of Tau on Total Endothelial Cell Antioxidant Capacity (T-AOC) After HS

Figure 8 show the difference in total antioxidant capacity of endothelial cells exposed to HS. HS decreased T-AOC of endothelial cells by 50.14%; The Tau groups difference was not significant ($P > 0.05$).

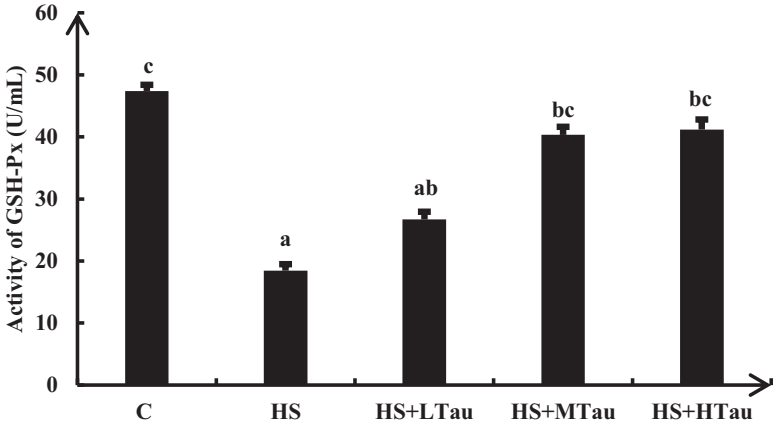

Fig. 6 Change of GSH-Px activity in EC's culture supernatant

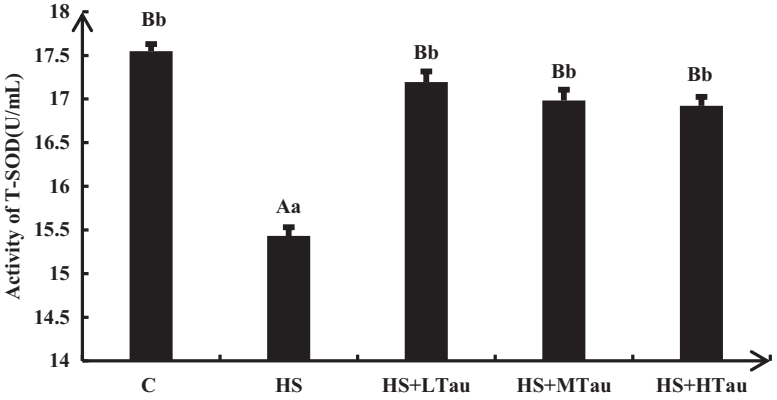

Fig. 7 Change of T-SOD activity in EC's culture supernatant

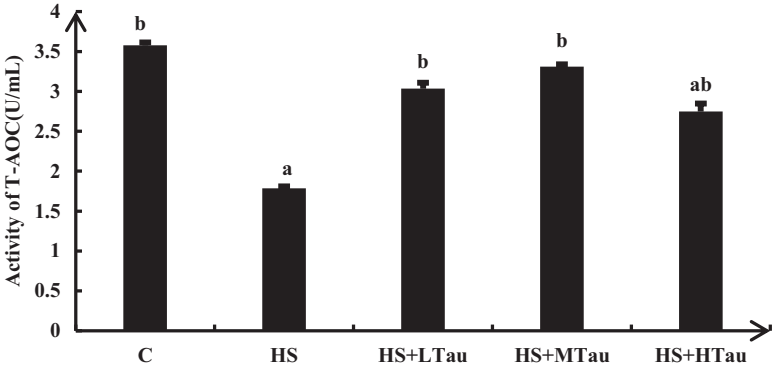

Fig. 8 Change of T-AOC in EC's culture supernatant

Compared with the HS group, T-AOC significantly increased ($P < 0.05$) activity by 69.27% and 84.92% in the LTau and MTau groups, respectively. This indicates that Tau mediates an increase in total antioxidant capacity in HS-treated aortic endothelial cells. There was no significant difference between the Tau groups ($P > 0.05$).

4 Discussion

4.1 Effects of Taurine on the Activity and Apoptosis Rate of Aortic Endothelial Cells in Heat Stressed Broilers

The physiological functions of endothelial cells include regulation of contraction and relaxation of blood vessels, maintenance of blood flow, selectively regulation of transport of physiological molecules and nutrients, construction of new blood

vessels and maintenance of a balance between local inflammatory regulators (Li 2015). Due to extensive coverage, endothelial cells were the first cell-type to suffer HS-induced damage. HS was found to destroy vascular integrity, increase vascular permeability, and aggravate endothelial cell damage. Broilers are very sensitive to heat stimuli due to defects in their own physiological function, having extremely low resistance to heat. At the same time, HS-induced oxidative damage is a common pathology of many diseases, such as diabetes, hypertension, and atherosclerosis (Haitao et al. 2013).

In this study, endothelial cell activity and the rate of apoptosis were measured by the MTT assay and AO/EB apoptotic fluorescence staining. After HS, survival of aortic endothelial cells in broiler chickens was significantly decreased, as the rate of apoptosis was significantly increased. The addition of Tau protected HS-treated endothelial cells by attenuating the degree of apoptosis.

The results of the MTT colorimetric assay and of AO/EB apoptosis fluorescence staining show that certain concentrations of Tau significantly inhibit heat stress-induced decrease in cell viability and increase in apoptosis. The result was consistent with previous studies (Sukhotnik et al. 2016; Gharibani et al. 2015).

4.2 Effects of Taurine on Antioxidant Function of Aortic Endothelial Cells in HSbroilers

Oxidative stress bears the hallmark of large production of ROS in cells, including increases in MDA content and decrease in the activities of antioxidant enzymes, such as SOD and GSH-Px. HS not only increases ROS production, but lowers the ability of cells to scavenge ROS by damaging the antioxidant enzymes (Liu et al. 2010), leading to increases in lipid peroxidation, damage to DNA, and ultimately promotes the failure of cells to resist oxidation (Babizhayev 2016). Acute heat stress requires that stressed individuals adjust to sudden changes in ambient temperature. As they have a robust basal metabolism, rapid rate of growth, subcutaneous and abdominal fat, and are covered with feathers and contain no sweat glands, broilers are highly sensitive to environmental changes. In particular, they are not very resistant to HS, resulting in serious cytotoxic effects, oxidation of body cells and cell death upon exposure to heat stress. Heat can also aggravate cell damage (Lugo-Amador et al. 2004). Some studies have found that endothelial cells may be one of the earliest targets of HS tissue damage (Gu et al. 2014). Studies in related animal models and cell lines have found that HS can directly damage tissues, cause direct cytotoxicity to vascular endothelial cells, inhibit the proliferation of endothelial cells, and induce extensive apoptosis of endothelial cells (Roberts et al. 2008; Jacob et al. 1997; Pan et al. 2012). Studies have shown that the increase in ROS caused by HS will lead to lipid peroxidation of cell membrane, resulting in a significant increase in MDA content, damage to cell membranes, and subsequent increases in LDH release. At the same time, the intracellular antioxidant capacity declines

significantly. The activity of indicators, such as SOD, GSH-Px, and T-AOC, is significantly reduced (Jinshun et al. 2014; Wenli et al. 2011; Cong et al. 2017). These experimental results are consistent with ours. In this experiment, the LDH, T-AOC, SOD and GSH-Px activities and MDA content in the culture medium of broiler endothelial cells were measured. It was found that the LDH activity and MDA content in HS group are higher than those of the C group ($P < 0.01$), indicating that endothelial cells develop severe oxidative damage after HS exposure. At the same time, T-SOD activity, GSH-Px activity and T-AOC, measures of antioxidant activity, were significantly lower than those in the normal control group ($P < 0.01$). However, LDH activity and MDA content in the endothelial cell medium of all the Tau groups were significantly decreased. At the same time, addition of Taurine also significantly increased the activities of antioxidant enzymes, SOD, GSH-Px and T-AOC, inhibited cellular oxidative stress and enhanced antioxidant capacity and protected against oxidative damage of endothelial cells. Cong et al. (2017) found that HS caused oxidative damage of bovine testicular cells, leading to a significant increase in MDA content and a significant decrease in SOD, GSH-Px and CAT activities. After treatment with puerarin, LDH activity and MDA content decreased, SOD, GSH-Px and CAT activities increased, and HS-induced oxidative damage was alleviated, showing that puerarin contained antioxidant activity. Tau appears to have the same antioxidant capacity as puerarin, playing a cytoprotective role by removing excess free radicals. HS-induced oxidative damage is consistent with the results of this study.

Liu Nannan et al. (2011) studied the treatment of manganese-treated rat hippocampal neurons with Tau. It was found that Tau can reduce oxidative damage caused by manganese and promote cell cytoprotection by inhibiting the massive production of ROS.

A large number of studies have also found that Tau inhibits the production of lipid peroxidation products, such as MDA and enhance the body's antioxidant enzymes, SOD and GSH-Px activity, thereby inhibiting the production of ROS (superoxide anion, hydroxyl radical, hydrogen peroxide, etc.) in the body. (Kalaz et al. 2014; Chowdhury et al. 2016).

Studies on rats with oxidative damage and toxicity caused by aluminum stress were found to benefit from taurine administration, which significantly reduced MDA content and improved SOD, glutathione peroxidase (GSH-Px), Na + K + -ATPase and Ca_2 + -ATPase activities.

This indicates that taurine plays a protective role as an antioxidant against damage to the ATPases of the brain and blood of rats exposed to aluminum (Qiao et al. 2015). Tau also provides protection against doxorubicin (DOX)-induced acute liver injury, decreasing DOX-mediated increases in serum LDH, ALT, AST activity, and decreasing SOD activity and GSH content of the liver. However, after Tau administration, all these changes were suppressed (Nagai et al. 2016). These studies are consistent with the antioxidant effects of Tau in this study.

5 Conclusion

Taurine significantly reduces LDH activity and MDA content of aortic endothelial cells, significantly increasing the activity of antioxidant enzymes, T-SOD, GSH-Px and T-AOC, in aortic endothelial cells, and inhibiting oxidative damage of aortic endothelial cells induced by heat stress.

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Hepatoprotective Activity of a Taurine-Rich Water Soluble Extract from *Octopus vulgaris* Meat

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Abstract In this study, we investigated the hepatoprotective activity of the water extract derived from *Octopus vulgaris* meat (OM). First of all, a water extract prepared from OM (OMW) showed the high extraction yield (48.22%) and the highest taurine content (39.84%) in free amino acids. OMW exhibited the high value of

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reducing power, ABTS and hydrogen peroxide radical scavenging activities in dose-dependent manner. The taurine-rich OMW also led to the reduced intracellular reactive oxygen species (ROS) generation with the increased cell survival in H_2O_2-treated Chang liver cells. In addition, OMW decreased the apoptotic phenomenon, including the formations of apoptotic bodies and sub-G_1 DNA contents by regulating the protein expressions of apoptosis-related molecules such as Bcl-2 and Bax. From these results, this study indicated the taurine-rich OMW protected hepatocytes against oxidative stress. These findings suggest that OWM may be a novel potential antioxidant resource.

Keywords Taurine · *Octopus vulgaris* · Hepatoprotective activity · Apoptosis

1 Introduction

Reactive oxygen species (ROS) and free radicals, generated by abnormal metabolism or immune function, means as unstable oxygen compounds or reactive oxygen compounds with free radicals that do not form a pair of electrons (Porter et al. 1995). Under the pathological condition, the unbalance between the formation and removal of ROS is affected to biomacromolecules. Typically, the formation of free radical containing superoxide anion (O_2^-), hydroxyl radical anion (OH^-), dioxygen (O_2) and hydrogen peroxide (H_2O_2) is very unstable and reacts with other substances in the human body. Generally, this free radical induced the oxidative stress and it led to damage of cell membranes, lipids, and proteins to cause swelling denature of cells as well as apoptosis (Kang 2013).

Apoptosis is the step that leads to death by genetic traits while maintaining cellular functional roles. The characteristic of apoptosis showed that it implies a decrease in the specific gravity of the cell, destruction of the cell membrane and condensation of the chromosome, together with apoptotic body formation and phagocytosis (Kaufmann and Hengartner 2001). Generally, Bcl-2 and Bcl-xl are known to improve the cell survival by preventing apoptotic cell death produced by oxidative stress, whereas, Bax and p53 has been shown to increase apoptosis in mammalian cells (Baek et al. 2000). Thus theses mediator may be illustrated that they are considered one of the many mechanisms by which oxidative stress can be caused the apoptosis.

Recently, many natural antioxidants without side effects have been studied in several years. Until now, a number of synthetic antioxidants such as BHA (butylated hydroxylanisol), BHT (butylated hydroxytoluene), PG (propyl gallate), and TBHQ (tertiarybutyl hydroquinone) are commonly used to reduce oxidative stress inducing damage (Khan et al. 2010). However, excessive use of these syntheses can cause the some side effects, such as toxicity, cancer and liver damage (Ko et al. 2012). Therefore, many researches have been conducted in the search for naturally derived antioxidants that induce no deleterious side effects.

Marine organisms have been composed potential rich source of biological compounds compared to land plants. Marine environments have unique metabolic process and various useful things, including fatty acids, minerals, vitamins, polysaccharides and bioactive compounds (Holdt and Kraan 2011). Biological active protein plays an important role in metabolic systems. These proteins can be used as potential materials based on their structure compositions and sequence of amino acids. In addition, it may be involved in various physiological activities (Najafian and Babji 2012).

Octopus vulgaris (*O. vulgaris*) is one of the cephalopod which inhabits in the warm and tropical water of the world, including South Korea, Southern coast, Honshu coast of Japan and Mediterranean Sea (Kang et al. 2009). Previous study has been shown that *O. vulgaris* contains abundant taurine (Villanueva et al. 2004). And taurine has beneficial effects such as antioxidative defense, cellular osmoregulation and fat digestion (Li et al. 2009). But the data about antioxidant of *O. vulgaris* extract is limited.

Therefore, to prove that *O. vulgaris* is suitable as functional material, we investigated the hepatoprotective activity of *O. vulgaris* on oxidative stress caused by stimulation of H_2O_2 in Chang liver cells.

2 Materials & Methods

2.1 Materials

All testing reagent including D-glucose, bovine serum albumin (BSA), gallic acid, folin-ciocalteau reagent, 2,2′-azobis (2-methylpropion-amidine) dihydrochloride (AAPH), 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), fluorescein sodium salt, 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), 3-(4–5-dimethyl-2-yl)-2–5-diphenyltetrazolium bromide (MTT) were bought from Sigma Chemical Co. (St. Louis, MO, USA). Chang liver cell line (CCL-13) was offered from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin/streptomycin, and other materials required for culturing the cells were bought from Gibco BRL (Paisley, UK).

2.2 Preparation of the OMW

O. vulgaris meat (OM) was purchased from Yeosu, South Korea. 4 g of OM was mixed with 200 ml of distilled water for 24 h at R.T, and the filtered water extract was freeze-dried and stored at −20°C until use. The OM water extract was named as OMW.

2.3 Measurement of the Carbohydrate, Protein, and Phenol Components

The protein content of OMW was measured by the Lowry et al. (1951) method. The carbohydrate content of OMW was measured using the method of Nielsen (2010). The total phenolic contents of OMW were determined by using the method of Singleton et al. (1999).

2.4 Measurement of the Amino Acid Component

Free amino acid component in OMW was measured by using an amino acid analyzer (S433-H, Sykam GmbH, Germany) with the previous indicated method (Han et al. 2017).

2.5 *Antioxidant Effect of OMW* in vitro

To investigate of antioxidant effect of OMW, effects of OMW on ABTS radical scavenging and reducing power were respectively evaluated according to the modified methods of previous studies (Han et al. 2017; Oyaizu 1986).

2.6 Antioxidant Effects of OMW in Chang Liver Cells

Effects of OMW on Cell Viability in H_2O_2-Stimulated Cells
The cell viability of OMW was checked MTT assay of modified method from Hasegawa and Hori (2017). Chang liver cells (1×10^5 cells/wells) were pretreated with OMW for 1 h and stimulated by 750 μM H_2O_2. After 24 h, the absorbance of formazan solved by MTT solution (5 mg/ml) was measured at 540 nm using a microplate reader (SpectraMax® M2/M2e, CA, USA).

Effects of OMW Against Intracellular ROS Generation in H_2O_2-Stimulated Cells
Chang liver cells (1.6×10^4 cells/wells) were pretreated with OMW for 1 h and stimulated by 750 μM H_2O_2 for an additional 1 h at 37°C. The generated intracellular ROS was detected by the adding ′2,7′-dichlorofluorescin at excitation 485 nm/emission 528 nm was measured.

Effects of OMW Against Apoptotic Body Formation in H_2O_2-Stimulated Cells
To identify whether OMW reduces the apoptotic body formation, Hoechst 33342 staining was performed. Chang liver cells (6×10^5 cells/wells) placed in 6 well plate. After 18 h incubation, cells were pre-treated with OMW (125, 250 and 500 μg/

ml), and then 750 μM H_2O_2 was added. After 12 h, the cells were stained with 2 μg/ml hoechst 33342 reagent. The morphological aspect of stained cells was measured with a florescent microscope (Olympus, Shinjuku, Japan).

Effects of OMW Against the Increment of Sub-G_1 Hypodiploid Cells in H_2O_2-Stimulated Cells

We conducted a flow cytometry (Chae et al. 2011) to determine the effects of OMW on the increment of sub-G_1 cells in H_2O_2-stimulated cells. Chang liver cells (6×10^5 cells/wells) placed in 6 well plate and incubated with OMW (125, 250 and 500 μg/ml) for 1 h and then stimulated by H_2O_2 (500 μM) for 24 h. The cells were stained in 500 μl of 2 mM PBS-EDTA containing propidium iodide (PI) (50 μg/ml) (Sigma, Missorui, USA) and RNase A (0.2 μg/ml) (Promega, Wisconsin, USA). The cell cycle was anlayzed using a CytoFLEX (Beckman coulter, California, USA).

Effects of OMW Against the Expression of Apoptosis Related with Molecules in H_2O_2-Stimulated Cells

To investigate effect of OMW on the expression levels of apoptosis molecules, western blot analysis was performed. Chang liver cells (5×10^5 cells/wells) were incubated with OMW (125, 250 and 500 μg/ml) and then incubated with 750 μM H_2O_2 for 24 h. The cytoplasmic proteins were extracted from the cells using RIPA buffers (Thermo, Rockford, IL) and the obtained proteins (40 μg) were applied to western blot analysis. The primary antibodies were Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.), Bax (1:1000 dilution, Cell Signaling Technology Inc.), p53 (1:1000 dilution, Cell Signaling Technology Inc.) and β-actin (1:3000 dilutions, Sigma) and the secondary antibodies were the HRP-conjugated anti-mouse IgG and anti-rabbit IgG (1:5000, Cell Signaling Technology Inc.). The bands were detected using an enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Canada, Burlington) reagents and analyzed using NIH Image J software (US National Institutes of Health, Bethesda, MD).

2.7 Statistical Analysis

Data was analyzed using the SPSS package (Version 21), one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Values were expressed as mean ± standard error (S.E). A *p*-value of less than 0.05 was considered significant.

3 Results

3.1 Extraction Yields and Approximate Components of OMW

As shown in Table 1, the extraction yield of OMW was the 48.22 ± 0.17%. OMW contained the higher protein content (72.45 ± 0.62%) compared to carbohydrates (3.75 ± 0.76%) and phenols (3.62 ± 0.06%).

Table 1 Yield, carbohydrate, protein and phenol contents (%) of OMW

Samples	Extraction yields	Protein	Carbohydrate	Phenol
OMW	48.22 ± 0.17	7.45 ± 0.62	3.75 ± 0.76	3.62 ± 0.06

Table 2 Free amino acid composition (%) of OMW

	OMW	
Amino acid	mg/100 g	% amino acid
Phosphoserine	N.D	N.D
Taurine	1430.05	39.84
Phosphoethanolamine	N.D	N.D
Urea	N.D	N.D
Aspartic acid	4.65	0.13
Hydroxyproline	N.D	N.D
Threonine	48.43	1.35
Serine	44.12	1.23
Asparagine	20.64	0.58
Glutamic acid	21.18	0.59
Sarcocine	N.D	N.D
α-aminoadipic acid	N.D	N.D
Proline	218.02	6.07
Glycine	42.89	1.20
Alanine	240.79	6.71
Citrulline	N.D	N.D
α-aminobutyric acid	N.D	N.D
Valine	111.00	3.09
Cystine	N.D	N.D
Methionine	79.00	2.20
Isoleucine	101.25	2.82
Leucine	140.03	3.90
Tyrosine	68.77	1.92
Phenylalanine	92.72	2.58
β-alanine	618.48	17.23
β-aminoisobutyric acid	N.D	N.D
γ-amino-n-butyric acid	N.D	N.D
Histidine	18.00	0.50
3-methylhistidine	N.D	N.D
1-methylhistidine	N.D	N.D
Carnosine	N.D	N.D
Anserine	N.D	N.D
Tryptopan	N.D	N.D
Hydroxylysine	N.D	N.D
Ornitine	N.D	N.D
Lysine	N.D	N.D
Ethanolamine	N.D	N.D
Arginine	289.05	8.05
Total	3589.07	100.00

3.2 *Free Amino Acids Composition of OMW*

Table 2 showed that OMW contained the various free amino acid components and especially, plentiful taurine, β-alanine, arginine, and alanine contents which accounted for 39.84%, 17.23%, 8.05% and 6.71%, respectively.

3.3 *Antioxidant Effect of OMW* In Vitro

In order to investigate the antioxidant activity of OMW, ABTS radical scavenging activity and reducing power assay were performed. Generally, ABTS radical scavenging activity was well known to apply antioxidant activity assay to both lipophilic and hydrophilic compounds (Erel 2004). As shown in Fig. 1a, OMW dose-dependently increased the ABTS radical scavenging activity. Also, Fig. 1b exhibited OMW increased the reducing power. As a result, OMW has the antioxidant effects.

3.4 *Effects of OMW on Cell Viability and Intracellular ROS Generation in H_2O_2-Stimulated Cells*

Figure 2a showed that OMW has no cytotoxic effects at the used all concentrations. Also, OMW led to the improvement of cell viability in H_2O_2-treated cells, in a dose-dependent manner (Fig. 2b). Our result clearly indicates that the application of OMW can affect to protect Chang liver cells under the exposure of H_2O_2.

The exposure of H_2O_2 causes oxidative stress as increasing intracellular ROS generation in cells. So, scavenging free radicals is an essential factor for antioxidant therapy (Finkel and Holbrook 2000). We evaluated the antioxidant effect of OMW

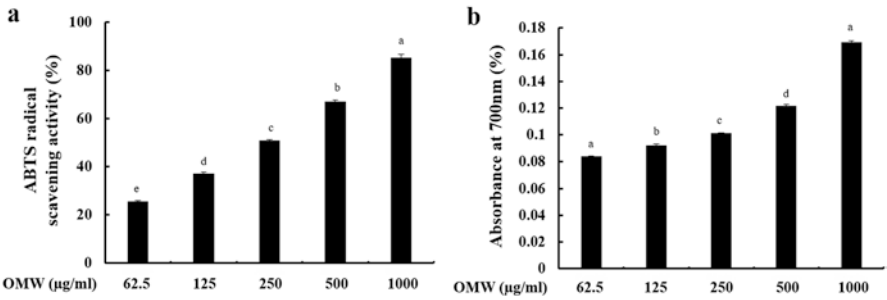

Fig. 1 ABTS radical scavenging activity (**a**) and reducing power of OMW (**b**). Values means ± S.E of three determinations. The data is presented as the means ± S.E of three experiments. [a–e]The bars with different letters are significantly different ($p < 0.05$)

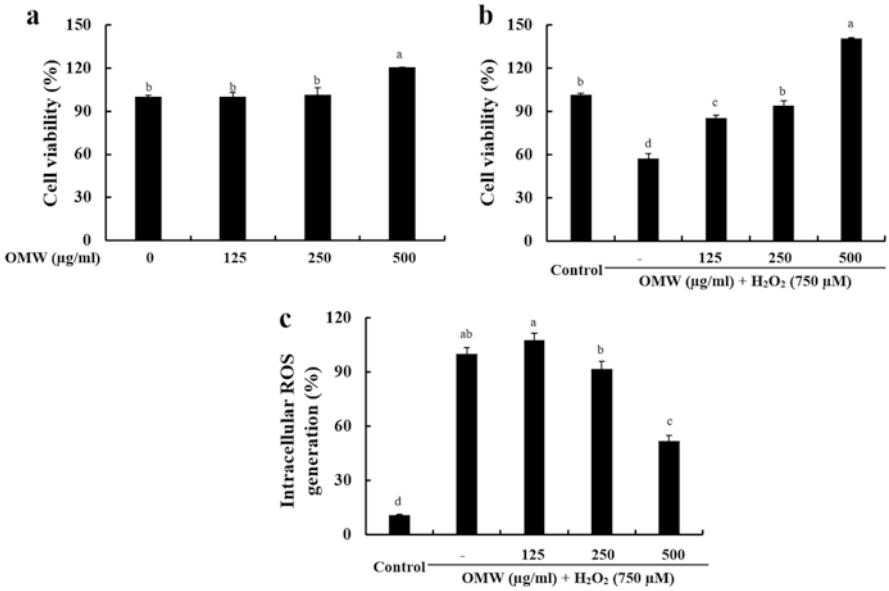

Fig. 2 Effects of OMW on cell viability (**a**, **b**) and ROS generation (**c**) in H_2O_2-treated Chang liver cells. Chang liver cells were pretreated with various dose of OMW (125, 250 and 500 μg/ml). Values means ± S.E of three determinations. [a–d]The bars with different letters are significantly different ($p < 0.05$)

in cells using DCFH-DA assay. Figure 2c indicated H_2O_2 stimulation markedly increased the intracellular ROS generation in Chang liver cells, whereas they were significantly and dose-dependently decreased by the pre-treatment of OMW. From this result, we indicate OMW improved the cell viability as inducing the antioxidant effect in H_2O_2-treated Chang liver cells.

3.5 Effects of OMW Against the Increment of Apoptosis Phenomenon in H_2O_2-Stimulated Cells

We confirmed the effects of OMW on nuclear morphological changes in Chang liver cells (Fig. 3a). As shown in Fig. 3a, the control cells displayed regular and round-shaped nuclei morphologies. However, the exposure to H_2O_2 increased the apoptotic body formation as well as the shrunken and condensed DNA in the nucleus. Interestingly, OMW rescued the DNA morphology damaged by H_2O_2 in Chang liver cells. Also, effect of OMW on the sub-G_1 cell populations was evaluated by PI staining assay in Chang liver cells (Fig. 3b, c). The hypoploid DNA contents of H_2O_2-induced cells were increased, compared to the control cells, whereas they dose-dependably inhibited by the pre-treatment of OMW. These results show that OMW inhibited the apoptosis phenomenon and led to the protective effect in Chang liver cells under the exposure of H_2O_2.

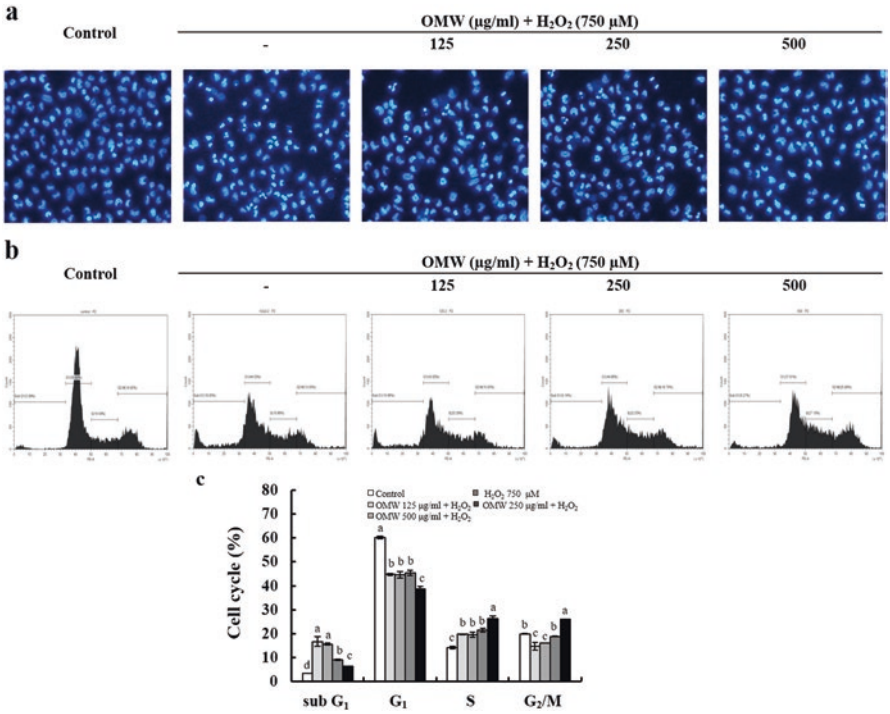

Fig. 3 The effect of OMW on apoptotic body formation (**a**) and cell cycle pattern (**b**) and percentage (**c**) induced by H_2O_2 in Chang liver cells. The apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and the cell cycle was observed with PI staining using flow cytometry. [a–d]The bars with different letters are significantly different ($p < 0.05$)

3.6 Effects of OMW Against the Expression Levels of Apoptosis Molecules in H_2O_2-Stimulated Cells

We examined whether OMW affect to the expression levels of anti-apoptosis molecules, Bcl-2, and a pro-apoptosis molecule, Bax and p53 by western blot analysis. The stimulation of H_2O_2 increased the expression level of Bcl-2, whereas increased those of Bax and p53, compared to the control cells (Fig. 4). These apoptosis molecules were modulated by the pre-treatment of OMW in a dose-dependent manner (Fig. 4). This results suggest that OMW has the protective effect against apoptosis caused by oxidative stress by modulating the expressions of apoptosis molecules.

4 Discussion

Liver cell damage caused by H_2O_2 treatment is regarded as an excellent model for analyzing liver damage caused by a number of metabolic conditions which cause an increase in the ROS generation. The chronic effects of ROS may cause a series of

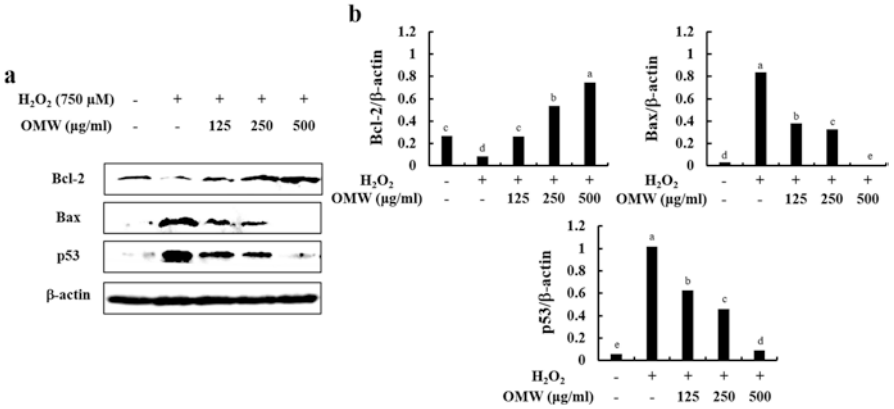

Fig. 4 Protective effect of OMW against H_2O_2-induced apoptosis in cultured Chang liver cells. The expression levels of apoptotic proteins, namely Bcl-2, Bax and p53 (**a**) and their densitometry analysis (**b**). Values means ± S.E of three determinations. [a–e]The bars with different letters are significantly different ($p < 0.05$)

complications and increase the liver cell damage ultimately resulting in cell death. The antioxidant defense mechanisms may not be sufficient to counteract the oxidative damage. Hence supplementation of antioxidant functional compounds may increase the ROS-defense against liver damage. The antioxidant effects could be direct or indirect. Although it is not evaluated in the present study, certain bioactive metabolites imply their therapeutic effects by stimulating the production of antioxidant enzymes such as SOD, GSH-Px, CAT, and HO-1 (Olorunnisola et al. 2011). These enzymes work against ROS neutralizing their reactivity to reduce cell damage. In previous studies, a large number of antioxidant peptides have been isolated from edible marine organisms (Sila and Bougatef 2016). Many of these proteins and peptides provide desirable health effects beyond their nutritional value.

Here, this study revealed the antioxidant and cytoprotective effects of a water-soluble extract from *O. vulgaris* meat (OMW). The extract contained 72.45 ± 0.62% of proteins, which was identified to be rich in the amino acid taurine. In fact, taurine is the most abundant free amino acid found in humans which maintain calcium homeostasis, bile acid conjugation, membrane stabilization, and osmoregulation. Taurine, a sulfur-containing β-amino acid biosynthesized from methionine and cysteine metabolites via the action of hypotaurine (Bouckenooghe et al. 2006). Also, taurine has been studied as a potent antioxidant and anti-inflammatory compound (Shimada et al. 2015; Marcinkiewicz and Kontny 2014). Based on current evaluations the taurine-rich extract OMW indicated prominent $ABTS^+$ and FRAP antioxidant activities. $ABTS^+$ is a free radical cation which can react with hydrogen donating groups (Aliaga and Lissi 1998). In FRAP assay, antioxidant power is calculated as a function of the ability to reduce ferric to ferrous at low pH. It is considered as a putative index of antioxidant activity or reducing the potential of a substance which determines oxidative stress (Benzie and Strain 1996). In addition, recent studies have indicated that taurine ensures proper function of the mitochon-

drial respiratory chain, thereby limiting ROS generation by the mitochondria (Jong et al. 2012, 2017). With these points, our data suggest that the taurine-rich OMW might have the antioxidant activity by ensuring proper function of the mitochondrial respiratory chain, thereby limiting ROS generation by the mitochondria.

Chang liver cells are widely used in research as a model for screening hepatoprotective effects of substances (Park et al. 2017). Substances which could reduce intracellular ROS levels could impose a protective effect towards oxidative stress conditions resulting from various factors. OMW treatment caused an increase in the cell viability showing a protective effect against H_2O_2-induced cytotoxicity. Prolonged levels of intracellular ROS cause cell death either by apoptosis or necrosis. The evaluation of apoptotic body formation and cell cycle analysis indicated that the H_2O_2-induced effects are mediated via apoptosis. Apoptosis is the typical cellular degradation process seen during the metamorphosis, differentiation, and physiological cell turnover. Apoptosis is mediated by a complex system of molecular mediators. The apoptotic events include chromatin condensation, DNA fragmentation, cell shrinkage, membrane blebs and finally the cell disassemble into apoptotic bodies. It is regarded as the important strategy for cell death as it does not cause detrimental effects on nearby cells (Fernando et al. 2018). As evident from Fig. 3, treatment of OMW decreased the apoptotic body formation which was induced by H_2O_2 treatment. This indicates that OMW could impose protective effects against the induction of apoptosis. The results of PI staining analysis draw a parallel relation to the apoptotic body formation as it increases the number of apoptotic hypodiploid cells evident by the increment of sub-G_1 cells.

Apoptosis involve two major signaling mechanisms including the intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated pathway) pathways (Park et al. 2016). This study evaluated the molecular mediators involved in the intrinsic apoptotic pathway. The key molecular mediators of apoptosis include Bcl-2 family proteins and caspases. Bcl-2 proteins can be of two major categories as anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) and pro-apoptotic proteins (Bid, Bad and Bax). Particularly the ratio in both proteins are considered as a key indicator of apoptosis (Fernando et al. 2018). p53 is regarded as an upstream mediator of Bax gene. The increased Bax and decreased Bcl-2 levels in the group treated with H_2O_2 suggests the induction of apoptosis in Chang liver cells. Moreover, the increase of p53 is associated with the increased levels of Bax upon H_2O_2 treatment. The effects were reversed upon the treatment of increasing concentrations of OMW suggesting its protective effects against the apoptosis induced by H_2O_2.

5 Conclusion

In conclusion, the water-based meat extract of *Octopus vulgaris* which was rich in taurine possesses potential hepatoprotective functionality. Such extracts could be marketed as food supplements due to their nutritional and functional values.

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Hot Water Extract of *Loliolus beka* Meat Attenuates H_2O_2-Induced Damage in Human Umbilical Vein Endothelial Cells

WonWoo Lee, Eui Jeong Han, Eun-Ji Shin, Hee-Jin Han, Ginnae Ahn, and Seon-Heui Cha

Abstract Blood vessels become less flexible with senescence; arteries narrow and become less flexible, disturbing blood circulation in aging and other vascular diseases. Mechanistically, vascular senescence plays an important role in the pathogenesis of normal aging and age-related vascular diseases. Vascular senescence also causes vascular dysfunction, resulting in damage to the vessel wall. Vascular aging involves the senescence of endothelial cells. Hydrogen peroxide is widely used to achieve oxidative stress-induced premature senescence. Here, we investigated the protective effects of a hot water extract of *Loliolus beka* meat (LBM) against H_2O_2-exposed HUVECs, a human umbilical vein endothelial cells line. The hot water extract of LBM protected cells against H_2O_2-induced cytotoxicity while reducing the expression of senescence markers, including β-galactosidase, p53, and p21. In addition, the hot water extract of LBM protected against H_2O_2-induced DNA

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Taurine theme: Taurine and Anti-oxidation and Anti-microbial

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damage. These findings suggest that the hot water extract of LBM protects HUVECs from H_2O_2-induced senescence by preventing cellular damage. LBM serve as a supplement or natural food with benefits against vascular disease.

Keywords Seaweed · *Loliolus beka* meat · Vasculadisease · Endothelial · Taurine · Oxidative stress

1 Introduction

Oxidative stress, caused by increased concentrations of reactive oxygen species (ROS) is one of the most important causal factors for the induction of cellular apoptosis (Holbrook and Ikeyama 2002). Oxidative stress has been implicated in the induction of cellular senescence, which result in decreased organ function (Brandl et al. 2011). During senescence, a number of physiological functions are altered, with the slowing or termination of cell division contributing to changes in cellular function, morphology and gene expression, which in turn promote the development of senescence-associated diseases, including hypertension, chronic coronary disease and diabetes. Vascular cell senescence, which accompanies aging, promotes endothelial cell dysfunction (Krouwer et al. 2012) and is associated with increased vascular risk (Northcott et al. 2017). Pathological states, including those observed linked to oxidative stress, invoke irreversible growth arrest in vitro within a few days, a term referred to as stress-induced premature senescence (SIPS) (Toussaint et al. 2002). Previous evidence suggests that premature senescence of endothelial cells may lead to endothelial dysfunction. Taurine is an endogenous compound found in substantial amounts in mammalian tissues. Taurine plays an important role in biophysiological functions, and taurine deficiency has been associated with various pathological conditions, including impaired cardiovascular regulation, antioxidation, diabetes and diabetic complications (Hansen 2001; Jong et al. 2012; Militante and Lombardini 2002; Militante et al. 2000). Thus, taurine-rich foods protect against various diseases, including vasculopathy and aging. In particular, dietary intake of taurine enhances the population of endothelial cells in smokers (Moloney et al. 2003). *Loliolus beka* gray is a kind of marine Mollusca, which contains plentiful amounts of taurine, and is consumed as a natural food in the Mediterranean, South American, and Asian countries. Extracts of LBM prepared at moderate temperature contain potent cytoprotective activity, an effect that may be related to the abundance of taurine (7%). When *Loliolus beka* gray meat is ingested as a food, it is cooked at high temperature. Therefore, it is unclear if cooked *Loliolus beka* meat (LBM) delivers enough taurine to ameliorate endothelial cell dysfunction. As a first step, we elucidated whether the hot water extract of LBM prevents H_2O_2-induced vascular senescence.

2 Materials and Methods

2.1 Preparation of Hot Water Extract of LBM

Loliolus beka were purchased at a market, rinsed with freshwater to remove salt, epiphytes and sand. LBM (20 g) was solubilized with 1 L of distilled water for 4 h under continuous shaking at 100 °C, and then the extract was filtered through a Whatman No. 6 filter paper and freeze-dried. The extract was stored at −70 °C until further use and dissolved in cell culture media for cell treatment.

2.2 Amino Acid Composition of LBM Extract

The amino acid composition of the hot water extract of LBM was analyzed using an amino acid analyzer (S433-H, Sykam GmbH, Germany). The hot water extract of LBM (50 mg) was hydrolyzed in 2 ml of 6.0 M HCl in a sealed vacuum ampoule at 110 °C for 24 h. HCl was removed by rotary evaporator and the final volume was adjusted at 10 ml with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated using a cation separation column (LCA K06/Na, 4.6 × 150 mm) at a flow rate of 0.45 ml/min (buffer) and 0.25 ml/min (reagent) and detected at wavelengths of 440 and 570 nm. For the determination of free amino acids, 2 g of LBM extract was centrifuged at 12,000 rpm twice for 2 min with 75% ethanol, followed by centrifugation at 2000 × g for 30 min. The supernatant solvents were removed by using a rotary evaporator, and dissolved in 8.0 ml of distilled water containing 5-sulfosalicylic acid (0.2 g) at 4 °C for 1 h. Then, the mixture was centrifuged at 2000 × g for 30 min, and 2 ml of the supernatant was transferred to a new tube containing 1 ml of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were determined.

2.3 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM (Welgene, Korea) supplemented with 10% FBS (Welgene, Korea), 100 U/ml penicillin and 100 μg/ml streptomycin (Welgene, Korea), and were maintained in a humidified incubator with 5% CO_2.

2.4 Assessment of Cell Viability

Cell viability was estimated using a cell counting kit (D-PlusTM CCK; Dongin LS, Korea) that measures mitochondrial dehydrogenase activity. For the D-PlusTM CCK assay, HUVEC cells (1×10^4 cells/wells) were seeded onto 96 well plates. After 16 h, the cells were treated with LBM extract and/or H_2O_2. We treated the cells with different concentrations of H_2O_2 (100, 200, 400 and 800 μM) for evaluation of cell viability after 24 h and the antitoxic actions of the LBM extract (125, 250, 500, and 1000 μg/ml) were evaluated after 24 h of exposure. To study the protective effects of the LBM extract, cells were pretreated with either vehicle (control) or 500 μg/ml LBM extract for 1 h and subsequently incubated with or without 400 μM H_2O_2 for 24 h at 37 °C. The D-PlusTM CCK solution was then added to the wells to a total reaction volume of 110 μl. After 1 h of the incubation, the absorbance was measured at a wavelength of 450 nm. The optical density of the formazan generated in the control cells represented 100% viability.

2.5 Quantitative Real Time PCR

Total RNA was extracted from cells using RNAiso plus (Takara Bio Inc., Japan), and cDNA was prepared using PrimeScriptTM cDNA synthesis kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. cDNA samples were analyzed by the SYBR® Premix ex TaqTM, ROX plus (Takara Bio Inc., Japan) on Bio-Rad cyclers (Hercules, CA). Gene expression was normalized to the endogenous housekeeping control gene, 18S rRNA, which was not influenced by LBM or H_2O_2. Relative expression was calculated for each gene using the $\Delta\Delta$ CT (where CT is the threshold cycle) method. Statistical analysis of PCR data was based on duplicate samples.

2.6 Western Blotting

HUVECs (1.5×10^5 cells/wells) were seeded onto 6 well plates, and the cells were incubated with vehicle (control) or 500 μg/ml LBM for 1 h and then further incubated with or without 400 μM H_2O_2 for 24 h. The cells were lysed using RIPA buffer (GeneDEPOT, Barker, TX) and protease inhibitor cocktail (GenDEPOT, Barker, TX) for 20 min on ice. The lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was used for Western blotting. Protein concentrations were measured using a DC protein assay kit (Bio-Rad, Hercules, CA). The lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were incubated with 5% skimmed milk for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 °C. After washing extensively, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). The signal was

detected using WESTSAVE (Ab Frontier, Korea), and the enhanced chemiluminescence system. Image J software was used to quantify band intensity of the Western blots. The primary antibodies used were anti-β-galactosidase (Invitrogen, Carlsbad, CA), anti-p53 (Cell signaling, Danvers, MA), anti-p21 (Cell signaling, Danvers, MA), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

2.7 Comet Assay

The alkaline comet assay was conducted according to the method of Samarakoon (2013). Briefly, the cell suspension was mixed with 75 µl of 0.5% low melting agarose, and added to slides pre-coated with 1% normal melting agarose. After solidification of agarose, the slides were covered with another 100 µl of 0.5% low melting agarose, and then immersed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid, 10 mM Tris, and 1% sodium lauroylsarcosine; 1% Triton X-100 and 10% dimethyl sulfoxide) for 30 min at 4 °C, then DNA was subjected to electrophoresis. Stained DNA (20 µg/ml ethidium bromide) was observed by fluorescence microscopy (BX-FLA; Olympus Optical Co. Ltd., Tokyo, Japan) and the image analyzed using Komet 5.0 imager (Liverpool, UK).

2.8 Statistical Analysis

All measurements were carried out in triplicate and all values represent means ± S.E. The results were subjected to an analysis of variance (ANOVA) with the Two-way and Tukey test to establish significant differences.

3 Results

3.1 Hot Water Extract of LBM Attenuates H_2O_2-Induced Cytotoxicity in HUVECs

To determine whether the hot water extract of LBM contains taurine, we analyzed its amino acid content. Taurine accounted for 29.66% of the total amino acid pool, which was present in the highest portion of amino acids as shown in Table 1. Taurine is known as a cytoprotective agent in a variety of tissues (Ito et al. 2012). So, to examine whether the hot water extract of LBM protected against H_2O_2-induced cytotoxicity, HUVECs were co-treated with the hot water extract of LBM and H_2O_2 or H_2O_2 alone. The order of the co-treatment was an initial preincubation with the hot water extract of LBM for 1 h and then further exposure to H_2O_2. The hot water extract of LBM alone showed no evidence of cytotoxicity to HUVECs at the

Table 1 Free amino acid compositions (%) from hot water extract of LBM

Amino acid name	% Amino acid
Taurine	38.22
Phosphoserine	0.29
Urea	10.75
Aspartic acid	0.98
Threonine	1.44
Serine	1.75
Glutamic acid	4.57
Proline	0.81
Glycine	0.86
Alanine	4.27
Citrulline	1.23
Valine	1.51
Methionine	1.36
Isoleucine	1.40
Leucine	3.28
Tyrosine	1.80
Phenylalanine	1.41
β-Alanine	1.81
γ-Amin-n-butyric acid	0.01
Histidine	0.52
Ornithine	0.94
Lysine	2.60
Arginine	17.58
Total	**100.00**

concentrations tested (125–1000 μg/ml) (Fig. 1a). However, the extract significantly diminished in a dose-dependent manner the loss of cell viability observed in HUVECs treated with H_2O_2 (Fig. 1b). Pretreatment with the hot water extract of LBM restored normal cell viability in the presence of 400 μM H_2O_2 for 24 h (Fig. 1c), indicating that taurine-rich-containing hot water extract of LBM possesses a cytoprotective effect against H_2O_2-induced damage of HUVECs.

3.2 Hot Water Extract of LBM Attenuates H_2O_2-Induced Senescence of HUVECs

Hydrogen peroxide exposure induces cellular senescence (Zdanov et al. 2006). Taurine is a potent anti-aging molecule that postpones the replicative senescence of stromal cells (Ji et al. 2012) and skeletal muscle (Ito et al. 2014). So, we wondered whether the taurine-rich hot water extract of LBM contained anti-senescence activity. First, examined the effect of LBM extract on the expression of β-galactosidase, a common marker of cellular senescence. Both mRNA and protein content of

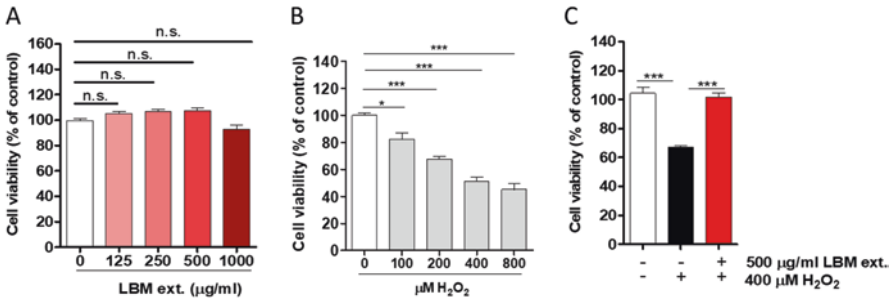

Fig. 1 Hot water extract of LBM attenuates H_2O_2-induced cytotoxicity in HUVECs. HUVECs were incubated with the indicated concentrations of the LBM extract (ext.) for 24 h (**a**). HUVECs were incubated with the indicated concentrations of H_2O_2 for 24 h (**b**). HUVECs were pre-treated with 500 μg/ml LBM for 1 h and then further incubated with or without 400 μM H_2O_2 for 24 h (**c**). CCK-8 assays were subsequently performed. Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s. no significance

β-galactosidase were elevated by treatment with H_2O_2, but β-galactosidase expression was decreased in cells pretreated with the hot water extract of LBM (Fig. 2a, b). In addition, p53-p21, well-known features of both replicative and stress-induced senescence markers also increased upon treatment with H_2O_2 but treatment with the hot water extract of LBM reduced both mRNA and protein content of p53-p21 (Fig. 2b–d), suggesting that the hot water extract of LBM attenuates H_2O_2-induced senescence of HUVECs.

3.3 Hot Water Extract of LBM Attenuates H_2O_2-Induced DNA Damage in HUVECs

DNA damage is a common mediator of premature cellular senescence induced by oxidative stress (Chen et al. 2007). Thus, we determined whether the hot water extract of LBM exerts protection against H_2O_2-induced DNA damage. As expected, DNA tail (meaning that DNA fragmentation occurs) was significantly increased by H_2O_2 treatment, whereas pretreatment with hot water extract of LBM significantly reduced the H_2O_2-induced DNA tail (Fig. 3), suggesting that the hot water extract of LBM attenuates H_2O_2-induced DNA damage.

4 Discussion

A number of studies have demonstrated that senescence is a risk factor for the development of vascular diseases. Cellular senescence is a process by which cells irreversibly exit the cell cycle and cease to divide in response to a variety of stresses, including oxidative states (Van Deursen 2014). However, it is not known whether

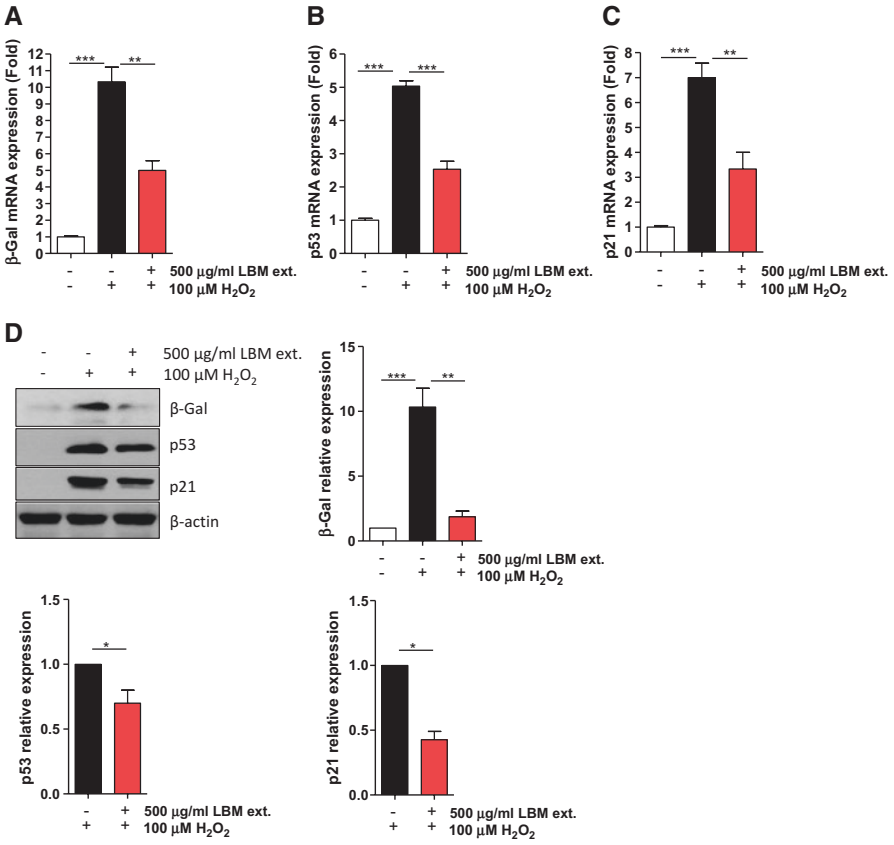

Fig. 2 Hot water extract of LBM attenuates H_2O_2-induced senescence in HUVECs. HUVECs were incubated with 500 μg/ml LBM extract (ext.) for 1 h and then further incubated with or without 100 μM H_2O_2 for 24 h. qRT-PCR and Western blot were performed for senescence markers of mRNA and protein expression. qRT-PCR was performed for β-Galactosidase (β-Gal) (**a**), p53 (**b**), and p21 mRNA expression (**c**). Protein expression of β-Gal, p53 and p21 (**d**). Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

taurine originated from *Loliolus beka* gray meat (LBM) might ameliorate senescence related diseases at all. In this study, we established an H_2O_2-induced senescent model in vitro using HUVECs to investigate the protective effect of LBM extract in cell senescence.

Cellular senescence refers to metabolically active cells having entered a state of permanent growth arrest. H_2O_2, the freely diffusible form of ROS, is generated by a variety of intracellular reactions (Cai 2005). It is an intermediate product of ROS degradation and when it is not hydrolyte becomes highly reactive molecule and major factor of oxidative stress. Therefore, the early onset of cellular senescence induced by H_2O_2 is termed stress-induced premature senescence. In this study, we

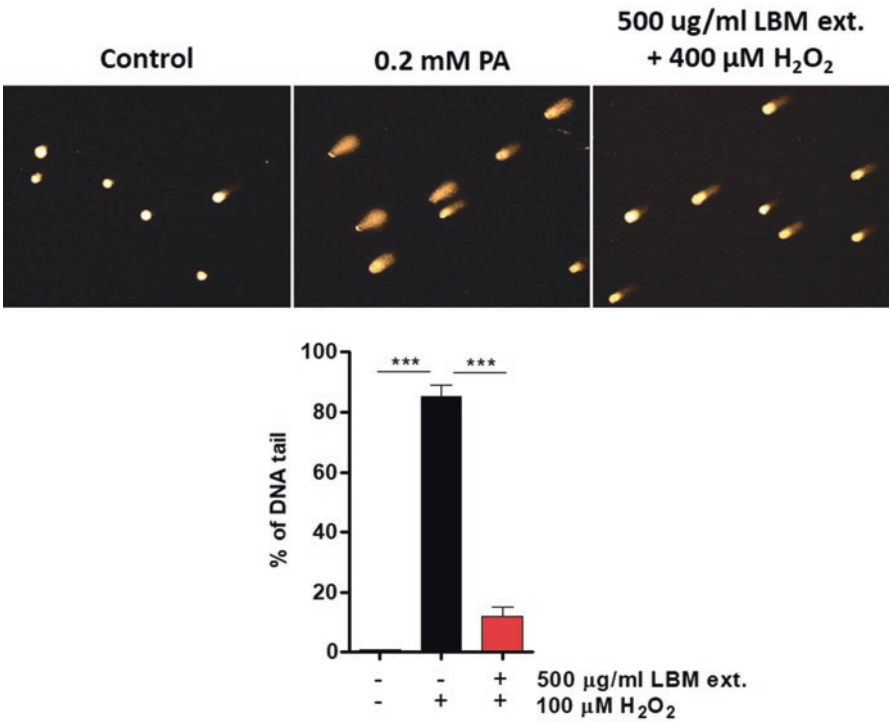

Fig. 3 Hot water extract of LBM protects against H_2O_2-induced DNA damage of HUVECs. HUVECs were incubated with 500 μg/ml LBM extract (ext.) for 1 h and then further incubated with or without 400 μM H_2O_2 for 24 h. Thereafter, the cells were harvested in low melting point agarose. Comet assays were subsequently performed. Experiments were performed in triplicate. $***p < 0.001$

observed cell viability which decreased by hydrogen peroxide was conserved by LBM extract treatment.

DNA damage has been suggested as one of the major casing factor to the senescence of cells and organisms (Arranz et al. 2015). Cellular DNA is constantly exposed to both extrinsic such as UV irradiation and other environmental toxic agents and intrinsic insults principally consist ROS. During proliferation of cultured cells, DNA is lost with each round of replication and is thought to be the major pathway that limits the cell proliferation capacity because DNA damage activate the senescence process (Shay and Wright 2000). Thus, senescence can be induced by DNA damaging agents (Von Zglinicki et al. 2001). Interestingly, we found that LBM extract was also able to directly protect DNA damage with attenuation of cellular senescence.

5 Conclusion

Our study shows that LBM extract can effectively protect HUVECs from toxicity induced by H_2O_2. Moreover, LBM extract attenuates DNA damage, as well cellular senescence markers. These results suggest that LBM extract may be an ingredient for functional foods for vascular aging and a source of pharmaceutical agents for treating senescence-related disease.

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Protective Effect of Hot Water Extract of Loliolus Beka Gray Meat Against Palmitate-Induced HUVEC Damage

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Abstract Endothelial dysfunction is a critical factor in the development of diabetes-mediated cardiovascular complications. Free fatty acids (FFA), such as palmitate, which are elevated in diabetes and obesity, have been shown to mediate endothelial dysfunction, perhaps related to oxidative stress and inflammation. Taurine ameliorates endothelial dysfunction induced by diabetes. However, there has been no reports on the effect of Loliolus beka gray meat extracts, which contain large amounts of taurine. Here, we investigated the protective effect of a hot water extract of Loliolus beka gray meat (LBM), on palmitate-induced cell damage in human umbilical vein endothelial cells (HUVEC). The LBM extract was found to inhibit palmitate-induced cytotoxicity and DNA damage. In addition, the LBM extract reduced the level of reactive oxygen species (ROS) and nitric oxide (NO), as well as pro-inflammatory cytokines in HUVEC. These results suggest that the LBM extract protects against

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palmitate-induced cytotoxicity in HUVECs. Therefore, potential therapeutic and/or inhibitors of vascular disease may be derived from the LBM extract.

Keywords Loliolus beka gray meat · Loliolus beka · Vascular disease · Taurine · Lipotoxicity

1 Introduction

Endothelial cell dysfunction (ECD) is a broad term which incorporates vascular diseases, including impairment of endothelial cell barrier function, vasodilation, disturbances in proliferative capacity, tube formation properties and antigenic properties, attenuation of synthetic function, and deterrence in the adhesion and diapedesis of white blood cells to endothelial cells (Goligorsky 2005). Endothelial cells (ECs) play a key role in the transport of metabolic substrates and cells between the blood and the interstitial space, including a complex signaling system that regulates innate and immune responses of the vascular bed (Cernuda-Morollon and Ridley 2006).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), including free radicals, are essential signaling molecules that regulate physiological cell function. However, the overproduction of ROS and/or RNS can lead to pathology by causing organellar stress, injury and cell death. Oxidative stress caused by overproduction of ROS/RNS is provoked in diabetes and contributes to the development of vascular diseases (He and Zuo 2015).

Moreover, elevations in circulating free fatty acids (FFAs) contributes to the pathogenesis of vascular diseases (Gruzdeva et al. 2014). Palmitate is a potent inducer of ROS (Sato et al. 2014).

Taurine (2-aminoethanesulfonic acid) has been reported to exert cytoprotective effects (El et al. 2011). Recently, the beneficial effects of taurine have emerged as treatment for the management of vascular diseases (Zulli 2011).

Loliolus beka gray, which contains large amounts of taurine, is a marine Mollusca that is consumed in the Mediterranean, South American, and Asian countries as a food.

Therefore, in this study, we elucidated whether the hot water extract of LBM prevents palmitate induced HUVECs damage mediated by exposure to high levels of fatty acids.

2 Materials and Methods

2.1 *Preparation of Hot Water Extract of* Loliolus beka gray *Meat (LBM)*

Loliolus beka was rinsed with freshwater to remove salt, epiphytes and sand. To prepare a hot water extract, 20 g of Loliolus beka meat (LBM) was solubilized with 1 L of distilled water, subjected to 4 h of continuous shaking at 100 °C, and then

filtered through a Whatman No. 6 filter paper and freeze-dried. The extract was stored at −70 °C until further use and dissolved in cell culture media for cell treatment.

2.2 *Amino Acid Composition of* Loliolus beka gray *Meat (LBM) Extract*

The amino acid composition of the LBM extract was determined using an amino acid analyzer (S433-H, Sykam GmbH, Germany). Briefly, 50 mg of LBM extract was hydrolyzed using 2 ml of 6.0 M HCl in a sealed vacuum ampoule at 110 °C for 24 h. Hydrochloric acid was removed by rotary evaporator and a final volume was adjusted to 10 ml with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were separated and detected using a cation separation column (LCA K06/Na, 4.6 × 150 mm) fixed at a flow rate of 0.45 ml/min (buffer) and 0.25 ml/min (reagent) and detected at wavelengths of 440 and 570 nm. For the determination of the free amino acids, 2 g of LBM extract was homogenized and then centrifuged twice at 12,000 rpm for 2 min with 75% ethanol, followed by centrifuging again at 2000 × g for 30 min. The supernatant solvents were removed by using a rotary evaporator and then the samples were dissolved in 8.0 ml of distilled water containing 5-sulfosalicylic acid (0.2 g) at 4 °C for 1 h. The mixture was centrifuged at 2000 × g for 30 min, and 2 ml of the supernatant was transferred to a new tube containing 1 ml of 0.2 M lithium citrate buffer (pH 2.2). Free amino acids were determined.

2.3 *Cell Culture*

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM (Welgene, Korea) supplemented with 10% FBS (Welgene, Korea), 100 U/ml penicillin and 100 μg/ml streptomycin (Welgene, Korea), and were maintained in a humidified incubator with 5% CO2.

2.4 *Assessment of Cell Viability*

Cell viability was estimated using a cell counting kit (D-Plus™ CCK; Dongin LS, Korea) that measures mitochondrial dehydrogenase activity. For the D-Plus™ CCK assay, HUVECs (1×10^4 cells/well) were seeded onto 96 well plates. After 16 h, the cells were treated with the LBM extract and/or PA. To examine the effect of fatty acids and the LBM extract, cells were exposed to different concentrations of palmitate (0.25, 0.5, 1 and 2 mM) and the LBM extract (125, 250, 500 and 1000 μg/ml) for

24 h. To study the protective effects of the LBM extract, cells were pretreated with vehicle (control) or 500 μg/ml LBM extract for 1 h, and subsequently incubated with or without 0.5 mM palmitate for 24 h at 37 °C. The D-Plus™ CCK solution was then added to the wells to a total reaction volume of 110 μl. After 3 h of the incubation, the absorbance was measured at a wavelength of 450 nm. The optical density of formazan generated in the control cells was considered to represent 100% viability.

2.5 Estimation of Intracellular Alkyl Radical Spectrum

Ins-1 cells (0.5×10^5 cells/wells) were seeded onto 48 well plates. The cells were treated with vehicle (0.2% BSA) or 500 μg/ml LBM, and 1 h later, 0.5 mM palmitate was added and the cells were incubated for 24 h. The cells were dissociated with trypsin and resuspended in PBS. Intracellular alkyl radical was detected by electron spin resonance. The dissociated cells were mixed with 10 mM 4-α-(4-pyridyl N-oxide)-N-tert-butylnitrone (Sigma, St. Louis, MO), incubated for 30 min at 37 °C in a water bath and transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C.

2.6 Estimation of Intracellular Hydroxyl Radical Content

Ins-1 cells (0.5×10^5 cells/wells) were seeded onto 48 well plates. The cells were treated with vehicle (0.2% BSA) or 500 μg/ml LBM, and 1 h later, 0.5 mM palmitate was added, and the cells were incubated for 24 h. The cells were dissociated with trypsin and resuspended in PBS. Intracellular hydroxyl radical was detected by electron spin resonance. The dissociated cells were mixed with 50 mM α-Phenyl-N-tert-butylnitrone and then incubated for 30 min at 37 °C in a water bath before being transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3470 G, modulation frequency 100 kHz, modulation amplitude 0.5 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C.

2.7 Preparation of Colloid Fe $(DETC)_2$

Sodium DETC (diethyldithiocarbamate, 4.5 mg, Sigma, St.Louis, MO) and $FeSO_4$ $7H_2O$ (2.8 mg, Daejung, Korea) were dissolved in two separate volumes (10 ml) of deoxygenated Krebs solution. Equal volumes of these parent solutions were rapidly

mixed and a 0.5 mM Fe $(DETC)_2$ colloid solution with a yellow-brownish color and slight opalescence to light was formed. No aggregate formation was observed at least by 30 min. The Fe $(DETC)_2$ colloid solution was used immediately after preparation.

2.8 Estimation of Intracellular Nitric Oxide (NO·) Spectrum

Ins-1 cells (0.5×10^5 cells/wells) were seeded onto 48 well plates. The cells were treated with vehicle (0.2% BSA) or 500 μg/ml LBM, and 1 h later, 0.5 mM palmitate was added and the cells were incubated for 24 h. The cells were dissociated with trypsin and resuspended in PBS. Intracellular nitric oxide was detected by electron spin resonance. The dissociated cells were mixed with 200 μM colloid $Fe(DETC)_2$, incubated for 20 min at 37 °C in a water bath, and then transferred to Teflon capillary tubes. The spin adduct was recorded using a JES-FA ESR spectrometer (JEOL Ltd. Japan) under the following measurement conditions: central field 3290 G, modulation frequency 100 kHz, modulation amplitude 5 G, microwave power 10 mW, gain 6.3×10^5, and temperature 25 °C.

2.9 Quantitative Real Time PCR

Total RNA was extracted from cells using RNAiso plus (Takara Bio Inc., Japan), and cDNA was prepared using PrimeScript™ cDNA synthesis kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. cDNA samples were analyzed by the SYBR® Premix ex Taq™, ROX plus (Takara Bio Inc., Japan) on Bio-Rad cyclers (Hercules, CA). Gene expression was normalized to the endogenous housekeeping control gene, 18S rRNA, which was not influenced by LBM or palmitate. Relative expression was calculated for each gene using the $\Delta\,\Delta\,C_T$ (where C_T is the threshold cycle) method. Statistical analysis of the PCR data was based on duplicate samples.

2.10 Statistical Analysis

All measurements were carried out in triplicate and all values are represented as the means ± S.E. The results were subjected to an analysis of variance (ANOVA) with the Two-way and Tukey test to analyze the differences.

3 Results

3.1 Taurine-Rich Hot Water Extract of LBM Attenuates Palmitate-Induced Cytotoxicity in HUVECs

To determine whether the LBM extract contains taurine, we analyzed the amino acid content. Taurine accounted for 38.22% of the total amino acids which were present in high levels as shown in Table 1. Taurine is known as a cytoprotective agent in various cells and tissues (Ito et al. 2012). Therefore, to exacted mine whether the LBM extract exerts protection against palmitate (PA)-induced cytotoxicity, HUVECs were treated with the LBM extract alone, PA alone or were preincubated with LBM extract for 1 h and then further incubated with various doses of PA. The LBM extract alone did not show any cytotoxicity of HUVECs over the concentration range tested (125–1000 μg/ml) (Fig. 1a). A significant dose-dependent decline in cell viability was observed for HUVECs treated with PA (Fig. 1b). Pretreatment with the LBM extract exhibited cell viability values similar to that of the control despite exposure to 0.5 mM PA for 24 h (Fig. 1c), indicating that the taurine-rich LBM extract possesses a cytoprotective effect against PA-induced damage in HUVECs.

3.2 Taurine-Rich Hot Water Extract of LBM Reduced Palmitate-Induced Alky, Hydroxyl Radical and Nitric Oxide in HUVECs

Taurine is a potent free radical scavenger that attenuates the damage caused by excessive oxygen free radicals (Sree and Sethupathy 2014). An excess production of PA can increase free radicals (Sato et al. 2014). So, we determined whether LBM extract can attenuate PA-induced overproduction radicals. As expected, alkyl (Fig. 2a), hydroxyl (Fig. 2b), and nitric oxide (Fig. 3) radicals were significantly increased by PA treatment, whereas pretreatment with LBM extract significantly reduced PA-induced overproduction of radicals (Figs. 2 and 3), suggesting that the LBM extract attenuates PA-induced overproduction of radicals.

3.3 Taurine-Rich Hot Water Extract of LBM Protects Against Palmitate-Induced Upregulation of Pro-inflammatory Cytokines in HUVECs

Taurine possesses potent anti-inflammatory activity, therefore, we determined whether the LBM extract attenuated the anti-inflammatory actions of PA. Using quantitative RT-PCR, mRNA levels of the inflammatory genes IL1-β, TNF-α and

Table 1 Free amino acid composition (%) of LBM hot water extract

Amino acid name	% Amino acid
Taurine	38.22
Phosphoserine	0.29
Urea	10.75
Aspartic acid	0.98
Threonine	1.44
Serine	1.75
Glutamic acid	4.57
Proline	0.81
Glycine	0.86
Alanine	4.27
Citrulline	1.23
Valine	1.51
Methionine	1.36
Isoleucine	1.40
Leucine	3.28
Tyrosine	1.80
Phenylalanine	1.41
β-Alanine	1.81
γ-Amin-n-butyric acid	0.01
Histidine	0.52
Ornithine	0.94
Lysine	2.60
Arginine	17.58
Total	**100.00**

COX-2 were to be elevated by palmitate treatment, but these effects were attenuated by exposure to the LBM extract (Fig. 4). These results indicate that the LBM extract attenuates PA-induced pro-inflammatory cytokine activation.

4 Discussion

Natural products have been used as an alternative treatment for vascular disease in many countries (Bonnefont-Rousselot 2016; Slevin et al. 2012). Additionally, taurine shows anti-vascular disease potential (Zulli 2011). However, it is not known whether *Loliolus beka* gray meat (LBM), which contains a great deal of taurine, might ameliorate vascular related diseases. In the present study, we investigated the protective effects of the LBM extract against palmitate-induced vascular dysfunction.

Increases in free fatty acids (FFAs) alone or in combination with hyperglycemia, have been shown to trigger vascular damage (Sena et al. 2013; Vorn and Yoo 2017).

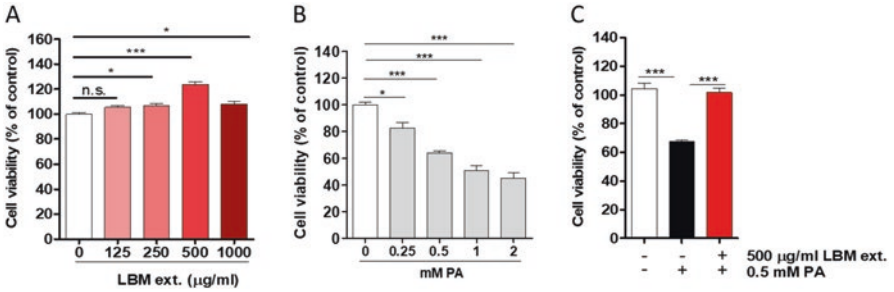

Fig. 1 Taurine-rich hot water extract of LBM attenuates palmitate-induced cytotoxicity in HUVECs. HUVECs were incubated with the indicated concentrations of LBM extract (ext.) for 24 h (**a**). HUVECs were incubated with the indicated concentrations of palmitate (PA) for 24 h (**b**). HUVECs were incubated with 500 μg/ml LBM for 1 h and then further incubated with or without 0.5 mM palmitate for 24 h (C). CCK-8 assays were subsequently performed. Experiments were performed in triplicate. $*p < 0.05$, $***p < 0.001$, n.s. no significance

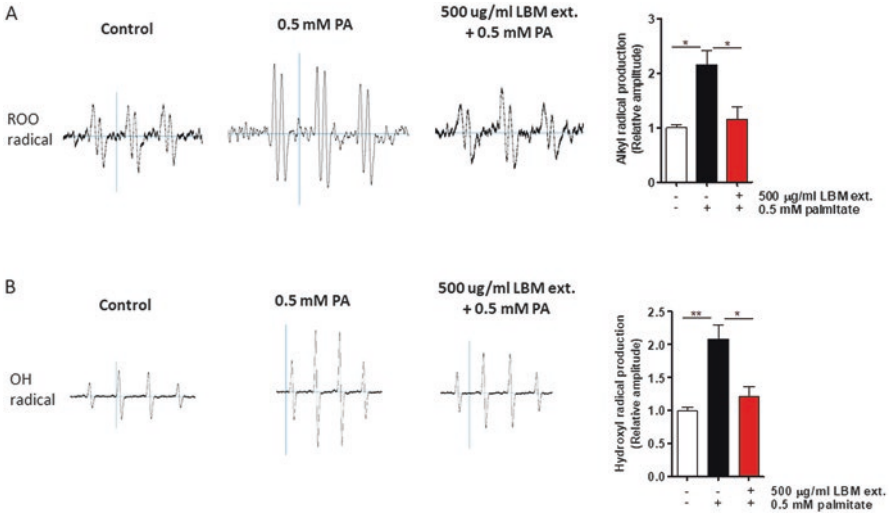

Fig. 2 Taurine-rich hot water extract of LBM reduces palmitate-induced ROS in HUVECs. HUVECs were incubated with 500 μg/ml LBM extract (ext.) for 1 h and then further incubated with or without 0.5 mM palmitate (PA) for 24 h. Thereafter, the cells were harvested and analyzed radical spectrum. Alkyl radical (**a**). Hydroxyl radical (**b**). Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$

In addition, lipotoxicity induced by prolonged exposure to elevated FFAs, in particular saturated FFAs such as palmitate, leads to endothelial cell dysfunction (Ghosh et al. 2017; Piro et al. 2008). In agreement with previous studies (Hu et al. 2017; Luo et al. 2017), our results show that exposure to palmitate induces significant cell HUEVC death. Nonetheless, we provide evidence that the LBM extract HUVEC death caused by palmitate.

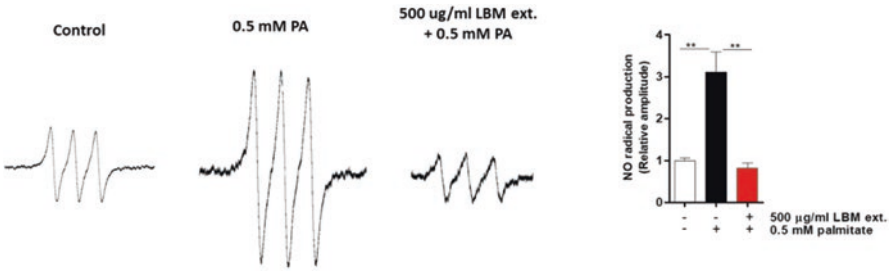

Fig. 3 Taurine-rich hot water extract of LBM reduces palmitate-induced NO in HUVECs. HUVECs were incubated with 500 μg/ml LBM extract (ext.) for 1 h and then further incubated with or without 0.5 mM palmitate (PA) for 24 h. Thereafter, the cells were harvested and analyzed radical spectrum. Experiments were performed in triplicate. $**p < 0.01$

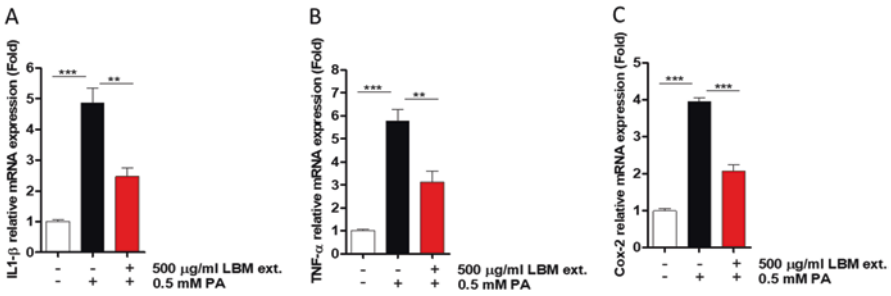

Fig. 4 Taurine-rich hot water extract of LBM protects palmitate-induced expression of pro-inflammatory cytokines in HUVECs. HUVECs were incubated with 500 μg/ml LBM extract (ext.) for 1 h and then further incubated with or without 0.5 mM palmitate (PA) for 24 h. qRT-PCR was performed for IL1-β (**a**), TNF-α (**b**), and COX-2 (**c**) mRNA expression. Experiments were performed in triplicate. $**p < 0.01$, $***p < 0.001$

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in humans and plays an important role in several essential biological processes. In particular, attenuation of apoptosis and antioxidant activity are seemingly crucial to its cytoprotective actions (Messina and Dawson 2000; Xu et al. 2008). In this study, 40% of amino acid composition of the LBM extract is taurine.

Oxidative stress is a major factor responsible for tissue damage in conditions such as infection, acute and chronic inflammation and aging. At the site of inflammation, oxidative stress is mediated by ROS and/or RNS generated primarily by activated leukocytes. ROS/RNS play a beneficial role in providing protection against pathogens, but in excess they are also responsible for tissue injury (Witter et al. 2016). A variety of antioxidants are involved in the prevention of oxidant-induced cell damage, including the oxidation of high molecular weight compounds, such as lipids, proteins and DNA.

Taurine is found at particularly high concentrations in tissues exposed to elevated levels of oxidants, supporting the view that it attenuates oxidative stress-mediated

injury Lu et al. (2009). Indeed, there have been reports implicating taurine as an effective antioxidant, with two actions of taurine responsible for this action. But the mechanism underlying its antioxidant activity remains unclear. One well established antioxidant action of taurine is neutralization of hypochlorous acid, an extremely toxic oxidant generated by the myeloperoxide/halide system (Marcinkiewicz and Kontny 2014). This activity explains the anti-inflammatory properties of taurine, as its reaction with HOCl results in generation of taurine chloramine (TauCl), a more stable and less toxic anti-inflammatory mediator. Taurine also regulates the activity of the mitochondrial respiratory chain, which also is responsible for the antioxidant activity of taurine (Jong et al. 2012, 2017). In our result, taurine-rich extract of LBM attenuated the elevation of pro-inflammatory cytokines, this also implies LBM would be a potent anti-inflammatory functional ingredient.

5 Conclusion

In conclusion, our study shows that the LBM extract can effectively protect HUVECs from toxicity induced by palmitate. Moreover, the LBM extract attenuates overproduction of radicals and pro-inflammatory cytokines in HUVECs exposed to palmitate. These results suggest that the LBM extract from a functional food contains an ingredient that may be included in supplements that benefit patients with vascular disease and may ultimately be used as a pharmaceutical action against vascular disease.

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Radio-Protective Effects of *Loliolus beka* Gray Meat Consisted of a Plentiful Taurine Against Damages Caused by Gamma Ray Irradiation

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Abstract Gamma ray irradiation causes immune suppression, in which oxidative stress reduces cell viability and damages immune cells. In the present study, we investigated whether *Loliolus beka* gray meat (LBM), which contains large amounts of taurine, protects against damage of murine splenocytes by oxidative stress. An aqueous extract of LBM (LBMW) was prepared, which contained plentiful levels of

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taurine. LBMW improved cell viability of gamma ray-irradiated murine splenocytes, an effect that was associated with significant reduction in the production of reactive oxygen species (ROS). We also showed that the production of nitric oxide (NO) and ROS in gamma ray-irradiated zebrafish embryos, as well as the death of the embryos, were diminished by LBMW. These data suggest that the consumption of taurine-rich foods, such as LBM, may be used in the protection of cells against oxidative stress.

Keywords *Loliolus beka* gray · Gamma ray irradiation · ROS · Zebrafish embryos

1 Introduction

Radioprotection is a term that attracts a great deal of interest in biomedical research. The Late-term effects of ionizing radiation to humans from large-scale and long-term cohort epidemiological studies have been examined. The 1945 atomic bombing of Hiroshima and Nagasaki in Japan during the World War II led to a surge in research focusing on the discovery of procedures to counter radiation sickness (Kamiya et al. 2015). Radiation exposure-related adverse health effects, such as epilation and skin burns, were first identified as early as 1896, shortly after the discovery of x-rays (Daniel 1896). Although increased doses of radiation are harmful to organisms, humans possess defense mechanisms to counter the low doses of background radiation present throughout the environment and from solar radiation and cosmic ray showers. Exposure to ionizing radiation induces cellular damage by reacting directly with essential biomolecules or indirectly by the synthesis of intracellular reactive oxygen species (ROS) (Oh et al. 2016). The dominant ROS are hydroxyl radicals generated by irradiation of water and intracellularly generated ROS, including superoxide radicals, singlet oxygen and hydrogen peroxide (Gligorovski et al. 2015). These ROS instantaneously react with the surrounding biomolecules, causing structural alterations.

Gamma ray is an ionizing shortwave electromagnetic radiation emitted secondary to alpha, beta, or positron emissions. Unlike alpha and beta particles, gamma ray photons possess more energy and can penetrate tissue, where they can cause damage. Direct exposure to large doses of radiation may result in death. Exposure to a low radiation dose produces chronic cytogenetic effects, such as chromosomal aberrations that can lead to mutations and cancer (Ray and Stick 2015). Radio-protective substances, such as amifostine and palifermin, have been approved by the FDA for treatment to protect against damaging doses of radiation by scavenging ROS or

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increasing the levels of the antioxidant defenses (Johnke et al. 2014). Recently, marine organisms, such as sponges, algae, anthozoans, fish, molluscans, and protozoans, were found to contain natural products with impressive bioactive activity (Imen et al. 2015). Among those with the greatest potential as therapeutic agents are the antioxidants, such as polyphenols of brown algae and the antioxidant peptides of marine invertebrates (Chai et al. 2017; Fernando et al. 2016)

Loliolus beka is a species of squid distributed in the temperate and tropical waters of the Western Pacific, East and South China Sea (Norman et al. 2014). In a recent study, the protective and antioxidant activities of *L. beka* meat (LBM) against oxidative stress in H_2O_2-mediated oxidative stress have been described (Han et al. 2017). The aqueous extract of LBM (LBMW) protects hepatocytes against oxidative stress-induced apoptosis.

The objectives of the present study using a zebrafish embryo model were to explore the antioxidant and radio-protective effects of LBMW against gamma ray induced ROS production and cell damage.

2 Materials & Methods

2.1 Materials

2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), diaminofluorescein-FM diacetate (DAF-FM DA) and acridine orange and 2-phenoxy ethanol were purchased from Sigma, Aldrich, USA. Other chemicals and regents used in these experiments are of the highest purity grade.

2.2 Preparation of Sample Extract

L. beka was purchased from Yeosu, South Korea. Samples were washed with tap water freeze-dried and pulverized. A weight of 100 g of the sample was extracted using 5 L of distilled water at 37 °C under continuous agitation for 24 h. The crude extract (LBMW) was obtained by lyophilization of the sample filtrate.

2.3 Maintenance of Zebrafish

Adult experimental zebrafish were purchased from a commercial dealer in South Korea. The fish were acclimated to the laboratory environment for 2 weeks. The embryos were obtained by stimulating the spawning by the onset of light in the morning.

2.4 Procedure for Gamma Ray Irradiation

Each group of fish was transferred to 1.5 ml micro test tube. It was irradiated to 20 Gy at a dose rate of 1.5 Gy/min at a source distance of 150 cm. Exposure was carried out using a ^{60}Co gamma ray irradiator (Theratron-780) (Lee et al. 2017).

2.5 Evaluation of Zebrafish Survival and Cytotoxicity by Observing Malformation

Zebrafish embryos were transferred to separate wells in a 12 well plate (15 eggs per well) and placed in embryo media. After acclimation for 1 h, different concentrations of LBMW were added to the wells. Following 2 h, the fish embryos were either irradiated or left blank. Survival was monitored each day for 5 days. Toxicity was assessed by measuring tail bending at three-days post fertilization (dpf).

2.6 Protective Effect of LBMW against Oxidative Stress Induced by Gamma Ray Irradiation

After treatment and irradiation, fish embryos were maintained for 3 days until the larvae is hatched. The hatched larvae (3 from each group) were transferred to 24 well culture plates and separately treated with different fluorescent dyes specific for identifying ROS (DCF-DA), NO production (DAF-FM DA) and cell viability (acridine orange). After incubation for specific time periods, the larvae were rinsed using embryo media, anesthetized using 2-phenoxy ethanol (x1000 dilution), and visualized by a fluorescent microscope integrated with a Moticam color digital camera.

2.7 Statistical Analysis

Data were analyzed using the SPSS package (Version 21). Values were expressed as means ± standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p-value less than 0.05 was considered significant.

3 Results

3.1 LBMW Enhances Survival and Counteracts Cytotoxicity Induced by Gamma Ray Irradiation in Zebrafish Larvae

Zebrafish embryos were treated with different concentrations of LBMW and exposed to gamma ray irradiation. The fish were daily monitored for 5 days. In the untreated group, gamma ray irradiation caused a drop in zebrafish survival (Fig. 1a). Treatment of different concentrations of LBMW caused a dose-dependent increase in survival. However, LBMW did not restore survival to control levels. Observations were made on malformations and behavioral changes, such as inability to stay upright, exhibit tail bending and bent spine (cyphosis and scoliosis), develop yolk sac edema, pericardial edema or uninflated swim bladder and undergo tissue necrosis. The tail bending morphologies are indicated in Fig. 1b. Accordingly gamma ray irradiation caused tail bending. Treatment of different concentrations of LBMW restored tail bending in fish.

3.2 LBMW Reduced Gamma Ray Irradiation-Induced ROS Production in Zebrafish Larvae

Gamma ray-irradiated zebrafish were treated with or without LBMW, after which intracellular ROS levels were determined by the fluorescence indicator, DCFH-DA. Treatment with increasing concentrations of LBMW slightly reduced fluorescence intensity in a dose-dependent manner, indicating that LBMW modestly reduced ROS levels originating from gamma ray irradiation (Fig. 2).

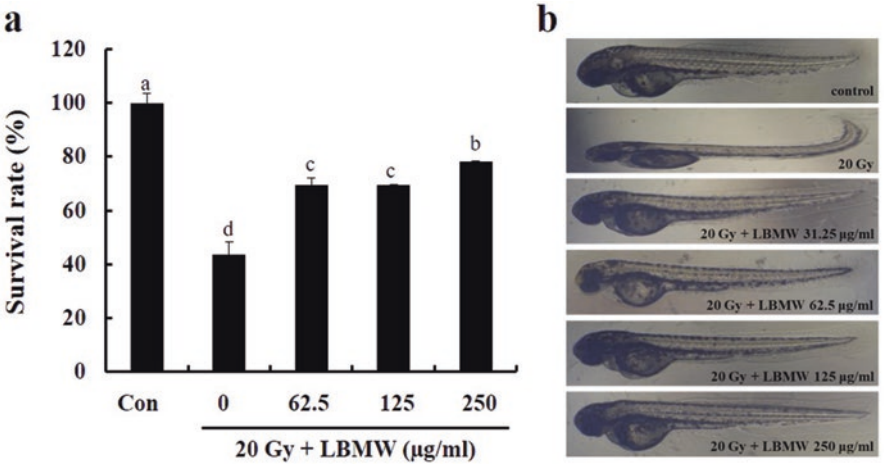

Fig. 1 Effect of LBMW on survival (**a**) and cytotoxicity (**b**) using a zebrafish model. [a–d]The Bars with different letters are significantly different ($p < 0.05$)

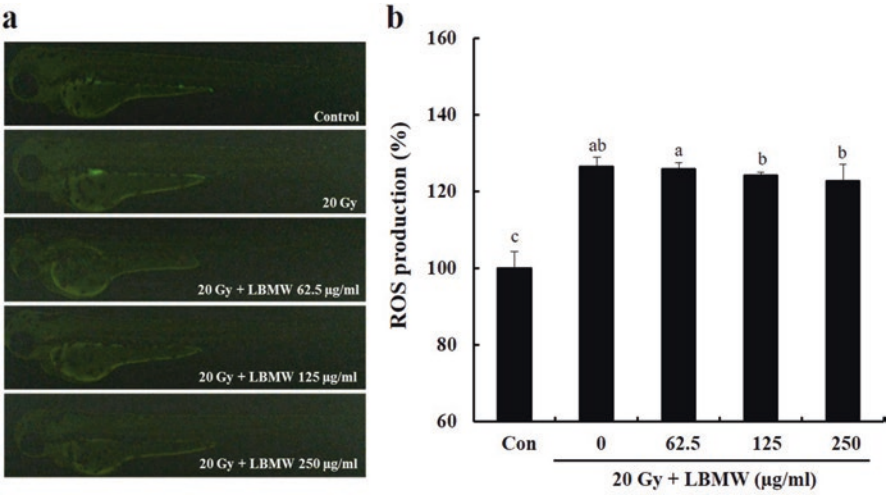

Fig. 2 Inhibitory effects of LBMW on ROS generation in zebrafish models (**a** and **b**). [a-c] The bars with different letters are significantly different ($p < 0.05$)

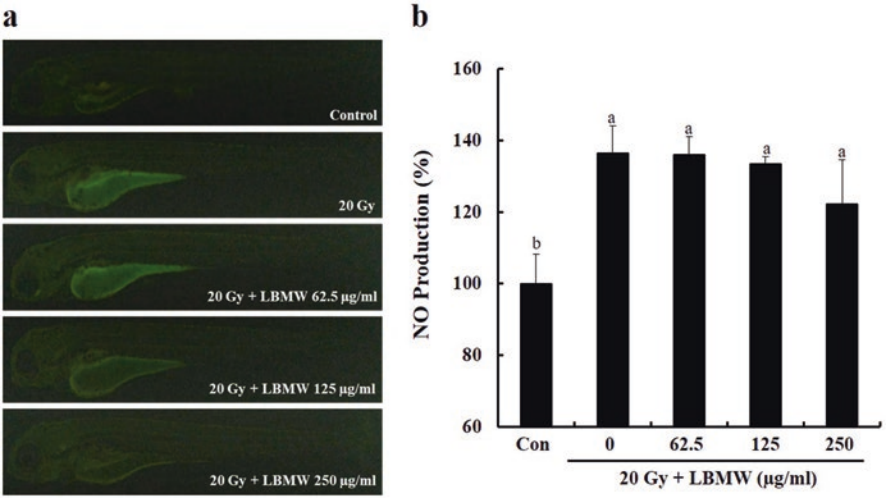

Fig. 3 Inhibitory effects of LBMW on NO generation in a zebrafish model (**a** and **b**). [a-b]The bars with different letters are significantly different ($p < 0.05$)

3.3 LBMW Reduced NO Production Levels in Gamma Ray-Irradiated Zebrafish Larvae

NO levels were identified using the fluorescent dye DAF-FM DA. Gamma ray irradiation increased NO production by zebrafish embryos, as evident by the increase in fluorescence intensity (Fig. 3). Treatment with increasing concentrations of LBMW

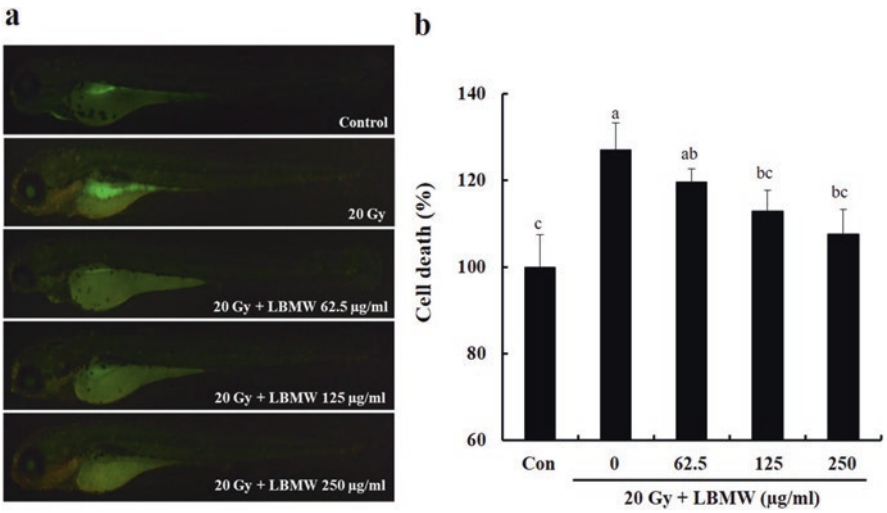

Fig. 4 Inhibitory effects of LBMW on cytotoxicity in zebrafish model. [a-c] The bars with different letters are significantly different ($p < 0.05$)

mediated a dose-dependent reduction in NO levels, as evident by the decrease in fluorescence intensity.

3.4 LBMW Reduces Cytotoxicity Induced by Gamma Ray Irradiation in Zebrafish Larvae

As indicated in Fig. 4, gamma ray irradiation increased cell death, as evidenced by the elevation in fluorescence intensity of acridine orange in the reaction solution. Acridine orange is a nucleic acid binding dye that can penetrate the cell membrane. Increasing concentrations of LBMW reduced the fluorescence intensity of acridine orange, indicating a reduction in cell death.

4 Discussion

The zebrafish animal model has become popular for assessing drug efficacy, safety, and toxicity. A large number of studies describe the similarities between the metabolic activity of mammals and zebrafish, revealing that zebrafish represent a convenient model for mimicking human disease conditions. The zebrafish model has several advantages over the conventional mouse model, including short lifespan, transparency (in larval stages), production of a large number of offspring, low cost, convenience in handling and genetic similarity to humans (McGrath and Li 2008). Several recent studies describe the use of the zebrafish embryo model in evaluating

the antioxidant effects of substances (Kim et al. 2014; Um et al. 2017). However, only a few studies describe the use of zebrafish in assessing the effects of radiation (Lee et al. 2017). In the present study, *L. beka* meat extract was prepared using distilled water. In our previous study, we have described the proximate composition of the meat extract which contained high levels of protein (63.29 ± 0.32%) and carbohydrate (30.86 ± 0.21%) (Han et al. 2017). According to Han et al., the free amino acid profile of the extract revealed high levels of L-lysine, L-phenylalanine, L-leucine, L-methionine, and taurine (Han et al. 2017). According to several previous studies, taurine is an antioxidant amino acid (Shimada et al. 2015; Jong et al. 2012). Based on the present observation, LBMW treatment improved gamma ray irradiation-induced mortality of zebrafish embryos. Although a substantial increase in survival was observed after LBMW treatment, the recovery was 70% at 250 μg/ml concentration compared to the control, which showed >95% survival. Compared to the survival of the fish irradiated with gamma rays (without sample treatment), the treatment of 250 μg/ml of LBMW significantly increased survival. In previous studies involving zebrafish, a sulfated polysaccharide purified from *Lactobacillus plantarum*-fermented Ishige okamurae restored survival to 83.3% after gamma ray irradiation (20 Gy) of zebrafish (3.13 μg/ml sample concentration) five-days post fertilization (dpf) (Lee et al. 2017). It is known that ROS generated during gamma ray irradiation can directly or indirectly damage cellular components ultimately causing death (Bing et al. 2014). This phenomenon could be used to explain the reduction in zebrafish survival upon gamma ray irradiation.

The next step was to analyze the radioprotective effects of LBMW in terms of ROS and NO levels in zebrafish embryos exposed to gamma ray irradiation. The oxidation sensitive fluorescent probe dye DCFH-DA was used to identify ROS levels. DCFH-DA can diffuse into cells where it gets hydrolyzed into the nonfluorescent intermediate 2′,7′-dichlorodihydrofluorescein (DCFH). Inside the cells, DCFH gets oxidized into highly fluorescent dichloroflurescein (DCF) by reacting with H_2O_2 produced during oxidative stress (Caldefie-Chézet et al. 2002). DCF fluorescence was then observed by the fluorescence microscope, where fluorescence intensity was taken as a measure of intracellular ROS. Gamma ray irradiation caused an increase in the intracellular ROS levels, as evidenced by an increase in fluorescence intensity (128%) compared to that of the control. Treatment with increasing concentrations of LBMW caused a dose-dependent minor decrease in ROS levels. The fluctuation in NO levels in gamma ray irradiated zebrafish were measured by using the fluorescent probe DAF-FM DA. NO plays a critical role in biological systems, as an intracellular molecular mediator which control various physiological responses, including neurotransmission, inflammation, immune regulation, and smooth muscle relaxation of blood vessels, pulmonary vessels, and sphincters. NO is synthesized from L-arginine by the action of NO synthase (Moncada et al. 1989). DAF analogs are widely used for the determination and bioimaging of NO in cells. Among DAF analogs, DAF-FM DA is widely used for NO assays. Inside the cells, the diacetate of DAF-FM (DAF-FM DA) converts into the fluorescence active triazole derivative DAF-FM in the presence of NO and O_2 (Itoh et al. 2000). As evident from the present studies the gamma ray irradiation significantly increase

NO levels in zebrafish embryos. LBMW treatment causes a dose-dependent reduction in NO levels.

Cell viability was determined using the fluorescence probe dye acridine orange. Acridine orange is rapidly absorbed by dying cells and binds with chromatin producing a fluorescent complex. Fluorescence intensity fluctuates based on the proportion of dead cells (Tucker and Lardelli 2007). As evident from the current analysis, the proportion of dead cells was high in gamma ray irradiated zebrafish embryos. LBMW treatment decreased the numer of dead cells in a dose-dependent manner, suggesting that it protects against gamma ray induced cell damage.

5 Conclusion

In conclusion, present study evidenced that the treatment of LBMW may have a positive impact on counteracting gamma ray induced oxidative stress in zebrafish embryos. It could be a potential alternative to developing radioprotective drugs and nutraceuticals.

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Protective Effect of Taurine on Paraquat-Induced Lung Epithelial Cell Injury

Shuangxing Li, Jinhua Wang, Bin Kai Wei, Guangtao Dong, and Xiujie Wang

Abstract The herbicide Paraquat induce oxidative stress-mediated lung injury. Taurine is a well-known antioxidant. This study was designed to explore the effect of taurine on paraquat-induced injury and its related mechanism in A549 cells. The cells were pretreated with various concentrations of taurine for 30 min prior to paraquat exposure. 24 h later, cell viability was examined by the MTT assay. The level of glutathione (GSH) and the activity of glutathione peroxidase (GPx) were analyzed. The results show that taurine treatment significantly attenuates the decrease in cell viability mediated by paraquat in A549 cells. Taurine also reversed paraquat-induced disturbances in GSH content and GPx activity. Taurine exerts protection against paraquat-mediated A549 cell toxicity likely through modulation of oxidative stress.

Keywords Taurine · Paraquat · Oxidative stress · Lung injury

Abbreviations

GSH	glutathione
GPx	Glutathione peroxidase
DMSO	dimethyl sulfoxide
DTNB	5,5′-dithiobis-2-nitrobenzoic acid

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1 Introduction

Paraquat (1-1′-diethyl-4′-bipyridylium dichloride), a quaternary ammonium herbicide, is widely used around the world, especially in developing countries. As a non-selective herbicide, this chemical compound has been used as an aquatic herbicide, a crop desiccant and defoliant, especially to control broad leaf weed (Lee et al. 1995; Zerin et al. 2012). Owing to the high morbidity and fatality rates of its use, the detrimental health effects of paraquat has caused great concerns among public and medical researchers(Wei et al. 2018; Elenga et al. 2018; Ikpesu 2015; Vadivelan et al. 2014).

After paraquat enters the body, it is distributed into several tissues and organs. The lungs are the main target organ, but also the site of accumulation and toxicity (Lee et al. 1999; Lin et al. 2011; Han et al. 2015). It has been reported that lung injury is the primary form of toxicity associated with paraquat poisoning (Mitsopoulos and Suntres 2010; Zerin et al. 2012; Han et al. 2015). Due to a paucity of effective treatment, paraquat toxicity is associated with a high mortality rate. Recent evidence shows that paraquat mediates these toxic responses through excessive generation of reactive oxygen species (Yamada et al. 2015; Meng et al. 2013; Kim et al. 2013). Therefore, antioxidant should prove effective in rescuing subjects from paraquat poisoning.

Taurine, a sulfur-containing-β-amino acid, is abundant in many mammalian tissues as a free amino acid (Batista et al. 2013). It is generally accepted that taurine is cytoprotective, reducing inflammation, fibrosis, apoptosis and hyperplasia in lungs and alveolar epithelial cells (Men et al. 2010; Schuller-Levis et al. 2003; Bhavsar et al. 2010, Schuller-Levis et al. 1995). Several studies have shown that it diminishes toxicity related to various poisonous substances (Das et al. 2009; Sun et al. 2014; Men et al. 2010). Thus, taurine is an attractive candidate to relieve paraquat-induced injury in lungs.

In the present study, the effect of paraquat and taurine on A549 cell viability was assessed by the MTT assay. Glutathione (GSH) levels and glutathione peroxidase (GPx) activity were examined in taurine/paraquat-treated cells. The aim of the study was to investigate the beneficial effect of taurine on paraquat-induced injury of A549 cells and its mechanism of action.

2 Methods

2.1 Cell Culture

The human non-small cell lung carcinoma A549 cell line was provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in DMEM medium with 10% FBS and 1% (v/v) antibiotic/

antimycotic cocktail (100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2 MTT Assay

Cell viability was assessed by examining the level of MTT reduction. A549 cells (2 × 105 cells/well) were plated in 96-well plate. Twenty-four hours later, A549 cells were treated with or without taurine (5 mM) for 30 min prior to addition of various concentrations of paraquat (0, 50, 100, 200, 400, 600 μM). Twenty-four hours later, 10 μl of 5 mg/ml MTT solution (in PBS) was added to the plate wells, and the cells were incubated at 37 °C for 4 h. Then, 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Following 30 min incubation, the absorbance was read at 570 nm. Cellular viability was expressed as a percentage relative to the control (Control %).

2.3 GSH Level Measurement

Glutathione (GSH) concentration was measured by the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) method using a commercially available kit. The rate of formation of TNB was monitored and GSH content was determined. A549 cells, plating in 6-well plates, were treated with taurine and paraquat. The cell pellets were collected after washing with PBS, then homogenized. After centrifugation for 10 min, 10,000 rpm at 4 °C, the supernatant was transferred to a new tube and used for the analysis of GSH level.

2.4 GPx Activity Assessment

One unit of GPx was calculated as the rate at which 1 mmol GSH was reduced in 1 min per milligram protein. Protein content was examined by the Coomassie blue protein-binding method. Bovine serum albumin was used as a standard.

2.5 Statistic Analysis

Data were expressed as the means ± SD and analyzed using SPSS 11.0 statistical software. Comparisons between groups were analyzed using one-way ANOVA followed by the LSK test; $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of Paraquat Exposure on A549 Cells

To evaluate the toxic effects of paraquat, paraquat-treated A549 cells were analyzed by the MTT assay. Exposure of A549 cells to paraquat for 24 h markedly decreased cell viability in a dose-dependent manner. The cell viability was reduced nearly 55% at a concentration of 0.2 mM paraquat. Therefore, 200 μM, of paraquat was used in subsequent experiments (Fig. 1).

3.2 Effect of Taurine Treatment on Cell Viability

To assess the protection of taurine on paraquat-induced toxicity, A549 cells were pretreated with 4 mM taurine for 30 min prior to paraquat exposure. As shown in Fig. 2, cell viability was decreased after paraquat exposure, indicating that paraquat is toxic to A549 cells. However, taurine pretreatment attenuated the paraquat-mediated reduction in cell viability, rescues A549 cells from paraquat-mediated cell death.

3.3 Effect of Taurine on GSH Level

We assessed GSH content using a commercial kit. Fig. 3 shows that paraquat exposure markedly reduced the level of GSH, whereas taurine treatment significantly attenuated the response to paraquat.

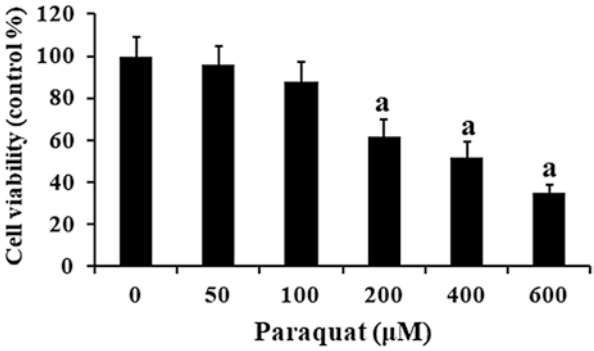

Fig. 1 Effect of paraquat on A549 cell viability. Data were presented as means ± SD. [a]$p < 0.05$, compared with control group

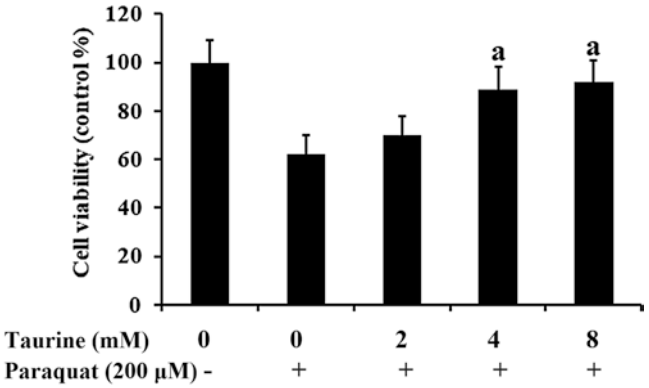

Fig. 2 Effect of taurine on paraquat-mediated decline in cell viability. A549 cells were pretreated with taurine prior to paraquat exposure. After 24 h, the MTT assay was performed to examine cell viability. Data were presented as means ± SD. [a]$p < 0.05$, compared with the control group

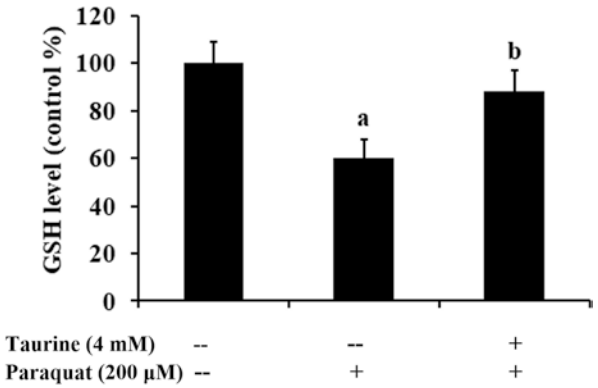

Fig. 3 Effect of taurine on GSH content of paraquat-exposed A549 cells. Data represent means ± SD. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with paraquat group

3.4 Effect of Taurine Treatment on GPx Activity

Figure 4 shows the effect of taurine treatment on GPx activity in paraquat-exposed cells. The results show that paraquat exposure markedly decreased the activity of GPx, while taurine treatment significantly attenuated the reduction, indicating a beneficial role of taurine against oxidative stress.

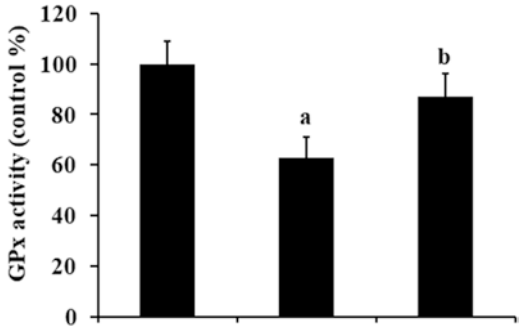

Fig. 4 Effect of taurine exposure on GPx activity of paraquat-treated A549 cells. Data are presented as means ± SD. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

4 Discussion

The present study examined the effect of taurine on paraquat-induced injury and the mechanism underlying the effect. It has been known that paraquat causes lung toxicity (Meng et al. 2013; Men et al. 2010). To support the conclusion, A549 cells were exposed to various concentrations of paraquat for 24 h before examining cell viability using the MTT assay. When compared with the control cells, the viability of A549 cells was decreased by paraquat in a dose-dependent manner. Cell viability was reduced ~55% at a paraquat concentration of 0.2 mM. However, cells pretreated with taurine prior to exposure to paraquat exhibit marked improvement in cell viability in comparison to cells treated with paraquat without taurine.

The over-production of free radicals and development of oxidative stress plays a vital role in the onset and development of toxicity of many toxicants (Zhang et al. 2014; Mershiba et al. 2013; Gao et al. 2013). It has been shown that GSH neutralizes the effects of several toxicants in lungs. Tobli et al. reported that iron-containing compounds can cause lipid peroxidation and the reductions in the activity of antioxidant enzymes in the lungs of rats (Toblli et al. 2017). Bai et al. reported that methamphetamine enhanced the level of reactive oxygen species (ROS) and decreased GSH level in the rat lungs (Bai et al. 2017). In the present study, our results showed that parquet reduced the level of GSH and the activity of GPx in A549 cells. These findings indicate that paraquat may result in cell injury by the disturbance of antioxidant system.

Taurine, the non-essential amino acid, is one of the important agents which preventing oxidative stress, modulate glutamatergic signaling and regulate osmolytes (Higuchi et al. 2012; Budhram et al. 2013). More important, taurine has protective effects against many kinds of lung injury(Men et al. 2010; Schuller-Levis et al. 2003; Das et al. 2009). In this study, the results showed that taurine exported protective effects on paraquat-induced injury and markedly inhibited the reduction of GSH and preserved of GPx activity. It has been suggested that taurine treatment protects against paraquat-induced cell injury, which relate to its antioxidant properties.

5 Conclusion

In summary, the present study shows that taurine treatment significantly attenuates the decrease in cell viability of paraquat-exposed A549 cells. Taurine also reverses paraquat-induced lowering of GSH levels and GPx activity. Thus, taurine protects against paraquat-induced damage to A549 cells, an effect that may involve the regulation of oxidative stress.

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Taurine Protects Against Arsenic-Induced Apoptosis Via PI3K/Akt Pathway in Primary Cortical Neurons

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Abstract Arsenate, a well known toxicant, can induce injury in nerve system via oxidative stress and apoptosis. This study was designed to explore the protective effect of taurine against arsenite-induced neurotoxicity and its related mechanism in primary cortical neurons. The cells were treated with arsenite with or without taurine. Twenty-Four hours later, cell viability was examined using the MTT assay. The activity of caspase-3 was analyzed and the level of Akt and p-Akt were examined by western blot. The results show that taurine treatment significantly attenuates the decrease in cell viability of arsenite-exposed primary cortical neurons. Taurine also reversed the arsenite-induced increase in caspase-3 activity. The decrease in p-Akt levels induced by arsenite exposure was prevented by taurine treatment. Thus, taurine attenuated the effect of arsenite on primary cortical neurons, an effect that may involve the Akt pathway.

Keywords Taurine · Arsenic · PI3K/AKT · Apoptosis

Abbreviations

As	arsenic
DMSO	dimethyl sulfoxide

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1 Introduction

Being a well known environmental contaminant, arsenic (As) is widely distributed, being present in nearly every country of the world. The metalloid was contained in contaminated drinking water, soil, fish and occupational poisons (Carlin et al. 2016). Both organic and inorganic arsenic compounds threaten the health of millions of people and as such has become a major public health issue throughout the world (Hata et al. 2012). Various epidemiologic investigations and lab studies have shown that As exposure affects a myriad of targets with multiple endpoints, such as cancer, neurologic deficits, psychiatric problems, kidney disease, diabetes, cardiovascular disease, respiratory outcomes, and reproductive abnormalities (Kuo et al. 2013; Navas-Acien et al. 2005; Peters et al. 2015). Recent evidence indicates that As exposure is linked to damage of neurons (Yorifuji et al. 2016; Chen et al. 2015; Liu et al. 2012). Several research studies show that apoptosis takes part in the onset and development of As-induced neurotoxicity.

Taurine, a sulfur-containing-β-amino acid, is present in many mammalian tissues as a free intracellular amino acid (Batista et al. 2013). A wide range of studies have shown that taurine treatment reduces inflammation, fibrosis, apoptosis and hyperplasia in various organs (Li et al. 2017; Zhang et al. 2014; Men et al. 2010). It protects many tissues and organs against oxidative stress and toxicity induced by various poisonous substances (Das et al. 2009; Sun et al. 2014; Men et al. 2010). Taurine is considered an attractive candidate to relieve As-induced neurotoxicity.

In the present study, the effect of arsenite and taurine on the viability of primary cortical neurons was assessed using the MTT assay. Apoptosis was assessed by measuring caspase-3 activity in taurine/arsenite-treated cells. The aim of the study was to investigate the beneficial effect of taurine on As-induced neurotoxity in primary cortical neurons.

2 Methods

2.1 Primary Cortical Neuron Culture and Treatment

Primary cortical neurons were obtained from embryonic rat brains and characterized according to methods reported previously (Teng et al. 2013) with slight modifications. Briefly, embryos were collected from pregnant rat at day 16–18. Brains were isolated and kept in basal media eagle containing 26.8 mM glucose, 2 mM glutamine, 20% fetal bovine serum at 37 °C for 10 min with a gentle shaking. Then, the cortices were passed through a 14-G cannula and dissociated. The resulting suspension was centrifuged at 200 × g for 5 min and cell pellet was collected. Cells were resuspended and seeded on poly-D-lysine (5 ug/ml) precoated dish in an incubator. Sodium arsenite was exposed on the second day with or without taurine for 24 h.

2.2 MTT Assay

Cell viability was assessed by examining the level of MTT reduction. Briefly, 10 μl of 5 mg/ml MTT solution (in PBS) was added to each well of a 96-well plate, and the cells were incubated at 37 °C for 4 h. Then, 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Following incubation for 30 min, the absorbance was read at 570 nm. Cellular viability was expressed as a percentage relative to the control (Control %).

2.3 Caspase-3 Activity Assessment

Caspase-3 activity was examined with a commercial kit (Beyotime, China) according to the protocols. The absorbance was measured at 405 nm and expressed as a percentage relative to the control (control %).

2.4 Western Blot

Cells were homogenized in lysis buffer with 1% proteinase inhibitors. The total cell lysis was loaded for SDS-PAGE to separate various proteins, then transferred to a PVDF membrane. The membrane was incubated with Akt or p-Akt primary antibodies (1:1000, Cell Signaling Technology, USA) overnight at 4 °C. Second horseradish peroxidase-conjugated antibody (1:5000, Sigma, USA) was used for visualizing.

2.5 Statistic Analysis

Data were analyzed with SPSS 11.0. Difference between various groups was analyzed by one-way ANOVA and LSD test.

3 Results

3.1 Effect of as Exposure on Cell Viability

To confirm the toxicity of As on primary cortical neurons, neurons were incubated with medium containing 0, 1, 3, 5, 7, 9 μM arsenite for 24 h. As shown in Fig. 1, viability of neurons was decreased in a dose-dependent manner by As exposure. Cell viability was reduced nearly 55% at a concentration of 5 μM arsenite. Therefore, 5 μM arsenite was used in subsequent experiments.

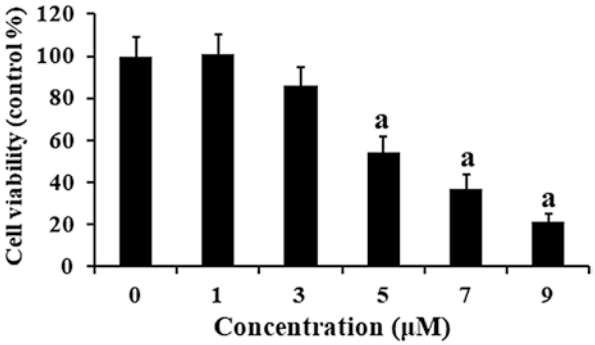

Fig. 1 The viability of arsenite-exposed primary cortical neurons. Data are represented as means ± SD. [a]$p < 0.05$, compared with the control group

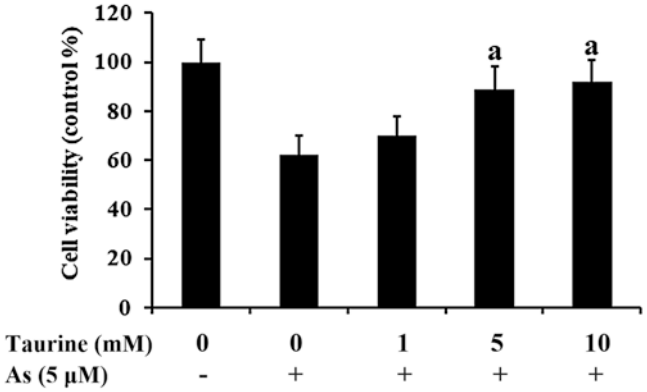

Fig. 2 Effect of taurine on arsenite-mediated drop in cell viability. Primary cortical neurons cells were co-treated with taurine and arsenite for 24 h. The MTT assay was performed to examine cell viability. Data represent means ± SD. [a]$p < 0.05$, compared with arsenite group

3.2 Effect of Taurine Treatment on Cell Viability

To assess the action of taurine on arsenite-induced neurotoxicity, primary cortical neurons were co-treated with taurine and arsenite. As shown in Fig. 2, cell viability decreased after 24 h of arsenite exposure, indicating that arsenite is toxic to neurons. However, taurine treatment significantly attenuated the decline in viability mediated by arsenite, suggesting that taurine functions as a cytoprotective agent against arsenite toxicity.

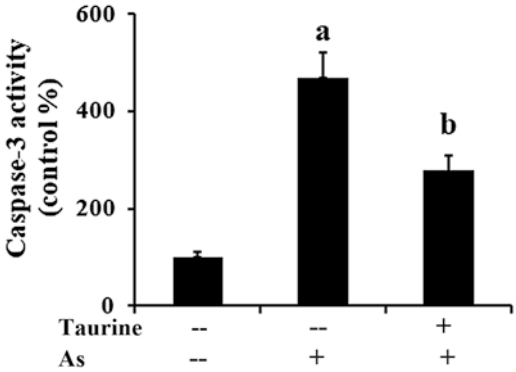

Fig. 3 Effect of taurine treatment on caspase-3 activity in arsenite-exposed primary cortical neurons. Data represent means ± SD. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with arsenite group

3.3 Effect of Taurine on Caspase-3 Activity

We assessed the activity of caspase-3 using a commercial kit. As shown in Fig. 3 arsenite exposure markedly increased caspase-3 activity, an effect significantly attenuated by taurine treatment.

3.4 Effect of Taurine Treatment on Akt and p-Akt Levels

We subsequently detected the effect of taurine on the levels of total Akt ltersand phosphorylated Akt (p-Akt) in primary cortical neurons. Figure 4 shows that neither arsenite exposure nor taurine treatment alters total Akt content. Compared with the control group, the level of p-Akt decreased significantly in arsenite-exposed primary neurons. Taurine treatment markedly reduced the response of arsenite, indicating that the Akt pathway may contribute to the beneficial effect of taurine on arsenite-induced neurotoxicity.

4 Discussion

Apoptosis plays an important role in the neurotoxicity of various toxicants (Li et al. 2017; Mao et al. 2016; Ghooshchian et al. 2017; Dai et al. 2017). It has been reported that the regulation of apoptosis is a positive means to protect disturbed nerves. Taurine, a well-known antioxidant, is potentially anti-apoptotic (Li et al. 2017; Das et al. 2009). In this study, the effect of taurine on arsenite-exposed neurons was assessed. Primary cortical neurons were exposed for 24 h to arsenite with or without taurine, and the cell viability was examined using MTT assay. When compared with the control cells, cell viability of arsenite-treated primary cortical neurons decreased in a dose-dependent manner. Interestingly, taurine co-treatment significantly

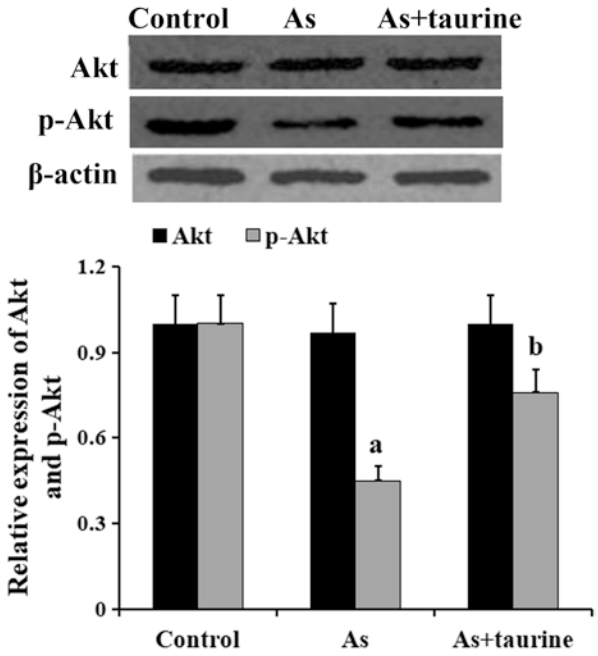

Fig. 4 Effect of taurine on Akt and p-Akt levels in arsenite-exposed primary cortical neurons. Data represent means ± SD. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with As group

inhibited the decrease of cell viability in arenite-exposed neurons. Das et al. reported that taurine protected against $NaAsO_2$-induced apoptosis and oxidative stress in rat testes (Das et al. 2009). It was reported that taurine attenuated apoptosis in the lung of a limb ischemia reperfusion rat (Men et al. 2010), supporting our results.

To establish the effect of taurine on arsenite-induced apoptosis, caspase-3, a mediator of apoptosis, was examined. The results show that arsenite exposure is associated with the increase in caspase-3 activity, while the effect was prevented by taurine. We also found that arsenite exposure induced a decrease in Akt phosphorylation level in primary cortical neurons. While the inhibited effect of arsenite in neurons was abolished by taurine treatment. These results suggest a possible link between the Akt pathway and the anti-apoptotic effect of taurine in arsenite-exposed primary cortical neurons. In future, we will block the activation of Akt and assess the cytoprotective activity of taurine to provide more evidence.

5 Conclusion

In summary, the present study shows that taurine treatment largely prevents the decrease in cell viability of arsenite-exposed primary cortical neurons. Taurine also attenuates arsenite-induced activation of caspase-3 activity and the decrease in Akt

phosphorylation. Thus, taurine treatment protects against arsenite-mediated apoptosis, an effect that may involve the activation of the Akt pathway.

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Thiotaurine: From Chemical and Biological Properties to Role in H_2S Signaling

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Abstract In the last decade thiotaurine, 2-aminoethane thiosulfonate, has been investigated as an inflammatory modulating agent as a result of its ability to release hydrogen sulfide (H_2S) known to play regulatory roles in inflammation. Thiotaurine can be included in the "taurine family" due to structural similarity to taurine and hypotaurine, and is characterized by the presence of a sulfane sulfur moiety. Thiotaurine can be produced by different pathways, such as the spontaneous trans-sulfuration between thiocysteine – a persulfide analogue of cysteine – and hypotaurine as well as in vivo from cystine. Moreover, the enzymatic oxidation of cysteamine to hypotaurine and thiotaurine in the presence of inorganic sulfur can occur in animal tissues and last but not least thiotaurine can be generated by the transfer of sulfur from mercaptopyruvate to hypotaurine catalyzed by a sulfurtransferase. Thiotaurine is an effective antioxidant agent as demonstrated by its ability to counteract the damage caused by pro-oxidants in the rat. Recently, we observed the influence of thiotaurine on human neutrophils functional responses. In particular, thiotaurine has been found to prevent human neutrophil spontaneous apoptosis suggesting an alternative or additional role to its antioxidant activity. It is likely that the sulfane sulfur of thiotaurine may modulate neutrophil activation via persulfidation

This work is dedicated to the memory of Professor Doriano Cavallini and Professor Carlo De Marco

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of target proteins. In conclusion, thiotaurine can represent a biologically relevant sulfur donor acting as a biological intermediate in the transport, storage and release of sulfide.

Keywords Thiotaurine · Hypotaurine · Sulfane sulfur · H_2S donor · H_2S signaling · Reactive sulfur species · Hydrogen sulfide · Antioxidant · Inflammation · Neutrophils

Abbreviations

APAP	Acetaminophen
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CDO	Cysteine dioxygenase
CN^-	Cyanide
CSAD	Cysteine sulfinate decarboxylase
CSE	Cystathionine γ-lyase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
GSSH	Glutathione persulfide
H_2S	Hydrogen sulfide
HSSH	Hydrogen persulfide
MDA	Malondialdehyde
MST	Mercaptopyruvate sulfurtransferase
NAC	*N*-acetylcysteine
PLP	Pyridoxal 5′-phosphate
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
RS^-	Thiolate anion
RSH	Thiol
RSO_2H	Sulfinate/Hypotaurine
RSO_2SH	Thiosulfonate/Thiotaurine
RSO_3H	Sulfonate/Taurine
RSOH	Sulfenate
RSSH	Persulfide/Thiocysteine
RSS_nSR	Polysulfide
RSSR	Disulfide
S^0	Zero-valent sulfur
S^{2-}	Sulfide
$-S_2O_2^-$	Thiosulfonate group
$S_2O_3^{2-}$	Thiosulfate
S_8	Elemental sulfur
SCN^-	Thiocyanate
SO_3^{2-}	Sulfite
STZ	Streptozotocin

1 Introduction

Thiotaurine, 2-aminoethane thiosulfonate, is a sulfur containing compound characterised by the presence of a thiosulfonate group ($-S_2O_2^-$) containing one sulfur bound to another sulfur atom, often referred as sulfane sulfur (Fig. 1).

Due to structural similarity to sulfinate and sulfonate related compounds, respectively hypotaurine (RSO_2H) and taurine (RSO_3H), thiotaurine (RSO_2SH) can be included in the "taurine family" (Fig. 2).

Interestingly, the presence of the sulfane sulfur modulates the biological properties of thiotaurine compared to that of sulfur biomolecules structurally related (Westley and Heyse 1971; Luo and Horowitz 1994; Acharya and Lau-Cam 2013; Capuozzo et al. 2015). Thiotaurine is a cysteine-derived metabolite, discovered in 1957 by Sörbo while studying the enzymatic reaction between mercaptopyruvate and sulfite in which thiosulfate is formed. When sulfite, as sulfur acceptor, is replaced in this transsulfuration reaction by the structurally related sulfinates, thiosulfonates are formed instead of thiosulfate. In particular, thiotaurine is generated by a sulfurtransferase that catalyzed the transfer of sulfur from mercaptopyruvate to hypotaurine (Scheme 1).

Afterwards, in 1959, Cavallini and co-workers (1959a) firstly reported the biological occurrence of thiotaurine in mammals as a metabolic product of cystine in vivo, by demonstrating that rats fed with a diet supplemented in cystine excreted taurine, hypotaurine and a newly unknown compound identified as the thiosulfonate analogue of taurine, thiotaurine. Thiotaurine was also detected by autoradiography of rat kidney in rats injected with ^{35}S-cystine (Cavallini et al. 1960a). Thiotaurine was also reported in 1986 in deep-sea symbiotic mussels in high concentrations along with hypotaurine and taurine (Alberic 1986), where its role seems to be related to the metabolism of the symbionts (Pruski et al. 1997).

The aim of the present review is to sum up the current knowledge of the biochemical properties of thiotaurine as well as looking to its antioxidant properties and to highlight the potential biological significance of thiotaurine in the H_2S signaling mechanisms.

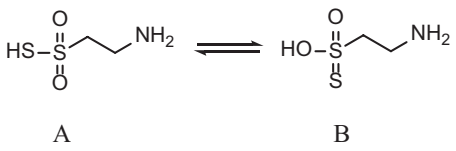


Fig. 1 Thiotaurine: thiosulfonate (**A**) and thiosulfoxide (**B**) tautomers

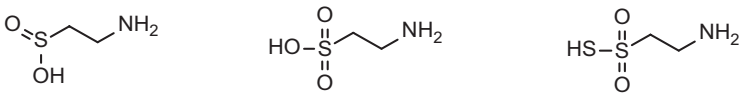
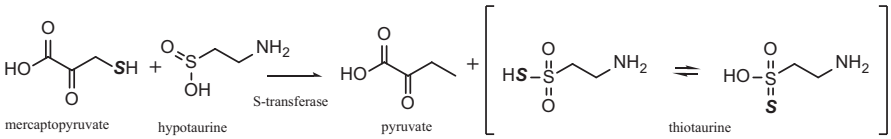


Fig. 2 Taurine family



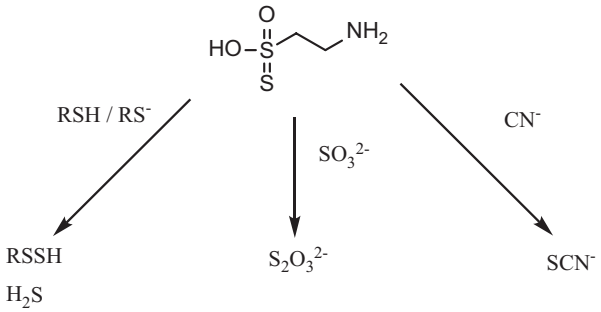
Scheme 1 Thiotaurine synthesis catalyzed by sulfurtransferase

2 Chemistry and Biochemistry of Thiotaurine

Thiotaurine contains a highly reactive sulfur atom, which has been defined in different ways, such as "zero-valent sulfur (S^0)", "sulfane sulfur", and "sulfur-bonded sulfur". Even if the chemical lability of sulfane sulfur has not yet been clarified, it seems to be correlated to its presence in a thiosulfoxide form (−S=S), either by structure, as in thiosulfate, or by tautomerization (Fig. 1) (Kutney and Turnbull 1982; Toohey 1989; Toohey 2011; Toohey and Cooper 2014). The sulfur in the thiosulfoxide form exists in an oxidation state of zero and it is easily removed by nucleophilic acceptors such as thiolate anion (RS^-), sulfite (SO_3^{2-}) and cyanide (CN^-) whereas, sulfane sulfur is also often named cyanolysable sulfur (Iciek and Włodek 2001; Toohey 2011). The thiosulfoxide form is a weak bond (Steudel et al. 1997) where the sulfur atom can be released as elemental sulfur and transferred to another sulfur atom or reduced to hydrogen sulfide (H_2S) by thiols, such as glutathione (GSH). Thiotaurine could exist in the tautomeric thiosulfoxide form that would then act as a perfect sulfane sulfur donor, however, neither experimental nor computational data support the existence of this tautomeric form. Anyhow, sulfane sulfur of thiotaurine can be readily removed and transferred to an appropriate acceptor (Westley and Heyse 1971). This transferable sulfur atom has an electrophilicity index intermediate between that of thiosulfate ion and that of the most reactive sulfur compounds including hydropersulfide, simply called here persulfide, (RSSH) and polysulfide (RSS_nSR). The sulfur-sulfur bond of thiosulfonates is readily cleaved by simple thiol compounds forming persulfide or by reduction to H_2S by thiols. Interestingly, it has been reported that the positive charged ammonium group of thiotaurine confers an advantage in this reactivity by diminishing the electron density of the sulfur-sulfur bond (Chauncey and Westley 1983) (Scheme 2).

From a biochemical point of view, thiotaurine is an intermediate in the cysteine/cystine metabolic pathway. Cysteine is the main source of sulfur in the animal and human body. It is metabolized via two metabolic routes. The first one, called the cysteine sulfinate-dependent (aerobic) pathway, is a series of oxidative steps leading to hypotaurine and taurine. The second one, a transsulfuration path, is independent of cysteine sulfinate (anaerobic pathway) and is a source of sulfane sulfur-containing compounds as well as hydrogen sulfide/sulfide (H_2S/S^{2-}) (Stipanuk and Beck 1982; Stipanuk and Ueki 2011).

As thiotaurine is formed by the direct reaction of hypotaurine with sulfide (Cavallini et al. 1963), it can act as a link between the aerobic and anaerobic metabolism of cysteine. Hypotaurine, the metabolic precursor of thiotaurine, is synthesized



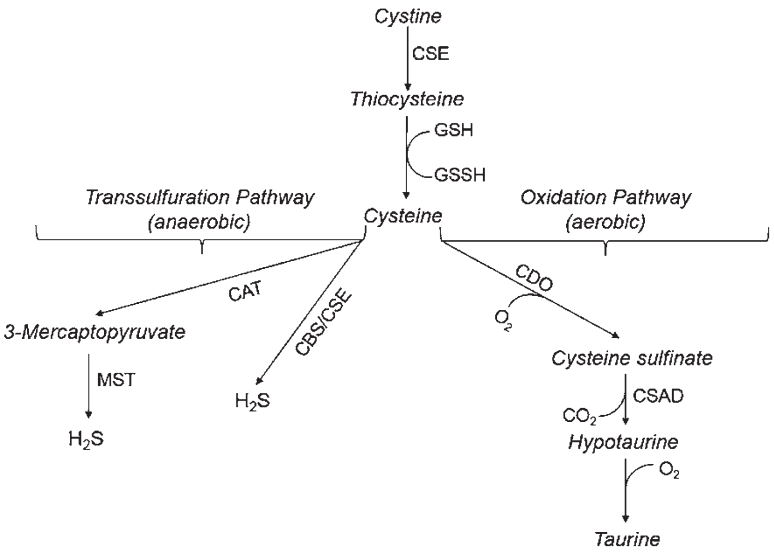
Scheme 2 Thiotaurine spontaneous organic/inorganic transsulfuration reactions

by the combined action of cysteine dioxygenase (CDO) that oxidizes the thiol group of cysteine to form cysteine sulfinate and of cysteine sulfinate decarboxylase (CSAD) that subsequently generates hypotaurine. Hydrogen sulfide (H_2S) is produced mainly from desulfhydration of L-cysteine (Stipanuk and Ueki 2011). The enzymes involved in the H_2S production include pyridoxal 5′-phosphate (PLP)-dependent cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) as well as cysteine aminotransferase (CAT) in conjunction with PLP-independent mercaptopyruvate sulfurtransferase (MST) (Scheme 3) (Kabil and Banerjee 2014; Beltowski 2015).

3-Mercaptopyruvate formed by the CAT-catalysed transamination of L-cysteine can play the role of the sulfur donor for different acceptors, including thiols and sulfite/sulfinates with the formation of persulfides and thiosulfate/thiosulfonates, respectively (Nagahara and Sawada 2006; Hildebrandt and Grieshaber 2008; Yadav et al. 2013). In turn, thiocysteine (the persulfide analogue of cysteine, RSSH) is produced from cystine by transsulfuration enzymes, CSE and CBS (Cavallini et al. 1960b, 1962a, b; Chiku et al. 2009; Singh et al. 2009; Majtan et al. 2018). Consequently, the sulfane sulfur of thiocysteine can be transferred to various acceptors (i.e. sulfite, hypotaurine). Rhodanese, an important enzyme known for its ability to utilize sulfane sulfur in cyanide detoxification, transfers sulfur from thiocysteine (RSSH) to sulfite or hypotaurine (RSO_2H) to generate thiosulfate or thiotaurine (RSO_2SH), respectively, and cysteine (RSH) (Reaction 1) (Cavallini et al. 1960b; Luo and Horowitz 1994).

$$SO_3^{2-} / \text{Hypotaurine} + \text{RSSH} \rightarrow S_2O_3^{2-} / \text{Thiotaurine} + \text{RSH} \quad (1)$$

According to this pathway, thiotaurine is produced in vivo from cystine (Cavallini et al. 1959a, 1960a) and is generated spontaneously by transsulfuration between the persulfide analogue of cysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961). In several animal tissues, thiol oxidation to sulfinates and thiosulfonates can enzymatically occur when inorganic sulfur is present (De Marco and Tentori 1961; Cavallini et al. 1961, 1963). Furthermore, thiotaurine is formed by a sulfurtransferase catalyzing sulfur transfer from mercaptopyruvate to hypotaurine (Sörbo 1957; Sörbo 1958; Chauncey and Westley 1983).



Scheme 3 Metabolic pathways of cystine/cysteine

3 Protective Roles of Thiotaurine

Different studies have been carried out to investigate the antioxidant activity of thiotaurine compared with taurine or hypotaurine, which are both known as powerful antioxidants (Acharya and Lau-Cam 2013; Budhram et al. 2013; Mathew et al. 2013; Pandya et al. 2013; Rosenberg et al. 2006; Yancey et al. 2002, 2009; Joyner et al. 2003; Inoue et al. 2008; Chaimbault et al. 2004).

One of the factors that seem to play a relevant role in the development of diabetic complications is the hyperglycemia and its close relation with the development of oxidative stress. In particular, hyperglycemia influences the production of reactive oxygen species (ROS) at the mitochondrial electron transport chain level (Brownlee 2005). To manage diabetes and its complications, numerous antioxidants can be used to improve the response against the excess of ROS during oxidative stress (Rahimi et al. 2005). Extensive work has been carried out to understand the role of thiotaurine as antioxidant in diabetic rats, from the biochemical and cellular alteration to the damage caused to the aorta and heart, and to the effect to on nephropathy associated with this disease (Budhram et al. 2013; Mathew et al. 2013; Pandya et al. 2013). Taurine has received extensive evaluation due to its ability to protect against oxidative damage and lately, also thiotaurine has been studied to understand the role of thiosulfonate group compared to the sulfonate group of taurine on diabetes (Acharya and Lau-Cam 2010; Acharya and Lau-Cam 2013).

The researches carried out by Lau-Cam and his collaborators evaluated the role of thiotaurine in male Sprague-Dawley rats treated with streptozotocin (Budhram

et al. 2013; Mathew et al. 2013; Pandya et al. 2013) to induce diabetes or with acetaminophen (APAP) to induce liver damage (Acharya and Lau-Cam 2013) targeting different biochemical and cellular compounds.

The protective effect of thiotaurine against hepatocellular damage has been investigated in male rats treated with a high dose of APAP, that can cause depletion of glutathione and of protein thiol groups and consequently a high oxidative/nitrative stress condition (Acharya and Lau-Cam 2013). The liver damage was analysed by evaluating plasma aminotransferases and lactate dehydrogenase and also oxidative stress indices such as malondialdehyde (MDA), catalase, reduced glutathione, superoxide dismutase, glutathione disulfide, peroxidase in plasma and liver homogenates from rats treated intraperitoneally with 2.4 mmol/kg dose of thiotaurine or taurine and using N-acetylcysteine (NAC) as comparison. The results showed a protective action of thiotaurine comparable to that exerted by NAC and better than that of taurine (Acharya and Lau-Cam 2013). Indeed, the MDA level in liver was attenuated in the presence of thiotaurine the reduction of glutathione/glutathione disulfide ratio was preserved.

In the three studies using the streptozotocin (STZ) (60 mg/kg, i.p., for 2 weeks), chronic type 2 diabetes has been induced in male Sprague-Dawley rats. From 15 days after STZ treatment and for 6 weeks, a daily dose of thiotaurine or taurine (2.4 mmol/kg) has been administered and isophane insulin at 4 U/kg as reference group has been used. On day 57 the rats were sacrificed and blood, plasma, urine, heart and thoracic aorta as well kidney samples have been collected. Different parameters have been tested such as the glutathione redox status, insulin and glycated hemoglobin levels, antioxidant enzymes activities, cholesterol and triglycerides, malondialdehyde, creatinine, sodium and potassium levels. Data from all these studies demonstrate that thiotaurine has generally an effect comparable to insulin against the changes associated with diabetes. A greater effect of thiotaurine has been shown on hyperglycemia, hypoinsulinemia and hyperlipidemia compared with taurine. These findings suggest a better antioxidant effect of thiotaurine due to the presence of the thiosulfonate group.

Studies performed on deep-sea animals have detected a high level of thiotaurine as well hypotaurine (Pruski et al. 1997; Yancey et al. 2009). These two sulfur amino acids seem to serve as osmolytes, to balance internal osmotic pressure with that of the ocean but mainly, thiotaurine seems to transport and/or detoxify sulfide (Rosenberg et al. 2006; Yancey et al. 2002). The studies suggested that hypotaurine could interact with the excess of sulfide, through a hypothesis not supported by experimental data where the oxygen of hypotaurine sulfinic group should interact with the sulfide radical to form thiotaurine. Being a toxic compound, hydrogen sulfide can interact with iron and consequently bind proteins resulting in cellular damage (Inoue et al. 2008).

The presence of thiotaurine in these animals seems to be correlated to their need to decrease the level of sulfide, consequently thiotaurine can be included in the mechanisms developed to contrast the presence of sulfide radical in the deep-sea animals. Moreover, thiotaurine has been proposed as a marker in animals with a

sulfide-based symbiosis. Inoue and co-workers (2008) have also proved that the taurine transport in the deep-sea mussel Bathymodiolus septemdierum has an affinity for thiotaurine and hypotaurine suggesting an involvement of this transporter in sulfide detoxification.

4 Thiotaurine: H_2S Donor/Reactive Sulfane-Sulfur Species

Thiotaurine (RSO_2SH) has the ability to release hydrogen sulfide (H_2S) in a thiol-dependent reaction. In particular, a thiosulfate reductase activity occurring in various animal cells uses electrons of thiols, such as glutathione, to reduce sulfane sulfur of thiosulfonates, such as thiotaurine (Koj et al. 1967; Chauncey and Westley 1983; Hildebrandt and Grieshaber 2008). The reaction mechanism involves the formation of a persulfide (RSSH) (Reaction 2) that spontaneously releases hydrogen sulfide (Reaction 3) in the presence of excess thiols (RSH).

$$RSO_2SH + RSH \rightarrow RSO_2H + RSSH \tag{2}$$

$$RSSH + RSH \rightarrow RSSR + H_2S \tag{3}$$

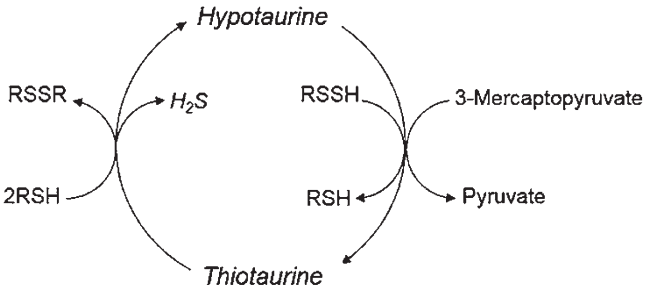
In general, H_2S can be easily released from sulfane sulfur compounds in the presence of reducing agents (Mikami et al. 2011). Accordingly, it has been observed that human neutrophils generate H_2S and hypotaurine (RSO_2H) from thiotaurine with GSH as catalyst (Capuozzo et al. 2013). As a result, thiotaurine can be considered a thiol-activated H_2S-donor molecule.

In absence of thiols, thiotaurine is quite stable, but exposed to irradiation decomposes to hypotaurine and elemental sulfur (Reaction 4) (Cavallini et al. 1959b).

$$RSO_2SH \rightarrow RSO_2H + S_8 \tag{4}$$

It is possible that thiotaurine is also involved in the sulfide oxidation pathways. In this reactions, H_2S is oxidized by the mitochondrial sulfide:quinone oxidoreductase (SQR) generating a protein-bound persulfide, which is subsequently transferred to acceptors, such as GSH or sulfite/sulfinate, resulting in the formation of GSH persulfide (GSSH) or thiosulfate/thiosulfonate (Kabil and Banerjee 2010, 2014; Jackson et al. 2012). In the latter case, the sulfane sulfur of thiosulfate/thiosulfonate can be remobilized by a sulfurtransferase, including thiosulfate reductase as in the Reactions 2 and 3 (Scheme 4).

According to these reactions, there is a close relationship between H_2S and the sulfane sulfur compounds, which are regarded as a large and highly regulated physiological H_2S reservoir in biological systems (Kimura 2011). In this regard, thiotaurine with its sulfane sulfur moiety can be part of the sulfur store pool and represent



Scheme 4 Transsulfuration pathway of thiotaurine

a biologically relevant sulfur donor. Recently, sulfane sulfur and H_2S have been included in the family of reactive sulfur species (RSS). The initial concept of RSS postulated by Giles et al. (2001) has now expanded to include a variety of sulfur-containing secondary metabolites, such as reactive sulfane-sulfur species, and consequently, thiotaurine can be considered as a reactive sulfane-sulfur species with regulatory functions in the cells (Giles et al. 2017). Besides thiosulfonates such as thiotaurine, the group of biologically reactive sulfane-sulfur species includes persulfide (RSSH) and hydrogen persulfide (HSSH), thiosulfate ($S_2O_3^{2-}$), organic (RSS_nSR, $n \geq 1$) and inorganic (H_2S_n, $n \geq 3$) polysulfides, and elemental sulfur (S_8).

5 Thiotaurine Involvement in H_2S Signaling

The biological role of thiotaurine in the cell regulatory processes resides in the close relationship between its ability to release H_2S in a thiol-dependent mechanism and its property of bearing a sulfane sulfur atom. Owing to these characteristics, thiotaurine takes part in the H_2S signaling pathways. H_2S is a gaseous signaling molecule able to act as neurotransmitter, modulator of inflammation, vasorelaxant (Abe and Kimura 1996; Zanardo et al. 2006; Yang et al. 2008; Whiteman and Winyard 2011; Predmore et al. 2012). However, the actual mechanism of H_2S-mediated signaling remains to be fully understood. One proposed mechanism whereby H_2S signals is the modification of protein cysteine residues by persulfidation, also called S-sulfhydration (Mustafa et al. 2009; Paul and Snyder 2012; Filipovic et al. 2018). Therefore, the transmission of sulfide-based signals includes activation or inactivation of enzymes via post-translational modification of reactive cysteine thiols (RSH) to persulfides (RSSH) (Toohey 2011; Yadav et al. 2016). To ensure that this post-translational modification occurs, H_2S must react with oxidized protein cysteine residues, such as sulfenates (RSOH) (Reaction 5) or disulfides (RSSR) (Reaction 6) to give persulfides (RSSH) (Kabil and Banerjee 2010; Finkel 2012; Francoleon et al. 2011; Cuevasanta et al. 2015).

$$\mathrm{RSOH} + \mathrm{H_2S} \rightarrow \mathrm{RSSH} + \mathrm{H_2O} \tag{5}$$

$$\mathrm{RSSR} + \mathrm{H_2S} \rightarrow \mathrm{RSSH} + \mathrm{RSH} \tag{6}$$

However, the reaction of disulfides with H_2S is unlikely to occur under physiological conditions, due to the low concentration in vivo and the non-sufficient reduction potential of H_2S (Kabil and Banerjee 2010; Toohey 2011). Conversely, sulfane sulfur (S^0) can be readily transferred to thiols (Reaction 7) and disulfides (Reaction 8) with the formation of persulfides and trisulfides, respectively, via the thiosulfoxide (−S=S) tautomer as transient intermediate (Toohey 1989; Toohey and Cooper 2014).

$$\mathrm{RSH} + \mathrm{S^0} \rightarrow \mathrm{RS(S)H} \rightarrow \mathrm{RSSH} \tag{7}$$

$$\mathrm{RSSR} + \mathrm{S^0} \rightarrow \mathrm{RS(S)SR} \rightarrow \mathrm{RSSSR} \tag{8}$$

Analogously, sulfane sulfur and a thiosulfoxide intermediate are involved in the direct reaction of thiols with H_2S (Chen and Morris 1972; Kotronarou and Hoffmann 1991; Toohey 2011). The reaction can be considered the combination of two reactions: the reaction of H_2S with oxygen to give rise to sulfane sulfur; and the transfer of the sulfane sulfur to thiols (Reaction 9).

$$\mathrm{RSH} + \mathrm{H_2S} + \tfrac{1}{2}\,\mathrm{O_2} \rightarrow \mathrm{RSSH} + \mathrm{H_2O} \tag{9}$$

Recent studies indicate that reactive intermediates other than H_2S react with thiols to generate persulfides (Ida et al. 2014; Mishanina et al. 2015). These reactive sulfur species are formed during sulfide oxidation reactions and sulfur amino acid metabolism. Some of these reactive intermediates have been identified among the sulfane sulfur compounds. In this regard, thiotaurine with its sulfane sulfur moiety can represent a biologically relevant sulfur donor intermediate in protein cysteine persulfidation reactions (Reactions 10 and 11).

$$\mathrm{RSO_2SH} + \mathrm{RSH} \rightarrow \mathrm{RSO_2H} + \mathrm{RSSH} \tag{10}$$

$$\mathrm{RSO_2SH} + \mathrm{RSSR} \rightarrow \mathrm{RSO_2H} + \mathrm{RSSSR} \tag{11}$$

Interestingly, persulfidation reactions occur at high level in the mitochondria during the H_2S oxidation pathway, where protein-bound persulfides and GSSH are formed (Jackson et al. 2012). Mitochondrial rhodanese-catalyzed reactions generate other sulfane sulfur intermediates, such as thiosulfates and possibly thiosulfonates from the sulfane sulfur of persulfides (Reaction 12) (Sörbo 1958; Hildebrandt and Grieshaber 2008; Libiad et al. 2014).

$$\mathrm{SO_3^{2-}} / \mathrm{RSO_2H} + \mathrm{RSSH} \rightarrow \mathrm{S_2O_3^{2-}} / \mathrm{RSO_2SH} + \mathrm{RSH} \tag{12}$$

Taking into account these reactions, sulfide oxidation pathways can be considered a way for generation of sulfane sulfur compounds, such as persulfides, polysulfides, thiosulfate and thiosulfonates. Sulfane sulfur generation can be perceived as a reservoir of H_2S and a pathway for elemental sulfur release. Consequently, the thiotaurine formation can be proposed as one of the methods of H_2S storage in cells, from which it can be released by thiosulfate reductase (Reactions 2 and 3). Furthermore, the body fluid and tissue concentration of this thiosulfonate can be considered as an indicator of H_2S biogenesis in the biological systems.

6 Thiotaurine and Inflammation

In the last years, the regulatory role of thiotaurine in the innate immune response has been reported. In particular, the ability of thiotaurine to act as a bioactive compound able to regulate inflammation has been investigated in human neutrophils, cells of the innate immune response that constitute the major players during acute inflammation. Leukocytes can eliminate pathogens by two different microbicidal mechanisms: an oxygen-dependent mechanism, which produces reactive oxygen species by NADPH oxidase complex, and an oxygen-independent mechanism, which acts through the production of antimicrobial peptides and proteolytic enzymes. In this context, it has been demonstrated that thiotaurine is an effective antioxidant agent due to its ability to counteract ROS generation and superoxide anion production in activated human neutrophils (Capuozzo et al. 2015). Moreover, it has been reported that thiotaurine prevents human neutrophil spontaneous apoptosis by inhibiting caspase-3 activation, suggesting an alternative or additional role to besides its antioxidant activity (Capuozzo et al. 2013). Interestingly, it has been showed that the effects exerted by thiotaurine on human neutrophil responses are significantly higher than taurine and its derivatives or related compounds (Capuozzo et al. 2015). Specifically, thiotaurine inhibits human neutrophil activation in response to PMA, a diacylglycerol substitute that activates protein kinase C (Capuozzo et al. 2015). In this regard, in a recent paper, the proteomic profiling of PMA-activated human neutrophils has been applied to study and identify proteins that change their expression level or undergo biochemical modifications after thiotaurine pre-treatment of granulocytes (Capuozzo et al. 2017). In particular, it has been demonstrated that thiotaurine affects glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and induces down-regulation of this enzyme in activated leukocytes. They suggest that thiotaurine plays an active role in the mechanisms underlying the inflammatory process influencing the energy metabolism of activated granulocytes, and propose an intriguing molecular mechanism by which thiotaurine modulates human leukocyte activation in which persulfidation of target proteins very likely occurs (Capuozzo et al. 2017).

7 Other Biological Effects of Thiotaurine

Thiotaurine participates in the endogenous detoxification of cyanide by the coupled action of two enzymes, CSE and rhodanese (Szczepkowski and Wood 1967). In this mechanism, the cyanide (CN^-) is eventually metabolized by the sulfurtransferase activity of rhodanese to the less toxic thiocyanate (SCN^-). The persulfide analogue of cysteine, thiocysteine, is produced from cystine by CSE, the first enzyme of the coupled system. Afterwards, thiocysteine transsulfurates hypotaurine to thiotaurine (Cavallini et al. 1960b). The formed thiotaurine is an excellent sulfur substrate for rhodanese-catalyzed transsulfuration of cyanide to thiocyanate (Luo and Horowitz 1994). Noteworthy, thiotaurine increases survival of mouse following a lethal dose of cyanide (Dulaney et al. 1989). Thiotaurine has been also tested in vivo as a sulfur donor for detoxification of cyanide in the chicken and it has been found that thiotaurine supported thiocyanate urinary excretion to a similar extent to other rhodanese sulfur donors (Mousa and Davis 1991). However, it has been also reported that prophylactic treatment with thiotaurine does not protect nearly as much against cyanide exposure as the sulfur donor thiosulfate (Marziaz et al. 2013).

Recently, the neuroprotective effect of thiotaurine in isolated cerebellar granule cells has been investigated. This is a well-established system to study cell survival and apoptosis. Thiotaurine protects mouse cerebellar granule cells by potassium deprivation-induced apoptosis by inhibiting caspase-3 (Dragotto et al. 2015). Thiotaurine showed also a displacing effect of ^{3}H-GABA binding to $GABA_A$ receptors in bovine brain cortical membranes (Costa et al. 1990). Thiotaurine is similar in displacing potency to taurine and hypotaurine, which for a long time have been investigated for a neurophysiological role (Oja and Kontro 1982). Interestingly, the thiotaurine higher homologue, homothiotaurine, exhibits also a good affinity for the GABA sites (Costa et al. 1990).

Several anionic sulfur compounds reduce the toxicity of mustard agents. Consequently, thiotaurine has been tested as a protective agent against DNA damage caused by sulfur and nitrogen mustards. Baskin and co-workers (2000) proposed that the interaction of thiotaurine with DNA could protect against sulfur mustard intoxication.

8 Conclusions

Thiotaurine is a thiosulfonate compound bearing a sulfane sulfur atom metabolically generated in body fluids and tissues. Thiotaurine constitutes an interconnection molecule between aerobic and anaerobic pathways of cysteine metabolism. Thiotaurine formed as a result of the reaction between hypotaurine and sulfide may be converted back to H_2S and hypotaurine. Thus, thiotaurine may be considered as a safe, non-toxic storage form of H_2S and an important key intermediate in the biochemical routes of transport, storage and release of sulfide. Sulfane sulfur-containing

compounds efficiently regulate the activity of enzymes and exhibit antioxidative properties. Interestingly, thiotaurine influences inflammatory processes modulating functional responses of human neutrophils and exhibits a protective effect against oxidative damage. In many papers is widely recognized that thiotaurine-related compounds such as taurine, hypotaurine and taurine chloramine exert a regulatory role in acute inflammation (Green et al. 1991; Marcinkiewicz and Kontny 2014; Kim and Cha 2014), whereas, thiotaurine shows a more powerful effect compared to the related compounds, hypotaurine and taurine (Capuozzo et al. 2015).

All these reasons suggest that thiotaurine is a signaling biomolecule exerting regulative functions in the cells. The influence of thiotaurine on biological cell responses can be attributed to its involvement in H_2S signaling, as H_2S donor and/or as reactive sulfane-sulfur species. Noteworthy, it has been reported that biological effects initially attributed to H_2S actually are caused by sulfane sulfur compounds (Ida et al. 2014; Mishanina et al. 2015). Protein persulfidation of key regulatory cysteine residues is considered as a major mechanism of sulfide signaling. In this regard, thiotaurine can represent a biologically relevant sulfur donor in persulfide formation in proteins. In summary, thiotaurine, due to its peculiar properties and its ability to modulate and control H_2S signal, seems to be a very interesting biomolecule whose regulatory pathways are worth to be investigated more in depth.

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Part VII
Taurine and Neuroprotection

Alteration of Placental Deiodinase 3 Expression by BDE 209 and Possible Protection by Taurine in Human Placenta-Derived JEG Cells Under Hypoxia

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Abstract Thyroid hormones are key hormones involved in growth and development. Changes in their levels can cause embryonic brain developmental damage in the first trimester. Studies have shown that polybrominated diphenyl ethers (PBDEs) have developmental neurotoxicity as environmental pollutants, and exposure during pregnancy can cause irreversible brain damage in offspring, similar to the interference effects of thyroid hormones, but its mechanism has not yet been understood. Since the physiological environment for placental cells is highly hypoxic, in the current study, the human placenta-derived JEG cells were cultured at 1% oxygen, 4% carbon dioxide and 94% nitrogen, to reflect in vivo scenario, and the possible protection of taurine on BDE 209-mediated toxicity in JEG cells was studied. Our data showed that different concentrations of BDE 209 can have profound effects on cell viability and placental deiodinase 3 expression under hypoxic culture condition. Taurine was found to improve BDE 209-induced reductions in cell viability and altered gene and protein expressions of placental deiodinases. The results

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provide a reference for the establishment of early biomarkers and effective preventive measures.

Keywords JEG cells · Hypoxia · Polybrominated diphenyl ethers (PBDEs) · Taurine · Deiodinases · Developmental neurotoxicity

Abbreviations

Dio	Deiodinase
JEG	Human trophoblastic JEG-3 cells
MTT	4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PBDEs	Polybrominated diphenyl ethers
TH	Thyroid hormone

1 Introduction

Polybrominated diphenyl ethers (PBDEs) are the general term for a large class of brominated flame retardants with 209 homologous compounds. Because of their good flame retardancy, they are being incorporated into a wide variety of commercial products, including electronic products, plastics and textiles (Mcdonald 2002). PBDEs can easily accumulate in the sediments, bioaccumulate and enrich along the food chain, and thus have been listed as persistent organic pollutants. China, a major manufacturing country, is the hardest attacked by PBDEs as a result of rapid development in the past 4 decades.

Decabromodiphenyl ether (BDE 209) is one of the PBDE congeners containing the largest number of bromine atoms in the polybrominated diphenyl ether homologue. It is used worldwide because of its low cost, superior performance and lowest acute toxicity among all brominated diphenyl ethers. BDE 209 is not only found in the environmental media, but also in human tissue samples in recent years, especially in the placenta, suggesting potential damage to embryonic and fetal development (Murphy et al. 2006). Placental tissue plays an crucial role in the delivery of nutrients during pregnancy, and its dysfunction can have adverse effects on the fetuses. Studies have shown that exposure to BDE 209 during pregnancy can lead to decreased spatial learning and spatial memory in offspring rats (female) indicating that PBDEs have developmental neurotoxicity (Tseng et al. 2013). Studies have also found that PBDEs have endocrine disrupting effects that can disrupt the thyroid system in adult and developing mammals (Stapleton et al. 2011). Thyroid hormones (TH) are closely related to the physical and the mental development. They mainly promote the growth and development of bones, brain, and changes in the TH before or during infancy have greater damage than adulthood. Congenital or juvenile lacks of TH are known to cause minor illness, brain hypoplasia and mental retardation

(Lafranchi et al. 2005). This is very similar to the developmental neurotoxic effects on offspring caused by in utero exposure to PBDEs during pregnancy. Different physiological cells of the body have different physiological requirements for TH, and thus it is necessary to regulate the TH from the circulation in the local tissue cells. Deiodinases (Dio) in the tissues play a key role in regulating the level of local TH. There are three kinds of Dio, Dio1, Dio2 and Dio3. Among them, Dio3 is highly expressed in the placenta during pregnancy, and its function is mainly to inactivate the T3 and T4 from the maternal circulation to avoid direct exposure of the fetus to the mother's high concentration of TH, which is of the significance in protecting the embryos and fetuses (Koopdonk-Kool et al. 1996). BDE 209 has been reported to induce developmental neurotoxicity both in vivo and in vitro (Qian et al. 2014). Therefore, for the health of future generations, it is indispensable to intervene the neurotoxicity induced by BDE 209 exposure.

Taurine can maintain normal physiological functions of the human body, and have the functions of promoting brain development, anti-lipid peroxidation and protecting nerve cells (Yang 2000). Taurine plays an significant role especially in the central nervous system. It can promote nerve conduction, improve intelligence, enhance and improve brain tissue development, enhance immunity, and strengthen memory (Kumari et al. 2013). Although it has been widely reported that the beneficial effects of taurine are due to its multiple effects on the cellular function, there is no information about the effect of taurine on the PBDEs-induced developmental neurotoxicity.

JEG cells are placental human chorionic trophoblast cells that under hypoxic state in vivo condition. In order to simulate the real situation in the body, in the current study, we set up an in vitro hypoxic environment (1% oxygen, 4% carbon dioxide and 95% nitrogen) to culture cells and carry out related experiments. Briefly, JEG cells were exposed to BDE 209 in vitro under hypoxia, and the effect of BDE 209 on cell proliferation and Dio3 expression were examined, and the possible protective effects of taurine were also investigated. It is expected that our study will contribute to reducing the damage of BDE 209 in the environment to the human body.

2 Methods

2.1 Chemicals

MEM (Gibco, USA); FBS (NQBB, AUS); RNAiso Plus kit (Takara, Japan); PrimeScript® RT Master Mix kits (Takara, Japan); SYBR Premix Ex TaqTM II kit (Takara, Japan); PMSF, RIPA, BCA Protein Asssay Reagent Kit (Beyotime Biotechnology, Nanjing, China); EasySee Western Blot Kit (TransGen Biotech, Beijing, China). All the other reagents were analytical grade.

2.2 Cell Culture

JEG cells are purchased from ATCC, USA. The cells were grown in minimum Eagle's medium (MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (NQBB, AUS) and 1% penicillin-streptomycin (Hyclone, USA). For continuous cell culture, cells were carefully digested with 0.25% Trypsin-EDTA (Beyotime, China), and grown in a 37 °C humid incubator at 4% CO2, 1% O2 and 95% N2.

2.3 Cell Viability

Changes in cell activity induced by BDE 209 were detected by MTT assay. The JEG cells suspension was adjusted to 5 × 104 cells/ml and inoculated into 96-well plates. The suspension was cultured for 24 h in an environment of 1% oxygen, 4% carbon dioxide and 95% nitrogen. For taurine protection, cells were pretreated with 1, 3 and 9 mM for 24 h, abandon supernatant absorption and then exposed to BDE 209 at 1 μM alone for 24 h in the same culture environment. After imbibing and discarding supernatant, a new 0.5 mg/ml MTT solution without serum MEM culture was added into each well. After 4 h, the supernatant was removed, and replaced with 100 μl DMSO for 30 min incubation. The absorbance of each well were measured at 595 nm with the enzyme marker.

2.4 RNA Extraction and qRT-PCR

Total RNA was extracted from the JEG cells with the RNAiso Plus kit (Takara, Japan). 1 μg of total RNA was transcribed into cDNA directed by Pr-imeScript® RT Master Mix kits (Takara, Japan). The PCR amplification was carried out according to the SYBR Premix Ex TaqTM II kit (Takara, Japan) instructions. All the primers used in this study were designed and s-ynthesized by Takara (TakaRa Bio, Dalian), the primers were as follows: GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-R: 5′-TGGTGAAGACGCCAGTGGA-3′, Dio3-F: 5′-CTTCGAGCGTCTCTATGTCATCCA-3′, Dio3-R: 5′-TGCAGTTGCTCATCATAGCGTTC-3′. Reaction c-onditions were listed as follows: an initial denaturation at 95 °C for 30 s, t-he PCR reaction at 95 °C for 5 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s. Results analysis: Prism Demo software was used to anal-yze the results, and the expression of countenances β-actin was used stand-ardize the results.

2.5 Western Blot Analysis

Western blot method was used to detect the effect of BDE 209 infected JEG cells on the protein expression of Dio3 at 24 h and the changes after the application of taurine protection. The treated JEG cells were collected, and the cells were washed with pre-cooled PBS, and RIPA lysate was added according to the proportion, which was lysed on the ice for 20–40 min, which the centrifuge tube was intermittently and lightly bounced to make the cracking sufficiently. After cracking, the mixture under 4 °C, 14000 r/min, centrifuged for 15 min. The supernatant was taken, the protein concentration was determined by BCA method and the protein standard curve was plotted. SDS-PAGE gel electrophoresis was performed by taking 40 μg protein samples, transfer film, after the transfer film with sealing fluid containing 5% skimmed milk enclosed 2 h at room temperature. Proteins were incubated overnight at 4 °C with primary antibodies against β-actin (1:2000, ZSGB-Bio, China), Dio3 (1:3000, Abcam, UK). The membranes were washed with TBST 15 min by 3 times next day. Then, after incubation at room temperature for 2 h with TBST diluted HRP-conjugated secondary antibodies (Proteintech, USA), the blots were washed 3 times with TBST for 15 min and developed using the ECL method.

2.6 Statistical Analysis

All data were generated from three experiments and each with three replicates. Data were described as the mean ± SD and the GraphPad Prism software version 3.03 was applied for the statistics (GraphPad Software Inc., San Diego, CA, USA). The differences were assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls posttest. The statistical significance was determined at $p < 0.05$.

3 Results

3.1 Effect of BDE 209 on the Proliferation of JEG Cells Under Hypoxia and the Protective Effect of Taurine

Using the MTT assay, we found that BDE 209 significantly reduced JEG cell survival ($p < 0.05$) in the hypoxic environment with a significant dose-dependent (Fig. 1). This indicates that BDE 209 has an inhibitory effect on the proliferation of JEG cells. After pretreatment of JEG cells with taurine for 24 h, the effect of 1 μM BDE 209 alone on cell proliferation was observed (Fig. 2). The results showed that pretreatment of JEG cells with taurine can ameliorate the decrease on cell viability caused by BDE 209 ($p < 0.05$).

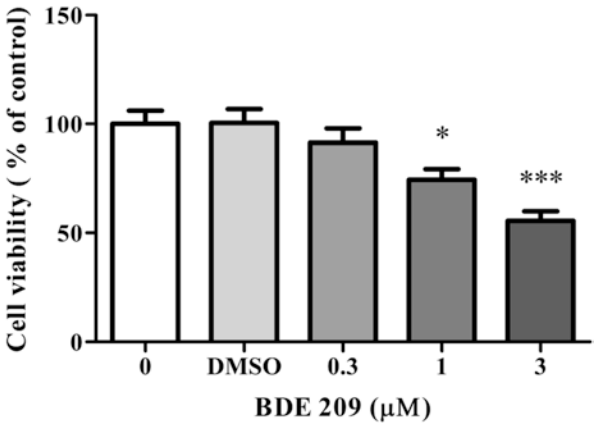

Fig. 1 Effect of BDE 209 on the proliferation of the JEG cells in the hypoxic environment

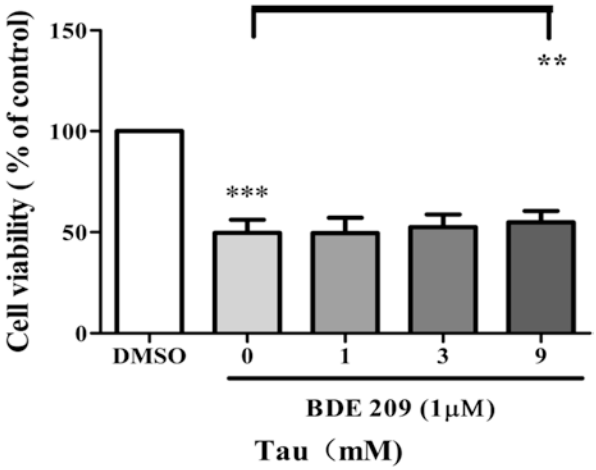

Fig. 2 Effect of BDE 209 on the survival rate of the taurine-pretreated JEG cells in the hypoxic environment

3.2 Effect of BDE 209 on Dio3 Protein Expression in JEG Cells Under Hypoxic Environment and Protective Effect of Taurine Against BDE 209 Toxicity

Western blot analysis was performed to analyze the effect of different concentrations of BDE 209 on the expression of Dio3 protein under hypoxic conditions (Fig. 3). The expression of Dio3 protein in JEG cells was detected after BDE 209

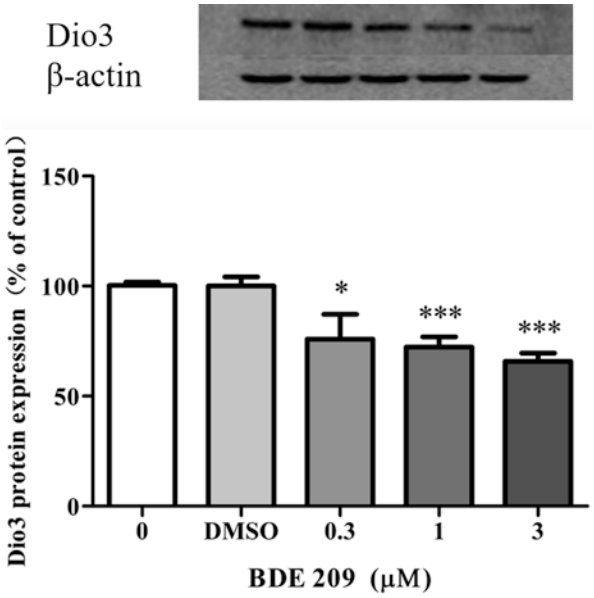

Fig. 3 Effect of BDE 209 on Dio 3 protein expression in JEG cells under hypoxic environment

was applied to JEG cells for 24 h. The expression of Dio3 protein in JEG cells gradually decreased with the increase of BDE 209 concentration ($p < 0.05$ and $p < 0.001$).

Taurine (1, 3 and 9 mM) pretreated JEG cells for 24 h. After 24 h of BDE 209 treatment, the Dio3 protein expression level was significantly improved in comparison to BDE 209 alone (1 μM) ($p < 0.05$) (Fig. 4), indicating that taurine improved the decrease of Dio3 protein expression in JEG cells caused by exposure to BDE 209 under hypoxic conditions.

3.3 Effect of BDE 209 on the Gene Expression of Dio3 in JEG Cells Under Hypoxic Conditions and Changes After Application of Taurine Protection

qRT-PCR analysis showed that Dio3 gene expression was down-regulated in different concentrations of BDE 209 in JEG cells for 24 h under hypoxic conditions ($p < 0.05$) (Fig. 5). Taurine pretreatment (1, 3 and 9 mM) restored Dio3 mRNA expression in JEG cells exposed to BDE 209 (1 μM, 24 h) (Fig. 6).

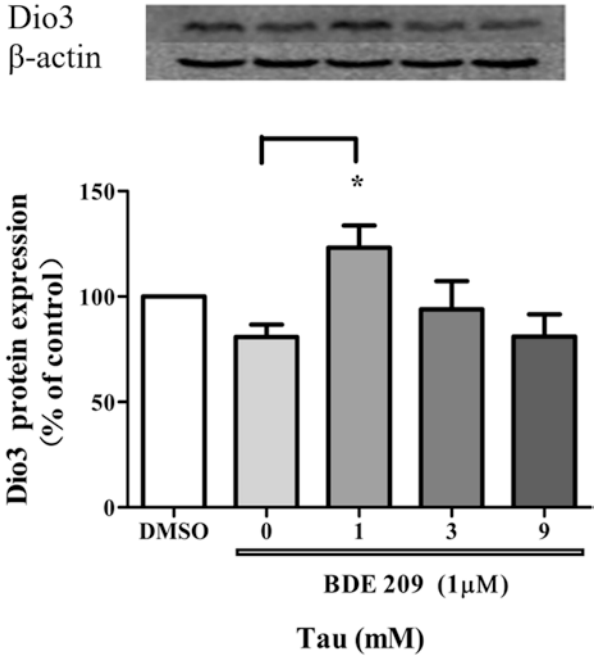

Fig. 4 Effect of BDE 209 on the expression of JEG Dio3 protein in taurine under hypoxic conditions

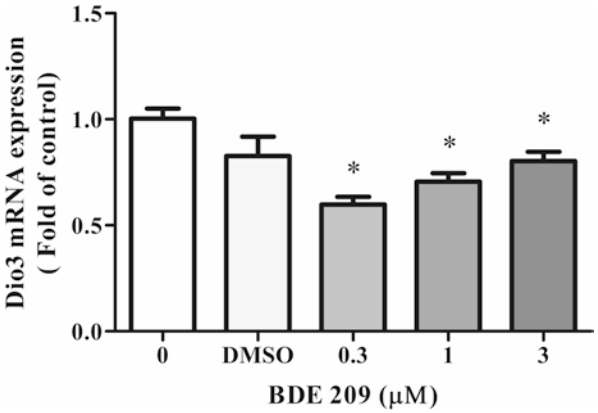

Fig. 5 Effect of BDE 209 on Dio 3 gene expression in JEG cells under hypoxic environment

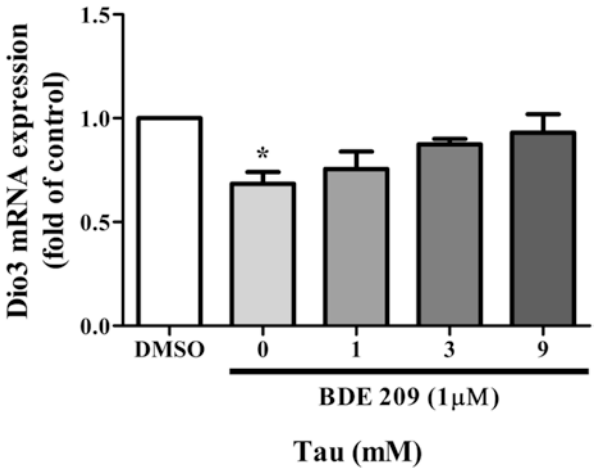

Fig. 6 Effect of BDE 209 on the expression of JEG Dio3 gene in taurine treatment in the hypoxic environment

4 Discussion

Studies have confirmed that PBDEs can damage the reproductive system, endocrine system, immune system, but it is more worrying that exposure to low concentrations of PBDEs in the environment will affect neurodevelopment, learning and memory skills. Experiments have shown that exposure to BDE 47 can cause abnormalities in motor behavior in developing rats, and the learning and memory abilities of these rats are significantly reduced in adulthood (Tagliaferri et al. 2010). In the zebrafish experiments, exposure to a series of PBDEs homologs resulted in changes in the spontaneous motor behavior of zebrafish (Yu et al. 2010). It is suggested that PBDEs have neurotoxic effects and affecting a variety of animals. Epidemiological studies such as Harley suggest that the level of cord blood PBDEs is negatively correlated with neonatal growth and development (Harley et al. 2011). PBDEs exposure during pregnancy can increase the risk of neonatal intrauterine growth retardation. Hence, ongoing studies for PBDEs-induced neurotoxicity are of practical significance.

According to the accumulation position BDE 209 may affect embryonic development by altering the expression of certain substances in the placental cells. The results of this study showed that BDE 209 significantly reduced the survival rate of JEG cells and inhibited the proliferation of JEG cells in a similar hypoxic environment. Studies with Viberg H have found that BDE 153 can cause nerve cell damage, which is manifested by a decrease in cell viability and consistent dose-dependent results (Viberg et al. 2013). A large number of experimental studies have confirmed that PBDEs can induce oxidative damage in cells (Tagliaferri et al. 2010). It is suggested that the inhibition of proliferation of BDE 209 may be one of the reasons for

its developmental neurotoxicity. A certain dose of taurine can improve the inhibition of proliferation caused by BDE 209, but the mechanism is still unclear.

Decreased or disordered thyroid function will affect important indicators such as growth and developmental behavior and intelligence level. Dio3 is the main thyroid hormone inactivating factor, which can convert T4 and T3 metabolism into trans-T3 (rT3) or diiodothyronine (T2) without physiological activity (Hernandez et al. 2016). Dio3 is mainly expressed in the uterus, placenta and most fetal tissues of humans and mice. The expression of Dio3 is mainly localized in the skin and central nervous system after birth. This pattern of expression suggests that Dio3 ensures normal fetal development by modulating thyroxine levels in different tissues of the fetus (Koopdonk-Kool et al. 1996). Therefore, we consider that PBDEs exposure during pregnancy may be due to the developmental neurotoxic effects of the placenta Dio3. The results of this experiment show that BDE 209 can down-regulate Dio3 protein and gene expression in placental cells under hypoxic conditions. The results indicate that Dio3 is lowly expressed in placental cells, and low expression of Dio3 can reduce T3 metabolic conversion and increase local T3 concentration, thus affecting the normal regulation of thyroid hormone. Taurine as a protective agent can improve Dio3 protein and genetic changes caused by BDE 209. This provides a good experimental basis for the study of taurine protection against neurodevelopmental toxicity.

5 Conclusion

Our data showed that different concentrations of BDE 209 can effects on cell viability and placental Dio3 expression in JEG cells cultured under hypoxia condition. Taurine was found to improve BDE 209-induced reductions in cell viability and altered gene and protein expressions of placental Dio3. The results provide a reference for the establishment of effective preventive measures. Further studies in seeking for the protective role of taurine in BDE 209 toxicity are still needed.

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Anti-apoptotic Effect of Taurine on Schwann Cells Exposed to High Glucose In Vitro

Kaixin Li, Inam-u-llah, Xiaoxia Shi, Mengren Zhang, Pingan Wu, Shuangyue Li, Raheel Suleman, Azhar Nisar, and Fengyuan Piao

Abstract It was reported that apoptosis of Schwann cells could increase in the diabetic rats. The studies showed that taurine inhibited apoptosis in a variety of cells. However, there were few reports on studying the protection of taurine against apoptosis of Schwann cells induced by high glucose (HG) and the underlying mechanism. In our study, the cells were divided into five groups: Control: the normal medium; HG group: 50 mM high glucose; T1: 50 mM high glucose+Taurine (10 mM) group; T2: 50 mM high glucose+Taurine (20 mM) group; T3: 50 mM high glucose+Taurine (40 mM) group. We used MTT and Tunel assays to measure the cell viability and apoptosis, respectively. Then, we also used western blotting to detect the protein levels of apoptosis-related protein. The results demonstrate that taurine promoted cell viability and decreased apoptosis in RSC96 cells exposed to HG. Furthermore, taurine markedly improved imbalance of Bax and Bcl-2, inhibited the translocation of Cytochrome C (Cyt C) from mitochondria to cytosol and reduced caspase-3 activity in HG-induced RSC96 cells. Our results indicate that taurine protect against apoptosis of Schwann cells induced by HG via inhibiting mitochondria-dependent caspase-3 pathway.

Keywords High glucose · Apoptosis · Schwann cells · Schwann cells · Anti-apoptotic effect

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Abbreviations

DM	Diabetes mellitus
HG	High glucose
MTT	4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
TUNEL	In situ TdT-mediated dUTP nick end labeling

1 Introduction

With the development of society and economy, the number of diabetic patients worldwide is increasing (Russell and Zilliox 2014). Therefore, diabetes mellitus (DM) is seriously threatening human health. Long-standing hyperglycemia in diabetes may cause various injuries on the body and lead to a lot of complications (Alam et al. 2014). Diabetic peripheral neuropathy (DPN) is considered as one of the most common, painful and long lasting complications of diabetes (Tesfaye and Selvarajah 2012). About 50% diabetic patients suffer from DPN, which induces a chronic pain sensation, impaired tactile, foot ulceration and eventual limb amputation (Edwards et al. 2008). The pathological characteristics of DPN are nerve demyelination and impaired remyelination (Bondan et al. 2006; Yagihashi et al. 2007). It is well known that Schwann cells can form myelin by winding axons during development and regeneration of the peripheral nervous system. Early study has shown that hyperglycemia in diabetic induced Schwann cell damages, leading to changes of segmental demyelination (Said 2007). In addition, it was found that the apoptosis of Schwann cells was increased in diabetic rats or under high glucose condition (Li et al. 2017; Zhang et al. 2017). These reports suggested that Schwann cells apoptosis may be involved in demyelination of DPN. Therefore, antagonizing the apoptosis of Schwann cells may be a novel treatment strategy of DPN.

Taurine (2-aminoethanesulfonic acid), a conditionally essential amino acid (Luca et al. 2015). It has a physiological function of resisting oxidative stress, scavenging free radicals and resisting lipid peroxidation. Recently some studies have reported that taurine could involve in regulation of anti-apoptosis. The study by Li et al. indicated that taurine significantly decreased the apoptosis of PC12 cells by METH-induced (Li et al. 2012a, b). Kaihong Zeng et al. also observed that taurine reduced the apoptosis ratio of retinal glial cells on the diabetes-induced or HG-induced retinopathy (Zeng et al. 2010). However, there are few reports that a protective effect of taurine on the apoptosis of Schwann cells in diabetes.

The purpose of our present study is tried to reveal the effect of taurine against apoptosis of Schwann cells induced by HG in vitro and explore the potential mechanisms. To simulate in a study model of DPN in vitro, RSC96 cells (A rat Schwann cell line) were exposed to high glucose (HG) with or without taurine. Our present study indicates that taurine may protect against apoptosis of RSC96 cells via inhibiting mitochondria-dependent caspase-3 pathway.

2 Methods

2.1 Cell Culture and Treatment

RSC96 cells (A rat Schwann cell line) were bought from Beijing Beina Chuanglian Institute of Biotechnology, China. Cells were cultured in DMEM (Hyclone, China) including 10% fetal bovine serum (FBS) (Gibco, USA), and incubation containing 5% CO2 at 37 °C. The RSC96 cells were divided into five groups: (1) Control group: the normal medium; (2) HG group: 50 mM high glucose; (3) T1 group: 50 mM high glucose + Taurine (10 mM) group; (4) T2 group: 50 mM high glucose + Taurine (20 mM) group; (5) T3 group: 50 mM high glucose + Taurine (40 mM) group. All treatments lasted for 48 h.

2.2 MTT Assay

After three passages, RSC96 cells were plated in 96-well plate (8.103 cells/well) for 24 h and then exposed to different dose of high glucose (10, 30, 50 mM HG) for 24 h, 48 h and 72 h, respectively. RSC96 cells were added to 100 μl MTT application solution (0.5 mg/ml) for 4 h. The medium was discarded and replaced with 200 μl DMSO. Then cells were incubated for 45 min without light. We used a microplate reader (Thermo Fisher Scientifc, USA) to measure the absorbance at OD 570 nm. The cells were cultured for 48 h in the taurine intervention experiment. The effect of HG and taurine on cell viability was compared as percentage to OD value at 570 nm of the control group.

2.3 TUNEL Assay

The apoptosis of RSC96 cells was detected by TUNEL assays with In Situ Cell Death Detection Kit (KeyGEN, China). We also used the DAPI staining to counter the number of cells nucleus. We randomly selected six fields, then the percentage of positive cells was determined as the apoptosis index (AI) through Image-Pro Plus software.

2.4 Flow Cytometry with Propidium Iodide/Annexing V Double Staining

RSC 96 cells were evenly planted in 100 mm dish (3 × 106 cells/well) and treated with 50 mM HG, as well as, 10, 20 and 40 mM taurine, respectively. Then we used the trypsin to digest the cells, washed it with PBS, and collected it. Then the cells

were re-suspended in 500 μl binding buffers and add to 10 μl PI and 10 μl AV at room temperature without light for 30 min. The percentage of cells stained by AV alone represented early apoptosis. Moreover the percentage of cells stained by both PI and AV represented late apoptosis. Apoptosis of RSC 96 cells was evaluated by FACS Calibur flow cytometer (BD, USA) as using a PI/AV-FITC kit (KeyGEN, China).

2.5 Western Blotting

RSC 96 cells grouping is in agreement with flow cytometry. These cells were used by the ice-cold RIPA cell protein extraction reagent (Beyotime, China) and 1% proteinase inhibitor mixture to homogenize, then incubated for 30 min on ice. The homogenization was centrifugation at 4 °C at 12,000 g for 15 min and then the supernatant was collected. The concentration of protein supernatant was assayed by BCA Protein Assay Kit (Beyotime, China). The proteins were separated on 12% SDS-PAGE and then transferred onto a PVDF membrane (Millipore, France). Antibodies used were Bcl-2 (1:1000, Beyotime, China), Bax (1:1000, Beyotime, China), Cytochrome C (1:1000, Beyotime, China), VDAC (1:1000, Beyotime, China) and β-actin (1:1000, ZS-Bio, China) overnight at 4 °C. Immunoreactivity was detected with second horseradish peroxidase-conjugated antibody (1:4000, Beyotime, China) and visualized by enhanced chemiluminescence (Beyotime, China). The immuno-labeling was performed using UVP BioSpectrum multispectral imaging system (UltraViolet Products Ltd., Upland, CA, USA).

2.6 Caspase-3 Activity Detection

We were used to the caspase-3 activity detection kit (Beyotime, C1116, China) to evaluate the activity of caspase-3 according to the manufacturer's protocol. RSC96 cells were treated and homogenized with lysis buffer for 15 min on ice, then centrifuged (20,000 g, 10 min, 4 °C) and collected the supermatants, mixed with both reaction buffer and Ac-DEVD-pNA (2 mM) followed by 2 h incubated at 37 °C. The absorption at 405 nm was measured with a microplate reader (Thermo Fisher Scientifc, USA).

2.7 Statistical Analysis

All data were analysed by one-way ANOVA using the program Graph Pad Prism 5.0. The data are expressed as the mean ± SEM. Values of $p < 0.05$ was considered significant.

3 Results

3.1 Effect of HG on the Apoptosis of RSC96 Cells

To study the effect of glucose on cells death, RSC96 cells were exposed to increasing dose of glucose (10–50 mM) for different cultural time, and then we were used a modified MTT assay to measure cell viability. As shown in Fig. 1a, RSC96 cells viability of HG-exposed groups decreased at 48 h and 72 h as compared to 24 h ($p < 0.05$). However, it was observed that the viability of RSC96 cells was no significant difference ($p > 0.05$) in 48 h and 72 h. Further exploring the protective effect of HG on apoptosis of RSC96 cells, we use TUNEL assay to detect the apoptosis cells. It was found that the TUNEL-positive cells increased in HG-exposed RSC96 cells and increasing in the dose-dependent manner ($p < 0.05$; Fig. 1b). Therefore, the RSC96 cells exposed to 50 mM HG for 48 h were used in the following experiments.

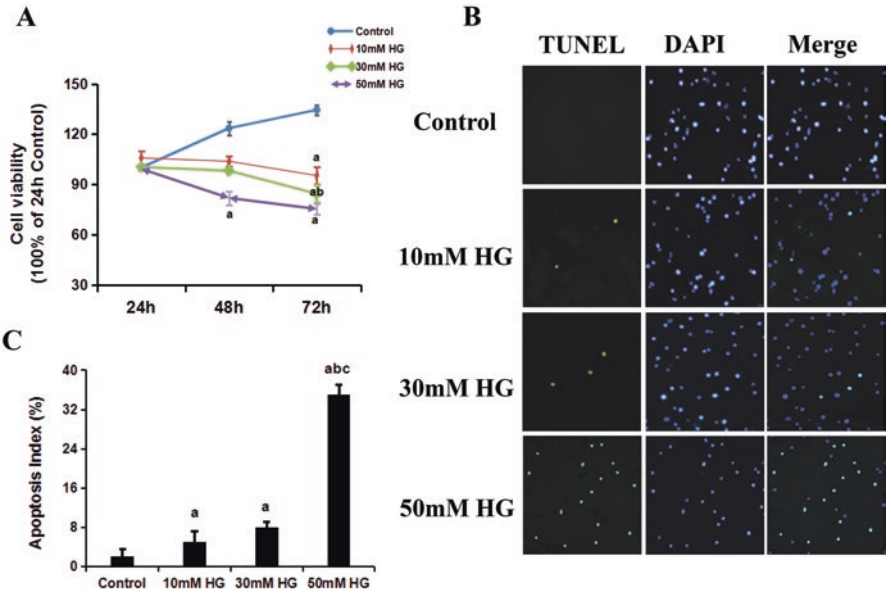

Fig. 1 The viability and apoptosis of RSC96 cells exposed different concentration of HG. (**a**) RSC96 cells were exposed with 0, 10, 30 and 50 mM HG by MTT analysis for 24 h, 48 h and 72 h, respectively. [a]$p < 0.05$, compared with 24 h; [b]$p < 0.05$, compared with 48 h. (**b**) Detection of RSC96 cells apoptosis by TUNEL and DAPI staining in different HG groups. Green color represented TUNEL-positive cells. Blue color represented cell nuclei. (**c**) The percentage of TUNEL-positive cells was quantified in different HG groups. [a]$p < 0.05$, compared with control group; [b]$p < 0.05$, compared with 10 mM HG group; [c]$p < 0.05$, compared with 30 mM HG group. Data was presented as mean ± SD (n = 3)

3.2 *Effect of Taurine Against Apoptosis of RSC96 Cells Induced by HG*

The RSC96 cells were exposed to 50 mM HG and then treated with different dose of taurine for 48 h. The viability of RSC96 cells was measured by the MTT assay. As shown in Fig. 2a, the results found that HG significantly decreased the viability of RSC96 cells compared with the control group ($p < 0.05$). However, the decreasing viability of RSC96 cells induced by HG was obviously reversed after taurine treatment in T2 and T3 groups ($p < 0.05$). Then, TUNEL staining was used to examine whether taurine affected the apoptosis of RSC96 cell induced by HG. As showed in Fig. 2b, c, the more TUNEL-positive cells were found in the HG group than that in control group. However, taurine treatment remarkably decreased the TUNEL-positive cells compare with HG group. Especially, the apoptosis index of RSC 96 cells was reduced from about 29% in the HG group to 4% in T3 group. In Addition, we also used the AV/PI doubles staining flow cytometry assay to examine the apoptosis rate in RSC96 cells. The apoptosis rates were 1.32 ± 0.05% in Control group, 25.87 ± 0.79% in HG group, 7.32 ± 1.06% in T1 group, 3.73 ± 0.54% in T2 group and 2.96 ± 0.69% in T3 group, respectively (Fig. 2d). These results indicated that taurine attenuates HG-induced apoptosis of RSC96 cells.

3.3 *Effect of Taurine on Expression of Bax and Bcl-2 in RSC96 Cells Induced by HG*

To explore the underlying mechanisms of taurine in the against HG-induced apoptosis of RSC 96 cell, the expression level of Bax and Bcl-2 was determined by Western blotting. As shown in Fig. 3, it was found that the protein expression of Bax in RSC96 cells was enhanced in HG group compare with control group ($p < 0.05$). Nevertheless, taurine supplement reduced Bax expression in T2 and T3 groups compare with HG group ($p < 0.05$; Fig. 3a). On the other hand, HG treatment reduced the expression of Bcl-2 in RSC96 cells, which were reversed in RSC96 cells with taurine treatment of T2 and T3 groups ($p < 0.05$; Fig. 3b). Then, the Bax/Bcl-2 ratio was measured in the present study. As shown in Fig. 3c, consistent with Bax protein expression, administration of HG elevated the Bax/Bcl-2 ratio in RSC96 cells, but taurine reduced the Bax/Bcl-2 ratio induced by HG. The results indicated that taurine decreased the expression of Bax and increased the expression of Bcl-2 in HG-induced RSC96 cells.

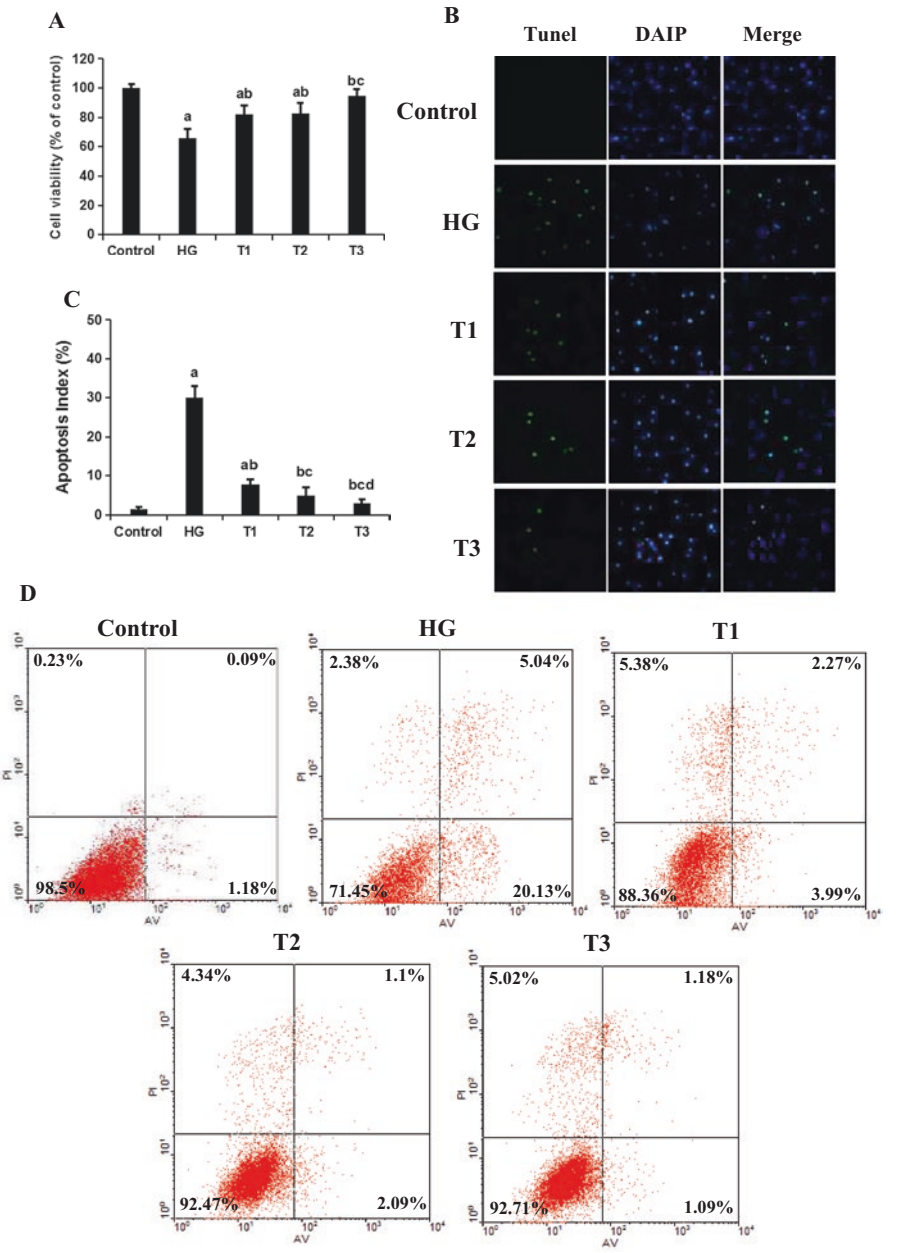

Fig. 2 The viability and apoptosis of RSC96 cells exposed HG alone or with taurine. (**a**) RSC96 cells were interpreted with 10, 20 and 40 mM of taurine with 50 mM high glucose for 48 h. MTT analysis was performed by Cell viability. (**b**) Apoptosis in RSC96 cells was measured by TUNEL assay. (**c**) The percentage of TUNEL-positive cells was quantified in different HG groups. (**d**) Flow-cytometry result of AV and PI double staining for cell apoptosis analysis in different groups. Data was presented as mean ± SD (n = 3). $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with T1 group; $^{d}p < 0.05$, compared with T2 group

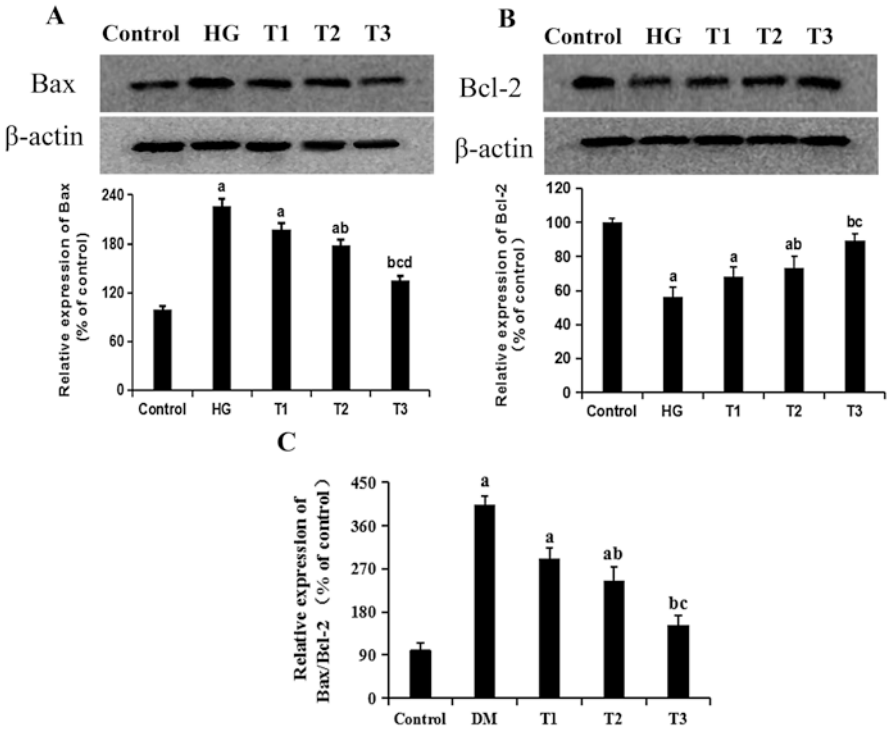

Fig. 3 The expression level of Bax and Bcl-2 in RSC96 cells exposed HG alone or with taurine. RSC96 cells were interpreted with 10, 20 and 40 mM of taurine with 50 mM high glucose for 48 h. (**a**–**b**) Western blotting analysis was used to detect Bax and Bcl-2 protein levels. (**c**) The ratio of Bax/Bcl-2 was analyzed. Data was presented as mean ± SD (n = 3). $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with T1 group; $^{d}p < 0.05$, compared with T2 group

3.4 Effect of Taurine on Expression of Cyt C in RSC96 Cells Exposed to HG

The expression level of Cyt C in mitochondria and cytosol in RSC96 cells was measured by western blotting. Our results showed that the protein expression of Cyt C in the mitochondrial was remarkably increased and that in cytosol was remarkably decreased as compared to control group ($p < 0.05$; Fig. 4(a–b)). Interestingly, T2 and T3 groups could reverse these changes. These results implied that taurine inhibited the translocation of Cyt C from mitochondria into cytosol in RSC96 cells induced by HG.

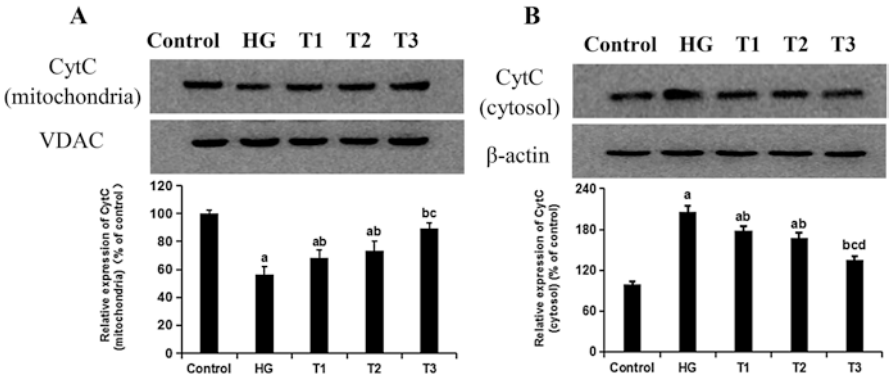

Fig. 4 Cyt C levels of RSC96 cells exposed to HG alone or with taurine. RSC96 cells were interpreted with 10, 20 and 40 mM of taurine with 50 mM high glucose for 48 h. (**a**–**b**) Western blot analysis was used to detect Cyt C levels in mitochondrial and cytosol fraction. Data was presented as mean ± SD (n = 3). $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with T1 group; $^{d}p < 0.05$, compared with T2 group

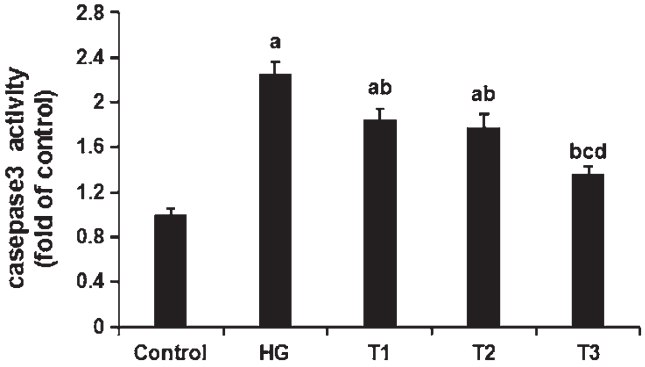

Fig. 5 Caspase-3 activity of RSC 96cells exposed HG alone or with taurine. The activity of caspase-3 was detected by caspase-3 activity detection kit in different groups. Data was presented as mean ± SD (n = 3). $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with T1 group; $^{d}p < 0.05$, compared with T2 group

3.5 Effect of Taurine on the Activity of Caspase-3 in RSC96 Cells Induced by HG

Due to caspase-3 is a key step in mitochondria-mediated apoptosis pathway, we probed into anti-apoptotic effect of taurine on activity of caspase-3 in RSC96 cells. The results showed that a markedly increase in caspase-3 activity was observed in HG-treated RSC96 cells compared with control group. However, it was found that the increasing caspase-3 activity in HG induced RSC 96 cells was significantly reduced after taurine treatment and decreasing in a dose-dependent manner (Fig. 5). It was indicated that taurine treatment could inhibit caspase-3 activity in HG-induced RSC96 cells.

4 Discussion

It is well known that apoptosis is regarded as a phenomenon of programmed cell death (PCD). Abnormal apoptosis was one of the pathogenesis of various diseases (Maiese et al. 2012; Sun et al. 2018). Li et al. reported that significant increase in the number of apoptotic Schwann cells in sciatic nerve of diabetic animals compared to control animals (Li et al. 2017). Jing et al. also found the apoptotic cell ratio markedly increased in RSC96 cells exposed to HG (Yu et al. 2015). In the current study, the results showed that HG decreased RSC96 cells viability and increased its apoptosis. However, taurine administration enhanced the viability of RSC96 cells induced by HG and reduced apoptosis of these cells in a dose-dependent manner. Matsushita et al. reported that taurine supplementation markedly decreased the number of apoptosis in the AR42J pancreatic acinar cell induced by DBTC (Matsushita et al. 2012). Wang also found that taurine significantly reduced hypoxia induced apoptosis of glial cells (Wang et al. 2015), implying anti-apoptotic effect of taurine. It is indicated that taurine protects against HG-induced apoptosis of RSC96 cells.

Apoptosis occurs via two main interacted pathways which are intrinsic (mitochondrial pathways) and extrinsic (endoplasmic reticulum pathways) pathways (Lee et al. 2015). Several studies have found that the mitochondrial pathway plays an important role in apoptosis induced by diabetes (Trudeau et al. 2012; Zhongmin Alex Ma and Turk 2014). It was reported that the Bcl-2 family is crucial regulator of mitochondria-mediated apoptosis. This family includes anti-apoptosis protein Bcl-2 and pro-apoptosis protein Bax. Their localization and balance determine a cell's susceptibility to apoptosis (Fan et al. 2017). In our study, the results showed that HG increased expression of Bax and decreased expression of Bcl-2 in the RSC96 cells as compare to control group. However, taurine recovered the abnormal changes in the expression of Bax and Bcl-2 in the RSC96 cells exposed to HG. Tu et al. also reported that taurine decreased the expression of Bax, whereas increased that of Bcl-2 in lung cancer A549 cells (Tu et al. 2018), being agreement with our results. Thus, we speculate that taurine inhibits HG-induced apoptosis in RSC96 cells by improving Bax/Bcl-2 balance.

Disturbance of Bax/Bcl-2 balance increases permeability of the mitochondrial membrane. This increased permeability leads to movement of Cyt C into the cytosol causing abundance of Cyt C in cytosol and deficiency in mitochondria (Wang et al. 2013). In our study, HG resulted in the decrease of Cyt C in mitochondria and the increase of Cyt C in cytosol in the RSC96 cells. As opposed this, taurine reversed these changes. Similar to our data, taurine prevent sodium laurate-induced rat large-intestinal epithelial cells apoptosis via repressing the release of Cyt C from mitochondria (Takayama et al. 2009). Moreover, it was also reported that taurine effectively reduced the release of Cyt C from mitochondria in diabetic rats induced by alloxan (Das et al. 2012). The results suggested that taurine significantly suppresses the translocation of Cyt C from mitochondria in the RSC96 cells.

It was reported that the release of Cyt C in mitochondrial lead to cascade activation of caspases (Concannon et al. 2001). Among these activated caspases, caspase-

3 is regarded as an important enzyme to induce apoptosis. Furthermore, its activation, a downstream key step, has been demonstrated as a downstream key step in the mitochondria-mediated apoptosis of various cells (Li et al. 2012a, b). In our current study, the results found that HG led to the activity level of caspase-3 in RSC96 cells. On the contrary, taurine supplementation significantly attenuated the increased activity level of caspase-3. It was also reported that taurine mitigated caspase-3 activity in EBSS-induced ARPE-19 cells apoptosis (Zhang et al. 2017), showing that taurine inactivates caspase-3. These results imply that taurine protects against apoptosis in RSC96 cells exposed to HG via inhibiting mitochondria-dependent caspase-3 pathway. It has been shown that Schwann cells are vulnerable hyperglycemia and easily undergo demyelination in the DPN (Li et al. 2017; Liu et al. 2016). Moreover, the studies showed that the increased apoptosis in SCs is associated with nerve demyelination in DPN. (Han et al. 2014; Li et al. 2017). All of the above results indicate that taurine may ameliorate demyelination in the DPN via its anti-apoptotic effect on Schwann cells.

5 Conclusion

The present study demonstrated that taurine inhibits the apoptosis of RSC96 cells induced by HG. Furthermore, treatment of taurine remarkably reversed these abnormal changes in the expression level of Bax and Bcl-2, the enhanced release of Cyt C from mitochondria to cytosol, and the caspase-3 activity in RSC96 cells induced by HG. These results demonstrate that taurine reduces apoptosis via inhibiting mitochondria-dependent caspase-3 pathway. In the future, it will need to confirm the protective effect of taurine against apoptosis of SCs in the DPN in vivo.

Conflict of Interest It is declared that there is no conflict of interest among the authors.

Research Subjects It is declared that there is no animal was harmed or hurt except according to ethics.

Informed Consent It is declared that this manuscript is submitting after consent from all authors are aware of this submission.

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Assessing the Anxiolytic Properties of Taurine-Derived Compounds in Rats Following Developmental Lead Exposure: A Neurodevelopmental and Behavioral Pharmacological Pilot Study

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Abstract Lead (Pb^{2+}) is a developmental neurotoxicant that causes alterations in the brain's excitation-to-inhibition (E/I) balance. By increasing chloride concentration through GABA-ARs, taurine serves as an effective inhibitory compound for maintaining appropriate levels of brain excitability. Considering this pharmacological mechanism of taurine facilitated inhibition through the GABA-AR, the present pilot study sought to explore the anxiolytic potential of taurine derivatives. Treatment groups consisted of the following developmental Pb^{2+}-exposures: Control (0 ppm) and Perinatal (150 ppm or 1000 ppm lead acetate in the drinking water). Rats were scheduled for behavioral tests between postnatal days (PND) 36–45 with random assignments to either solutions of Saline, Taurine, or Taurine Derived compounds (i.e., TD-101, TD-102, or TD-103) to assess the rats' responsiveness to each drug in mitigating the developmental Pb^{2+}-exposure through the GABAergic system. Long

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Evans Hooded rats were assessed using an Open Field (OF) test for preliminary locomotor assessment. Approximately 24-h after the OF, the same rats were exposed to the Elevated Plus Maze (EPM) and were given an i.p. injection of 43 mg/Kg of the Saline, Taurine, or TD drugs 15-min prior to testing. Each rat was tested using the random assignment method for each pharmacological condition, which was conducted using a triple-blind procedure. The OF data revealed that locomotor activity was unaffected by Pb^{2+}-exposure with no gender differences observed. However, Pb^{2+}-exposure induced an anxiogenic response in the EPM, which interestingly, was ameliorated in a gender-specific manner in response to taurine and TD drugs. Female rats exhibited more anxiogenic behavior than the male rats; and as such, exhibited a greater degree of anxiety that were recovered in response to Taurine and its derivatives as a drug therapy. The results from the present psychopharmacological pilot study suggests that Taurine and its derivatives could provide useful data for further exploring the pharmacological mechanisms and actions of Taurine and the associated GABAergic receptor properties by which these compounds alleviate anxiety as a potential behavioral pharmacotherapy for treating anxiety and other associated mood disorders.

Keywords Anxiety · Elevated plus maze · Lead poisoning · Developmental neurotoxicity · Taurine · GABAergic system · Psychopharmacotherapy

Abbreviations

Pb^{2+}	Lead
TD	Taurine derivatives
E/I	Excitation-to-inhibition
BLL	Blood lead levels
GAD	Glutamic acid decarboxylase
PND	Postnatal day
EDTA	Ethylenediaminetetracetic acid
ASV	Anodic stripping voltammetry
OF	Open field
EPM	Elevated plus maze
OTC	Open-to-closed ratio

1 Introduction

Environmental lead (Pb^{2+}) poisoning continues to be a public health problem for many developed countries and arguably more detrimental for underdeveloped countries (Shinkuma and Huong 2009; Chen et al. 2011; Eneh and Agunwamba 2011; Laidlaw and Taylor 2011; Yang et al. 2013). The consequences of exposure to environmental Pb^{2+}-sources during the development of the nervous system remains an

ongoing global health issue. During the last century, elevated environmental Pb^{2+}-exposures were caused mostly by leaded or lead-containing paints and leaded-gasoline (Brody et al. 1994). However, today Pb^{2+}-contaminated water supplies (Edwards et al. 2009) and electronic waste (i.e., E-waste and E-recycling) has both remained and emerged as a new form of Pb^{2+}-expousres and other metal poisoning sources. One example of E-waste can arise from environmental byproducts that occur from metallurgical processes (i.e., heating and reducing electronics down to reusable metal amalgams), which has created resurgent forms of environmental Pb^{2+}-exposures worldwide (Hernberg 2000; Huo et al. 2007; Leung et al. 2008; Zheng et al. 2008; Guo et al. 2010; Chen et al. 2011; Eneh and Agunwamba 2011; Laidlaw and Taylor 2011; Yan et al. 2013; Yang et al. 2013). In underdeveloped countries, such metallurgical or electronic refinery processes are important sources of income; but, unfortunately, they are variants of modern smelting factories that release toxic emissions into the local environments (Leung et al. 2008; Chen et al. 2011; Yan et al. 2013; Yang et al. 2013). Children and young adolescents who reside or work near the toxic exposure sites are the most at risk populations for developmental neuropathologies (Leung et al. 2008; Chen et al. 2011; Yan et al. 2013: Yang et al. 2013). Extensive investigations on the sources of exposure to Pb^{2+} have shown that Pb^{2+} is both an environmental contaminant as well as a central nervous system neurotoxicant (Bellinger 2008; Lanphear et al. 2000). From an epidemiologically perspective, elevated levels of environmental Pb^{2+}-exposure (i.e., > 10 μg/dL in the USA) are considered a threat to the environment. From a neurotoxicology and neuropsychology perspective, both high- and low-levels of exposure to environmental Pb^{2+} can cause a wide-range of neuropathologies. Further, low-level Pb^{2+}-exposure may be beneficial for the environment, but these same low-level Pb^{2+}-exposures cause brain damage. Thus, although low-level Pb^{2+}-exposures in the environment may improve living conditions according to public health standards; the same low-levels of Pb^{2+}-exposure can significantly impact children's neurodevelopment in utero and during critical periods from birth through the first few years of postnatal life (Neuwirth 2018).

Consistent with the scientific evidence of neurotoxicant Pb^{2+}-exposures, the rationale for lowering environmental Pb^{2+}-exposure action levels from the current 5 μg/dL to 2 μg/dL (i.e., in the US) has been vehemently debated (Gilbert and Weiss 2006). Because the neurotoxicity of Pb^{2+} has been well documented, it follows that no environmental Pb^{2+}-level should be deemed "safe" for children (Bellinger and Dietrich 1994; Barbosa Jr. et al. 2005; Lidsky and Schneider 2006). Despite the ongoing important discussions, low-level Pb^{2+}-exposure remains both a challenge and a risk for children because trace metals are neurotoxicants regardless of exposure levels (Grandjean and Hertz 2015). In fact, based on neurotoxicology studies, the relationship between the damage inflicted upon the central nervous system and Pb^{2+}-exposures is quantitatively curvilinear (Needleman and Gatsonis 1990; Schwartz 1994; Bellinger and Needleman 2003; Canfield et al. 2003; Lidsky and Schneider 2006). At low- and high-levels of exposures, Pb^{2+} can cause a range of neuropathological and neuropsychological syndromes with varied behavioral and cognitive symptoms and/or signatures (Wong et al. 1991; Bellinger and Dietrich

1994; Schütz et al. 1996; Bleecker et al. 1997; Krieg Jr. et al. 2005; Lidsky and Schneider 2006; Bergdhal and Skerfving 2008; Rentschler et al. 2012). Although, moderate Pb^{2+}-exposures levels may appear less damaging than low- and high-level Pb^{2+} exposures, they are equally detrimental to the developing child's brain (Needleman 2004). Notably, even across this curvilinear range of Pb^{2+}-exposures, children that absorb Pb^{2+} from their environment show a consistent pattern of an inverse relationship between elevated blood Pb^{2+}-levels (BLLs) and a reduced intelligence quotient (IQ) (Needleman and Gatsonis 1990; Schwartz 1994; Bellinger and Needleman 2003; Canfield et al. 2003; Lidsky and Schneider 2006). Thus, as their circulating blood accumulates higher levels of Pb^{2+} their intellect and capacity to learn and function behaviorally decreases. At present, chelation therapy is an effective treatment for most children that experience metal toxicity at high-level Pb^{2+}-exposures (i.e., > 39 μg/dL). However, chelation therapy is inappropriate for lower levels of Pb^{2+}-poisoning which often are the current Pb^{2+}-exposure problems faced by children within the US (Rogan et al. 2001; Dietrich et al. 2004). Therefore, in order to help reduce the neurotoxicant impacts of Pb^{2+}-exposure to low-levels, new therapeutic approaches are warranted to best address children that continue to face these low-level Pb^{2+}-exposures (i.e., < 39 μg/dL) in the US, as well as, elsewhere.

The present pilot study sought to build upon prior reports in which developmental Pb^{2+}-exposure induced E/I imbalances that caused learning and memory deficits and were recovered by acute taurine treatment through the GABA-AR system (Neuwirth 2014, 2018; Neuwirth et al. 2017). Early disruption of the brain's E/I balancing between the Glutamatergic (i.e., excitatory) and GABAergic (i.e., inhibitory) systems have been consistently identified as a contributing neurodevelopmental risk factor for seizure and other closely related neuropathologies (Ben-Ari 2002; Ben-Ari et al. 2012). Taurine has been increasingly shown to mitigate against brain E/I imbalances in animal models of epilepsy (El Idrissi et al. 2003) through upregulation of glutamic acid decarboxylase (GAD) and interactions with the GABA-AR β2/β3 subunits (L'Amoreaux et al. 2010). In addition, other reports have shown that taurine provides neuroprotection by sustaining GABAergic signaling during senescence where, on the other side of the developmental continuum, the E/I balance begins to weaken with age (El Idrissi et al. 2013) with evidence supporting cognitive improvement in learning (El Idrissi 2008; Neuwirth et al. 2013) and motor abilities (Santora et al. 2013) of aged animals.

In addition to taurine psychopharmacological therapeutic approaches, the present pilot study evaluated the effects of developmental Pb^{2+}-toxicity on locomotion and anxiety, which are partially regulated by the GABAergic system. Consistent with previous reports (Neuwirth et al. 2017; Neuwirth 2014), the present pilot study explored whether the acute administration of taurine and taurine derivatives would recover the Pb^{2+}-induced neurobehavioral aberrations in the rat model. Furthermore, the present pilot study sought to evaluate sex-based differences in Pb^{2+}-vulnerabilities and taurine as well as taurine derivatives to recover sex-specific alterations of the GABAergic mediated behaviors. Lastly, Pb^{2+}-dosage was examined to determine the extent of GABAergic dysfunctions that could be assessed by their functionally associated behaviors in response to developmental Pb^{2+}-exposure and the potential

for taurine as well as taurine derivatives as a psychopharmacological treatment option for low-level Pb^{2+}-exposures (i.e., < 39 μg/dL).

2 Methods

2.1 Subjects

In accordance with The SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans Norwegian hooded male (N = 10) and female rats (N = 20) (Taconic, N.J.) were paired for breeding and their male and female F1 generations were used for future behavioral pharmacological experimentation. Rat litters were culled to 8–10 pups in order to control for maternal social influences on neurodevelopmental and behavioral outcomes that would be studied in later development. All rats were fed regularly with Purina rat chow (RHM1000 # 5P07) *ad libitum*. However, control rats were provided regular water, while the experimental rats were fed water containing lead acetate (Sigma Aldrich, St. Louis, MO) from pairing throughout gestation and continued through weaning at postnatal day (PND) 22 (i.e., constituting a Perinatal developmental Pb^{2+}-exposure model). At PND 22, the Pb^{2+}-exposures ceased and all rats returned to a regular water regimen. Rats assigned to the Peri-22 150 ppm group (drank a lead acetate water of [363.83 μM]) and the Peri-22 1000 ppm group (drank a lead acetate water of [2.43 mM]) and all treatments were administered *ad libitum*. Prior to behavioral testing, all rats were handled for 10-min per day for 1-week. Between PND 36–45 rats were assigned to the open field test and 24-h later, the elevated plus maze test.

2.2 Blood Pb^{2+}-Level Analyses

At PND 22 immediately following the end of Pb^{2+}-exposure, a separate group of male and female rats (i.e., with a representative sample culled from litter) were sacrificed (n = 4 per gender, per Peri-22 150 ppm and Peri-22 1000 ppm treatment group) and their blood samples were collected and analyzed consistent with previous reports (Neuwirth 2014; Neuwirth et al. 2017, 2018, 2019a, b). Briefly, blood samples were collected within 2 mL anti-coagulant ethylenediaminetetracetic acid (EDTA) coated syringes (Sardstedt, Germany), mixed to prevent coagulation, and then frozen at −80 °C. Blood samples were analyzed using a commercial ESA LeadCare II Blood Lead Analyzer system (Magellan Diagnostics, North Billerica, MA) to determine the amount of Pb^{2+} in the blood sample by electrochemical anodic stripping voltammetry (ASV) to eliminate any potential for experimenter bias. The ASV method was conducted by taking 50 μL of whole blood mixed with 250 μL of hydrochloric acid solution (0.34 M) and then applying the final mixture to the lead

sensor strip and inserted into the ESA LeadCare II Blood Lead Analyzer system to determine BLLs. After 3 min, the BLLs were reported from the instrument in μg/dL with a low sensitivity cut off value of 3 μg/dL and a high sensitivity cut off value of 65 μg/dL (i.e., SEM ± 1.5 μg/dL sensitivity detection level).

2.3 The Open Field Test

Between PND days 36–45 a series of naivë rats from the F1 generation offspring ($N = 159$) comprised of both males ($n = 80$) and females ($n = 79$) were subjected to an open field test (OF). The treatment groups were as follows: Control males ($n = 30$), Peri-22 150 ppm Pb^{2+} males ($n = 32$), and Peri-22 1000 ppm Pb^{2+} males. Control females ($n = 18$), Peri-22 150 ppm Pb^{2+} females ($n = 30$), and Peri-22 1000 ppm Pb^{2+} females ($n = 19$), respectively. All rats were examined during 10-min of locomotor exploration in the OF apparatus (376 mm H × 914 mm W × 615 mm L) in a dark room illuminated with red lighting (30 Lux) to promote locomotor activity in order to assess any motor disruption as a consequence of Pb^{2+}-exposure. Locomotor variables included *Total Distance Traveled* measured in meters (m) and *Overall Average Speed* measured in meters/second (m/s).

2.4 Taurine and Taurine Derivative Drug Preparations and The Elevated Plus Maze Test

The next day following the OF assessment, the male and female rats were randomly assigned to one of six Drug Treatment conditions (i.e., No Drug, Saline, Taurine ($NH_2CH_2CH_2SO_3H$ – FW: 125.15 g/mol) (Sigma Aldrich, St. Louis, MO), Taurine Derivative (TD)-101 ($C_3H_7NO_2$ 89.09 g/mol), TD-102 (CH_5NO_3S 111.12 g/mol), or TD-103 ($C_6H_7NO_3S$ 173.19 g/mol), respectively (Fig. 1). All Taurine and TD compounds were dissolved in physiological buffered saline (PBS) with a pH of 7.4 as a final systemic concentration of [10 mM] and were then sterilized by syringe filtration (0.2 μm) prior to being administered.

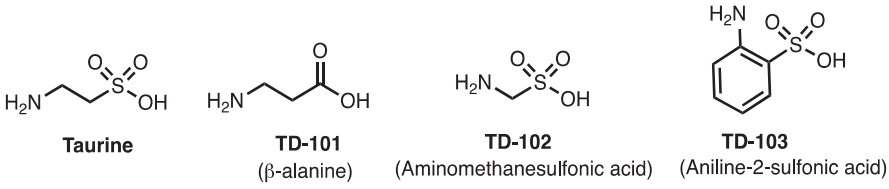

Fig. 1 Comparisons of the chemical structure for taurine and taurine derivatives (TD), TD-101, TD-102, and TD-103

Males were assigned as follows: No Drug (n = 20), Saline (n = 11), Taurine (n = 13), TD-101 (n = 14), TD-102 (n = 11), and TD-103 (n = 14). Females were assigned as follows: No Drug (n = 17), Saline (n = 10), Taurine (n = 11), TD-101 (n = 11), TD-102 (n = 13), and TD-103 (n = 13), respectively. Rats were administered their randomly assigned drug treatment as a triple-blind procedure via i.p. injection 15-min prior to EPM testing. Drugs were administered as equivalent 43 mg/kg drug injections (*i.e.,* to standardized against Taurine as a reference) across all treatments to draw appropriate comparative outcomes. All rats were examined during 10-min of anxiety-like behavioral assessments in the EPM. The EPM apparatus (external dimensions: 800.1 mm H × 1104.9 mm W × 1104.9 mm L; closed arm dimensions: 101.6 mm W × 1104.9 mm L × 304.8 mm H walls; open arm dimensions: 101.6 mm W × 1104.9 mm L; the platform was elevated off the floor by 495.3 mm H) was within a brightly illuminated room (300 Lux) to promote an anxiogenic response. The anxiogenic behaviors were evaluated in order to assess the effects of Pb^{2+}-exposure to evoke anxiety-like behaviors and the potential for Taurine and TDs treatments to provide anxiolytic pharmacotherapy within the EPM. Anxiety-like behavioral variables included the *Open-to-Closed (OTC) Ratio* and a representative group mean heat plot to assess activity across the 10-min of the EPM.

2.5 Data Analyses

Data were recorded in real-time and analyzed using the Anymaze® video tracking software (Stoelting Co., Wood Dale, IL) transmitted via a ceiling mounted Logitech C310 Hi-speed USB 2.0 web camera (High-definition video with 1280 × 720 pixels and 5 MP photo quality). The web camera was relayed to a standard Dell D16M Inspiron 3847 Desktop computer equipped with Windows 10 64-bit operating systems, 8 GB Dual Channel DDR3 1600 MHZ (4 GB × 2), 1 TB 7200 PRM Hard Drive, and a 4th Generation Intel® CoreTM i3–4170 Processor (3 M Cache, 3.70 GHz), and displayed through a Dell 20" E2016H monitor with an optimal resolution of 1600 × 900 pixels at 60 Hz. Data were recorded as digital videos that were analyzed using AnyMaze® software. Animal tracking was based on contrast relative to background. Different zones were labeled and indicated on the monitor for both the OF and EPM. Three tracking points were specified one on the rat's head, the center of the rat's body, and the rat's tail. A Microsoft Excel spreadsheet was generated containing all the parameters specified for both the OF and EPM tests, respectively.

2.6 Statistical Analyses

All behavioral data were collated in Microsoft Excel and later analyzed in IBM SPSS V. 24 (IBM, Inc. Armonk, NY). For the OF tests, a *Repeated Measures ANOVA* was conducted using *Time* and *Pb^{2+}-Exposure* as the within-subjects factors and *Pb^{2+} Exposure* as the between-subjects factors for the dependent variables of *Total Distance Travelled* (meters) and *Overall Average Speed* (meters/second). For the EPM tests, a *Multi-Factorial ANOVA* with *Treatment* and *PPM* as fixed-factors was used to evaluate the dependent variables of the *OTC ratio* and *Drug Treatment Condition*. The criteria for significance was set at $\alpha = 0.05\%$ with a 95% confidence interval (CI) and data are presented as the mean ± SEM. Significant differences were determined by an *unequal Tukey's HSD post hoc* multiple comparisons tests along with a partial Eta-square (η_p^2) for determining effect sizes where applicable.

3 Results

3.1 BLLs as a Function of Pb^{2+}-Dose and -Exposure Cessation Prior to Behavioral Testing

A separate set of rats was used to determine BLLs ($n = 4$ males and $n = 4$ females for both the Peri-22 150 ppm and Peri-22 1000 ppm Treatment groups). Rats were sacrificed at PND 22 when their Pb^{2+}-exposure ceased. Animals were then anesthetized with 50 mg/kg of sodium pentobarbital via i.p. injection, and once non-reflexive, a cardiothoracic blood draw was taken and analyzed with the LeadCare II system as stated above. The results showed no differences in BLLs as a function of sex. Each Pb^{2+}-treatment at the time of sample collection resulted in BLLs ranging from 3.3 to 10.7 µg/dL (SD ± 1.57) for Peri-22 150 ppm rats ($p < 0.001^{***}$) and from 9.0 to 17.8 µg/dL (SD ± 2.86) for Peri-22 1000 ppm ($p < 0.001^{***}$), respectively. The control rats were Pb^{2+}-negative. Thus, the BLL samples obtained in this study were less than the 39 µg/dL chelation therapy limit. The BLL samples from the behaviorally tested rats were also drawn at PND 55 days following the conclusion of the study; however, their BLLs were below the 3.3 µg/dL detection limit. The reduction in circulatory BLLs would be due to a combination of clearing from the body as well as bodily tissue absorption of Pb^{2+} from the blood. This is consistent with reports from the U.S. Agency for Toxic Substances and Disease Registry (ATSDR, 2007) that Pb^{2+} is not uniformly distributed in bone, blood, and soft mineralizing tissues; thus, requiring careful medical management in children.

3.2 *Developmental Pb^{2+}-Exposure Showed No Difference in Locomotor Activity Irrespective of Pb^{2+}-Dose or Sex*

The OF was used as a preliminary assessment for locomotor disruption to evaluate the potential for any confounding behavioral effects that might influence the interpretation of anxiogenic and anxiolytic behaviors within the subsequent EPM test. As such, a preliminary locomotor assessment was first conducted to determine whether there were any sex-based differences in the OF. The within-subject factors for the *Total Distance Travelled* (m) assessment showed a significant effect of *Time* $F_{(9,58)} = 81.125$, $p < 0.001^{***}$, $\eta_p^2 = 0.583$ (Fig. 2a), but no significant *Time X Gender* interaction $F_{(1,58)} = 0.771$, $p = 0.563$ n/s (Fig. 2a). The between-subjects assessment of *Total Distance Travelled* (m) was also not significant $F_{(1)} = 0.41$, $p = 0.839$ n/s (Fig. 2a). The rats *Overall Average Speed* (m/s) was also assessed, and the within-subject factors revealed a significant effect of *Time* $F_{(9,58)} = 78.992$, $p < 0.001^{***}$ (Fig. 2b), $\eta_p^2 = 0.577$, but no significant *Time X Sex* interaction was observed $F_{(1,58)} = 0.633$, $p = 0.671$ n/s (Fig. 2b). The between-subjects assessment of *Overall Average Speed* (m/s) was also not significant $F_{(1)} = 0.069$, $p = 0.793$ n/s (Fig. 2b).

Following the sex-based preliminary assessment of locomotor activity, each sex was separately examined to determine whether any within-sex effects were observed as a function of 150 ppm and 1000 ppm Pb^{2+}-exposures. For the OF assessment of *Total Distance Travelled* (m) in male rats, the within-subject factors revealed a significant effect of *Time* $F_{(9,77)} = 79.136$, $p < 0.001^{***}$, $\eta_p^2 = 0.07$ (Fig. 3a), but there was no significant *Time X Pb^{2+}-Exposure* interaction $F_{(2,77)} = 1.112$, $p = 0.349$ n/s (Fig. 3a). The between-subjects assessment of *Total Distance Travelled* (m) was also not significant $F_{(2)} = 1.694$, $p = 0.190$ n/s (Fig. 3a). In addition, the *Overall Average Speed* (m/s) was assessed in female rats and the within-subject factors revealed a significant effect of *Time* $F_{(9,77)} = 79.174$, $p < 0.001$***, $\eta_p^2 = 0.507$ (Fig. 3c), but there was no significant *Time X Pb^{2+}-Exposure* interaction $F_{(2,77)} = 1.115$, $p = 0.347$ n/s (Fig. 3c). The between-subjects assessment of *Overall Average Speed* (m/s) was also not significant $F_{(2)} = 1.698$, $p = 0.190$ n/s (Fig. 3c).

In contrast, the OF assessment of *Total Distance Travelled* (m) in female rats, the within-subject factors revealed a significant effect of *Time* $F_{(9,76)} = 90.058$, $p < 0.001^{***}$, $\eta_p^2 = 0.542$ (Fig. 3b), but there was no significant *Time X Pb^{2+}-Exposure* interaction $F_{(2,76)} = 0.947$, $p = 0.482$ n/s (Fig. 3b). The between-subjects assessment of *Total Distance Travelled* (m) was also not significant $F_{(2)} = 2.471$, $p = 0.091$ n/s (Fig. 3b). In addition, the *Overall Average Speed* (m/s) was assessed in female rats and the within-subject factors revealed a significant effect of *Time* $F_{(9,76)} = 88.481$, $p < 0.001$***, $\eta_p^2 = 0.538$ (Fig. 3d), but there was no significant *Time X Pb^{2+}-Exposure* interaction $F_{(2,76)} = 1.042$, $p = 0.405$ n/s (Fig. 3d). The between-subjects assessment of *Overall Average Speed* (m/s) was also not significant $F_{(2)} = 2.449$, $p = 0.093$ n/s (Fig. 3d).

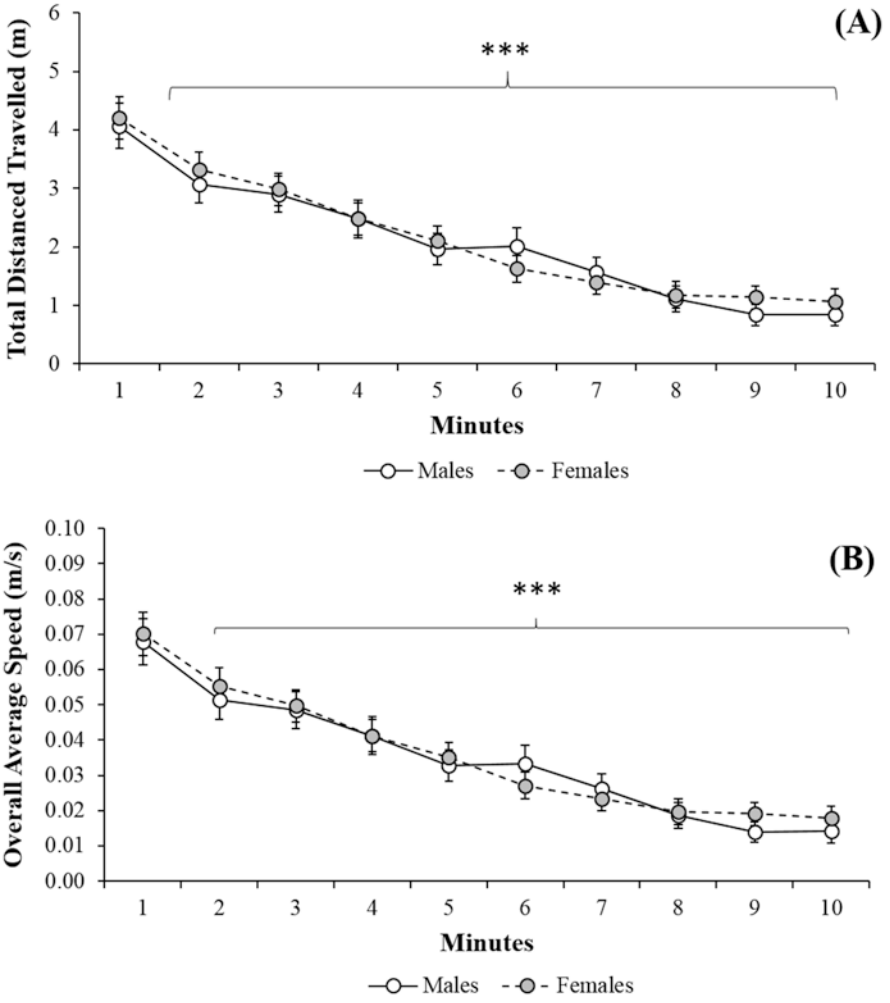

Fig. 2 Preliminary assessment of rat locomotor activity in the OF as an effect of *Sex* **(males = open circles; females = grey circles)**. Data show for both *Total Distance Travelled (m)* (**A**) and *Overall Average Speed (m/s)* (**B**), that there were no significant differences in rat locomotor activity in the OF as a function of *Sex*. However, as a function of *Time*, there was a significant effect across the 10-min of the OF in which the rats gradually shift from high-to-low locomotor activity as they habituated to the OF (***p* < 0.001∗∗∗**). Thus, indicating that both rat *Sexes* were equal in their locomotor behavioral profiles

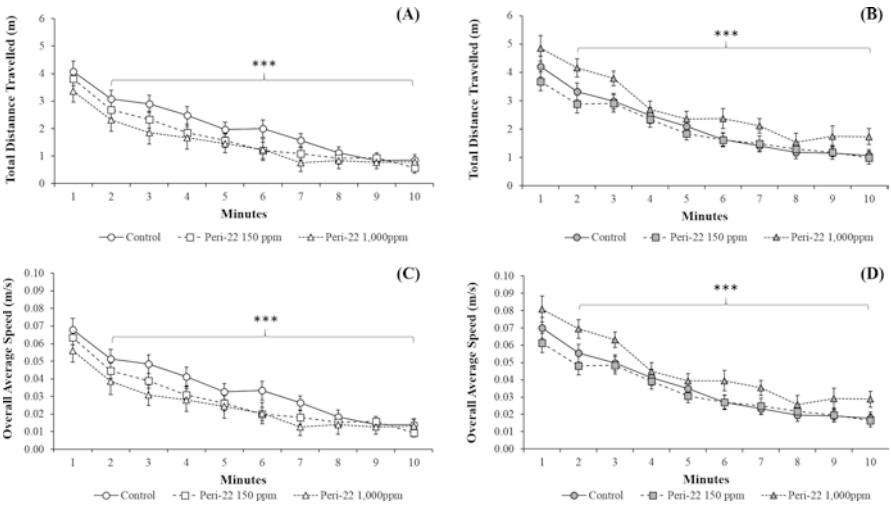

Fig. 3 Assessment of Pb^{2+}-exposure **(150 ppm = squares; 1000 ppm = triangles)** on rat locomotor activity in the OF and its influences within-*Sex* **(males = open symbols; females = grey symbols)**. Data show for both *Total Distance Travelled (m)* (**A** & **B**) and *Overall Average Speed (m/s)* (**C** & **D**), that there were no significant differences in locomotor activity in the OF as a function of *Pb^{2+}exposure* nor *Sex*. However, as a function of *Time*, there was a significant effect across the 10-min of the OF in which the rats gradually shift from high-to-low locomotor activity as they habituate to the OF (***p* < 0.001*****). Thus, indicating that both rat *Sexe*s were not influenced by *Pb^{2+}-exposure* in their locomotor behavioral profiles

3.3 *Developmental Pb^{2+}-Exposure Induced Sex-Based Differences in Anxiogenic Behaviors That Were Recovered by Taurine and Taurine Derivative Anxiolytic Drug Treatments*

After 24 h following the OF, rats were subjected to the EPM to compare the within-sex differences in response to both developmental Pb^{2+}-exposure as a function of *PPM* and *Drug Treatment Condition* effects on the *OTC ratio*. Male rats showed no significant effect of *Treatment* for the *OTC ratio* $F_{(1)} = 1.177$, $p = 0.282$ n/s (Fig. 4a), yet revealed a significant effect of *Treatment* and *PPM* for the *OTC ratio* $F_{(1,2)} = 153.452$, $p < 0.001^{***}$, $\eta_p^2 = 0.684$ (Fig. 4a). Interestingly, despite these Pb^{2+}-induced differences, male rats showed no significant effects of *Drug Treatment Condition* for the *OTC ratio* $F_{(5)} = 0.673$, $p = 0.645$ n/s (Fig. 4a), nor any significant effect on *Drug Treatment Condition* and *PPM* for the *OTC ratio* $F_{(5,3)} = 0.014$, $p = 1.000$ n/s (Fig. 4a). Also, male rats exhibited no significant *Treatment X Drug Treatment Condition* interaction for the *OTC ratio* $F_{(1,2)} = 0.043$, $p = 0.999$ n/s (Fig. 4a), nor any significant *Treatment X Drug Treatment Condition X PPM* interaction for the *OTC ratio* $F_{(1,2,5)} = 0.014$, $p = 1.000$ n/s (Fig. 4a).

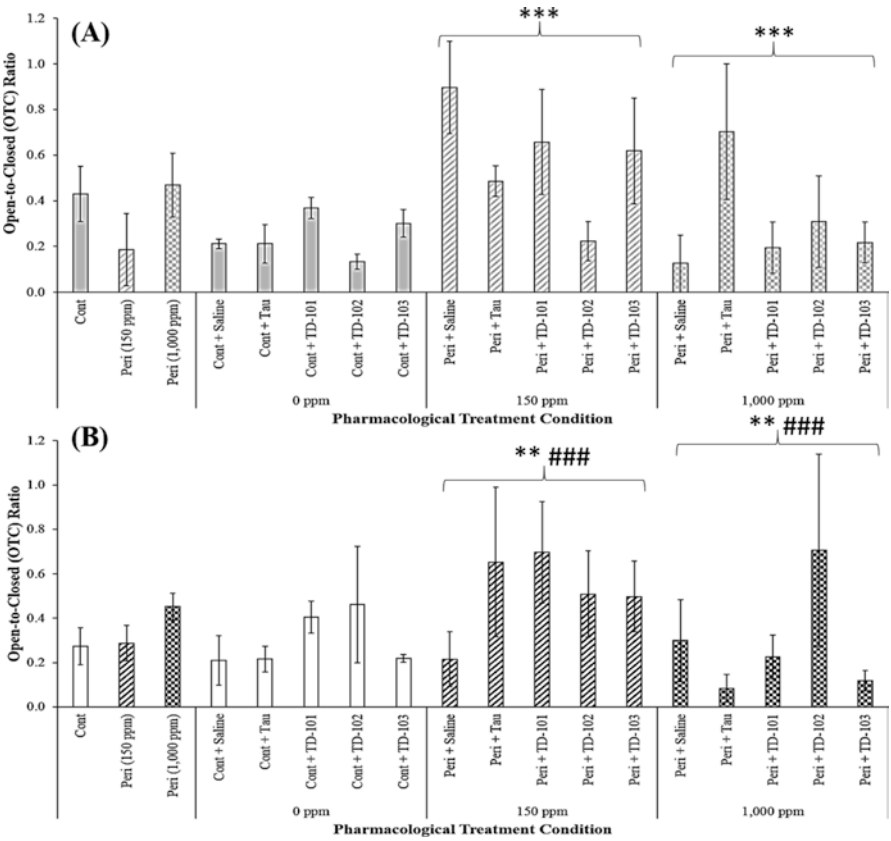

Fig. 4 Effects of Pb^{2+}-exposure **(150 ppm = diagonal line bar pattern; 1000 ppm = checkered bar pattern)** on *Open-to-Close (OTC) ratio* in the EPM and its influences within-*Sex* **(males = upper panel A; females = lower panel B)**. Data show for that there was an effect of *PPM* in both male rats *($p < 0.001$∗∗∗)* and female rats **($p < 0.01$∗∗)**, respectively. However, male rats did not show a significant effect of *Drug Treatment Condition*, yet female rats did show a significant effect of *Drug Treatment Condition* **($p < 0.001^{\#\#\#}$)**. The data suggest that female rats were more responsive to Taurine and Taurine Derivative pharmacotherapy than male rats. However, through this pilot study, there was an emerging trend that dependent upon the amount of Pb^{2+}-exposure (*PPM*) and *Sex*, perhaps different taurine derivatives may prove to be useful in facilitating recovery of Pb^{2+}-induced behavioral anxiety in the EPM

In contrast, female rats showed no significant effect of *Treatment* for the *OTC ratio* $F_{(1)} = 0.168$, $p = 0.683$ n/s (Fig. 4b), yet revealed a significant effect of *Treatment* and *PPM* for the *OTC ratio* $F_{(1,2)} = 10.017$, $p < 0.01$**, $\eta_p^2 = 0.124$ (Fig. 4b). Remarkably, female rats exhibited Pb^{2+}-induced anxiogenic differences, and showed more sensitivity to the *Drug Treatment Conditions*, when compared to male Pb^{2+}-exposed rats. Specifically, female rats showed significant effects of *Drug Treatment Condition* for the *OTC ratio* $F_{(5)} = 2.951$, $p < 0.05$*, $\eta_p^2 = 0.077$ (Fig. 4b), and a significant effect on Drug Treatment Condition and *PPM* for the *OTC ratio*

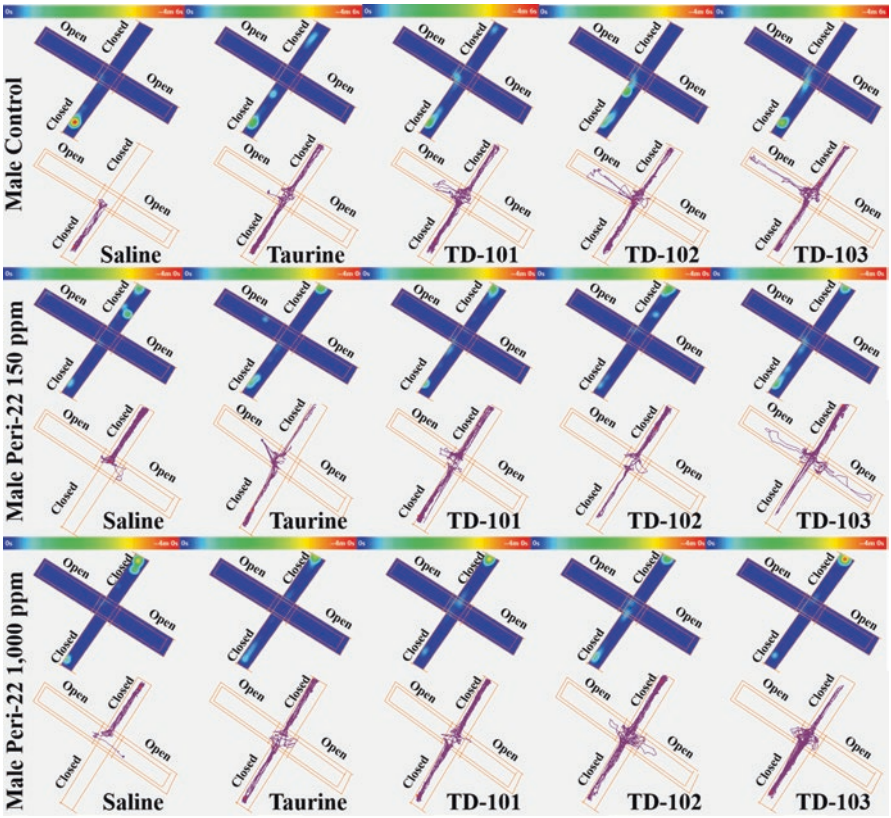

Fig. 5 A visual representation of an individual rat track plot from each treatment condition and their group mean activity average across the 10-min EPM test for male rats. Data are shown as a function of Pb^{2+}-exposure (*PPM*; upper panel 0 ppm; middle panel 150 ppm; and lower panel 1000 ppm). In addition, data are organized by *Drug Treatment Condition* vertically from left-to-right (Saline; Taurine; TD-101; TD-102; TD103). Control male rats show an increased anxiolytic response in the EPM to taurine and taurine derivatives. However, Pb^{2+}-exposed rats show less sensitivity and selectivity to drug treatments with perhaps less potential for taurine and taurine derived pharmacotherapy

$F_{(5,3)} = 14.659$, $p < 0.001^{***}$, $\eta_p^2 = 0.292$ (Fig. 4b). Furthermore, female rats exhibited a significant *Treatment X PPM X Drug Treatment Condition* interaction for the *OTC ratio* $F_{(1,2,5)} = 2.896$, $p < 0.05^{*}$, $\eta_p^2 = 0.166$ (Fig. 4b). To further illustrate the EPM data, Fig. 5 (males) and Fig. 6 (females) shows a representative individual rat track plot in addition to the *OTC ratio* as a function of group mean activity during the 10-min of the EPM. Low activity (i.e., anxiogenic responses) can be visualized by the dark-blue inactive freezing responses. In contrast, high activity in the EPM (i.e., anxiolytic responses) can be visualized by the increase in color shades shifting from light blue to green, yellow, orange, and red activity responses.

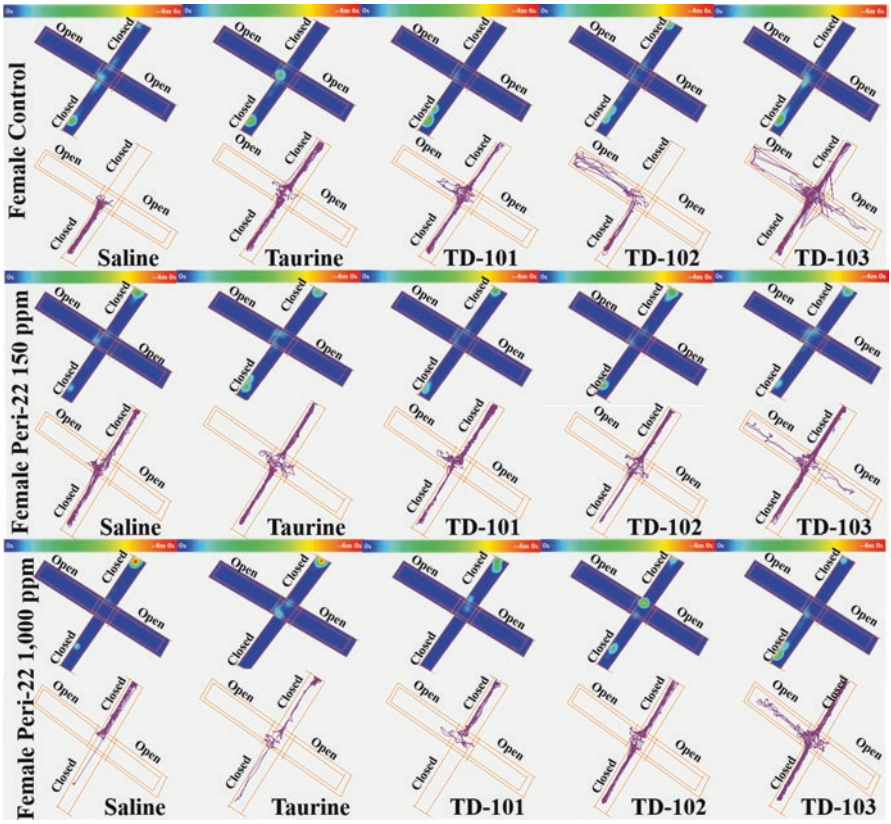

Fig. 6 A visual representation of an individual rat track plot and their group mean activity average across the 10-min EPM test for female rats. Data are shown as a function of Pb^{2+}-exposure (*PPM*; upper panel 0 ppm; middle panel 150 ppm; and lower panel 1000 ppm). In addition, data are organized by *Drug Treatment Condition* vertically from left-to-right (Saline; Taurine; TD-101; TD-102; TD103). Control female rats show an increased anxiolytic response in the EPM to taurine and taurine derivatives. Notably, Pb^{2+}-exposed rats show both a sensitivity and selectivity to certain taurine derivatives with the potential for more anxiolytic effects than taurine

4 Discussion

The present pilot study sought to examine the effects of developmental Pb^{2+}-exposure on locomotor activity within the OF and anxiogenic behaviors within the EPM as a function of *Treatment*, *Pb^{2+}-dose* (i.e., *PPM*), and *Sex*, as well as the pharmacological treatment by Taurine and Taurine Derived *Drug Treatment Conditions*. In the OF, no differences were observed in males or females with respect to the *Total Distance Travelled* (m) or the *Overall Average Speed* (m/s) as measures of locomotor activity. Furthermore, despite developmental Pb^{2+}-exposure, no differences in any of these OF measures were observed. This suggests that at the 150 ppm and 1000 ppm Perinatal exposure time-period of development in the Long

Evans Hooded rat, the Pb^{2+}-exposure produced no observable behavioral deficits or excesses that would have been deemed as abnormal locomotor activity. Thus, no evidence of issues with locomotor activity (i.e., that would have otherwise interfered with interpreting anxiety-like behaviors within the EPM) can be traced to the developmental Pb^{2+}-exposure from the OF preliminary assessment for the age of the rats tested herein.

In the EPM, the within-sex effects were assessed for anxiogenic behaviors that were evoked by the EPM testing apparatus and bright lighting effects. Female rats were observed to be more sensitive to the EPM and exhibited less *OTC ratios* when compared to males in the Control treatment conditions. However, when comparing the within-sex effects as a function of Pb^{2+}-exposure, male rats showed no differences in their *OTC ratios*, when compared to Control males. In comparison, female rats that were exposed to Pb^{2+} also showed no differences in their *OTC ratio* relative to Control females. Thus, it would appear that Pb^{2+} causes no anxiogenic behaviors in the EPM. However, the *OTC ratio* is a different dependent measure that is arguably more sensitive to drug effects in the EPM (Walf and Frye 2007). As such, it assesses the reduction or anxiolytic properties of the rat's exploratory behavior to inhibit fear and approach the open arms more than the closed arms. Traditional EPM dependent measures, such as *Time in the Closed Arm* or *Time in the Open Arm*, are fair indicators of anxiogenic behaviors, but require careful interpretation. First, most studies using the EPM may only report one of these dependent measures, which only describe half of the anxiogenic profile of the animal model under study. Second, because the rats could be moving freely or freezing, "*Time*" alone is an insufficient descriptor of an animal's behavior. Thus, unless clearly operationally defined, "*Time*" variable offers more obscurity than one would hope when interpreting EPM analyses. Lastly, the traditional EPM values do prove informative when carefully examined, operationalized, and interpreted when a full anxiogenic profile can be provided.

The present pilot study, sought to assess the effectiveness of Taurine and its derivatives in *Drug Treatment Conditions* for anxiolytic behavioral pharmacological effects on rats in the EPM. In this context, the *OTC ratio* served as a very sensitive dependent measure as it targets the increase in activity into the open arms relative to the activity into the close arms. Higher *OTC ratios* results in more anxiolytic behavioral responses in the rat. Conversely, the lower the OTC ratio the more anxiogenic the rat's behavioral response. The effects of the *Drug Treatment Conditions* revealed in this pilot study that the Control male rats were most sensitive to Taurine Derivatives TD-101 and TD-102, whereas the Control female rats were most sensitive to Taurine Derivatives TD-101 and TD-103. The Peri-22 150 ppm male rats seem to be sensitive and responsive to Taurine and each of the Taurine Derivative drugs, whereas Peri-22 1000 ppm male rats were only sensitive to TD-102 in promoting anxiolytic *OTC ratios*. Remarkably, the Peri-22 150 ppm female rats showed sensitivity to Taurine and each of the Taurine Derivatives, except for TD-102; whereas Peri-22 1000 ppm females were sensitive to Taurine and only TD-102.

Taken together, these findings suggest that Pb^{2+}-exposure perhaps changed the GABA-AR subunit arrangement by altering the sensitivity to the phamacodynamic properties of the receptor activation states—that is functionally different in both a sex-specific manner and in response to the amount of Pb^{2+}-exposure endured during development. Furthermore, the type of direct neurotoxicant impact that Pb^{2+} inflicts upon the developing nervous system during the critical stages of GABAergic neural development (Ben-Ari 2002, Ben-Ari et al. 2012; Neuwirth et al. 2017, 2018; Neuwirth 2014), could also alter the GABAergic tone and responsivity to GABAergic agonist drugs. Additionally, the mature GABAergic systems are critical for cortical inhibition/disinhibition in relationship with frontoexecutive functions and are significantly impacted by developmental Pb^{2+}-exposures (Neuwirth et al. 2019a) and are also ameliorated by taurine co-treatment (See Ch. 70 Neuwirth et al. 2019b). The Taurine derivatives used in this pilot study present a novel and, perhaps, a pioneering approach to the development and evaluation of new Taurine-like compounds that might foster more precise neuromodulatory actions of the GABA-AR to counteract against the neurotoxicant impacts of environmental Pb^{2+}-exposure to the developing central nervous system.

5 Conclusion

In summary, this pilot study shows that developmental Pb^{2+}-exposure can have neurodevelopmental impacts that persist across the lifespan. In addition, based on the Taurine derivatives used in this study, the amount of developmental Pb^{2+}-exposure can, perhaps, influence the arrangement of the GABA-AR in ways that alter its pharmacodynamics and subsequent responsivity to GABA-AR agonists. Furthermore, the chemical structure of the Taurine Derivatives provide new insights into examining specific drug treatments that might be uniquely matched to different Pb^{2+}-exposure levels, and may be further customized to accommodate sex-specific needs given the different sensitivity to Taurine and Taurine Derived compounds through the EPM. Although, this pilot study was limited to the EPM, future research may look to explore the effects of these Taurine Derivatives across a range of other behavioral test conditions to evaluate other cognitive and behavioral neurological conditions impacted by environmental Pb^{2+}-exposure (see Ch. 70 Neuwirth et al. 2019b). As such, this pilot study paves the way for new research in investigating possible drug treatments that are safe, effective, and precisely match the underlying neurobiological problems induced by neurotoxicants such as Pb^{2+}. Future Pb^{2+} research should make a concerted effort to provide children with psychopharmacotherapies that may improve their quality of life across their lifespan; especially if they are unable to be removed from sources of environmental Pb^{2+}-exposures or environments that continue to contain both historical and modern forms of Pb^{2+}-sources of exposure.

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Early Neurodevelopmental Exposure to Low Lead Levels Induces Fronto-executive Dysfunctions That Are Recovered by Taurine Co-treatment in the Rat Attention Set-Shift Test: Implications for Taurine as a Psychopharmacotherapy Against Neurotoxicants

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Abstract Lead (Pb^{2+}) is a developmental neurotoxicant that causes lifelong cognitive dysfunctions. In particular, Pb^{2+}-induced frontoexecutive dysfunctions emerge later in life when the cortex is fully myelinated, thereby permitting the ability to assess the extent to which Pb^{2+} has developmentally impacted higher order cognitive and behavioral systems. The present study evaluated the effects of developmental Pb^{2+}-exposure (150 ppm lead acetate in the drinking water) in Long Evans

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Hooded rats through the Attention Set-Shift Test (ASST) between postnatal days (PND) 60–90. Treatment groups were comprised of Control (0 ppm), Perinatal (150 ppm), and Perinatal+Taurine (150 ppm + 0.05% Taurine in the drinking water) rats (N = 36; n = 6 per treatment group for each sex). Frontoexecutive functions were evaluated based on *trials-to-criterion* (TTC) and *errors-to-criterion* (ETC) measures for simple and complex discriminations (SD & CD), intradimensional and extradimensional shifts (ID & ED), as well as reversals (Rev) of the CD, I-, and ED stages, respectively. Post-testing, the prelimbic (PrL), infralimbic (IL), orbital ventral frontal (OV), orbital ventro-lateral (OVL), and hippocampal (HP) brain regions were extracted and processed through Liquid Chromatography/Mass Spectrophotometry (LC/MS) for determining the GABA and Taurine ratios relative to Glutamate, Dopamine, Norepinephrine, Epinephrine, and Serotonin. The ASST data revealed that Perinatal rats are negatively impacted by developmental Pb^{2+}-exposures evidenced by increased TTC and ETC to learn the SD, ID, and ID-Rev with unique sex-based differences in frontoexecutive dysfunctions. Moreover, Perinatal+Taurine co-treated rats exhibited a recovery of the frontoexecutive dysfunctions observed in Perinatal rats to levels equivalent to Control rats across both sexes. The LC/MS data revealed altered brain sub-region specific patterns across the PrL, IL, OV, OVL, and HP in response to developmental Pb^{2+}-exposure that produced an altered neurochemical signaling profile in a sex-dependent manner, which may underlie the observed frontoexecutive dysfunctions, cognitive inflexibility, and associated motivation deficits. When taurine co-treatment was administered concurrently for the duration of developmental Pb^{2+}-exposure, the observed frontoexecutive dysfunctions were significantly reduced in both ASST task performance and neurochemical ratios that were comparable to Control levels for both sexes. Altogether, the data suggest that taurine co-treatment may facilitate neuroprotection, mitigate neurotransmitter excitability balancing, and perhaps ameliorate against neurotoxicant exposures in early development as a potential psychopharmacotherapy.

Keywords Attention-set-shift test · Lead poisoning · Developmental neurotoxicity · Taurine · Frontoexecutive functions · Psychopharmacotherapy

Abbreviations

AD/HD	attention deficit/hyper activity disorder
ASST	attention set-shift test
ASV	anodic stripping voltammetry
BLL	blood lead level
CD	complex discrimination
CD-ReAcq	compound discrimination re-acquisition
CD-Rev	complex discrimination reversal
ED	extradimensional shift
ED-Rev	extradimensional shift reversal

EDTA	ethylenediaminetetracetic acid
ETC	errors to criterion
HP	hippocampal area
HPLC	high performance liquid chromatography
ID	intradimensional shift
ID-ReAcq	intra-dimensional shift re-acquisition
ID-Rev	intradimensional reversal
IL	infralimbic area
LC/MS	liquid chromatography/mass spectrophotometry
OV	orbital ventral frontal area
OVL	orbital ventral lateral area
Pb^{2+}	lead
PND	postnatal day
PrL	prelimbic area
SD	simple discrimination
Tau	taurine
TTC	trials-to-criterion
WCST	Wisconsin Card Sorting Task

1 Introduction

In review of the lead poisoning (Pb^{2+}) neurotoxicology literature, the majority of studies have focused on hippocampal-dependent learning and memory systems in animal models. Although the hippocampus is important for learning to occur, the prefrontal cortex is also a critical brain region necessary for learning and memory and is further responsible for tightly regulating attentional mechanisms. It can be argued that without proper functioning attentional mechanisms, that sensory information cannot be adequately identified, focused upon, examined with greater visual detail, and attended to long enough to evaluate this information past sensory selection filters to reach high-order memory systems. These attentional mechanisms have been uniquely defined as frontoexecutive functions that encompass focused attention, planning, organizing action, goal-directed behaviors, valuing incentives, and cognitive flexibility (for review see Tait et al. 2018). When these prefrontal cortico-striatal systems are damaged, altered or disrupted by a developmental neuropathy or later life brain injury, the resulting condition is referred to as a frontoexecutive dysfuntion and can be tested through neuropsychological assessments (Lezak et al. 2004). In order to bridge the gap between human neuropathologies and animal models simulating human pathological states of the frontal lobes, Birrell and Brown (2000) created a derivation of the human Wisconsin Card Sorting Task (WCST) neuropsychological assessment (Milner 1963) for rats called the Attention Set-Shift Test (ASST). Consistent with the WCST, the ASST permits similar yet equally sensitive evaluation of the prefrontal cortical dysfunctions during real-time decision-making behaviors.

Since the inception of the ASST, a range of research groups have shown it to be a valid test procedure across frontoexecutive dysfunctions in animal models of

attention-deficit/hyperactivity disorder (AD/HD) (Aron and Poldrack 2005), stress (Arnsten 2009), emotional fear and schizophrenia (Gilmartin et al. 2014), and developmental Pb^{2+} neurotoxicity (Neuwirth et al. 2019a). Thus, providing good generalizability, face and external validity for the ASST. Moreover, Dalley et al. (2004) suggested that animal frontoexecutive dysfunction models could be used to assess different brain sub-cortical regions involved in the attentional control mechanisms of the ASST that may differentially contribute to frontoexecutive functions within- and between-animal models (for review see Bizon et al. 2012; Chudasama and Robbins 2006). Thus, studies are warranted in providing clearer relationships between the prefrontal attentional mechanisms that guide frontoexecutive behaviors and their neurochemical correlates, to better understand how they work both differentially and cooperatively in guiding context-specific behaviors in normal functioning mammals, as well as, in neuropathological states.

The present study sought to examine the effects of developmental Pb^{2+}-exposure in relation to later-life frontoexecutive dysfunctions in the rat model through performance measures in the ASST. The purpose of this study was to elucidate the rats' prefrontal attentional mechanisms and to compare the findings with reports found in the literature regarding children with low-level Pb^{2+}-exposure who present with frontoexecutive dysfunction as they age (Roy et al. 2009; Canfield et al. 2003, 2004, 2005). Further, these findings could be also evaluated against datasets that specifically evaluated Pb^{2+}-neurotoxicity in children and frontoexecutive results from clinical neurological assessment data (Lidsky and Schneider 2006; Lidsky and Schneider 2003). Lastly, taurine a potent $GABA_{AR}$ and partial $NMDA_R$ agonist with established neuroprotective properties, was employed as a co-treatment during the time-period of developmental Pb^{2+}-exposure to evaluate its potential as a psychopharmacotherapy agent in mitigating Pb^{2+}-neurotoxicity. Altogether, the present study examined the extent to which chronic taurine psychopharmacotherapy could be used to reduce the altered neurochemical excitability balance and frontoexecutive dysfunctions as a result of developmental Pb^{2+}-exposure consistent with prior reports on acute taurine therapeutic approaches (Neuwirth 2014; Neuwirth et al. 2017, 2019a; Neuwirth 2018).

2 Methods

2.1 Subjects

In accordance with The SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans Norwegian hooded male ($N = 3$) and female rats ($N = 6$) (Taconic, N.J.) were paired for breeding and their male and female F1 generation offspring were used for the present study. Rat litters were culled to 8–10 pups in order to control for maternal social influences on neurodevelopmental and behavioral outcomes that were later examined. Rats were randomly assigned to the following breeding groups: Control, Perinatal, or Perinatal+Taurine exposures, respectively.

All rats were fed regularly with Purina rat chow (RHM1000 # 5P07) *ad libitum*. However, Control rats were provided with regular water, while the experimental rats were fed water containing lead acetate (Sigma Aldrich, St. Louis, MO) from pairing throughout gestation and continued through weaning at postnatal day (PND) 22 (*i.e.,* constituting a Perinatal developmental Pb^{2+}-exposure model). At PND 22, Pb^{2+}-exposures ceased and all rats returned to a regular water regimen. Rats assigned to the Perinatal group drank a lead acetate water $(C_2H_3O_2)_2Pb{\cdot}3H_2O$ [363.83 μM] and the Perinatal+Taurine group drank the identical lead acetate water, but it was additionally supplemented with 0.05% Taurine $C_2H_7NO_3S_1$ [4 mM] (Sigma Aldrich, St. Louis, MO). All water solutions were administered *ad libitum*. Prior to behavioral testing, all rats were handled for 20-min per day for 2-weeks. Between postnatal days (PNDs) 60–90 (*i.e.,* when the prefrontal cortex has fully matured in rats) male and female rats were randomly selected from the litters and then assigned to the ASST. The following samples sizes were used within the ASST: $n = 6$ Control, $n = 6$ Perinatal, and $n = 6$ Perinatal+Taurine for both males and females, respectively.

2.2 Blood Lead Level Analyses

At PND 22 immediately following the end of Pb^{2+}-exposure, a separate group of male and female rats (*i.e.,* with a representative sample culled from the same litters) were sacrificed ($n = 4$ per gender, per treatment group) and their blood samples were collected and analyzed consistent with previous reports (Neuwirth 2014; Neuwirth et al. 2017, 2018, 2019a, b). Briefly, blood samples were collected within 2 mL anticoagulant *ethylenediaminetetracetic acid* (EDTA) coated syringes (Sardstedt, Germany), mixed to prevent coagulation, and then frozen at −80 °C. Blood samples were analyzed using a commercial ESA LeadCare II Blood Lead Analyzer system (Magellan Diagnostics, North Billerica, MA) to determine the amount of Pb^{2+} in the blood by electrochemical anodic stripping voltammetry (ASV) to eliminate any potential for experimenter bias. The ASV method was conducted by taking 50 μL of whole blood mixed with 250 μL of hydrochloric acid solution (0.34 M) and then applying the final mixture to the lead sensor strip and inserted into the ESA LeadCare II Blood Lead Analyzer system to determine BLLs. After 3 minutes, the BLLs were reported from the instrument in μg/dL with a lower sensitivity cut off value of 3 μg/dL and a high sensitivity cut off value of 65 μg/dL (*i.e.,* SEM ± 1.5 μg/dL sensitivity detection level).

2.3 Establishing Operation for Motivational Learning

At PND 55 a naivë set of Control ($n = 6$), Perinatal ($n = 6$), Perinatal+Taurine ($n = 6$) male and female rats were scheduled for dig training and subsequently the ASST. In order to ensure that the rats had the necessary motivation to search for and consume

a reward the following procedures were implemented as in the original ASST paper of Birrell and Brown (2000) and the methods of Neuwirth et al. (2019a): (1) rats were given a highly preferred food reward that consisted of a half piece of Kellogg's® Froot Loops® cereal; and (2) were placed on an approved National Institute of Health (NIH) (2017) Guidelines for Diet Control in Behavioral Studies (http://oacu.od.nih.gov/ARAC/dietctrol.pdf). This NIH approved food restriction schedule served to ensure that rats were maintained at a healthy 80% of their *ad libitum* body weight. The food restriction consisted of providing four food pellets to male and three food pellets to female rats daily. This procedure served to create a steady metabolic state and an establishing operation of motivation to search for and consume a food reward, during both the training and test session components comprising the ASST. The weights for each rat were taken as a baseline value prior to being placed on food restriction and continually monitored by being weighed every Monday, Wednesday, and Friday until testing was completed.

2.4 Dig Training

Following the establishment of the necessary motivational level for learning, at PND 55 male and female rats were scheduled for dig training. Dig training consisted of a rat searching within an acrylic bowl (711.2 mm L x 431.8 mm W x 406.4 mm H) in order to retrieve a half of a Kellogg's® Froot Loops® cereal piece within an increasing amount of shredded paper (*i.e.,* the digging medium). Training consisted of rats being shaped through a sequence of five forward-chained behaviors during a 2-min trial: (1) empty bowls were sprinkled with ground Kellogg's® Froot Loops® cereal dust and half a cereal piece was placed in the center of the bowl; (2) bowls were prepared as before, but 25% of the bowl was filled with shredded paper; (3) bowls were prepared as before, but 50% of the bowl was filled with shredded paper; (4) the bowls were then filled to 75% with shredded paper; and (5) the bowl was then 100% filled with shredded paper. Rats had to complete 10-trials successfully for each digging sequence before moving to the next sequence to meet the criteria for being adequately dig trained. All dig trainings were completed in a single training session.

2.5 Attention Set-Shift Test

The ASST was implemented consistent with the procedures of Birrell and Brown (2000) (for review of ASST methodology see Tait et al. 2018) and Neuwirth et al. (2019a) using the Neuwirth™ ASST apparatus. Between PNDs 56–90 dig-trained rats were subjected to a 4-day test schedule that was necessary to provide a test break for the Perinatal rats (*i.e.,* negative reinforcement) consistent with the

procedures of Neuwirth et al. (2019a). Briefly, the rats were given a two-choice pair stimulus presentation in which the bowls were lightly covered with ground Kellogg's® Froot Loops® cereal dust to prevent the rat from identifying the food reward based on scent alone. The criterion for a rat to move from one ASST condition to another was to complete 6-consequetive trials without an error.

On Test Day 1, the rat was presented with a first set of novel stimuli parings as a two-choice presentation procedure. Each two-choice presentation consisted of discriminating between a pair of novel odors to the bowls (*i.e.,* 20 μL of aromatic oils) and/or a pair of novel tactile medium (*i.e.,* digging materials) within the acrylic bowls (see Table 1). The rats were then tasked to associate which stimulus was paired with the food reward (*i.e.,* relevant stimulus) in comparison to the other stimulus/stimuli that was not paired with a food reward (*i.e.,* irrelevant stimulus/stimuli). This served as either a simple discrimination (SD) between 2-stimuli pairings of either two-odors (*i.e.,* an odor discrimination [OD]) or two-digging materials (*i.e.,* a digging medium discrimination [MD]) (Table 1).

On Test Day 2, rats had to generalize what they learned from the first set of novel stimuli parings for the OD and MD trainings using a new second set of novel stimuli pairings to make a SD. Then the rats frontoexecutive functions were further challenged by being tasked to make a complex discrimination (CD) (*i.e.,* now the two-choice presentation of bowls consisted of a combination of two odors and two digging mediums at once [4-stimuli pairings] (Table 1). Following the CD, the rats cognitive flexibility was now challenged to ignore the previously relevant stimuli that was associated with the food reward and shift its attention to the previously irrelevant stimuli that was now paired with the food reward; thus, constituting a complex discrimination reversal (CD-Rev) task (Table 1).

Table 1 The odor exemplar pairing used in the attention set-shifting task

Training	*Odors*		*Digging medium*	
Paring 1	O1-Cumin	O2-Paprika	M1-Shredded paper	M2-Polystyrene
Testing	*Odors*		*Digging medium*	
Pairing 2 SD, CD, CD-Rev	O3-White thyme	O4-Texas cedar wood	M3-Small beads	M4-Small gravel
Pairing 3 CD-ReAcq, ID, ID-ReAcq	O5-Clove buds	O6-Rosemary	M5-Fine wood shavings	M6-Large wood shavings
Pairing 4 ID-Retent, ED, ED-ReAcq	O7-Spearmint	O8-Cinnamon	M7-Dirt with wood shavings	M8-Mulch

Abbreviations are defined as follows: *Pairing 2* comprised the *SD* Simple Discrimination, *CD* Compound Discrimination, and the *CD-Rev* Compound Discrimination Reversal stages, *Pairing 3* comprised the *CD-ReAcq* Compound Discrimination Re-acquisition, *ID* Intra-dimensional Shift, and the *ID-Rev* Intra-dimensional Reversal stages, and *Pairing 4* comprised the *ID-ReAcq* Intra-dimensional Shift Re-acquisition, *ED* Extra-dimensional shift, and the *ED-Rev* Extra-dimensional Shift Reversal stages (Consistent with the procedures of Neuwirth et al. 2019a)

On Test Day 3, the CD-Rev stage was re-tested (*i.e.,* a learning re-acquisition probe [CD-ReAcq]) to re-establish behavioral momentum through the ASST due to the required test break between test days. After the CD-ReAcq stage, the rat was presented with a third set of novel stimuli pairings and it was tasked with following the same relevant stimulus dimension (*i.e.,* odor or digging medium from the prior day) in solving another CD, which served as an intradimensional shift (ID) (*i.e.,* odor-to-odor or medium-to-medium "in the same relevant stimulus dimension as the prior test day to generalize learning"). This was followed by an intradimensional reversal (ID-Rev) (Table 1).

On Test Day 4, the ID-Rev was re-tested again with a learning re-acquisition probe (*i.e*., ID-ReAcq) to ensure behavioral momentum. Then the rat was presented with a fourth new set of novel stimuli pairings and it was tasked with following the previously irrelevant stimulus dimension (*i.e.,* if the rat previously was following an odor stimulus it would now have to shift to a digging medium stimulus) serving as the extradimensional shift (ED). This was followed by an extradimensional reversal (ED-Rev) (Table 1).

2.6 Brain Extractions and Sub-Region Dissections

Immediately following the ASST, rats were deeply anesthetized using Isoflurane, then sacrificed, and their brains were extracted in cold physiological buffered saline (PBS) pH 7.4 in under 2-min. The rat whole brains were then transferred into a coronal sectioning steel brain matrix for 175–300 g rodents (Stoelting, Inc. Wood Dale, IL). The whole rat brains were then manually sectioned into 1 mm thin slices using two sterile single-edged razor blades, transferred into Petri dishes containing cold PBS, and the following brain sub-regions were then manually dissected and collected into 1.5 mL tubes using a dissection microscope: prelimbic (PrL), infralimbic (IL), orbital ventral frontal (OV), orbital ventro-lateral (OVL), and hippocampal (HP) areas, respectively. The collected brain sub-regions were stored at ⁻80 °C until ready for subsequent neurochemical assessments.

2.7 Neurotransmitter Profile and Ratio Assessment

The brain sub-regions were then manually homogenized with sterile glass homogenizers (*i.e.,* total volume 3 mL) using a 10 mg/100 μL (1:10) dilution of 100% acetonitrile (CHC_3N) (Sigma-Aldrich, St Louis, MO) as a miscible (*i.e.,* fully dissolvable solution) with a dielectric constant to study the separation of chemicals by mass charge and polarity. Post homogenization, samples were sonicated for 30 sec with a pulse on:off time of 10 sec at an amplitude of 20%, then centrifuged at 14.8

RPM for 5-min at 4 °C, and the supernatant collected and stored at ⁻20 °C until ready for LC/MS. The supernatant was injected (*i.e.,* 10 μL of pure brain sub-region sample) into a DC cell of a Shimadzu Liquid Chromatography/Mass Spectrophotometer (LC/MS) 8030 (Shimadzu Scientific Instruments, Columbia, MD) to assess the GABA and Taurine ratios to the following neurotransmitters of interest: glutamate, norepinephrine, dopamine, serotonin, and epinephrine. Neurotransmitters were separated by High Performance Liquid Chromatography (HPLC) using a C18 reverse phase column. An acetonitrile gradient (0–100% acetonitrile in 0.1% TFA containing HPLC water) was used to separate different neurotransmitters. The mass/charge (m/z) values of neurotransmitters were monitored and peak heights were obtained to compare the amount of neurotransmitters within- and between-samples. The elution was performed with a flow rate of 0.2 mL/min and the neurotransmitters that were eluted from the column were detected in the positive ion mode. The spray voltage was kept at 5 kV and the capillary temperature was set at 250 °C while the sheath gas (nitrogen) was set at 60 units. Standards for LC/MS were made at a concentration of 1 mg/1 mL 100% acetonitrile from TLC grade (97–99.99%) chemicals from Sigma-Aldrich (St. Louis, MO) for the following neurotransmitters: γ-aminobutyric acid $C_4H_9NO_2$ (103.4 g/mol), Dopamine hydrochloride $(HO)_2C_6H_3CH_2CH_2NH_2{\cdot}HCL$ (153.85 g/mol), (−)-Epinephrine $C_9H_{13}NO_3$ (165.95 g/mol), D-glutamic acid $C_5H_9NO_4$ (147.90 g/mol), (−)-Norepinephrine $C_8H_{11}NO_3$ (151.85 g/mol), Serotonin hydrochloride $C_{10}H_{12}N_2O{\cdot}HCL$ (159.95 g/mol), and Taurine $C_2H_7NO_3S_1$ (125.75 g/mol) (Fig. 1).

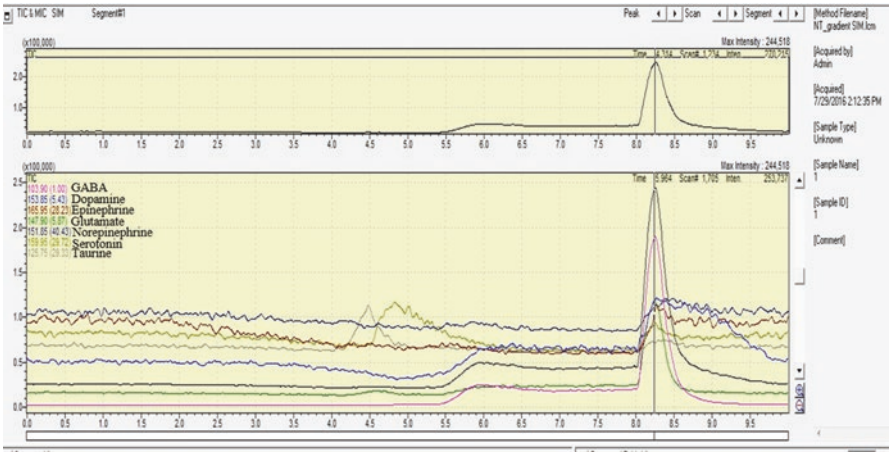


Fig. 1 Illustrates the LC/MS detection profiles of the Sigma-Aldrich (St. Louis, MO) standards for the following neurotransmitters: GABA (103.4 g/mol), Dopamine (153.85 g/mol), Epinephrine (165.95 g/mol), Glutamate (147.90 g/mol), Norepinephrine (151.85 g/mol), Serotonin (159.95 g/mol), and Taurine (125.75 g/mol). Standards were made at a concentration of 1 mg/mL 100% acetonitrile

2.8 Data Analyses

Data were recorded in real-time and analyzed using the Anymaze® video tracking software (Stoelting Co., Wood Dale, IL) transmitted via a ceiling mounted Logitech C310 Hi-speed USB 2.0 web camera (High-definition video with 1280 × 720 pixels and 5 MP photo quality). The web camera was relayed to a standard Dell D16M Inspiron 3847 Desktop computer equipped with Windows 10 64-bit operating systems, 8 GB Dual Channel DDR3 1600 MHZ (4 GB × 2), 1 TB 7200 PRM Hard Drive, and a fourth Generation Intel® Core™ i3-4170 Processor (3 M Cache, 3.70 GHz), and displayed through a Dell 20" E2016H monitor with an optimal resolution of 1600 × 900 pixels at 60 Hz. Data were recorded as digital videos that were analyzed using AnyMaze® software. Animal tracking was based on contrast relative to the background. Different zones were labeled and indicated on the monitor. Three tracking points were specified by one on the rat's head, center of its body, and the last on its tail. An excel spreadsheet was generated containing all the parameters specified. The dependent variables of interest were the number of *trials-to-criterion* (TTC) and the number of *errors-to-criterion* (ETC). Additionally, data were analyzed using a cumulative record to observe the *correct* and *error response* differences in the *rate-of-learning* during each test condition of the ASST.

Data for the LC/MS samples were analyzed by taking the average intensity values of the neurotransmitter value (*i.e.,* all values within +1 and − 1), then divided all values by GABA to find the GABA:Neurotransmitter ratio. The same procedure was done for Taurine, by taking the average intensity value of the neurotransmitter and then dividing all values by Taurine to find the Taurine:Neurotransmitter ratio. A Microsoft Excel spreadsheet was generated containing all the respective GABA:Neurotransmitter and Taurine Neurotransmitter ratios specified.

2.9 Statistical Analyses

All behavioral data were collated in Microsoft Excel and later analyzed in IBM SPSS V. 24 (IBM, Inc. Armonk, NY). For the ASST tests, an *ANOVA* was conducted using the *ASST Test Condition* as the within-subjects factors and *ASST Test Condition* and *Treatment* as the between-subjects factors for the dependent variables of *TTC* and *ETC*. For the LC/MS data, an *ANOVA* with *Treatment* and *Brain Region* as fixed-factors was used to evaluate the dependent variables of the *GABA:Neurotransmitter* and *Taurine:Neurotransmitter Ratios.* The criteria for significance was set at $\alpha = 0.05\%$ with a 95% confidence interval with the data presented as the mean ± SEM. Significant differences were determined by an equal *Tukey's HSD post hoc multiple* comparisons tests along with a *partial Eta-square* (η_p^2) for determining *pairwise comparisons* and *effect sizes* where applicable.

3 Results

3.1 *Developmental Pb^{2+}-Exposure Caused No Difference in Learning Odor and Digging Medium Simple Discriminations, While Co-treatment with Taurine Caused Learning Delays in a Sex-Dependent Manner*

The BLL data showed that Perinatal rats exhibited a range between 5.3–15 μg/dL at PND 22, with no significant differences as a function of taurine treatment. Between PNDs 56–90 after the rats had completed the ASST, their final blood draw reported BLLs below the <3 μg/dL detectable limit. This suggests that the Pb^{2+}-exposure that was circulating throughout their cardiovascular system throughout development had been absorbed by bodily tissues and/or eliminated from the system after having already disrupted neurodevelopmental processes that would later contribute to frontoexecutive dysfunctions.

Prior to the ASST, rats were trained to dig through a medium to associate a reward through both odor (OD) and digging medium (MD) discriminations to examine their learning differences measured by the *TTC* and *ETC*. Control and Perinatal male rats showed no differences in learning the OD or MD for both *TTC* and *ETC* (Fig. 2a, b). However, Perinatal+Taurine male rats had significant difficulty in learning to make the OD and MD with *Treatment* effects for the *TTC* $F_{(2)} = 4.817, p < 0.01^{\#\#}, \eta_p^2 = 0.243$ and the *ETC* $F_{(2)} = 6.023, p < 0.01^{\#\#}, \eta_p^2 = 0.286$ when compared to Control and Perinatal male rats (Fig. 2a, b). The data suggest that taurine co-treatment with developmental Pb^{2+}-exposure may have induced a learning delay in these rats, but they were still capable of completing the ASST training. In contrast, Control, Perinatal, and Perinatal+Taurine female rats showed no differences in their OD and MD learning for the *TTC* or the *ETC* (Fig. 3a, b). Taken together, these data suggest sex-based differences in learning as a function of developmental Pb^{2+}-exposure and taurine co-treatment.

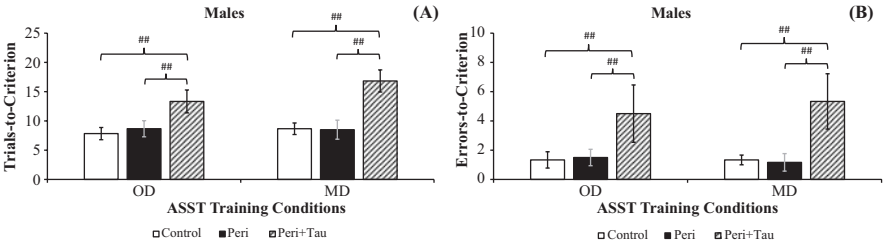


Fig. 2 Illustrates the differences in male rats' ability to learn odor (OD) and digging medium (MD) simple discriminations. The *TTC* (**a**) and the *ETC* (**b**) show that Control and Perinatal male rats learned at comparable rates. However, taurine co-treatment caused learning delays when compared to both Control and Perinatal male rats ($p < 0.01^{\#\#}$), respectively

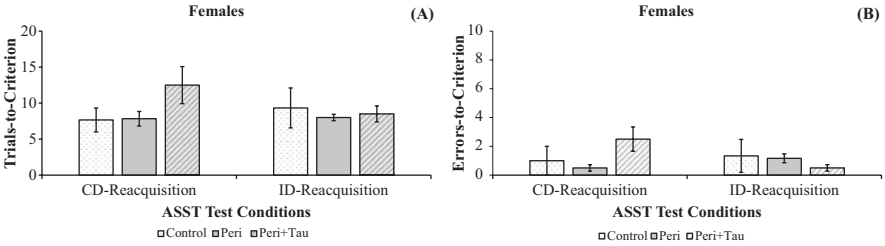
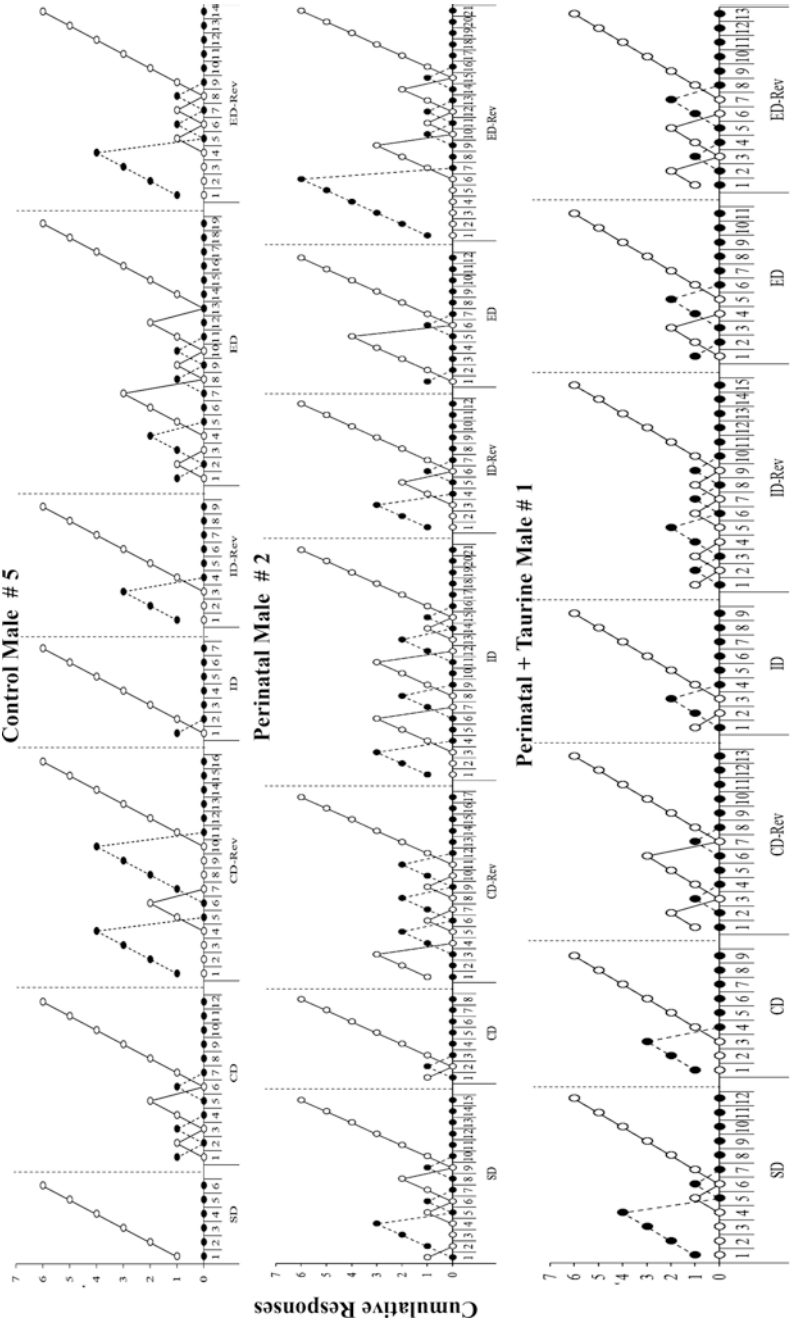


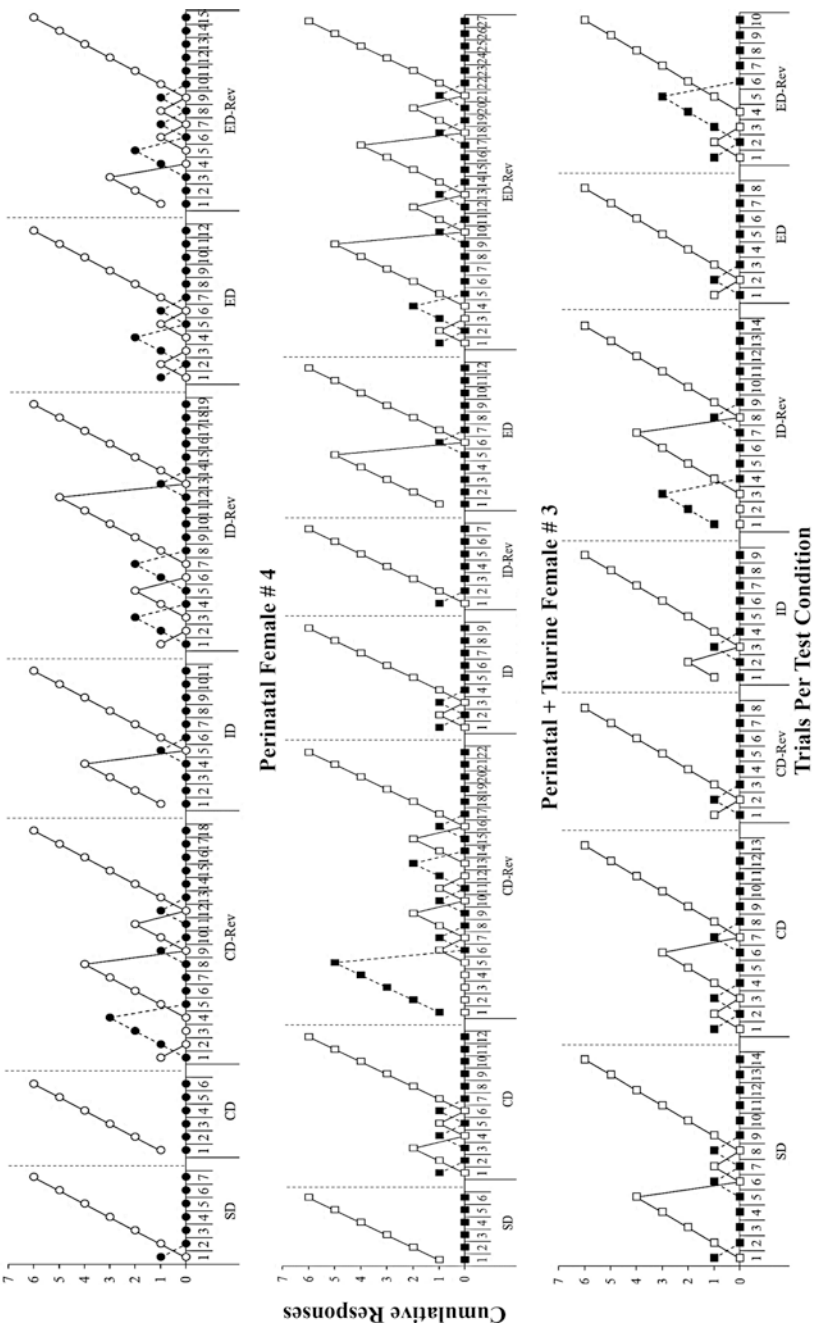
Fig. 3 Illustrates the differences in female rats' ability to learn odor (OD) and digging medium (MD) simple discriminations. The *TTC* (**a**) and the *ETC* (**b**) show that Control and Perinatal female rats learned at comparable rates

3.2 Developmental Pb^{2+}-Exposure Frontoexecutive Dysfunctions That Persisted in the Mature Rats' Rate-of-Learning Behavioral Patterns Were Improved by Taurine Co-treatment

At PND 22 the perinatal Pb^{2+}-exposed rats were removed from the neurotoxicant exposure for the remainder of the study. The effects of this developmental Pb^{2+}-exposure caused persistent frontoexecutive dysfunctions in a sex-dependent manner that was observed within the ASST. In order to examine the individual rats' ASST performance differences, a representative sample from each gender and treatment condition were randomly selected. The individual rats' performance data regarding their *correct* and *error* response differences during their *rate-of-learning* cumulative records across the test conditions of the ASST, showed that developmental Pb^{2+}-exposure caused significant frontoexecutive impairments and delays in and accuracy of *correct responses* for both male (Fig. 4) and female rats (Fig. 5). Female rats required a greater number of trials to complete the ASST with the most difficulty observed in the ED-Rev test condition. The data suggest that female rats were more negatively affected by Pb^{2+}-exposure than males as evidenced by increased trials

Fig. 4 Illustrates the *rate-of-learning* cumulative records for a single representative male rat from the Control (upper panel), Perinatal (middle panel), and Perinatal+Taurine (lower panel) treatment groups. The data show the 7-test conditions of the ASST (separated within each panel by the vertical dashed phase-lines) along the x-axis and the number of cumulative responses on the y-axis, with the *correct responses* (open circles with solid lines) and the *error responses* (black circles with dashed lines) are depicted as the rats' *rate-of-learning*. Control male rats make fewer errors throughout the 7-test conditions of the ASST, when compared to the Perinatal male rats. Control male rats' make sequential errors during the CD-Rev, ID, ID-Rev, ED, and ED-Rev ASST stages. In contrast, the Perinatal male rat makes sequential errors in the SD, CD-Rev, ID, ID-Rev, and ED-Rev ASST stages. Interestingly, the Perinatal + Taurine male rat exhibited a quicker *rate-of-learning* with less sequential errors during the SD, CD, ID, ID-Rev, ED, and ED-Rev ASST stages. The data suggest that developmental Pb^{2+}-exposure induces lasting frontoexecutive dysfunctions in the mature rats' *rate-of-learning* behavioral profile, which improved by the co-treatment of Taurine 0.05% developmentally during Pb^{2+}-exposure


Control Male # 5
Perinatal Male # 2
Perinatal + Taurine Male # 1
Cumulative Responses
Trials Per Test Condition
SD
CD
CD-Rev
ID
ID-Rev
ED
ED-Rev
-O- Correct - ● - Error


Control Female # 1
Perinatal Female # 4
Perinatal + Taurine Female # 3
Cumulative Responses
SD
CD
CD-Rev
ID
ID-Rev
ED
ED-Rev
Trials Per Test Condition
–□– Correct - ■ - Error

required to complete the ED and ED-Rev test conditions of the ASST. Interestingly, these individual within-subject behavioral performances showed significant improvements in response to taurine co-treatment; thereby, mitigating Pb^{2+}-exposure in reducing these frontoexecutive dysfunctions.

3.3 Developmental Pb^{2+}-Exposure Caused Deficits in the ASST Re-acquisition Learning Performance That Was Recovered by Taurine Co-treatment

During the ASST, a test break procedure was implemented consistent with reports by Neuwirth et al. (2019a). As such, a re-acquisition learning probe was used for the CD and ID (*i.e.,* CD-ReAcq and ID-ReAcq) to ensure the behavioral momentum to evaluate the rats' cognitive flexibility in shifting could be maintained. Perinatal male rats showed a significant increase in *TTC* for OD and MD as a *Treatment* effect $F_{(2)} = 7.405$, $p < 0.001***$, $\eta_p^2 = 0.331$, when compared to Control male rats (Fig. 6a). Further, Perinatal+Taurine male rats showed a recovery from the *TTC* reacquisition learning impairment for both the OD ($p < 0.01^{\#\#}$) and MD ($p < 0.01^{\#\#}$) (Fig. 6a). Additionally, Perinatal male rats showed a significant decrease in *ETC* for OD and MD as a *Treatment* effect $F_{(2)} = 3.458$, $p < 0.05*$, $\eta_p^2 = 0.187$, when compared to Control male rats, as well as, an *ASST Stage X Treatment* interaction $F_{(6,2)} = 4.031$, $p < 0.05*$, $\eta_p^2 = 0.212$ (Fig. 6b). Consistent with the *TTC* reacquisition learning data, Perinatal+Taurine male rats showed a fewer *ETC* errors in both the OD ($p < 0.05^{\#}$) and MD ($p < 0.05^{\#}$), corroborating the finding that taurine co-treatment improved reacquisition learning deficits (Fig. 6b). In contrast, Control, Perinatal, and Perinatal+Taurine female rats showed no differences in both *TTC* and *ETC* OD and MD performances, respectfully (Fig. 7a, b). Taken together, the data suggest that developmental Pb^{2+}-exposure caused reacquisition learning deficits in a sex-dependent manner with males being most affected, and these impairments were recovered in males by taurine co-treatment.

Fig. 5 Illustrates the *rate-of-learning* cumulative records for a single representative female rat from the Control (upper panel), Perinatal (middle panel), and Perinatal+Taurine (lower panel) treatment groups. The data show the 7-test conditions of the ASST (separated within each panel by a vertical dashed-line) along the x-axis and the number of cumulative responses on the y-axis, with the *correct responses* (open squares with solid lines) and the *error responses* (black squares with dashed lines) are depicted as the rats' *rate-of-learning*. Control female rats make fewer errors throughout the 7-test conditions of the ASST, when compared to the Perinatal female rats. Control female rats' make sequential errors during the CD-Rev, ID-Rev, ED, and ED-Rev ASST stages. In contrast, the Perinatal female rat makes sequential errors in the CD-Rev and ED-Rev ASST stages. Interestingly, the Perinatal + Taurine female rats exhibited a quicker *rate-of-learning* with less sequential errors during the ID-Rev and ED-Rev ASST stages. The data suggest that developmental Pb^{2+}-exposure induces lasting frontoexecutive dysfunctions in the mature rats' *rate-of-learning* behavioral profile, which improved by the co-treatment of Taurine 0.05% developmentally during Pb^{2+}-exposure with more sensitivity when compared to male rats

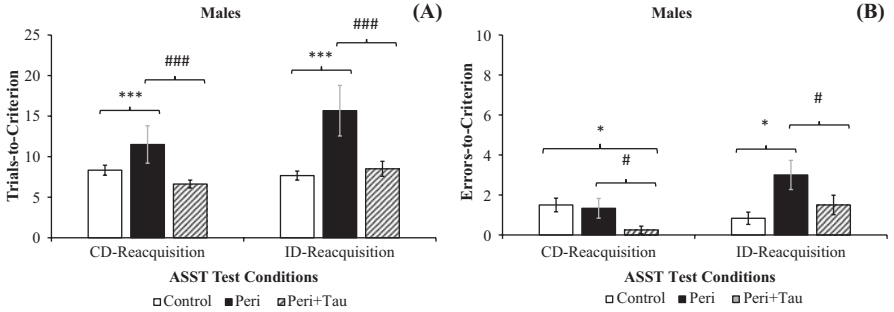


Fig. 6 Illustrates the male rat reacquisition learning data between test days to ensure their behavioral momentum when advancing to the next ASST condition. The reacquisition learning data show the *TTC* (**a**) and *ETC* (**b**) performances, respectively. Perinatal male rats showed a significant increase in the *TTC* required to complete the CD-ReAcq and ID-ReAcq ($p < 0.001***$), as well as the *ETC* in the ID-Reacquisition ($p < 0.05*$), when compared to Control male rats. Interestingly, co-treatment with taurine recovered reacquisition learning performance deficits to rates comparable to Control male rats for both the CD-ReAcq and ID-ReAcq in the *TTC* ($p < 0.001^{\#\#\#}$) and *ETC* ($p < 0.05^{\#}$). Thus, the data suggest that co-treatment with taurine improved ASST reacquisition learning performance in Perinatal Pb^{2+}-exposed rats

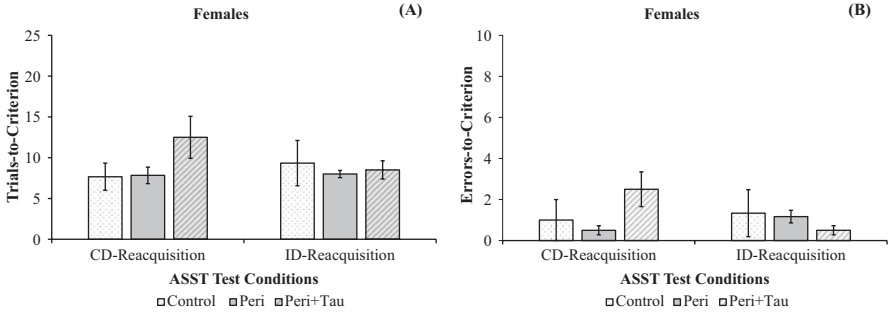


Fig. 7 Illustrates the female rat reacquisition learning data between test days to ensure their behavioral momentum when advancing to the next ASST condition. The reacquisition data show the *TTC* (**a**) and *ETC* (**b**) performances, respectively. There was no differences observed in female rats *TTC* and *ETC* as a function of treatment for both the CD-ReAcq or ID-ReAcq. Thus, unlike male rats, developmental Pb^{2+}-exposure did not impair female rats' ASST reacquisition learning performance

3.4 Developmental Pb^{2+}-Exposure Caused Frontoexecutive Dysfunction Impeding Rats' ASST Performance in a Sex-Dependent Manner That Was Recovered by the Co-treatment of Taurine

The ASST is a very sensitive behavioral test for frontoexecutive (dys)functions in rats. Consistent with reports by Neuwirth et al. (2019a), Perinatal male rats showed a significant *Treatment* effect in both *TTC* $F_{(2)} = 7.260$, $p < 0.01**$, $\eta_p^2 = 0.121$ and

ETC $F_{(2)} = 5.648$, $p < 0.01**$, $\eta_p^2 = 0.097$ performances (Fig. 8a, b). Perinatal male rats showed the most difficulty at the SD, ID, and ID-Rev ($p < 0.01**$) for the *TTC* and the SD and ID ($p < 0.01**$) for the *ETC* test conditions. Moreover, taurine co-treatment recovered these deficits to performance levels comparative to Control males with the most recovery observed in the *TTC* during the CD-Rev, ID, and ED ($p < 0.01^{\#\#}$) and in the *ETC* during CD-Rev and ID ($p < 0.01^{\#\#}$) (Fig. 8a, b). In contrast, the Perinatal females showed a significant effect of *ASST Stage* $F_{(6)} = 7.107$, $p < 0.001***$, $\eta_p^2 = 0.289$, but no significant *Treatment* effects for *TTC* and a significant *ASST Stage X Treatment* interaction for the *TTC* $F_{(6,2)} = 8.277$, $p < 0.001**$, $\eta_p^2 = 0.486$. Additionally, there was a significant effect of *ASST Stage* $F_{(6)} = 7.030$, $p < 0.001***$, $\eta_p^2 = 0.287$, *Treatment* $F_{(2)} = 5.638$, $p < 0.01**$, $\eta_p^2 = 0.097$, and a significant *ASST Stage X Treatment* interaction $F_{(6,2)} = 5.846$, $p < 0.001***$, $\eta_p^2 = 0.401$ for *ETC* (Fig. 9a, b). Perinatal female rats showed increased performance at the ID-Rev ($p < 0.05*$), and increased difficulty at the ED ($p < 0.01**$) and ED-Rev ($p < 0.001***$) for the *TTC*. In contrast, Perinatal female rats showed increased performance in the ID-Rev ($p < 0.05*$) and increased difficulty at the ED-Rev ($p < 0.001***$) for the *ETC* test conditions. Moreover, taurine co-treatment recovered these deficits to performance levels comparative to Control males with the most recovery observed in the *TTC* during the ED and ED-Rev ($p < 0.05^{\#}$) and in the *ETC* during ED and ED-Rev ($p < 0.05^{\#}$) (Fig. 9a, b). Taken together, the data suggest that the ASST is very sensitive in detecting the frontoexecutive dysfunctions caused by developmental Pb^{2+}-exposure and the recovery of these cognitive behavioral performance deficits by the co-treatment of taurine in a sex-dependent manner.

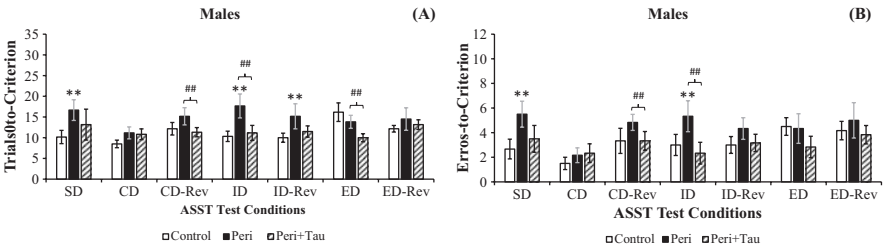


Fig. 8 Illustrates the male rats ASST performance for *TTC* (**a**) and *ETC* (B) performance, respectively. Perinatal male rats showed a significant increase in the *TTC* required to complete the SD, ID, and ID-Rev ($p < 0.01**$), as well as the *ETC* in the SD and ID ($p < 0.01***$), when compared to Control male rats. Interestingly, co-treatment with taurine recovered ASST performance to rates comparable to Control male rats for both the CD-Rev, ID, ID-Rev, and ED in the *TTC* ($p < 0.01^{\#\#}$) and in the *ETC* ($p < 0.01^{\#\#}$). Thus, the data suggest that co-treatment with taurine recovered ASST frontoexecutive functions in Perinatal Pb^{2+}-exposed rats

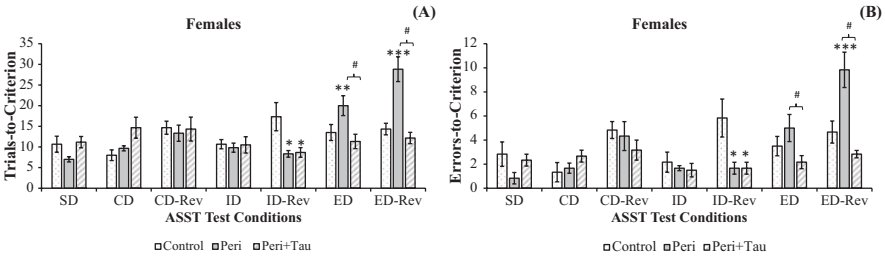


Fig. 9 Illustrates the female rats ASST performance for *TTC* (**a**) and *ETC* (**b**) performance, respectively. Perinatal female rats showed a significant decrease in the *TTC* required to ID-Rev ($p < 0.05*$) and a significant increased to complete the ED ($p < 0.01**$), and ED-Rev ($p < 0.001***$), as well as significant decrease in the *ETC* in the ID-Rev ($p < 0.05*$) and a significant increase to complete the ED-Rev ($p < 0.001***$), when compared to Control female rats. Interestingly, co-treatment with taurine recovered ASST performance to rates comparable to Control female rats for both the ED and ED-Rev in the *TTC* ($p < 0.05^{\#}$) and in the ED ($p < 0.05^{\#}$), and ED-Rev in the *ETC* ($p < 0.05^{\#}$). Thus, the data suggest that co-treatment with taurine recovered ASST frontoexecutive functions in Perinatal Pb^{2+}-exposed rats

3.5 Developmental Pb^{2+}-Exposure Caused Altered Neurochemical Profiles in Brain Regions That Serve to Regulate Frontoexecutive Functions and Are Recovered by Taurine Co-treatment

To corroborate the frontoexecutive functions observed at the behavioral level, LC/MS analyses were conducted from the brain tissues of rats that completed the ASST. The brain regions examined were selected since they are known to play a critical role in frontoexecutive control and learning and memory. The data for the male rats *GABA:Neurotransmitter* ratio showed an effect of *Treatment* that was observed for *GABA:Taurine* $F_{(2)} = 4.044$, $p < 0.01**$, $\eta_p^2 = 0.142$, *GABA:Glutamic Acid* $F_{(2)} = 13.456$, $p < 0.001***$, $\eta_p^2 = 0.355$, *GABA:Dopamine* $F_{(2)} = 17.880$, $p < 0.001***$, $\eta_p^2 = 0.422$ (Fig. 10). In addition, the *GABA:Dopamine* ratio showed a significant effect of *Brain Region* for the *IL* $F_{(4)} = 3.741$, $p < 0.01**$, $\eta_p^2 = 0.234$, with a *Treatment X Brain Region* interaction $F_{(2,4)} = 2.796$, $p < 0.01**$, $\eta_p^2 = 0.313$ (Figs. 10 and 13). Additionally, the data for male rats *Taurine: Neurotransmitter* ratio an effect of *Treatment* was observed for *Taurine:GABA* $F_{(2)} = 5.156$, $p < 0.01**$, $\eta_p^2 = 0.177$, *Taurine:Glutamic Acid* $F_{(2)} = 9.701$, $p < 0.001***$, $\eta_p^2 = 0.288$, *Taurine:Dopamine* $F_{(2)} = 23.600$, $p < 0.001***$, $\eta_p^2 = 0.496$, *Taurine:Serotonin* $F_{(2)} = 4.419$, $p < 0.01**$, $\eta_p^2 = 0.155$, and *Taurine: Epinepherine* $F_{(2)} = 8.305$, $p < 0.001***$, $\eta_p^2 = 0.257$ (Figs. 12 and 13). For the *Taurine:Neurotransmitter* ratio, an effect of *Brain Region* for the HP was observed for *GABA:Taurine* $F_{(4)} = 4.512$,

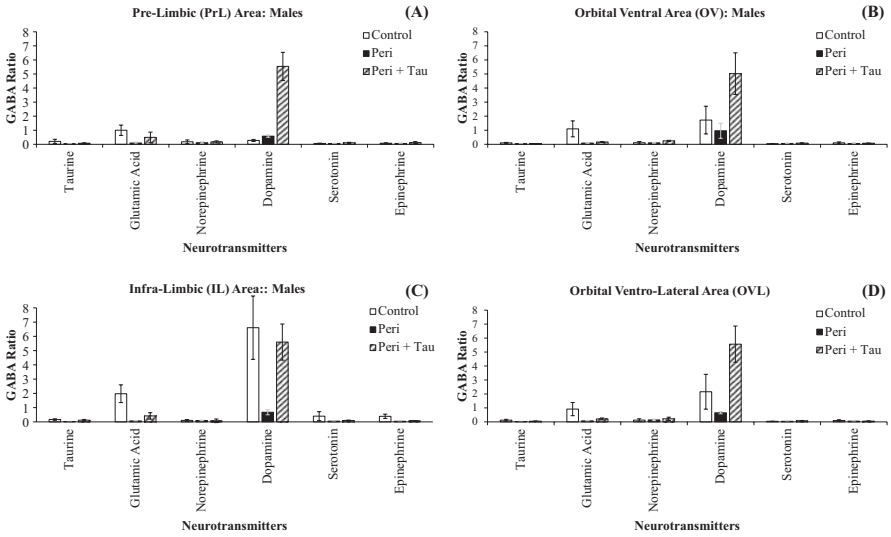


Fig. 10 Illustrates the male rats LC/MS GABA:Neurotransmitter ratios in the prelimbic (PrL) (**a**), the orbital ventral (OV) (**b**), infralimbic (IL) (**c**), and the orbital ventro-later (OVL) (**d**) areas of the prefrontal cortex that regulate frontoexecutive functions. The data reveal that in the IL, OV, and OVL Perinatal Pb^{2+}-exposures reduce GABA:Dopamine ratios. Following taurine co-treatment, these GABA:Dopamine ratios are reversed back to levels comparable to or exceeding those of Control males. The data suggest that Pb^{2+}-exposure negatively effects GABA:Dopamine frontoexecutive signaling at the neurochemical level, which could reduce motivational states at the behavioral level

$p < 0.001***$, $\eta_p^2 = 0.273$, *Taurine:Serotonin* $F_{(4)} = 4.115$, $p < 0.01**$, $\eta_p^2 = 0.255$, and *Taurine: Epinepherine* $F_{(4)} = 9.710$, $p < 0.001***$, $\eta_p^2 = 0.447$ (Fig. 13).

In contrast, for female rats the *GABA:Neurotransmitter* ratio showed an effect of *Treatment* that was observed for *GABA:Taurine* $F_{(2)} = 6.242$, $p < 0.01**$, $\eta_p^2 = 0.301$, *GABA:Glutamic Acid* $F_{(2)} = 4.127$, $p < 0.01**$, $\eta_p^2 = 0.216$, *GABA:Norepinephrine* $F_{(2)} = 5.089$, $p < 0.01**$, $\eta_p^2 = 0.260$, *GABA:Serotonin* $F_{(2)} = 5.789$, $p < 0.01**$, $\eta_p^2 = 0.278$, and *GABA:Epinephrine* $F_{(2)} = 4.597$, $p < 0.01**$, $\eta_p^2 = 0.235$ (Figs. 11 and 13). In regards to the female rats *Taurine:Neurotransmitter* ratio, an effect of Treatment was observed for *Taurine:Glutamic Acid* $F_{(2)} = 4.560$, $p < 0.01**$, $\eta_p^2 = 0.239$, while an effect of *Brain Region* was observed for the HP in *Taurine:Norepinepherine* $F_{(4)} = 2.814$, $p < 0.05*$, $\eta_p^2 = 0.327$, *TaurineSerotonin* $F_{(4)} = 3.129$, $p < 0.01**$, $\eta_p^2 = 0.350$, and *Taurine:Epinepherine* $F_{(4)} = 3.809$, $p < 0.01**$, $\eta_p^2 = 0.396$ (Figs. 13 and 14).

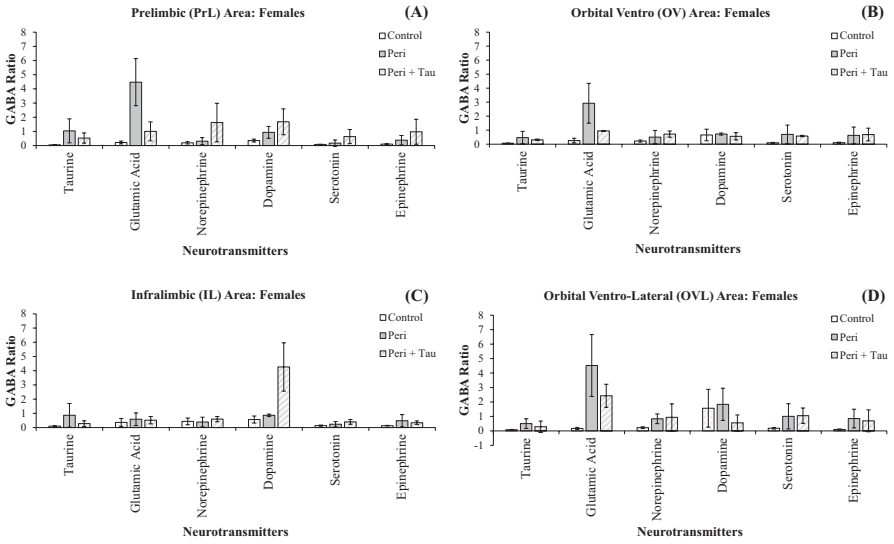


Fig. 11 Illustrates the female rats LC/MS GABA:Neurotransmitter ratios in the prelimbic (PrL) (**a**), the orbital ventral (OV) (**b**), infralimbic (IL) (**c**), and the orbital ventro-later (OVL) (**d**) areas of the prefrontal cortex that regulate frontoexecutive functions. The data reveal that in the Prl, OV, and OVL Perinatal Pb^{2+}-exposures increases GABA:Glutamic Acid ratios.. Following taurine co-treatment, these GABA:Glutamic Acid ratios are reversed back to levels comparable to or exceeding those of Control females. Additionally, in the IL an elevation in GABA:Dopamine was observed following taurine co-treatment. The data suggest that Pb^{2+}-exposure negatively effects GABA:Glutamic Acid and GABA:Dopamine frontoexecutive signaling at the neurochemical level, which could reduce motivational states at the behavioral level

4 Discussion

The present study showed that developmental Pb^{2+}-exposure caused significant frontoexecutive dysfunctions that persisted later in life when the mature rats were tested in the ASST. Further, these frontoexecutive dysfunctions are consistent with an environmentally induced developmental neuropathological disorder in response to a neurotoxicant such as Pb^{2+}. The changes observed in the rat during the ASST on the behavior level corroborated with frontoexecutive altered neurochemical signaling across the PrL, IL, OV, and OVL, as well as, HP signaling as it related to learning and memory frontoexecutive dysfunctions induced by developmental Pb^{2+}-exposure in a sex-dependent manner. Developmental Pb^{2+}-exposure has been reported to cause changes in the expression of adrenergic and dopaminergic receptors in the forebrain and striatum of rats (Rossouw et al. 1987) and chronic Pb^{2+}-exposure has been shown to differentially affect dopamine synthesis across brain regions (Jason and Kellogg Jason and Kellogg 1981; Lucchi et al. 1980; Govoni et al. 1979), as well as, glutamatergic and GABAergic altered brain excitability balancing (Strużyñska and Sulkowski 2004). In a clinical case study of chronic lead

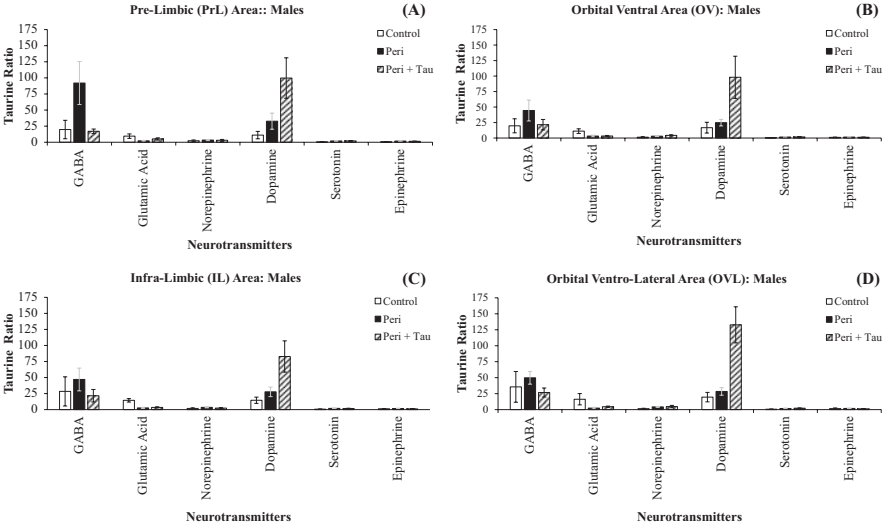


Fig. 12 Illustrates the male rats LC/MS Taurine:Neurotransmitter ratios in the prelimbic (PrL) (**a**), the orbital ventral (OV) (**b**), infralimbic (IL) (**c**), and the orbital ventro-later (OVL) (**d**) areas of the prefrontal cortex that regulate frontoexecutive functions. The data reveal that in the PrL Perinatal Pb^{2+}-exposures increase Taurine:GABA ratios. Following taurine co-treatment, these Taurine: GABA ratios are reversed back to levels comparable to or less than Control males. Additionally, taurine co-treatment elevated Taurine:Dopamine levels across all four brain regions. The data suggest that Pb^{2+}-exposure alters Taurine:GABA and Taurine: Dopamine frontoexecutive signaling at the neurochemical level, which could reduce motivational states at the behavioral level

exposed patients in Saudi Arabia, they found in their blood plasma levels elevated GABA, 5-HT, and DA with associated autism diagnoses when compared to healthy age-matched controls (El-Ansary et al. 2011). Moreover, prior reports have also eluded to the potential overlap between autism and environmental Pb^{2+}-exposure as a subset of childhood case studies exhibiting autism or autism developmental symptoms that could be assessed via neuropsychological testing (Lidsky and Schneider 2005). Consistent with these clinical reports, prior studies regarding the molecular changes observed in response to developmental Pb^{2+}-exposure and its translation with the behavioral and cognitive system levels, have been shown to disrupt inhibitory learning with observed increases in impulsivity under fixed-interval of scheduled behaviors (Cory-Slechta et al. 1998). Moreover, these findings were also shown to corroborate with Pb^{2+}-induced learning impairments because of changes to the dopaminergic, cholinergic, and glutamatergic neurotransmitter systems (Cory-Slechta 1995). Thus, the effects of low-level developmental Pb^{2+}-exposure can significantly affect dopaminergic systems that regulate incentive, motivation, mood balancing, along with other neurotransmitter systems that facilitate heighted arousal states in which to cognitively engage with one's environment. Further, it is suggested that through such a psychological profile, one could benefit from psychotropic medication that could prevent frontoexecutive dysfunction by regulating directly or indirectly dopamine tone in the frontal lobes.

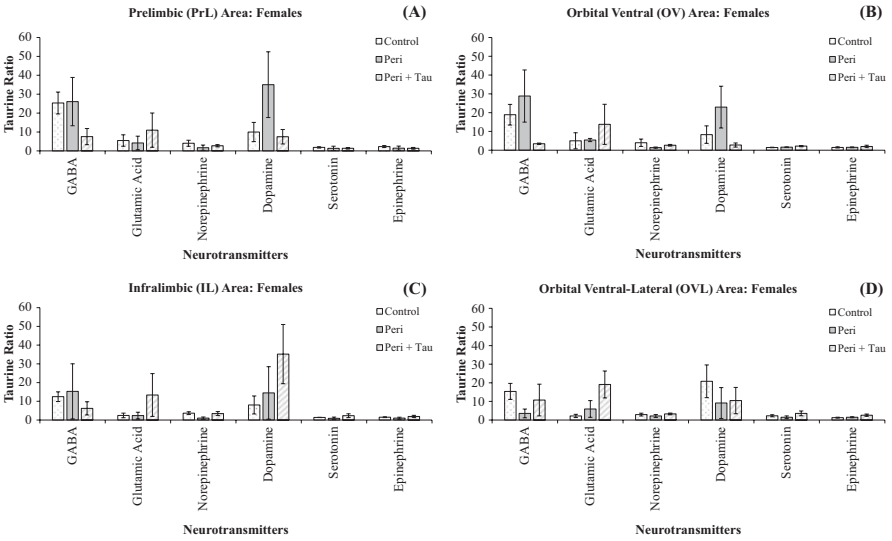


Fig. 13 Illustrates the female rats LC/MS Taurine:Neurotransmitter ratios in the prelimbic (PrL) (**a**), the orbital ventral (OV) (**b**), infralimbic (IL) (**c**), and the orbital ventro-later (OVL) (**d**) areas of the prefrontal cortex that regulate frontoexecutive functions. The data reveal that in the PrL and OV Perinatal Pb^{2+}-exposures increase Taurine:Dopamine ratios. Following taurine co-treatment, these Taurine: Dopamine ratios are reversed back to levels comparable to or less than Control females. The data suggest that Pb^{2+}-exposure alters Taurine: Dopamine frontoexecutive signaling at the neurochemical level, which could reduce motivational states at the behavioral level

The data obtained from the present study are in agreement with the findings from earlier reports. Thus, Pb^{2+}-exposure appears to effect a cluster of neurotransmitter systems differentially across neurodevelopment in a sex-specific manner. Earlier reports on developmental Pb^{2+}-neurotoxicity restricted their reports to one sex, thereby limiting comparative analyses as those produced herein. Further, taurine was shown to be effective in mitigating or at least reducing most of the Pb^{2+}-induced frontoexecutive dysfunctions that were observed in the Perinatal rats. Developmental Pb^{2+}-exposure also caused sex-based differences in the ASST performance that were far less dysfunctional following taurine co-treatment with distinct improvements in working memory and reacquisition learning performance, and more focused learning performances with less errors. Thus, taurine may provide a wide-range of neuroprotection within and across the neurodevelopmental signaling pathways that later govern frontoexecutive functions. Consistent with prior reports, taurine may serve to prevent brain excitability, by balancing the GABA-shift in early development (Ben-Ari 2002; Ben-Ari et al. 2012) and ensuring an adequate level of neurotransmitter tone across the establishment and maintenance of neurochemical signaling (Chan et al. 2014), emotional and age-dependent signaling (Neuwirth et al. 2013, 2015). Further, studies have also shown taurine's role in contributing to the regulation of context-dependent goal-directed behaviors (Neuwirth et al. 2013, 2017, 2019a Neuwirth 2014), with no apparent adverse

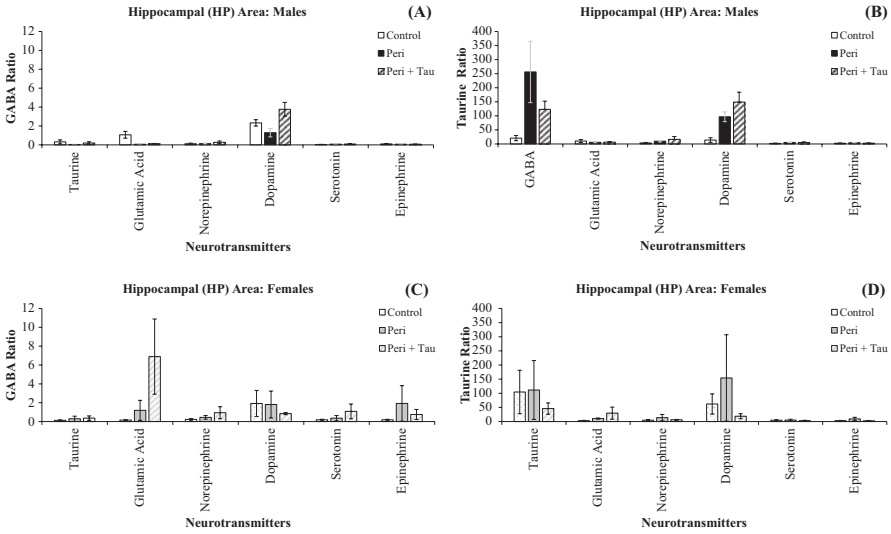


Fig. 14 Illustrates the male (**a**, **b**) and female (**c**, **b**) rats LC/MS GABA: Neurotransmitter (**a**, **c**) and Taurine:Neurotransmitter (**b**, **d**) ratios in the hippocampal (HP) areas that regulate learning and memory. The data reveal that in males the GABA:Dompamine ratios are reduced and the PrL in response to Pb^{2+}-exposure, and female HP are less affected (**a**, **c**). In female rats, the GABA:Glutamic acid ratio is elevated in response to taurine co-treatment (**c**). In contrast, perinatal Pb^{2+}-exposure elevated the Taurine:GABA ratio in male HP and not in females (**b**, **d**), whereas the female rates showed no differences in response to Pb^{2+}-exposure or taurine. The data suggest that Pb^{2+}-exposure alters GABA:Glutamate, GABA:Dopamine, and GABA:Taurine hippocampal signaling at the neurochemical level, which could reduce learning and memory states at the behavioral level

effects on locomotor activity or anxiety behaviors at a dosage of 43 mg/kg or 0.05% in the drinking water (Santora et al. 2013; El Idrissi et al. 2011; El Idrissi et al. 2009). Thus, taurine may prove useful as a psychopharmacotherapy for treating or counteracting against neurotoxicants such as Pb^{2+} (See Chap. 69 Neuwirth et al. 2019b).

5 Conclusion

In summary, this study shows that perinatal Pb^{2+}-exposure can cause frontoexecutive dysfunctions in the rat model that persists across the lifespan. These frontoexecutive dysfunctions effect males and females in a sex-dependent manner, which require further study. Moreover, the sex-dependent neuropsychological profiles could be observed at both the behavioral (*i.e.,* in the ASST) and the neurochemical levels (*i.e.,* LC/MS data). Although, individual rat differences in frontoexecutive dysfunction could be observed, group differences were also observed in this study, thereby suggesting that the ASST is sensitive in revealing frontoexecutive

dysfunction at the behavioral level in rats. This is significant as most reports on low-level Pb^{2+}-exposure historically shows reduced sensitivity at the behavioral level for showing significant hippocampal learning deficits. Thus, perhaps frontoexecutive behavioral tests of attentional mechanisms may prove more useful than hippocampal test in revealing a fine-grained analysis of Pb^{2+}-impacts during neurodevelopment. Further, taurine co-treatment revealed a sex-dependent recovery in the rats exposed to perinatal Pb^{2+}-exposure. Therefore, Pb^{2+} has been shown to disrupt GABAergic mediated networks that are, in part, responsible for regulating emotional-dependent learning and memory behaviors, and less is known regarding its involvement in frontoexecutive functions (Neuwirth et al. 2019a). Thus, this study presents a case for considering taurine as a psychopharmacotherapy for treating neurodevelopmental Pb^{2+}-exposure as a means to improve one's frontoexecutive functions across their lifespan. The present study serves to open a new dialogue for clinical trials to consider using taurine therapy in treating Pb^{2+}-exposed children that remain in environments that reside Pb^{2+}-contaminated (Neuwirth et al. 2018).

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Conflicts of Interest LSN discloses a public domain trademark used under common law as the Neuwirth™ ASST apparatus. Otherwise, the authors declare no other conflicts of interest.

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Effect of Taurine on Alterations in Deiodinase 3 Expression Induced by BDE 209 in Human Neuroblasoma-Derived SK-N-AS Cells

Xiaona Shang, Xiaoying Niu, Ruonan Wang, Zhenwei Li, Xiaoji Hao, Xiaohui Liu, Jing Shao, Lu Han, and Yachen Li

Abstract PBDEs (stands for polybrominated diphenyl ethers) are extensively utilized flame retardants, and BDE 209 is one of the most widely used congeners. Studies have suggested the general toxic effects of PBDEs on the endocrine system and neural development. Our previous studies found that BDE 209 changed Type 3 iodothyronine deiodinase (Dio 3) expression in human SK-N-AS neuroblastoma cells. The current study was designed to examine the potential protection of taurine on alterations of Dio 3 expression induced by BDE 209 in SK-N-AS cells. Briefly, SK-N-AS cells were pretreated with taurine prior to the BDE 209 treatment, and the cell viability was evaluated by the MTT (methyl-thiazolyl-tetrazolium) assay. The disturbance or restoration in the levels of Dio 3 proteins and mRNA were observed separately by the western blot and qRT-PCR. Our data showed that taurine moderately attenuated BDE 209-mediated the loss of cell viability. Also, taurine moderately prevented the reduction in the Dio 3 protein expression and mRNA expression induced by BDE 209 in the SK-N-AS cells. Our findings indicated that taurine potentially provide the protection on PBDEs-induced toxicity on endocrine and neuro-development.

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Keywords SK-N-AS cells · Polybrominated diphenyl ethers (PBDEs) · Taurine · Neurotoxicity · Deiodinase 3 (Dio 3)

Abbreviations

BDE 209	Decabromodiphenyl ether
Dio	Deiodinase
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
SK-N-AS	Human SK-N-AS neuroblastoma cells

1 Introduction

Polybrominated diphenyl ethers (PBDEs) are currently the most common brominated flame retardants (Ni et al. 2013). Because of its high thermal stability and low price, it is widely used in several commercial products such as electrical appliances, chemicals, transportation, building materials, plastic products, textiles and petroleum (Kim and Kim 2016). Due to its strong biological enrichment effect and biological toxicity, PBEDs migrate, diffuse and accumulate in the environment and organisms (Harley et al. 2017). PBDEs seriously endanger human health such as liver toxicity, endocrine toxicity, interfering of thyroid hormone levels, neurotoxicity, carcinogenicity and reproductive toxicity after entering the human body, which has attracted worldwide attention (Besis et al. 2016). There are 209 PBDE congeners, and among them penta-BDE, octa-BDE and deca-BDE represent three major industrial products (Jiang et al. 2011). In May 2009, the United Nations Environment Programme formally listed pentabromo and octa-bromodiphenyl ethers as persistent organic pollutants in the Stockholm Convention, which therefore have been banned ever since. Decabromodiphenyl ether (BDE 209) toxicity has become a research hotspot, because of its high fat-solubility, low volatility and not easy degradation (Shen et al. 2009). Studies have confirmed that BDE 209 is not highly toxic, but its metabolic toxicity is much higher than its own toxicity (Zhang et al. 2015). According to reports, BDE 209 may have toxic effects on the endocrine system and the central nervous system, particularly the neurobehavioral effects. Studies have found that BDE 209 can induce neurodevelopment and neurobehavioral disorders. Therefore, there is an urgent need to identify alternatives or preventions to protect human health from BDE 209 toxicity. Both human epidemiological evidence and animal research have proven that taurine has a good protective effect on nerve cells and protects neurons from contaminant-induced cytotoxicity through neuronal proliferation and synaptogenesis (Han et al. 2016).

Taurine is considered an antioxidant, and essential for the growth and development of the central nervous system. Studies show that taurine plays an important

role in the development of the nervous system (Kumari et al. 2013). But it is unclear whether taurine may provide protection in relieving BDE 209-caused neurotoxicity.

In the current study, we chose human neuroblastoma SK-N-AS cells as an *in vitro* model. SK-N-AS cells were incubated with taurine for 24 h prior to BDE 209 exposure, the effect of taurine on the BDE 209-mediated disturbance on the cell viability, the levels of Dio 3 protein and mRNA were assessed by the Western blot analysis and by the qRT-PCR. Consequently, the possible protection of taurine on SK-N-AS cells treated with BDE 209 can be deduced.

2 Methods

2.1 Chemicals

BDE 209 (purity >98%) was purchased from Sigma, USA. TRIzol reagent was supplied by the Takara, Japan. BCA protein assay kit was acquired from the Beyotime Biotechnology, China. Anti-Dio 3 antibody was bought from the Abcam, UK. Mouse Anti-β actin mAb was bought from the ZSGB-Bio, China. Anti-Rabbit antibody and Anti-Mouse antibody were bought from the Proteintech, USA. The primers of GAPDH and Dio 3 were designed and synthesized at the TaKaRa, Japan. All the other reagents were analytical grade.

2.2 Cell Culture

The human brain neuroblastoma cell line (SK-N-AS) was bought from the Cell Bank of BNCC (Beijing, China). SK-N-AS cells can differentiate easily, but are difficult to attach to the culture surface. The cells were grown in the DMEM (Hyclone, USA), which was made up by 10% FBS (NQBB, USA) and 1% penicillin/streptomycin (Hyclone, USA), and maintained at 37 °C in a humid incubator of 5% CO_2, 95% air. Cells were digested with 0.25% Trypsin (Solarbio, China) and 0.125% EDTA (Beyotime, China) for passaging.

2.3 Determination of Cell Viability

SK-N-AS cells were plated in 96-well plates at a density of 8×10^3 /ml. After cultured for 24 h at 37 °C, 5% CO_2 incubator, the cells were exposed to BDE 209 for 24 h. Then 100 μl MTT diluted by the serum-free medium (of 0.5 mg/mL concentration) was added to the culture and incubated for 4 h. Following discarding the supernatant, 100 μl dimethylsulfoxide (DMSO) per well was added and incubated

for 1 h in the dark. The OD value was measured at wavelength of 595 nm on the ELISA Reader (Thermo Fisher Scientific, MA USA).

2.4 Quantitative Real Time PCR

Five×10^6 SK-N-AS cells were inoculated into 10 cm culture dishes, and when the cell fusion rate reached 70%, they were treated with 1, 3 and 9 mM of taurine for 24 h. Then, the taurine protection solution was discarded, and each dish was added with 3 μM BDE 209 for exposure. After 24 h, total RNA was extracted from the cells by RNAiso Plus (Takara, Japan), and the quality of RNA was measured by ultraviolet spectrophotometer, ensuring the integrity of RNA. We used 800 ng of total RNA as the template for reverse transcription of the cDNA with the PrimeScript RT kit with gDNA Eraser (Perfect Real Time). Two μl of cDNA was used as a template, and amplified on a Thermal Cycler Dice Real Time System using the SyBr premix EX-Taq. GAPDH was used as a standardized internal reference, and the following primers were used to amplify them by qPCR: GAPDH-F: 5'-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-R: 5'-TGGTGAAGACGCCA GTGGA-3′; DIO 3-F: 5'-CTTCGAGCGTCTCTATGTCATCCA-3′, DIO 3-R: 5'-TGCAGTTGCTCATCATAGCGTTC-3′. We acquired the CT values of each group and calculated the relative expression of Dio 3 mRNA using $2^{-\Delta\Delta ct}$ method.

2.5 Protein Extracts and Western Blot

SK-N-AS cells at 5 × 10^6 were seeded in a 10 cm culture dish. After treatment with taurine and BDE 209, cells were collected and nuclear proteins were extracted by the RIPA (Beyotime, China) of 1 mM PMSF, and the protein concentrations were determined by the BCA protein assay kit (Beyotime, China), proteins were adjusted to the same concentration with 3d·H_2O and Loading Buffer, and Dio 3 protein expression was determined by western blot.

Protein extracts were separated by 12% (w/v) SDS-PAGE (100 V, 90 min) and transferred to PDVF membrane (220A, 120 min). The membranes were placed in 5% milk for 2 h, and then incubated with the primary antibodies (rabbit-derived Anti-Dio 3 antibody, 1:1000; mouse-derived Anti-β actin antibody, 1:1000) overnight at 4 °C. The PDVF membranes were incubated with secondary antibodies (Anti-rabbit antibody, 1:5000; Anti-mouse antibody 1:1000) for 1 h at room temperature. Each time when the antibody was applied, the blots were washed with 0.1% (v/v) Tween 20 in TBS for 30 min. The results were analyzed using the Image J software after capturing the Western blot image in the UVP Chemiluminescent Gel Image Analysis System (USA).

2.6 Statistical Analysis

Each data represents the mean ± standard error of the mean (SEM) of at least three independent experiments in all cases. Results were analyzed using the GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The differences were assessed by the one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoctest, and statistical significance was defined at $p < 0.05$.

3 Results

3.1 Effect of Taurine on Cytotoxicity in Neuronal Differentiated SK-N-AS Cells Treated with BDE 209

Figure 1 shows that the cell viability of SK-N-AS cells was significantly reduced with a dose-dependency when treated with 0, 1 and 3 μM of BDE 209 for 24 h ($p < 0.05$).

To evaluate the protective effect of taurine, the SK-N-AS cells were pretreated with 1, 3 and 9 mM of taurine for 24 h followed by the treatment with 3 μM BDE 209 for 24 h. As shown in Fig. 2a, microscopic observations on SK-N-AS cells showed that BDE 209 alone could lower the number of cells, and led to cell shrinkage, while the healthy cells could be found in the control and the taurine-pretreated group. Compared to the control group, all doses of taurine could enhance BDE 209-induced decrease in the cell viability, and the difference was particularly significant in the 3 mM taurine-pretreated group ($p < 0.01$) (Fig. 2b).

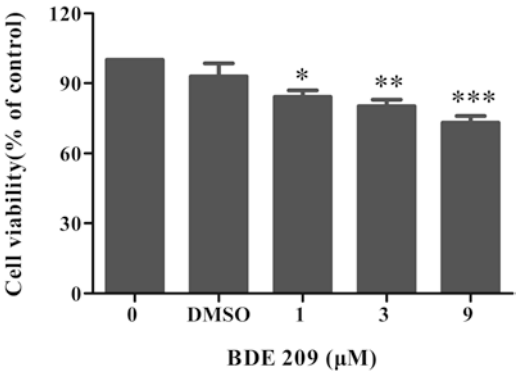

Fig. 1 BDE 209-induced cytotoxicity in SK-N-AS cells. Data were performed as mean ± SEM from three independently performed tests. ∗$p < 0.05$, ∗∗$p < 0.01$, and ∗∗∗$p < 0.001$, compared to the control (0)

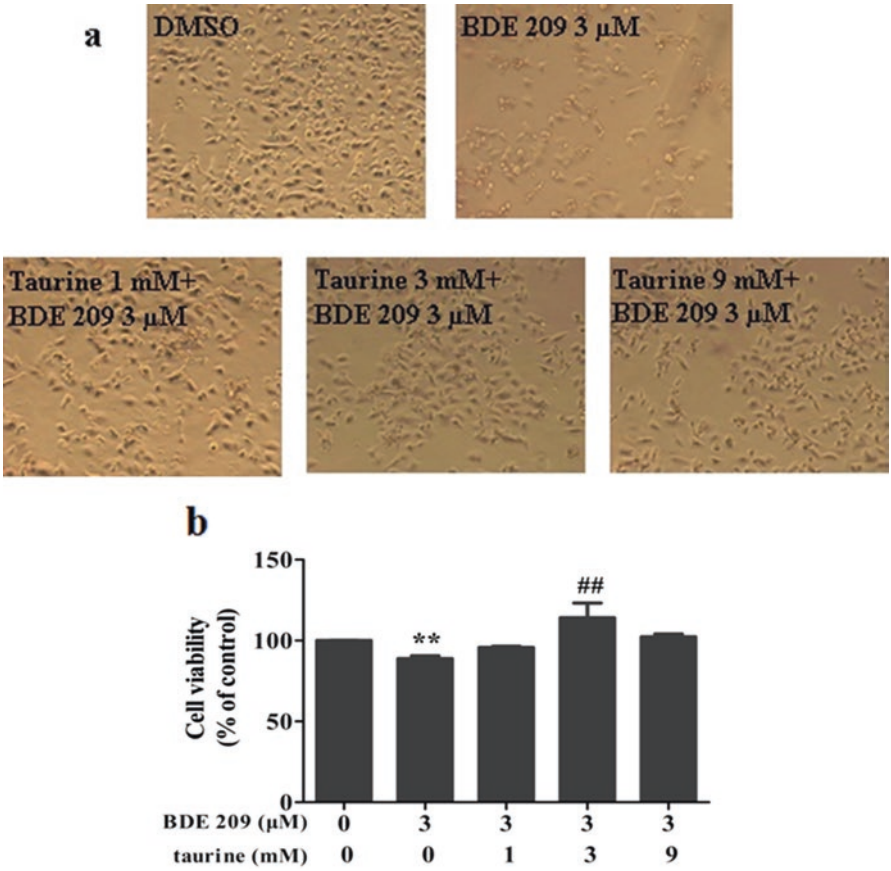

Fig. 2 Protection of taurine on BDE 209-induced cytotoxicity in SK-N-AS cells. (**a**). Microscopic observations. (**b**). Statistical analysis: data were performed as mean ± SEM from three independently performed tests. $^{**}p < 0.01$, compared to the control, and $^{\#\#}p < 0.01$, compared to BDE 209 exposure alone

3.2 Taurine Moderately Restored Gene Expressions of Dio 3 in SK-N-AS Cells Induced by BDE 209

Deiodinases (Dios) are the key regulation factors of Thyroid hormone activity, and are divided into three types (Dio 1, 2, 3). High expression of Dio 3 can cause deactivation of thyroid hormone and further affect neural development. As shown in Fig. 3, compared with the DMSO control, BDE 209 could dramatically increase the gene expression of Dio 3 ($p < 0.001$). By preincubating the SK-N-AS cells with 1, 3 and 9 mM taurine for 24 h, we found that the expression of Dio 3 mRNA in these cells was reduced by taurine. Specifically, when all doses of taurine can decrease BDE 209-induced Dio 3 mRNA overexpression, taurine at 1 mM showed the most

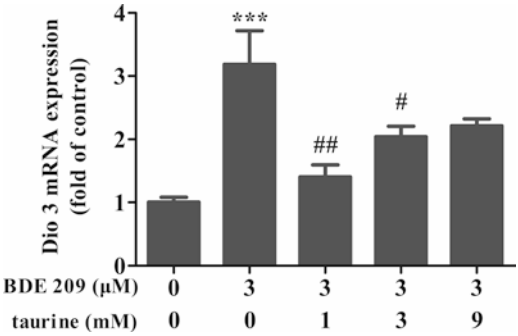

Fig. 3 Protection of taurine on BDE 209 induced Dio 3 mRNA expression in SK-N-AS cells. Data were performed as mean ± SEM from three independently performed tests. *** $p < 0.001$ relative to the control, # $p < 0.05$ and ## $p < 0.01$ as compared to BDE 209 exposure alone

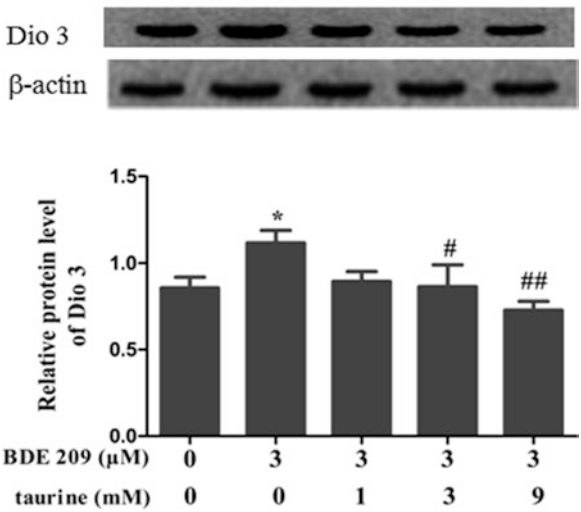

Fig. 4 Protection of taurine on BDE 209-induced Dio 3 protein expression in SK-N-AS cells. Data were performed as mean ± SEM from three independently performed tests. * $p < 0.05$ relative to the control, # $p < 0.05$ and ## $p < 0.01$ as compared to BDE 209 exposure alone

protective effect ($p < 0.01$), and 3 mM taurine showed moderate protection ($p < 0.05$); however, no statistical significance was found in 9 mM taurine group, indicating that the high doses of taurine may have saturation effects.

3.3 Taurine Partially Restored Protein Expression of Dio 3 in SK-N-AS Cells Induced by BDE 209

Meanwhile, we tested the effect of BDE 209 on the expression of Dio 3 protein in SK-N-AS cells through Western Blot analysis. As displayed in Fig. 4, compared to the DMSO control, BDE 209 could moderately increase the protein expression of Dio 3 ($p < 0.05$), although the dramatic mRNA overexpression was found as stated earlier; while all doses of taurine pretreatment would reduce BDE 209-induced increase in the expression of Dio 3 protein, the statistical significance was detected

in the 3 and 9 mM taurine groups ($p < 0.05$ or $p < 0.01$), suggesting that, the lack of significance in 1 mM taurine group may be due to experimental inconsistency which will be further tested in the future study, or there may exist a post-transcriptional regulation on Dio 3 protein production.

4 Discussion

PBDEs are brominated flame retardants that are widely added to various household essentials (Lim et al. 2014). The extensive use has caused global environmental pollution. It has been reported that PBDEs can damage the functions of various organs of animals, especially the function of the central nervous system (Costa et al. 2014). In this current study, a decrease in the survival rate of SK-N-AS cells exposed to BDE 209 indicates that BDE 209 has cytotoxicity on the neuronal cells, and the data are consistent with the other researchers on the neurotoxicological studies of the decabromodiphenyl ether (Wu et al. 2009). Epidemiological investigation found that BDE 209 can damage the central nervous system function, for example, PBDEs exposure during pregnancy may pass through the placenta and induce thyroxine interference, which leads to developmental nerve damage (Bo 2015). *In vitro* experiments found that BDE 209 can induce apoptosis of neural stem cells within a certain concentration range (Wu et al. 2018). Hence, the intervention approaches on BDE 209-induced neurotoxicity are the urgent study topics.

Taurine is a conditionally essential amino acid that can be related to the maintenance of central nervous system function (Ye et al. 2013). It is specifically distributed in the cerebral cortex, hippocampus and cerebellum, and plays an important role in cells, such as osmotic pressure regulation and cytoplasmic calcium levels, which have a wide range of cytoprotective effects for regulation, growth and development (Gharibani et al. 2013). Related studies have shown that taurine protects cell membranes, mitochondria and endoplasmic reticulum in the central nervous system by inhibiting the excitotoxicity of neurons and mitochondrial energy failure (Kumari et al. 2013). Taurine has the function of regulating intracellular free calcium levels, and by inhibiting calcium influx, weakens the depolarization tendency of cells, and protects the structure and function of cells (Liu et al. 2012). Taurine can also protect neuronal cells by inhibiting Caspase 9 activation (Yang et al. 2014).

In this experiment, by adding taurine to the BDE 209-infected SK-N-AS cell model, the growth of nerve cells was improved, and the apoptosis rate was significantly decreased. The dramatic elevation in both gene and protein expressions of Dio 3 were induced by BDE 209, in particular in the Dio 3 mRNA, indicating that the regulation on the translational process of Dio 3 protein may explain the differential effect by BDE 209. Further, when cells were pretreated with taurine, attenuations on both Dio 3 mRNA and protein overexpressions were observed, though with some discrepancies, suggesting there exist either the saturation effect by high taurine loading, or experimental inconsistency. And again, the post-transcriptional regulation on Dio 3 protein expression may partially explain the

discrepancy in protective effect on the mRNA and protein expressions of Dio 3. In any case, we have observed that taurine can significantly reduce the damage on cell viability and reverse the BDE 209-mediated overexpreesions in both Dio 3 mRNA and protein in neuronal cells, and therefore, it has a good neuroprotective effect, which provides a theoretical basis for the application of taurine in nervous system diseases.

5 Conclusion

Taurine moderately attenuated the loss of cell viability and partially restored alterations in the gene and protein expressions of Dio 3 in SK-N-AS cells induced by BDE 209. Taurine may have protective effect on PBDEs-induced developmental neurotoxicity during early fetal development.

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Glucose-Taurine Reduced Exerts Neuroinflammatory Responses by Inhibition of NF-κB Activation in LPS-Induced BV2 Microglia

Hwan Lee, Dong-Sung Lee, Kyung Ja Chang, Sung Hoon Kim, and Sun Hee Cheong

Abstract We want to find the anti-neuroinflammatory action of the taurine derivative Glucose-Taurine Reduced (G-T-R). The anti-neuroinflammatory action by G-T-R were investigated in lipopolysaccharide (LPS)-induced BV2 microglia. G-T-R inhibited the production of nitric oxide and prostaglandin E2, and down-regulated the protein expression of inducible NO synthase and cyclooxygenase-2. In addition, G-T-R reduced the cytokines secretion such as tumor necrosis factor (TNF-α), interleukin (IL) -1β and IL-6, in BV2 microglia treated with LPS. In addition, G-T-R dose-dependently decreased the activation of nuclear factor-kappa B. These findings confirmed the anti-neuroinflammatory activity of G-T-R, which may exert protective effects against neuroinflammatory-related diseases.

Keywords Glucose-taurine reduced (G-T-R) · BV2 microglia · Anti-neuroinflammatory effects

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Abbreviations

NF-κB	nuclear factor-kappa B
BCA	bicinchoninic acid
CNS	central nervous system
COX-2	cyclooxygenase-2
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGTA	ethylene glycol tetra-acetic acid
FBS	fetal bovine serum
G-T-R	glucose-taurine reduced
HRP	horseradish peroxidase
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NO	nitric oxide
PBS	phosphate buffered saline
PGE_2	prostaglandin E2
ROS	reactive oxygen species
TNF-α	tumor necrosis factor–alpha
α-MEM	alpha-minimum essential medium

1 Introduction

In recent years, the population of Asia, including South Korea, has undergone rapid aging; similarly, the incidence of neurodegenerative diseases has rapidly increased (Sohn and Sohn 2011). One cause of neurodegenerative diseases related to the central nervous system (CNS) is the excessive production of active oxygen induced by excessive stress, smoking, and drinking (Satoh et al. 2006). In organisms that respire aerobically, including humans, reactive oxygen species (ROS) are generated by oxidation-reduction reactions using oxygen molecules, immune cell responses to antigens, external radiation, or chemicals. ROS, including superoxide, hydroxyl, peroxyl, and nitric oxide, which are derived from oxygen, are naturally produced during respiration and cell metabolism and participate in the control of various cell responses (Kim et al. 2009; Yoon et al. 2010). However, excessive accumulation of ROS and strong oxidative reactivity causes oxidative stress or inflammatory injury when antioxidant function deteriorates (Coyle and Puttfarcken 1993). According to reports, oxidative stress and inflammatory injury cause nerve cell destruction and dysfunction (Satoh and Lipton 2007). The function of microglial cells as immune cells of the CNS that act as the main regulators of neuro-inflammation. In response to several negative stimuli including free radicals and tissue or organ damage, microglia are represent a reactive condition characterized by swelling and constriction of the

microglial process (Nakamura et al. 1999; Beynon and Walker 2012). During inflammation of the CNS, the response of microglia may be mediated by certain proinflammatory cytokines such as, lipopolysaccharide (LPS) (Romero et al. 1996).

Taurine (2-aminoethanesulfonic acid) is present in the brain, heart, eyeball, muscle tissue, and liver, and is known to be involved in the neuromodulation, cell membrane stabilization, detoxification, antioxidant, and osmoregulation activities of these organs (Hoffmann and Lambert 1983; Pasantes-Morales and Cruz 1985; Lim and Kim 1995). In addition, taurine is responsible for the function of lowering blood cholesterol and triglyceride concentration including bile acid in the liver through the excretion of substances into the intestines to help absorb the fat intake (Park and Lee 1997). It is also well known as an active substance in the body involved in a wide variety action including brain development, retinal function, cardio-protective action, reproductive function, growth development, and liver function protection (Gandhi et al. 1992; Park and Lee 1998). However, no study has evaluated anti-neuroinflammatory mechanisms by glucose-taurine reduced (G-T-R) in BV2 microglia or investigated whether these mechanisms were involved in nuclear factor-kappa B (NF-κB) regulation. Therefore, we investigated the inhibition of neuroinflammation by G-T-R, as mediated via the NF-κB pathway in BV2 microglia.

2 Methods

2.1 Chemical Reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and alpha-minimum essential medium (α-MEM) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, PGE2, IL-6, and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The taurine-carbohydrate derivative, synthesized G-T-R, was received from Prof. Sung Hoon Kim (Department of Chemistry, Konkuk University, Seoul, Republic of Korea).

2.2 Cell Culture and Cytotoxicity Measurements

The cells were grown in α-MEM medium supplemented with streptomycin (100 mg/mL), penicillin G (100 U/mL), l-glutamine (2 mM), and 10% heat-inactivated FBS. The cells were then incubated at 37 °C in 95% air and 5% CO_2. Cytotoxicity measurements were performed by using the MTT assay in accordance with the method of Roy et al. (2018).

2.3 Nitrite Assay

To measure the inhibitory effect of NO production, the NO concentration was evaluated by using the Griess reaction. Briefly, the Griess reaction method comprised mixing the supernatant (100 μL) with Griess's reagent (100 μL) and measurement of the absorbance at 570 nm with an ELISA plate reader from BIO-RAD (Hercules, CA, USA).

2.4 PGE_2, TNF-α, and IL-1β Assays

The cells were cultured for 6 h and then treated with G-T-R. After 3 h, the cells were stimulated with LPS for 18 h. Subsequently, the supernatants of stimulated cells was collected and analyzed for TNF-α, IL-1β, and PGE_2 concentrations by using commercial ELISA kits from R & D Systems (Minneapolis, MN, USA).

2.5 Western Blotting

Briefly, for western blotting of BV2 cells, the cells were first pelleted via centrifugation (200 × *g* for 3 min) and washed with phosphate buffered saline (PBS). The cells were then lysed with 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitor and the supernatant was transferred into tubes containing 5 mg/mL pepstatin A, 5 mg/mL aprotinin, 1 mg/mL chymostatin, and 0.1 mM phenylmethanesulfonyl fluoride. Protein concentration was quantified by using a BSA protein test kit and equal amounts of each sample was electrophoresed on 12% SDS-polyacrylamide gel and transferred to a nitrocellulose (NC) membrane. Non-specific binding to the NC membrane was blocked by incubation with blocking buffer (5% skim milk and 0.1% Tween 20 in Tris-buffered saline); subsequently, antibodies to iNOS, COX-2, and p65 antibody were diluted 1:1000 and reacted for 2 h. Secondary antibodies were diluted to 1:1000 and allowed to react for 1 h. The electrochemiluminescence (ECL) solution was added to the NC membrane at a ratio of 1:1 and the luminescence of the bound antibodies was observed.

2.6 Preparation of Cytosolic and Nuclear Cell Fractions

Cells were homogenized (1:20, w:v) with PER-Mammalian Protein Extraction Buffer of Pierce Biotechnology (Rockford, IL, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (EMD Biosciences, San Diego, CA, USA). Subsequently, centrifugation was performed at 15,000 × *g* for

30 min at 4 °C. The supernatant from the centrifuged tube was stored at −80 °C for use as a cytoplasmic fraction. The remaining precipitate was washed with PBS, centrifuged, and the supernatant was suctioned. Subsequently, RIPA buffer was added, mixed with the residues and stored at 4 °C for 15 min, centrifuged at 15,000 × *g* for 30 min at 4 °C, and the supernatant from the centrifuged tube, used as a nuclear fraction, was stored at −80 °C in a deep freezer.

2.7 NF-κB DNA Binding Activity

The cells were cultured in a 60 mm dish for 6 h, treated with G-T-R for 3 h, and exposed to LPS (1 μg/mL) for 1 h. DNA binding activity of NF-κB was determined using a Trans AM kit from Active Motif (Carlsbad, CA, USA).

2.8 Statistical Analysis

All data obtained from the experiment were expressed as the mean ± S.D. of three independent experiments. Statistical analyses were computed by using GraphPad Prism version 3.03 (GraphPad Software Inc. San Diego, CA, USA). The mean difference was assessed by the one-way ANOVA with the Newman-Keuls post-hoc test and statistical significance was defined as $P < 0.05$.

3 Results

3.1 The Effects of G-T-R on Nitrite, PGE_2, iNOS, and COX-2 Regulation in BV2 Microglia.

BV2 microglia are known to be activated by LPS, resulting in the production of NO, PGE_2, proinflammatory cytokines, and toxic ROS (Dinarello 1999; Li et al. 2005; McGeer et al. 1993). In this study, inflammatory factors and cytotoxicity were measured in BV2 microglia stimulated with LPS to examine whether G-T-R exerted an anti-neuroinflammatory action. The toxicity of G-T-R in BV2 microglia were evaluated using MTT assay were not cytotoxic to BV2 microglia (Fig. 1a). To detect the anti-inflammatory effects, the regulation of iNOS and COX-2 protein in BV2 microglia and the production of NO and PGE_2 as by-products was measured. First, BV2 microglia cells were incubated with G-T-R for 3 h, then treated with LPS, and the production of NO and PGE_2 was evaluated. G-T-R dose-dependently reduced NO and PGE_2 production (Fig. 1b, c). We also checked the protein expression of iNOS and COX-2 using western blotting. G-T-R also dose-dependently inhibited the expression of iNOS and COX-2, as expected (Fig. 2).

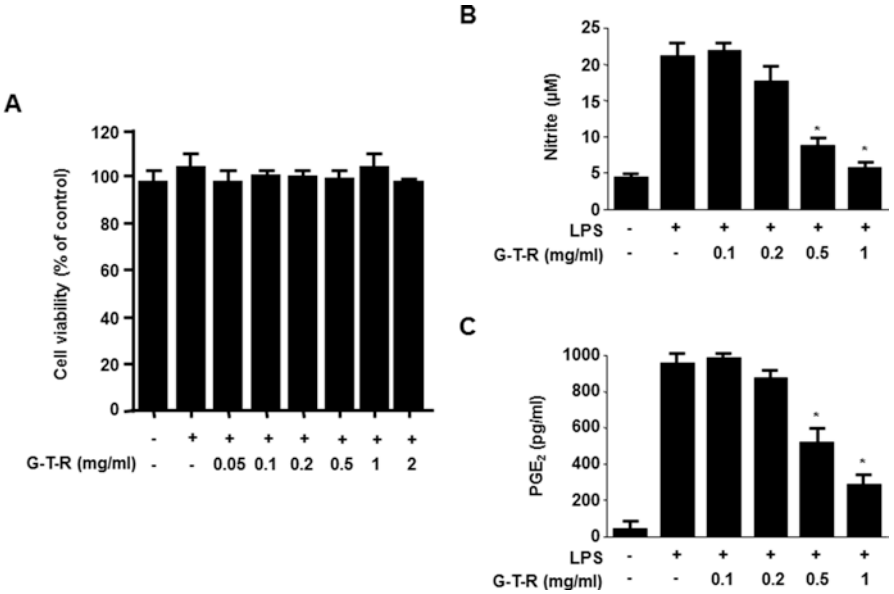

Fig. 1 The effects of G-T-R on cell viability, as determined by the MTT assay (**a**), nitrite (**b**), and PGE_2 (**c**) production in BV2 microglia. The cells were incubated for 48 h with various concentrations of G-T-R (0.1–1 mg/mL) (**a**). Cells were pretreated with labeled concentrations of G-T-R for 3 h and stimulated with LPS for 24 h (1 μg/mL) (**b, c**). The data were expressed as the mean ± S.D. values of three independent experiments. ∗$p < 0.05$ compared with the LPS-treated group

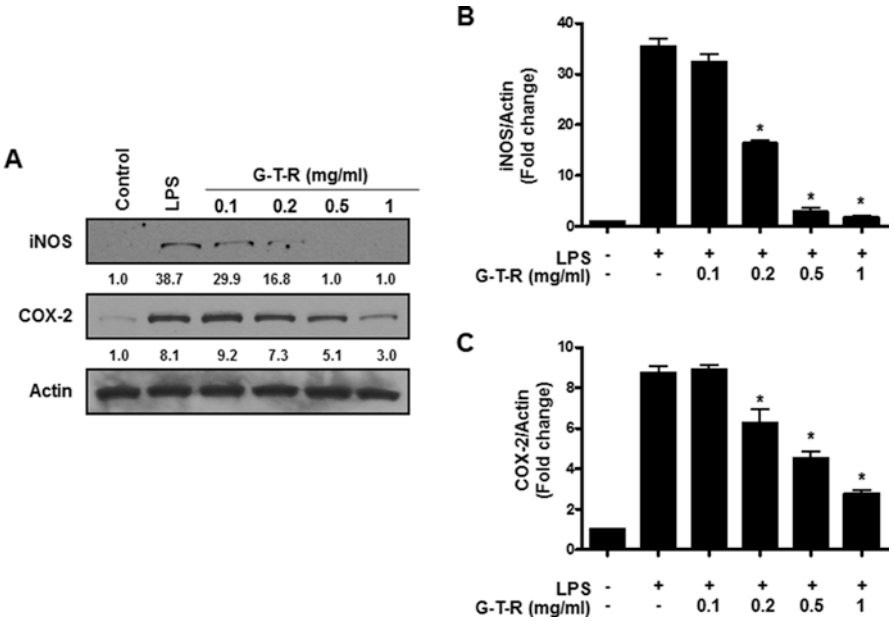

Fig. 2 The effects of G-T-R on iNOS and COX-2 protein expression in BV2 microglia. Cells were pretreated with labeled concentrations of G-T-R for 3 h and stimulated with LPS for 24 h (1 μg/mL). Representative blots from three independent experiments are shown. The data are presented as the mean ± S.D. values of three experiments. ∗$p < 0.05$ compared with the LPS-treated group

3.2 *The Effects of G-T-R on TNF-α (A), IL-1β (B), and IL-6 (C) Expression in BV2 Microglia*

Next, we examined the G-T-R-mediated regulation of TNF-α, IL-1β, and IL-6, which are representative inflammatory cytokines activated by microglia. BV2 microglia were treated with G-T-R and LPS. Subsequently, cytokines TNF-α and IL-1β were measured. G-T-R was found to concentration-dependently decrease TNF-α, IL-1β, and IL-6 (Fig. 3).

3.3 *The Effects of G-T-R on the NF-κB Regulation in BV2 Microglia*

We also examined activity of NF-κB transcription factor, which mediates inflammatory responses through the regulation of iNOS, COX-2, and other inflammatory-related genes (Xie et al. 1994; Baldwin 1996; Roshak et al. 1996). As shown in Fig. 4, the cytoplasm and nucleus of BV2 microglial cells were separated and observed. When treated with LPS (1 μg/mL) on BV2 microglia, p65, a subunit of NF-κB, were increased into the nucleus, however dose-dependently inhibited by

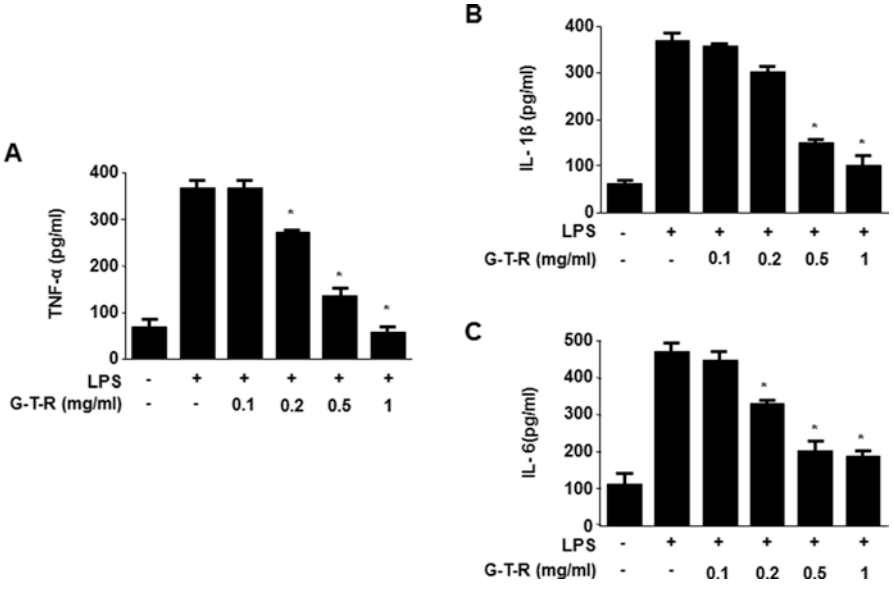

Fig. 3 The effects of G-T-R on TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) levels in BV2 microglia. Cells were pretreated with labeled concentrations of G-T-R for 3 h and stimulated with LPS for 24 h (1 μg/mL). The concentration of TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) was determined as described in Materials and Methods. The data are presented as the mean ± S.D. values of three experiments. $*p < 0.05$ compared with the LPS-treated group

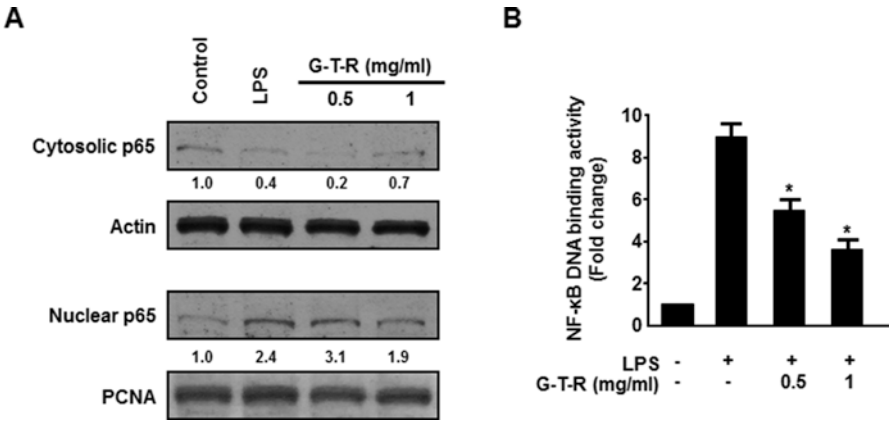

Fig. 4 The effects of G-T-R on the nuclear translocation of NF-κB p65 (**a**) and NF-κB DNA binding activity (**b**) in BV2 microglia. Cells were pretreated with labeled concentrations of G-T-R for 3 h and stimulated with LPS for 24 h (1 μg/mL). The representative blots of three independent experiments are shown (**a**). A commercially available NF-κB ELISA (Active Motif) kit was used to test the nuclear extracts and determine the degree of NF-κB DNA binding (**b**). The data shown are the mean ± S.D. values of three independent experiments. ∗$p < 0.05$ compared with the LPS-treated group

G-T-R treatment. In contrast, in the cytoplasm, the LPS-induced reduction of NF-κB p65 was restored by G-T-R (Fig. 4). This was consistent with the results that the LPS-induced increases in the expression of iNOS, COX-2, TNF-α, IL-1β, and IL-6 were suppressed by G-T-R (Figs. 2 and 3). It has been suggested that this occurs due to inhibition of NF-κB by G-T-R.

4 Discussion

Owing to the rapid aging of society, the occurrence of neurodegenerative diseases is increasing rapidly; consequently, the social burden has also become serious. Neurodegenerative diseases are classified into conditions such as including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, Lou Gehrig's disease, according to the main symptoms and the affected brain area (Stanzione and Tropepi 2011). Oxidative stress and excessive inflammation of the CNS are known to be associated with neurodegenerative disorders (Yang et al. 2016). The increasing incidence of neurodegenerative diseases has necessitated, the identification of the causes of diseases and the development of therapeutic techniques. Neuronal cells in the brain have a variety of functions, which vary depending on their location, and microglial cells play key role in brain inflammation or degeneration. Therefore, it is possible to control degenerative brain disease by controlling the inflammatory reactions that occurs in microglial cells (Nakamura et al. 1999). Taurine is a sulfur amino acid with a key role in the human body and brain (Lim and Kim 1995);

however, there are no suggestions of the involvement of glucose-taurine reduced (G-T-R) in anti-neuroinflammation mechanisms. Therefore, we aimed to evaluate the anti-neuroinflammatory response of glucose-taurine reduced (G-T-R) via the regulation of nuclear factor-kappa B (NF-κB).

Prolonged inflammation can cause a variety of neurodegenerative disorders. Excessive inflammatory reactions can also interfere with tissue function and cause disease pathogenesis (Medzhitov 2010). The exposure of microglia in neurons to particular agonists activates complex signaling cascades that immediately trigger the production of pro-inflammatory cytokines, especially interleukin-6 (IL-6), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), plays a crucial role of commencing neuroinflammatory response (Malyshev and Shnyra 2003; Lynch 2009; Kim et al. 2015; Lee and Jeong 2016). Therefore, we observed that G-T-R reduced the NO, PGE_2, iNOS and COX-2 (Figs. 1 and 2). As inflammatory mediators including NO and PGE_2 are produced by the expression of iNOS and COX-2 mediated by inflammation inducers, such as LPS or cytokines, the regulation of iNOS and COX-2 is recognized as a key mechanism for regulation of inflammation (Liao et al. 2004). In our study, we showed that the pre-treatment of BV2 microglial cells with G-T-R, suppressed the LPS-induced production of TNF-α, IL-6, and IL-1 β in microglia (Fig. 3). NF-κB binds to IκB and is present in the cytoplasm in an inactive state. After stimulation, NF-κB separates from IκB, the subunit p65 migrates to the nucleus (Roshak et al. 1996), and it activate the transcription of inflammation-related genes (Xie et al. 1994; Baldwin 1996). In our results, G-T-R inhibited the NF-κB p65 to the nucleus. In addition, we checked the NF-κB DNA-binding activity and noted that G-T-R dose-dependently inhibited the rise in NF-κB DNA-binding activity in activated BV2 microglial (Fig. 4). From these findings, we proposed that the NF-κB pathway would be the crucial target for G-T-R to suppress the induction of neuro-inflammatory inducers in microglial cells.

5 Conclusion

In summary, we determined the neuro-biological activity of G-T-R, a derivative of taurine. Overall, our results showed that G-T-R possessed anti-neuroinflammatory responses that were associated with its inhibitory effects on the NF-κB pathway in LPS-activated BV2 microglia. These results suggested that the taurine derivative, G-T-R, may be a favorable therapeutic agents for further development into a remedy for a range of neuroinflammatory diseases. Further detailed investigations into the anti-neuroinflammatory effects of G-T-R in *in vitro* and *in vivo* models of neuronal diseases will assist in the development of its therapeutic potential.

Acknowledgements This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2015R1C1A1A02036465 & NRF-2018R1C1B6001913).

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Protection of Taurine Against Neurotoxicity Induced by Arsenic in Primary Cortical Neurons

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Abstract Our group previously reported that taurine has a protective capacity on the hippocampus and cerebellum of arsenic (As)-exposed mouse. In the present study, we explore whether taurine demonstrates protection against As toxicity in primary cortical neurons. Primary cortical neurons were exposed to various concentrations of arsenite and cell viability was assessed to confirm the toxicity of As on cortical neurons. The protection of taurine was examined after primary cortical neurons were treating with arsenite and taurine for 24 h. The cell viability was examined by MTT and caspase-3 activity assay. The expression of Bax and Bcl-2 was determined by western blot. The results showed that As exposure reduced cell viability and enhanced the activity of caspase-3, which were markedly inhibited by taurine treatment. The expression of Bax and Bcl-2 were disturbed by As exposure, which were reversed by taurine. These results indicated that taurine expose protective effect on As-exposed primary cortical neurons and its mechanism maybe involved the regulation of Bax/Bcl-2.

Keywords Taurine · Arsenic · Neuroprotection · Apoptosis

1 Introduction

Arsenic (As), being a kind of toxic metalloid, is widely distributed around the world. Industrial and geological pollution is the major source of As which was found in contaminated water resource, food, ambient air and dust (Carlin et al. 2016).

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Humans are exposed to the toxin mainly through skin contact, digestive tract, and inhalation. The toxin presents as an organic compound or inorganic compound which involved with a myriad of disease, including cancer, neurologic deficits, psychiatric problems, kidney disease, diabetes, cardiovascular disease, respiratory outcomes, and reproductive abnormalities (Kuo et al. 2013; Navas-Acien et al. 2005; Peters et al. 2015). When arsenic was absorbed into the body, it would distribute into several tissues and organs. Our group previously reported that As exposure induced injury in the hippocampus and cerebellum of mice (Li et al. 2017; Zhang et al. 2014a). Cerebrum is another major target of As which is the vital organ in body. Impaired nerve system was observed in various As-exposed models at environmental relevant levels (Sinczuk-Walczak 2009; Zhang et al. 2013).

Taurine, a sulfur-containing-β-amino acid, presents in many mammalian tissues being a major free intracellular amino acid (Batista et al. 2013). Several studies indicated that taurine treatment reduced inflammation, fibrosis, apoptosis and hyperplasia in lungs and alveolar epithelial cells (Men et al. 2010; Schuller-Levis et al. 2003; Bhavsar, Patel and Lau-Cam 2010; Schuller-Levis et al. 1995). It protects many tissues and organs of body against apoptosis and toxicity induced by various poisonous substances(Men et al. 2010; Zhang et al. 2014b; Higuchi et al. 2012). Taurine is considered an attractive candidate to relieve arsenic-induced injury in neurons.

In the present study, the effect of As and taurine on the viability of primary cortical neurons was assessed by MTT and capase-3 assay. Bax and Bcl-2 levels were examined in taurine/arsenic-treated cells by western blot. The aim of the study was to investigate the beneficial role of taurine on arsenic-induced neurotoxicity in primary cortical neurons.

2 Methods

2.1 Primary Cortical Neuronal Culture and Groups Tested

Embryonic rats were used to isolate brains and obtain primary cortical neurons according to the methods previously reported (Teng et al. 2013) with some modifications. In brief, day 16–18 embryos were collected from pregnant rats and washed with PBS. Brains were isolated and kept in basal media eagle containing 26.8 mM glucose, 2 mM glutamine, 20% fetal bovine serum at 37 °C for 10 min with a gentle shaking. Then, a 14-G cannula was used to pass the cortices and make cell suspension. Then the suspension was centrifuged at 200 × g for 5 min and seeded on poly-D-lysine (5 μg/ml) precoated dish in incubator. Sodium arsenite in various concentrations was exposed on the second day with or without taurine for 24 h.

2.2 MTT Assay

Briefly, 10 μl of 5 mg/ml MTT solution (in PBS) was added to each well of 96-well plate, and the cells were incubated at 37 °C for 4 h. Then, 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Followed by incubation for 30 min, the absorbance was read at 570 nm. Cellular viability was expressed as a percentage relative to the control (control %).

2.3 Western Blot

Cells were homogenized in lysis buffer with 1% proteinase inhibitors. The total cell lysis were loading for SDS-PAGE to separate various proteins, then transferred to PVDF membrane. The membrane was incubated with Bad, Bcl-2 or p-Akt primary antibodies (1:1000, Cell Signaling Technology, USA) overnight at 4 °C. Second horseradish peroxidase-conjugated antibody (1:5000, Sigma, USA) was used for visualizing.

2.4 Statistic Analysis

Data were analyzed with SPSS 11.0. Difference between various groups was analyzed with one-way ANOVA and LSD test. P value <0.05 was considered significant.

3 Results

3.1 As Exposure Deduced the Viability of Primary Cortical Neurons in a Concentration-Dependent Manner

The toxic effect of As on primary cortical neurons was assess by MTT assay. As shown in Fig. 1, the viability of primary cortical neurons was decreased markedly in a dose-dependent manner after 24 h As exposure. The cell viability was reduced nearly 55% with 5 μM arsenite. So, this concentration, 5 μM arsenite, was used in the following experiments.

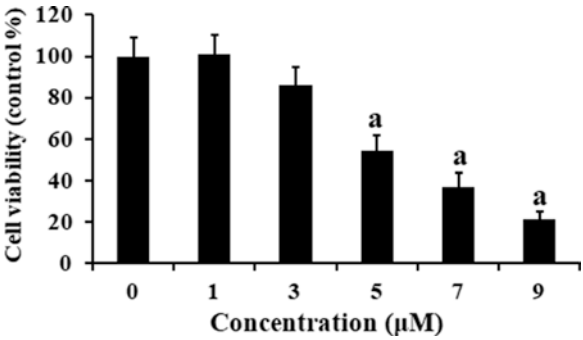

Fig. 1 Effect of As on the viability of primary cortical neurons. Data were presented as mean ± SD. [a]$p < 0.05$, compared with control group

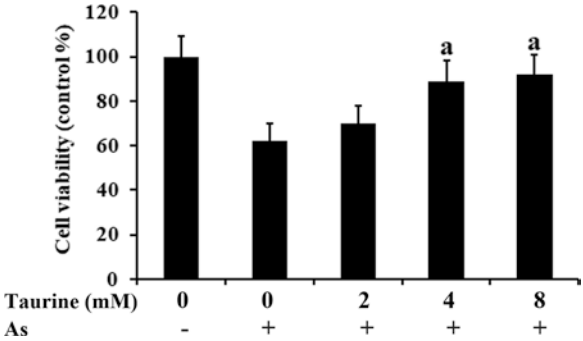

Fig. 2 Effect of taurine on the viability of As-exposed primary cortical neurons. Data were presented as mean ± SD. [a]$p < 0.05$, compared with As group

3.2 Taurine Treatment Protect Primary Cortical Neurons Against as Neurotoxicity

To confirm the protection of taurine against As neurotoxicity in primary cortical neurons, the cells were treated with 5 μM arsenite with or without taurine for 24 h. The results showed that the activity of primary cortical neurons was decreased after As exposure compared with control group, indicating arsenic has neurotoxicity in primary cortical neurons. Interestingly, the decreased viability was significantly reversed with taurine treatment. The reversion level is related with the concentration of taurine. With 4 mM taurine treatment, the viability of cells is about 90% compared with the control group. It is suggest that taurine have neuroprotection against As-exposed primary cortical neurons (Fig. 2).

3.3 Taurine Treatment Prohibited the Disturbance of the Level of Bax and Bcl-2 in As-Exposed Primary Cortical Neurons

The protein level of Bax and Bcl-2 in primary cortical neurons was examined with western blot. As shown in Fig. 3, Bax level was markedly increased in As exposure cells than that in control group. Interestingly, the enhancement of Bax expression was reduced apparently after taurine treatmeng. With the treatment of taurine, Bax

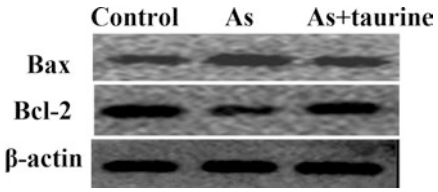

Fig. 3 Effect of taurine on the expression of Bax and Bcl-2 in As-exposed primary cortical neurons

level was significantly reduced. At the same time, compared with control group, As exposure significantly reduced the level of Bcl-2 in primary cortical neurons, which was markedly reversed after taurine administration.

4 Discussion

The present study explored the protective effect of taurine against As neurotoxicity in primary cortical neurons. Our group previously reported that As exposure induced injury in the hippocampus and cerebellum of mice (Li et al. 2017, Zhang et al. 2014a). To confirm the neurotoxicity of arsenite on primary cortical neurons, various concentration of arsenite were exposed to primary cortical neurons for 24 h and the cell viability was examined by MTT assay. The results showed that the viability of primary cortical neurons was decreased in a dose-dependent manner with arsenite exposure. To identify the neuroprotective capacity of taurine against arsenic-induced injury, cells were treated with a range of doses of taurine and arsenite. We found that taurine treatment markedly enhanced cell viability in a dose-dependent way. It is suggested that arsenite exerts harmful effects on primary cortical neurons and taurine has protective effects against arsenite neurotoxicity in primary cortical neurons.

Bax and Bcl-2 are the key regulators of cell viability which take part the activation of apoptosis (Wang et al. 2013). Disturbance of Bax or Bcl-2 expression would lead the release of mitochondrial Cyt C into the cytosol, which eventually activates the caspase cascade and apoptosis. Bax is pro-apoptotic regulator and Bcl-2 is an anti-apoptotic one (Braun 2012). In the present study, the results showed that taurine significantly prohibited the disruption of Bax and Bcl-2 in As-exposed primary cortical neurons. The results demonstrate that taurine elicited protective effects on arsenic-induced injury and markedly inhibited the disturbance of Bax and Bcl-2 expression. It is suggest that taurine treatment has a protective effect on arsenic-induced primary cortical neuron injury, which is related to altered Bax/Bcl-2 ratio.

5 Conclusion

In summary, the present study shows that taurine treatment significantly inhibited the decrease of cell viability in arsenic-exposed primary cortical neurons. Taurine reversed arsenite-reduced viability of primary cortical neurons. Taurine possesses neuroprotective capacity in arsenic-exposed primary cortical neurons involving the regulation of oxidative stress.

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Protective Effect of Taurine on Apoptosis of Spinal Cord Cells in Diabetic Neuropathy Rats

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Abstract Diabetes mellitus (DM) is a condition characterized by chronic hyperglycemia, which leads to diabetic neuropathy and apoptosis in the spinal cord. Taurine has been found to ameliorate the diabetic neuropathy and control apoptosis in various tissues. However, there are few reports that discuss the direct relationship between spinal cord and anti-apoptotic effect of taurine. In this study, DM was induced in male SD rats with STZ @ 25 mg/Kg of body weight in combination with high fat diet. After 2 weeks, they were divided into four groups as DM: diabetic rats, T1 (0.5%), T2 (1%) and T3 (2%) taurine solution, while control group was non-diabetic rats (no treatment). The results showed that DM increased apoptosis, decreased phosphorylated Akt and Bad. DM decreased expression of Bcl-2 and increased the Bax. Moreover, the release of cytochrome c into cytosol was increased in DM and activation of caspase-3 was also increased. However, taurine reversed all these abnormal changes in a dose dependent manner. Our results suggested the involvement of Akt/Bad signaling pathway and mitochondrial apoptosis pathway in protective effect of taurine against apoptosis in the spinal cord of diabetic rats. Therefore, taurine may be a potential medicine against diabetic neuropathy by controlling apoptosis.

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1 Introduction

Diabetes mellitus (DM) is a multifactorial disease, which is characterized by chronic hyperglycemia. There are two major forms of the disease that are: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is typically resulted by an autoimmune attack on the β-cells, with an increasing invasion of the pancreatic islets by mononuclear cells, causing an inflammatory process called insulitis (Cnop et al. 2005). T2D is a disruption of metabolism, and characterized by chronic hyperglycemia and dyslipidemia that is related to wide range of complications (Hoogwerf et al. 2006). Among these, diabetic neuropathy (DN) is the most common and troublesome complication of DM (Yasuda et al. 2003). The emerging evidence showed that central nervous system (CNS) is involved in DN. Several long-lasting consequences of chronic hyperglycemia on the brain have been described, like decreased synaptic plasticity (Reagan 2012; Revsin et al. 2009), impairment in learning and memory, and neuroinflammation, etc. (Hawkins and Davies 2001; Verdile et al. 2015; Zhang et al. 2008). It was showed that the spinal cord was also affected in DN (Selvarajah et al. 2006). Postmortem study of Reske and colleagues found axonal loss and atrophy, gliosis, and demyelination in the spinal cord of DN (Reske-Nielsen et al. 1970). Hyperglycemia caused apoptosis in liver (Rashid et al. 2013) in kidney (Das and Sil 2012) and brain (Li et al. 2002). Recently, it was reported that diabetes induced apoptosis in the spinal cord of rats (Lu et al. 2017). Apoptosis is a natural program of cell death (Singh and Anand 1994), which is responsible for cell renewal. However, an increased apoptosis causes a tissue damage or abnormal cell death (Mandrup-Poulsen 2003) and diabetes increased the apoptosis (Zhang et al. 2008). Previous studies revealed that apoptosis is responsible for high glucose that can cause neural dysfunction as well as cell death in vitro and in vivo studies (Lelkes et al. 2001; RUSSELL et al. 2002). Therefore, controlling apoptosis in the spinal cord in DN may be a novel strategy for treatment of DN.

Taurine (2-aminoethanesulphonic acid) is an endogenous amino acid that is found in a moderately high concentrations(mM) in the mammalian tissues (Huxtable 2002). Being a semi-essential amino acid, taurine has a significant role in the mammalian CNS. Taurine has a critical role in a variety of processes, e.g., neuromodulation, osmoregulation, membrane stabilization and cell propagation (De Luca et al. 2015; Oja and Saransaari 2007). Taurine has also been found to reduce apoptosis in cardiomyocytes (Takatani et al. 2004), in liver (Rashid et al. 2013), and in kidney (Das and Sil 2012). Moreover, it was found that taurine has reduced the neuronal apoptosis and improved the memory of diabetic rats (Rahmeier et al. 2016). However, there are a few of reports on anti-apoptotic effect of taurine in the spinal cord and its protective mechanism.

Owing to these studies, it was hypothesized that taurine may prevent apoptosis in the spinal cord of diabetic rats. Hence, we investigated the effect of taurine administration on STZ-induced diabetic rats. We further tried to explore the underlying mechanism for protective action of taurine against DN. It was considered that taurine can be a potential medicine against diabetes-induced apoptosis in spinal cord and may be helpful to attenuate diabetic neuropathy.

2 Material and Methods

2.1 Chemicals

Akt mouse monoclonal antibody, Akt- phospho- S473 mouse monoclonal antibody and Bad rabbit polyclonal antibody were obtained from Protein Tech, Inc. (USA). Mouse polyclonal anti-cytochrome c was purchased from Abcam Inc. (Cambridge, MA, USA). Mouse polyclonal anti-β-actin, goat-anti-rabbit horse radish peroxidase (HRP)-conjugated IgG and goat-anti-mouse horse radish peroxidase (HRP)-conjugated IgG were purchased from ZSGB Biotechnology, Inc. (Beijing, China). Rabbit polyclonal anti-VDAC, phospho-Bad (Ser112) mouse antibody, DAPI, RIPA lysis buffer, BCA Protein Assay Kit, ECL enhanced chemiluminescence kit were purchased from Beyotime Biotechnology, Inc. (Shanghai, China). TUNEL assay kit was bought from Key Gen Bio Tech, Inc. (Nanjing, China). All other chemicals were of the highest grade commercially available.

2.2 Ethics Statement

The research was conducted according to the Animal Guidelines and agreement with the Ethical Committee of Dalian Medical University, Dalian, China (Permit number:SCXK (liao) 2015–2003). An appropriate care was taken to lessen the number of animal subjects used and ameliorate the suffering and appropriate measures were taken to reduce pain and distress taking in accounts the endpoints of humans for animal suffering.

2.3 Establishment of Animal Model

Sixty male SD rats (180–200 g) were purchased from animal center at Dalian Medical University, Dalian. These animals were acclimatized in animal room for 2 weeks at 22 °C with 50% humidity at 12 h light dark-cycle. After acclimatization, rats were divided randomly into two groups, control (n = 12) and experimental (n = 48). Control group was provided with normal diet and simple water for whole

study period while experimental group was fed special diet (25% sucrose +15% oil +1.5% cholesterol +1% bile acid +57.55% normal diet) for 4 weeks and then administered with single dose of STZ at 25 mg/Kg of body weight (Lin et al. 2010), prepared freshly in 1% citrate buffer solution, while control was injected with citrate buffer only and blood glucose was checked after 72 h. If blood glucose was ≥16 mmol/L (Lin et al. 2013), it was considered as diabetic. Diabetic rats were kept on normal diet and normal water for 2 weeks and then randomly divided into to four groups i.e. DM, diabetic rats were given normal water. T1 (0.5% taurine solution for drinking), T2(1% taurine solution) and T3 (2% taurine solution) while normal feed was provided to all the groups. After 8 weeks, animals were sacrificed, and the organs were preserved for further analysis.

2.4 Detection of Apoptosis

The spinal cord tissue was taken, 4% paraformaldehyde was used to fix the spinal cord tissue sample of each group, then submerged in 15, 20 and 30% sucrose. After that, the sample was cut into 6 μm slices for the next procedure. The apoptosis of spinal cord cells was checked by TUNEL assay, the cell nuclei were stained by DAPI. The images were taken by the fluorescence microscope manufactured by Olympus, Japan, Model No. IX-70. Counting was done for the number of TUNEL-positive cells in six random areas at 200 × field.

$$\text{The apoptosis index}\left(\text{AI}\right) = \frac{\text{The number of apoptotic cells}}{6 \times 100} \times 100\%$$

2.5 Assay for Cytochrome c (cyt-c)

The isolation of mitochondria from spinal cord of lumbar vertebra was done by using Tissue Mitochondria Isolation Kit (Beyotime, Shanghai, China) as per manufacturer's protocol. The spinal cord of lumbar vertebra was homogenized in Mitochondria Isolation Reagent A at a ratio of 10 μl/mg tissue. Homogenized samples were centrifuged (machine detail) at 600 g for 5 min at 4 °C, and the supernatant was subsequently centrifuged again at 11,000 g for 10 min at 4 °C. The pellet was suspended; the supernatant (cytosol fraction) was transferred to another tube.

2.6 Determination of Cyt-c Protein Expression

To obtain cytosolic and mitochondrial fractions, spinal cord of lumbar vertebra was collected; cytoplasmic and mitochondrial fractions were extracted with a Tissue Mitochondria Isolation Kit (Beyotime, Shanghai, China) as mentioned above. The

proteins (20 μg/lane) were blended with an equal volume of SDS-PAGE loading buffer and separated by SDS-PAGE under non-reducing conditions using 12% SDS-PAGE Gels, and then electro transferred to Hybond-P PVDF membrane. Defatted milk containing blocking buffer was used for the blocking the membrane for 1 h and incubated at 4 °C for a night with cytochrome c polyclonal antibody (1:1000), β-actin and VDAC (1:1000) were used as loading control for mitochondrial proteins and cytosolic proteins, respectively. Membrane was washed thrice with Tris buffered saline having 0.05% Tween-20 (TBST) for 10 min and then incubated at room temperature for 2 h with horse radish peroxidase-conjugated goat anti-mouse IgG (1:5000). The signals were viewed by an ECL enhanced chemiluminescence kit (Beyotime, Shanghai, China) and quantification was done densitometrically using UVP BioSpectrum Multispectral Imaging System (Ultra-Violet Products Ltd. Upland, CA, USA).

2.7 Western Blotting

Chilled Spinal cord tissues were cut and the tissue was homogenized in ice-cold RIPA buffer with 1% protease inhibitor and 1% phosphatase inhibitors. Then incubated at 4 °C for 30 min, and the mixture was blended after every 10 min. The homogenate was centrifuged at 14,000 g for 15 min at 4 °C and the supernatant was collected. Concentration of protein was determined using the BCA method (Beyotime). The method of western blotting followed the protocols of the ECL plus western blotting detection reagents, except for the incubation time of the primary antibody. We changed the incubation time of the primary antibody from 1 h to overnight. Detection was performed using the ECL plus western blotting detection reagents. The results of western blotting were quantitatively analyzed using Image J.

2.8 Determination of Caspase-3 Activity

Caspase-3 activity kit was used for determination of caspase-3 activity (Beyotime, Shanghai, China) following the manufacturer's protocol. Protein was isolated from spinal cord using the lysis buffer supplied with the kit. Assays were conducted on 96-well microtitre plates, Samples were analyzed by a microplate-reader (detail) at 405 nm absorbance.

Statistical analysis

All results were expressed as mean ± S.D., and the statistical analysis was performed with one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test, or Student's t test using the program GraphPad Prism 5.0. and the difference was regarded as statistically significant ($P \leq 0.05$).

3 Results

3.1 Effect of Taurine on Diabetes Induced Apoptosis

Being a natural system of cell death (Singh and Anand 1994) apoptosis is responsible for cell renewal and turnover. Abnormal apoptosis may cause tissue damage or loss of cells (Mandrup-Poulsen 2003). We found that diabetes increased apoptosis in spinal cord. Fluorescent microscopy revealed that apoptosis was increased in STZ-induced diabetic rats. However, taurine reduced apoptosis in a dose dependent manner (Fig. 1a). AI is given in ($P < 0.05$) (Fig. 1b). It is evident that apoptotic index was significantly different among the groups. The highest apoptotic index was seen in DM and decreased with increasing dose of taurine. A direct indicator of apoptosis is caspas-3. Taurine caused down-regulation of caspase-3 in diabetic rats ($P < 0.05$) (Fig. 1c).

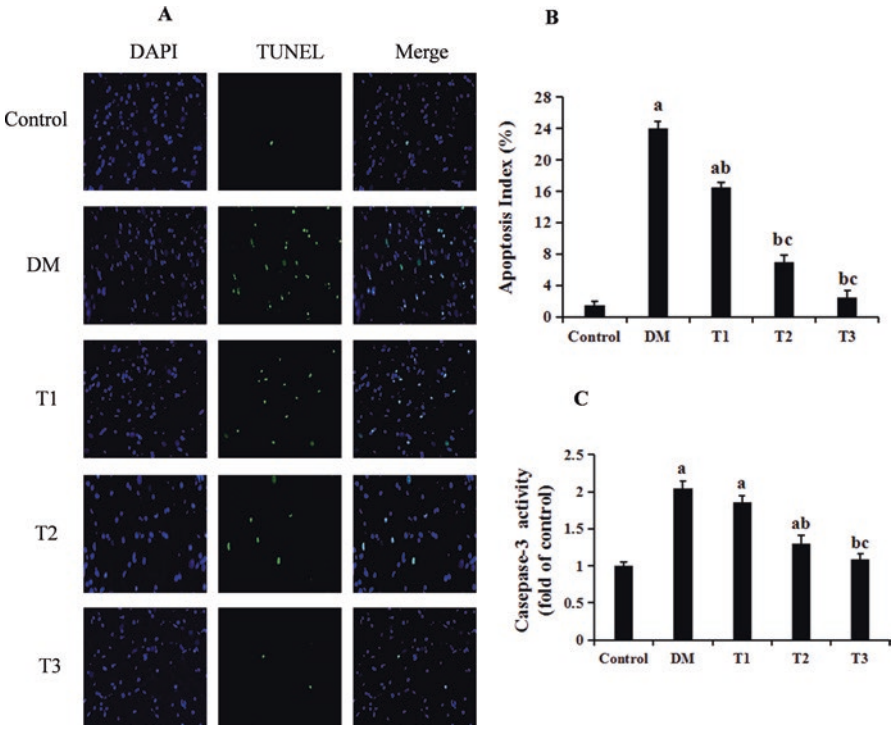

Fig. 1 Effect of taurine against apoptosis in spinal cord of STZ-induced diabetic rats. (**a**) Representative images of TUNEL stained cells are shown in the control group (Con) and the experimental groups. Green fluorescence represents the apoptosis cells, blue fluorescence represents the nuclei of the cells T1:0.5% Taurine, T2:1% Taurine and T3:2% Taurine solution, (**b**) Apoptotic index among groups (**c**) Caspas-3 activity among groups. Data were shown as the mean ± SD, $^{a}p < 0.05$, compared with Con group; $^{b}p < 0.05$, compared with DM group; $^{c}p < 0.05$, compared with T1

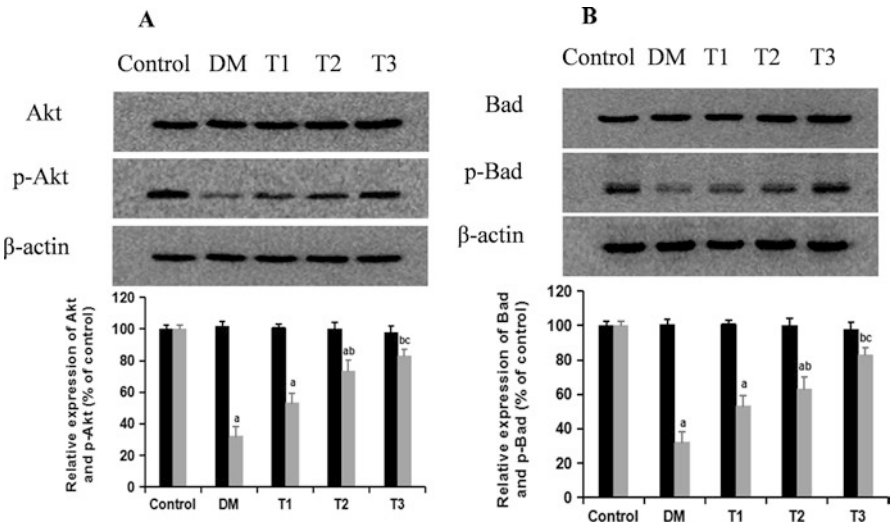

Fig. 2 Akt/Bad expression in experimental groups (**a**) Akt/p-Akt among different groups (**b**) Bad/p-Bad among different groups. Data is shown as the mean ± SD, $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with DM group; $^{c}p < 0.05$, compared with T1

3.2 *Effect of Taurine on Akt and Bad Regulation in Spinal Cord of Diabetic Rats*

Akt/p-Akt activity is important in cell survival (Danciu et al. 2003) and phosphorylation of Bad (p-Bad) is responsible for cell maintenance (Steenbergen et al. 2003). We found that activation of Akt (p-Akt) was decreased in diabetes. Nonetheless, taurine increased expression of p-Akt in diabetic rats in a dose dependent manner ($P < 0.05$) (Fig. 2a). Akt phosphorylates Bad (p-Bad). Our results found that Bad was not significantly different but p-Bad showed a significant difference among groups. Taurine caused an increase in expression of p-Bad in diabetic rats according to dose ($P < 0.05$) (Fig. 2b).

3.3 *Effect of Taurine on Expression of Bax and Bcl-2*

Bax promotes apoptosis and Bcl-2 stops it. Being downstream mediators of Akt they are of great importance (Hanke 2000). Our result showed that Bax was up-regulated in diabetic rats, however, taurine reduced this up-regulation gradually with dose ($P < 0.05$) (Fig. 3a). On the contrary Bcl-2 was down-regulated in diabetic rats and gradually increased with taurine in a dose dependent manner. There was a significant difference among the groups ($P < 0.05$) (Fig. 3b).

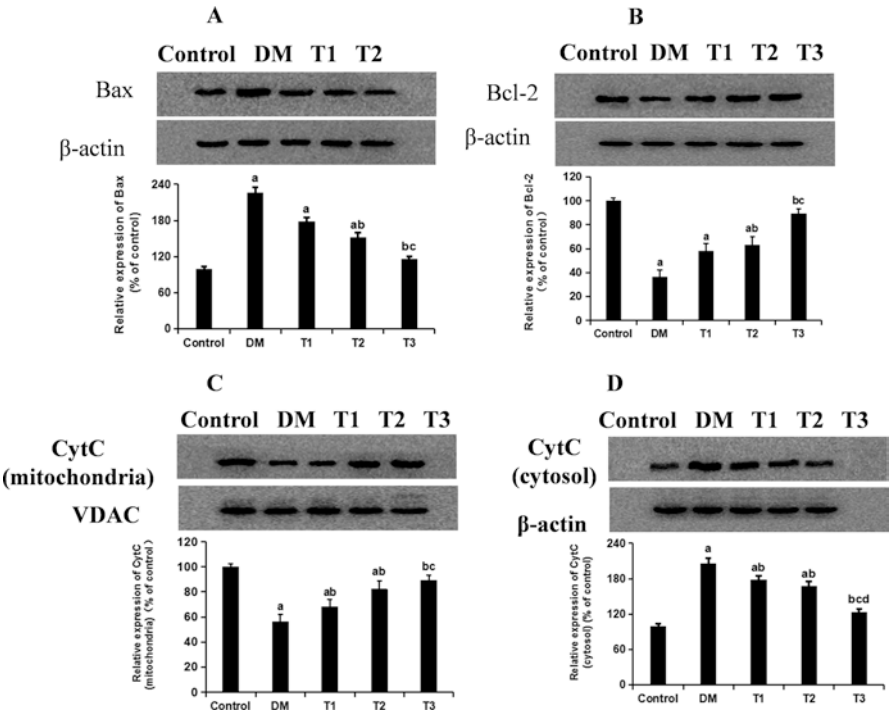

Fig. 3 Expression of different proteins (**a**) Bax expression (**b**) Bcl-2 expression (**c**) Mitochondrial cytochrome c expression, (D) Cytosolic cytochrome c expression. Data were shown as the mean ± SD, $^{a}p < 0.05$, compared with control group; $^{b}p < 0.05$, compared with DM group; $^{c}p < 0.05$, compared with T1

3.4 Effect of Taurine on Expression of Mitochondrial and Cytosolic Cytochrome c

The movement of cytochrome c from the mitochondria into the cytosol activates caspase-3 that causes apoptosis (Kamboj et al. 2010). Our results indicated that mitochondrial cytochrome c was down-regulated in diabetic rats, but taurine reversed this in a dose dependent manner (Fig. 3c), a significant difference ($P < 0.05$) was seen among the groups. Diabetes up-regulated cytosolic cytochrome c in rats, however, taurine stopped this change according to dose (Fig. 3d), difference among groups was significant ($P < 0.05$).

4 Discussion

Neuropathy is one of the serious complications causing a major economic burden in both T1DM and T2DM. Its occurrence is estimated to be around 8% in newly identified patients and more than 50% in patients having chronic disease (Boulton et al.

2005). Diabetes leads to neuropathy (Hoogwerf et al. 2006) and spinal cord is considered to be a participating organ in this abnormality (Eaton et al. 2001). Diabetes causes apoptosis in spinal cord (Lu et al. 2017) thus, apoptosis may participate in neuropathy of spinal cord induced by hyperglycemia. Consequently, it is adversely needed to have some medicine to ameliorate this abnormality and taurine is such a compound. Previous studies showed that taurine exhibited hypoglycemic effect (Winiarska et al. 2009) and it ameliorated diabetes induced neuropathy (Ford et al. 2003), and protected against diabetes-induced apoptosis in liver tissues (Rashid et al. 2013). These studies provoked us that taurine may ameliorate the diabetic neuropathy owing to its anti-apoptotic potential. In this study, we investigated the outcomes of taurine against apoptosis in diabetic rats and tried to explore the mechanism involved in this process. Our findings are promising and support our idea that taurine can be useful against diabetes induced apoptosis.

It had already been reported that diabetes induced apoptosis in the spinal cord (Lu et al. 2017). We also found that diabetes increased apoptosis in the spinal cord of rats. However, taurine decreased apoptosis in the spinal cord of diabetic rats (Fig. 1a) in a pattern depending upon dose. These results indicated that taurine may decrease apoptosis of the spinal cord tissues in diabetic rats. These findings are similar to previous studies, which showed that the taurine had protective effect against ischemia induced apoptosis in cardiomyocytes (Takatani et al. 2004), diabetes induced apoptosis in liver (Rashid et al. 2013) as well as in kidney (Das and Sil 2012) and neuronal apoptosis (Rahmeier et al. 2016). We further tried to explore the underlying mechanism.

In literature, Akt activity is responsible for the transduction of important signals involved in survival of some types of cell (Danciu et al. 2003; Dudek et al. 1997; Mangi et al. 2003). Previous studies indicated that activation of Akt (p-Akt) promoted cell survival and reduced apoptosis while reduction in phosphorylation of Akt was associated with apoptosis (Takatani et al. 2004) and it was suspected that same mechanism would be involved in our results. Hence, for confirmation of this hypothesis, Akt and its mediators were determined. Our results showed that expression of p-Akt (phosphorylated) and p-Bad was decreased in diabetic rats. However, taurine reversed this decrease in a dose dependent manner. This result suggested that taurine activated Akt, which may have participated in controlling apoptosis of the spinal cord tissues in STZ-induced diabetic rats. Our results are similar with previous results that taurine ameliorated ischemia-induced apoptosis by regulating Akt activation in cardiomyocytes (Takatani et al. 2004) and diabetes induced apoptosis in liver (Rashid et al. 2013). Bad is involved in survival of cell and it is a downstream mediator of Akt. The activation of Akt phosphorylates Bad (p-Bad) (Datta et al. 1997) and phosphorylation of Bad (p-Bad) is considered to improve conservation of cell (Steenbergen et al. 2003). We found that p-Bad expression was reduced in the spinal cord of diabetic rats. However, taurine controlled this decline in expression of p-Bad significantly. Moreover, hyperglycemia causes an inactivation of Akt, which causes reduction in phosphorylation of Bad and activation of caspases in cultured muller cells (Xi et al. 2005). In current study, we found that taurine activated Akt, which promoted phosphorylation of Bad suggesting involve-

ment of Akt/Bad in the mechanism of taurine for apoptosis control in the spinal cord of diabetic rats.

Bax and Bcl-2 are downstream mediators of Akt. Bax is a pro-apoptotic and Bcl-2 is anti-apoptotic protein and up-regulation of Bcl-2 indicates a strong resistance against apoptosis. Hence, mutual balance of Bax/Bcl-2 is responsible for cell survival and decides the fate of the cells that receive a death signal (Garchon et al. 1994; Hanke 2000). Our results also showed that diabetes increased Bax expression and decreased that of Bcl-2 consequently Bax/Bcl-2 was disturbed. Nonetheless, taurine controlled the disturbance of Bax/Bcl-2 according to dose and suppressed apoptosis. Taurine up-regulated Bcl-2 and down-regulated Bax consequently improved Bax/Bcl-2 suppressing apoptosis. These results are supported by study of Rashid and colleagues (Rashid et al. 2013). They also showed that taurine reduced expression of Bax and increased that of Bcl-2 under hyperglycemia. Moreover, Das et al. also found that taurine caused a reduced expression of Bax and increased expression of Bcl-2 (Das et al. 2012). Thus, taurine can regulate Bax/Bcl-2 balance under hyperglycemia.

Bax/Bcl-2 disruption leads to increase in Mitochondrial Membrane Permeability (MMP) causing release of cytochrome c from mitochondria, which activates cascade of caspases (Wang et al. 2013). Our study showed that expression of cytosolic cytochrome c was increased and that of mitochondrial cytochrome c was decreased in diabetic rats indicating high release of cytochrome c. However, taurine reduced cytosolic cytochrome c and increased mitochondrial cytochrome c in the spinal cord of diabetic rats. Our results are supported by the findings of Das and others. They showed that diabetes caused a release of cytochrome c in kidney under hyperglycemia which was controlled by taurine (Das and Sil 2012). However, taurine did not show any effect on cytochrome c in cardiomyocytes under ischemia (Takatani et al. 2004). In our study, change in cytochrome c expressions is possibly the result of Bax/Bcl-2 improvement. This improvement of Bax/Bcl-2 stabilized mitochondrial membrane and reduced release of cytochrome c from mitochondria in diabetes rats. The reduction of cytochrome c release caused a decline in activation of caspase-3 and consequently apoptosis. Kamboj stated that activation of caspase-3 plays a role in diabetic neuropathy and taurine successfully controlled it in our study (Kamboj et al. 2010)

In this study, it was found that diabetes caused apoptosis in the spinal cord of diabetic rats. However, taurine controlled this apoptosis by activating Akt, which in turn increased Bad phosphorylation. As a result, expression of Bax was decreased and Bcl-2 was increased resulting in improvement of Bax/Bcl-2 balance which in turn stabilized mitochondrial membrane. Stabilization and reduction of permeability in mitochondrial membrane reduced the liberation of cytochrome c consequently that caused a reduction in the activation of caspase-3. Decline in activation of caspase-3 caused a decrease in apoptosis. Our findings proposed that taurine may have controlled apoptosis by involving Akt/Bad signaling and mitochondrial pathway. These results support the concept that taurine can be useful in ameliorating neuropathy in diabetic rats by controlling apoptosis in the spinal cord.

5 Conclusions

Abnormal apoptosis is a damaging process causing cell death and it is increased in neuropathy. Our study showed that diabetes caused apoptosis resulting in cell death in the spinal cord of rats. Nevertheless, taurine controlled this apoptosis by mediating Akt/Bad signaling pathway as well as mitochondrial apoptotic pathway. Hence, we can deduce that taurine can ameliorate diabetic neuropathy by controlling apoptosis in the spinal cord. This study suggested that taurine has the capability to serve as a protective medicine against diabetes induced neuropathy.

Conflict of Interest It is declared that there is no conflict of interest among the authors.

Research Subjects It is declared that no animal was harmed or hurt except according to ethics.

Informed Consent It is declared that this manuscript is submitted after consent from all authors and all authors are aware of this submission.

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Taurine Ameliorates High Glucose Induced Apoptosis in HT-22 Cells

Pingan Wu, Xiaochi Chen, Inam-u-llah, Xiaoxia Shi, Mengren Zhang, Kaixin Li, Raheel Suleman, Muhammad Shahbaz, Shahid Alam, and Fengyuan Piao

Abstract Diabetes causes memory loss. Hippocampus is responsible for memory and increased apoptosis was found in diabetes patients. Taurine improved memory in diabetes condition. However, mechanism is unclear. In current study, hippocampal cell line HT-22 cells were subjected to analysis as five groups i.e. Control, High glucose (HG) at concentration of 150 mM, HG + 10 mM (T1), 20 mM (T2) and 40 mM (T3) taurine solution. TUNEL assay showed that HG increased the number of apoptotic cell significantly while taurine reduced apoptosis. Taurine increased phosphorylation of Akt in HT-22 cell treated with HG, and increased phosphorylation of Bad (p-Bad) was seen suggesting involvement of Akt/Bad signaling pathway. Expression of Bcl-2 was reduced in HG group but taurine improved this. Bax expression showed opposite trend. This indicated that taurine may reduce apoptosis by controlling balance of Bcl-2 and Bax. When the activation of Akt was blocked by using of perifosine, the effect of taurine disappears either partially or altogether. Thus, it was clear

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that taurine reduces apoptosis via Akt/Bad pathway in HT-22 cells exposed to HG which further improves downstream balance of Bcl-2 and Bax. This mechanism may be involved in apoptosis of hippocampus cells in diabetic condition.

Keywords High glucose · Apoptosis · HT-22 cell · Taurine · Anti-apoptosis

Abbreviations

DM	Diabetes mellitus
MTT	4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
HG	High glucose
TUNEL	In situ TdT-mediated dUTP nick end labeling

1 Introduction

Diabetes mellitus (DM), a chronic metabolic disorder is the most severe and prevail cause of morbidity and mortality in modern civilization (Chinenye et al. 2012). This disorder is the consequence of long time durability of high glucose (HG) level in blood and metabolic defects of ipivotal biomolecules such as carbohydrates, proteins and lipids (Baynes 1991). There are two types of diabetes i.e. type I, associated with impairment in insulin secretion and type II is resulted by resistance of insulin (Chuhwak and Pam 2007). There are various influences caused by chronic hyperglycemia on the body and central nervous system (CNS). Several chronic effects on the brain have been expounded, such as decreased synaptic plasticity (Reagan 2012; Revsin et al. 2009) neurotoxicity, neuroinflamation, decreased cell proliferation, increased neuronal apoptosis (Hawkins and Davies 2001; Verdile et al. 2015; Zhang et al. 2008). Indeed, some evidence showed that diabetes causes cognitive deficits (McCrimmon et al. 2012). In this regard, some adults with diabetes performed learning and memory impairment (Tonoli et al. 2014) and mainly cognitive, memory and learning deficits (Ojo and Brooke 2015; Reagan 2012; Revsin et al. 2009; Stranahan et al. 2008; Vieira et al. 2015).

The hippocampus, located between the thalamus and the medial temporal lobe of the brain, is responsible for such functions as orientation and memory, but many aspects of molecular and cellular mechanisms regarding its physiological function and changes in pathological conditions remain to be elucidated. For example, the dementia and Alzheimer's disease (AD), has been shown that there were severe pathological changes in the hippocampus, leading to progressive memory loss and other cognitive decline (Eustache et al. 2001). On the other hand, some paper showed that apoptosis occurs in hippocampal cells in diabetes which is associated with memory impairment (Li et al. 2002). So, we had an idea that if apoptosis in hippocampus of diabetic condition is controlled then we can effectively alleviate memory deficits in the diabetic conditions.

Taurine, a semi-essential amino acid, has several important functions, especially on the mammalian CNS acting in various processes such as osmoregulation, neuromodulation, membrane stabilization and cell proliferation (De Luca et al. 2015). Kim et al. (2012) discussed that taurine ameliorated hyperglycemia and dyslipidemia. Diabetic nerve injury was aggravated by taurine depletion (Stevens et al. 1993). It was found that taurine prevented sensory as well as motor conduction velocity deficits in STZ induced diabetic rats (Pop-Busui et al. 2001). Taurine also addressed apoptosis in hepatic cells (Rashid et al. 2013), in renal tubules (Hizoh and Haller 2002) and cardiac myocytes (Takatani et al. 2004). It is clear from above data that taurine has anti-apoptotic and anti-diabetic properties and also ameliorates diabetes-induced neuropathy. But mechanism of taurine of against diabetes induced apoptosis in hippocampus is yet unclear.

Owing to properties of taurine it was hypothesized that it may prevent apoptosis in the hippocampal cells during diabetes and which may be beneficial against memory impairment in diabetes and can be used as therapeutic strategy against diabetes-induced neuropathy. Hence, this study was designed to check the anti-apoptotic effect of taurine and explore its mechanism in HT-22 cells, hippocampal cell line, exposed to HG.

2 Methods

2.1 HT-22 Cells Cultures

HT-22 cells were purchased from BeNa Culture Collection (Kunshan city, Jiangsu province, China). Cells grown in Dulbecco's Modifed Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 1% streptomycin (100 mg/ml), penicillin G (100 U/ml), and then incubated at 37 °C with 5% CO_2.

2.2 Experimental Grouping

The cells were divided into five groups, Con group: control group, cells treated with normal culture medium. HG group: cells were cultured by normal medium with 150 mM glucose. Three taurine treatment groups: cells were co-treated of glucose (150 mM) with 10 mM, 20 mM and 40 mM taurine as T1, T2 and T3 groups, resprectively. To intervention experiments of pathway inhibitor, cells were treated with Akt inhibitor (Perifosine). Con group: cells were cultured by normal medium. HG group: cells were exposed to 150 mM glucose. HG + T group: cells were cultured with normal medium co-treatment of 150 mM glucose with 40 mM taurine. HG + T + Perifosine group: The 150 mM glucose, 40 mM taurine and 5 μM Perifosine were added into the normal medium to culture the cells.

2.3 MTT Assay

Cell viability was measured by using MTT Assay. In brief, HT-22 cells were seeded in 96-well culture plates at the density of 8×10^3 cells/well for 24 h (h). And then treated as Con, HG, T1, T2 and T3 groups for 48 h. After treatment, added 100 μl (0.5 mg/ml) MTT to each well and incubated for 4 h at 37 °C with 5% CO_2. Then each cell culture medium was replaced with 100 μl DMSO. The ELISA microplate reader was used to test the value of OD at 570 nm. The viability was presented as the percentage survival rate taking control as 100%.

2.4 Tunel Assay

The apoptosis in HT-22 cells was checked by TUNEL assay, the cell nucleus was stained by DAPI. The images were taken by the fluorescence microscope made by Olympus Japan (Model No IX-70). In the results, the apoptosis of cells were stained as green. Count TUNEL-positive cells in 100 cells in 200 × field of view, calculated the number of apoptotic cell in six random fields, and the apoptotic index (AI) = (the number of apoptotic cell/(6 × 100) × 100%).

2.5 Caspase-3 Activity

Caspase-3 Colorimetric Assay Kit (Beyotime, China) was used to evaluate caspase-3 activity. All steps are followed the kit instructions. The related outcomes were showed as a ratio to control.

2.6 Western Blot Analysis

The HT-22 cells were seeded in 100 mm dishes at the density of 2×10^6 cells/dish for 24 h. And then control group was given DMEM without FBS. HG group was given DMEM with glucose (150 mM), glucose (150 mM) +10 mM taurine as T1, glucose (150 mM) +20 mM taurine as T2 and glucose (150 mM) +40 mM taurine as T3. After 48 h incubation, the cells were collected to homogenize in ice cold RIPA Protein Extraction Reagent (Beyotime, China), mixed with 1% proteinase inhibitor and phosphatase inhibitor. The concentration of lysates was quantified by a BCA Protein Assay Kit (Beyotime, China). The lysates (40 μg of protein) were separated by electrophoresis in 12% SDS-PAGE transferred to PVDF. The membranes were blocked with 5% skimmed milk in TBS-T. Then incubated the first

antibody like Akt/p-Akt, Bad/p-Bad, Bcl-2, Bax and β-actin at 4 °C overnight. The membranes were rinsed three times with 1 × TBS-T and then incubated for 2 h with HRP-conjugated secondary antibody. The immuno-labeling were detected using the UVP Bio-Spectrum Imaging System.

2.7 Statistical Analysis

All data were analyzed by one-way ANOVA, followed by Bonferroni's post-hoc test using the program Graph Pad Prism 5.0. The results were present by mean ± SD, and the difference was regarded as statistically significant when $p \leq 0.05$.

3 Results

3.1 Effect of Taurine on Cell Viability of HT-22 Cells Treated with HG

Cell viability was measured by using MTT assay. The result showed that the cell viability in HG group was reduced compared to the Con group. The cell viability was no significant difference between T1 group and HG group, while, it was significantly increased ($p < 0.05$) in T2 and T3 groups than that in HG group. These results indicated that HG could decreased the HT-22 cells viability, but taurine improved the viability of HT-22 cells treated with HG (Fig. 1).

3.2 Effect of Taurine on HT-22 Cell Apoptosis Induced by HG

The apoptotic cells were detected by TUNEL Assay. The TUNEL-positive cells were stained in green color, and the cell nuclei were stained by DAPI (Fig. 2a). Our results showed that the number of apoptotic cell have significantly increased in HG group ($p < 0.05$) as compared to Con group (Fig. 2b). In contrast, amounts of TUNEL-positive cells significantly reduced in T2 group and T3 group than that in HG group ($p < 0.05$). It was revealed that taurine attenuated the apoptosis of HT-22 cells induced by HG.

Caspase-3 activity was measured by caspase-3 assay kit, as shown in Fig. 2c, caspase-3 activity was increased in HG group compared to the Con group, while caspase-3 activity was decreased in T2 and T3 groups compared with HG group. It suggested that taurine could decreased caspase-3 activity in HT-22 cells exposed to HG.

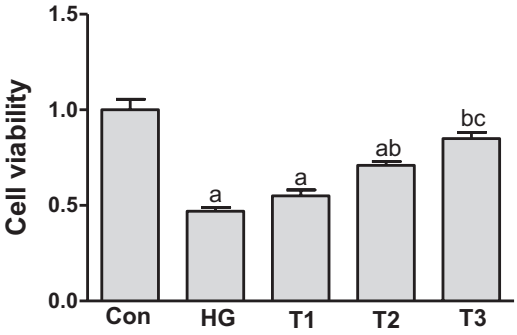

Fig. 1 The effect of taurine on viability of HT-22 cells treated with HG. The results were present by mean ± SD. Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the T1 group

3.3 Effect of Taurine on Expression of Akt/p-Akt, Bad/p-Bad

The Akt is an important regulator to control apoptosis by activating downstream signaling molecules. Bad, as a downstream mediator of p-Akt, could improve the cell survival when it is activated. In order to ensure the Akt/Bad signaling pathway whether involving in controlling apoptosis in HT-22 cells induced by HG, the level of expression in Akt/p-Akt and Bad/p-Bad in HT-22 cells were measured by western blot (Fig. 3a, b). The results showed that the expression of Akt and Bad has no significant difference among whole groups, however, the expression of p-Akt was significantly increased in T2 and T3 groups compared with the HG group. Similar expression trend was found in p-Bad ($p < 0.05$), except the T1 group, which had no significant difference compared to the HG group. These results indicated that taurine up-regulated the expression of p-Akt and p-Bad in HT-22 cells induced by HG.

To confirm the expression changes of p-Bad being mediated by Akt in HG-induced HT-22 cells treated with taurine, the expression of Bad/p-Bad was measured in HG/taurine-treated HT-22 cells in the presence of perifosine. As shown in Fig. 3c, the expression of p-Bad in HG + T + Perifosine group was significantly decreased, comparing with HG + T group ($p < 0.05$), which implied the up-regulating expression of p-Bad being mediated by Akt/Bad pathway.

3.4 Effect of Taurine on Expression of Bcl-2 and Bax

Bcl-2 family members are the key factor in apoptosis signals especially anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax. The balance between them directly decides the fate of the cells accepting death message, and the ratio of Bax/Bcl-2 determines the susceptibility of a cell to apoptosis. The changes in expression

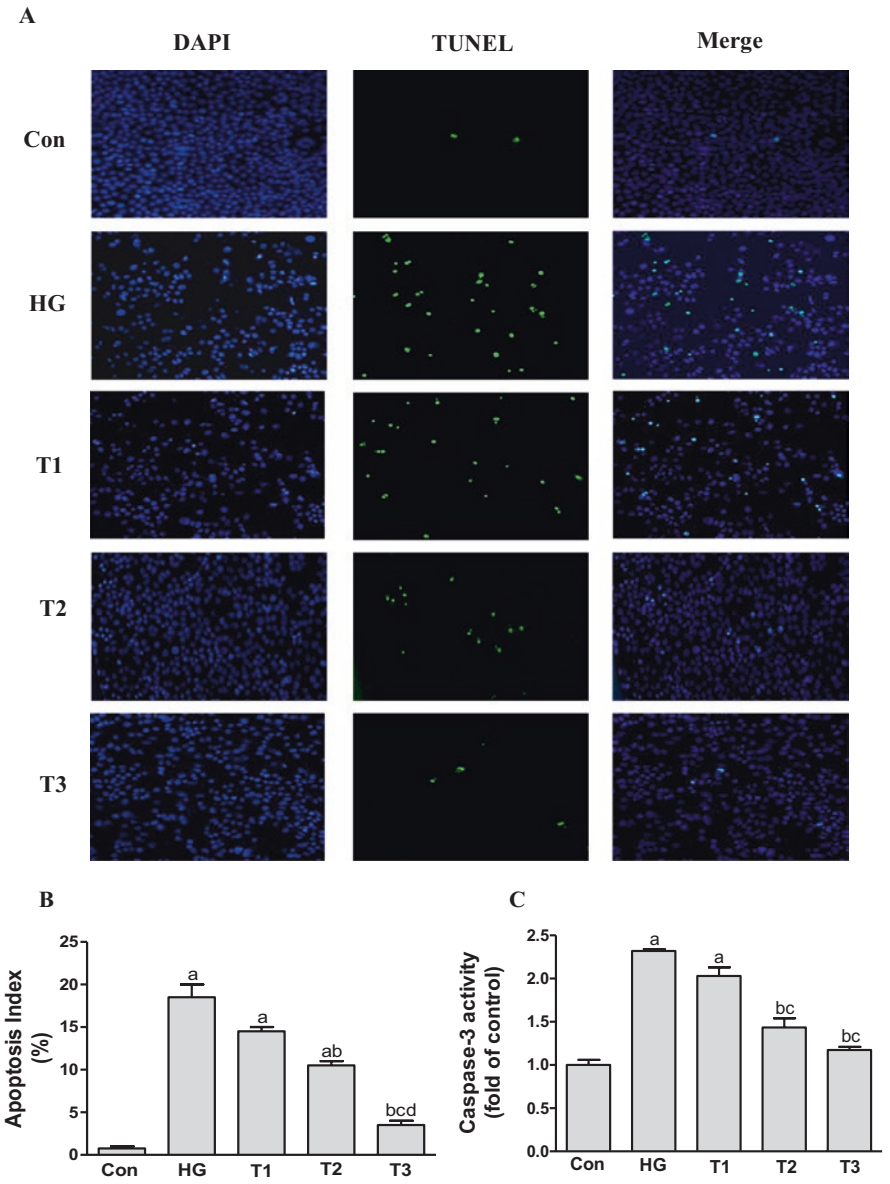

Fig. 2 The effect of taurine on apoptosis of HT-22 cells induced by HG. (**a**) Representative images of TUNEL stained cells are shown for the Con group, HG group and T1, T2 and T3 groups. (**b**) Quantify on data of the apoptotic cell. (**c**) Caspase-3 activity was detected in each group. Data are shown as the mean ± SD (n = 3 per group). Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the T1 group, $^{d}p < 0.05$ with respect to the T2 group

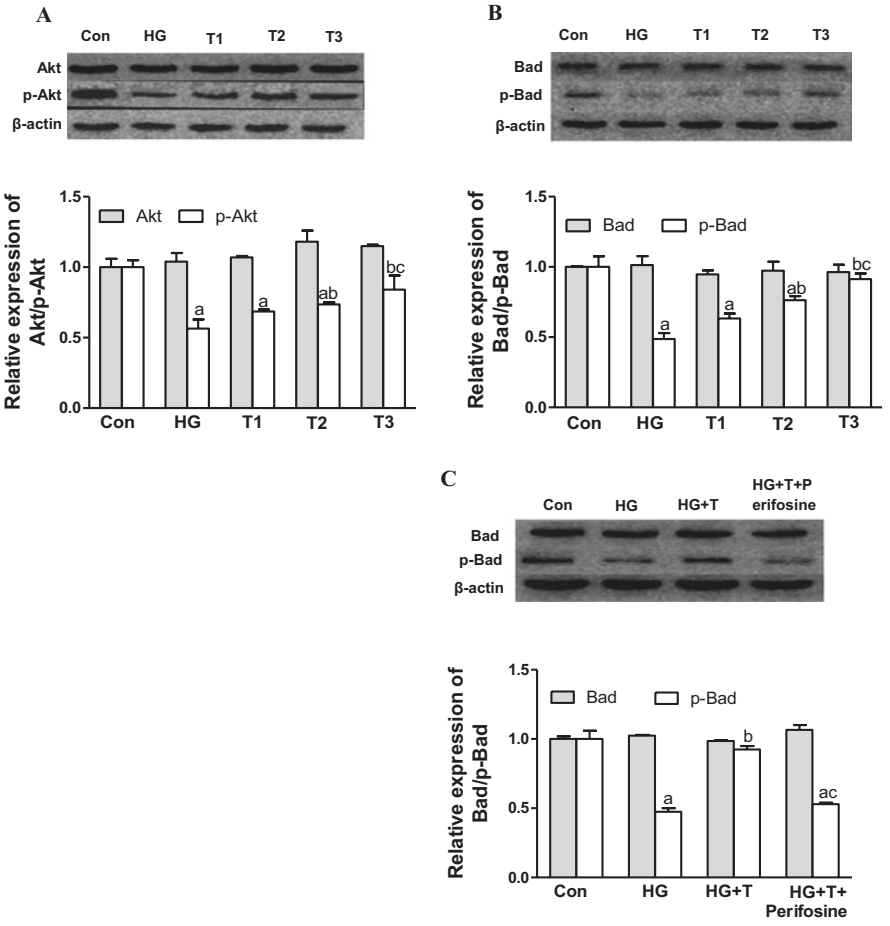

Fig. 3 The effect of taurine on expression of Akt/p-Akt (**a**), Bad/p-Bad (**b**). The expression of protein was analyzed by Western blot. The relative proteins were expressed by the ratio of sample to β-actin. Data obtained from three separated analyses are expressed as mean ± SD (n = 3 per group). Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the T1 group. The effect of taurine on expression of Bad/p-Bad of HT-22 cells treated with Perifosine (**c**). Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the HG + T group

of Bcl-2 and Bax were measured by western blot (Fig. 4a, b). Results showed that the expression of Bcl-2 decreased in HG group but the level of Bcl-2 was increased in T2, T3 groups compared to the HG group ($p < 0.05$). The expression of Bax showed the reverse condition. The ratio of Bax/Bcl-2 was showed in Fig. 3c, the ratio was significantly increased in HG group compared with Con group, the ratio of Bax/Bcl-2 was decreased in T2 and T3 groups compared to the HG group. The level of Bcl-2, Bax and the ratio of Bax/Bcl-2 treated with Perifosine was showed

as Fig. 4d–f. Compared to the Con group, the expression of Bcl-2 was decreased but Bax was increased in HG groups ($p < 0.05$), the expression of Bcl-2 was decreased but Bax was increased in HG/taurine co-treated HT-22 cells in the precence of Perifosine. These results revealed that taurine up-regulated the expression of Bcl-2 and down-regulated the expression of Bax in HG-induced HT-22 cells by Akt/Bad pathway.

3.5 Inhibition of Akt Abolishes the Anti-apoptotic Effects of Taurine

The central role of Akt signaling in the anti-apoptotic effect of taurine prompted us to test whether inhibition of Akt was capable of blocking taurine-enhanced cell survival. The TUNEL assay and caspase-3 activity were tested with or without Perifosine. The results showed that the number of apoptotic cell have significantly increased in HG group ($p < 0.05$) as compared to Con and HG + T groups (Fig. 5a, b), compared with the HG + T group, the number of apoptotic cell has increased in HG + T + Perifosine group. The caspase-3 activity showed the same condition as the apoptosis index (Fig. 5c). It was indicated that the anti-apoptotic effects of taurine on HG-induced HT-22 cells were mediated by Akt/Bad pathway.

4 Discussion

Some evidences indicate that diabetes causes learning and memory deficits (McCrimmon et al. 2012). The hippocampus, which plays a critical role in cognitive function, it had been found that there were structural changes according to some imaging studies in the diabetic patients (Verdile et al. 2015). Moreover, it was shown that apoptosis occurs in hippocampal cells in diabetes which is associated with memory impairment (Li et al. 2002). It prompted us that anti-apoptosis may ameliorate the cognitive impairment caused by diabetes. On the other hand, taurine improved memory in diabetic rats (Rahmeier et al. 2016). So we presumed that taurine may play a role of ameliorating cognitive deficits through controlling apoptosis of hippocampus neuronal cells in diabetic rats. In current study, we tested the cell viability and apoptosis by MTT, TUNEL assay and caspase-3 activity kit. The results showed that administration of HG decreased cell survival rate and increased apoptosis drastically as compared to control same like (Hajializadeh et al. 2014), but addition of taurine successfully checked this decline, improved cell survival and decreased apoptosis rate in a dose dependent manner. It was also reported that taurine reduced apoptosis in PC12 exposed to HG in vitro (Maher et al. 2005), being in accordance with our study. These results indicate that taurine protects against HG-induced apoptosis in HT-22 cells.

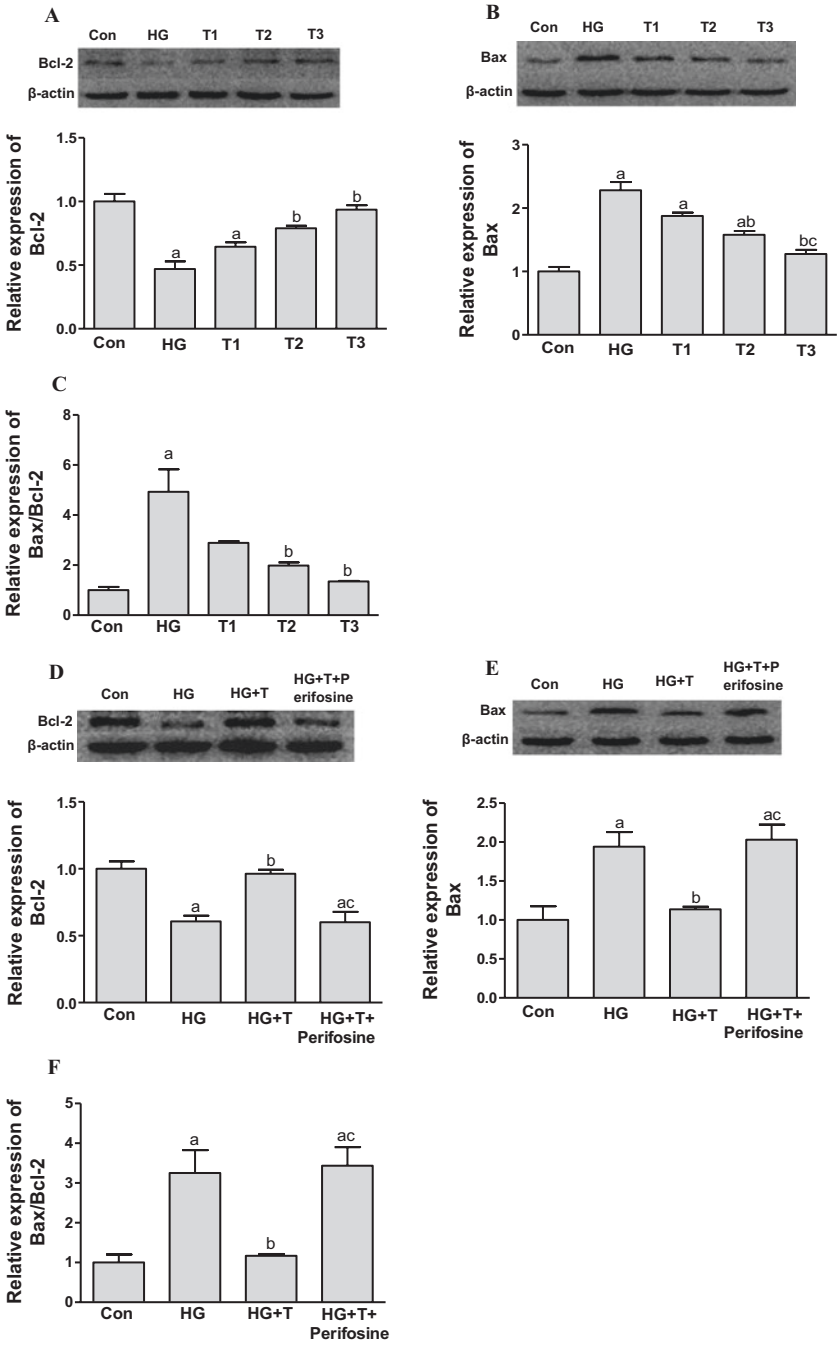

Fig. 4 The effect of taurine on expression of Bcl-2 (**a**), Bax (**b**) and ratio of Bax/Bcl-2 (**c**) of HT-22 cells treated with HG. The relative proteins were expressed by the ratio of sample to β-actin.

Akt is a regulator of cell processes and cell survival (Srivastava et al. 2008) and can regulate cell apoptosis by activating a series of downstream signaling molecules. The studies showed that the phosphorylation of Akt inhibited apoptosis and the inhibition of Akt phosphorylation induced apoptosis (Takatani et al. 2004). To investigate whether the anti-apoptotic effect of taurine is associated with phosphorylation of Akt, the expression levels of Akt and p-Akt were measured by Western blot assay. HG significantly decreased the expression of p-Akt in HT-22. However, taurine recovered this HG induced down-regulation of p-Akt. Takatani et al. reported that treatment of taurine significantly reversed decrease in phosphorylation level of Akt in cardiac myocyte cells taken from ischemic rats (Takatani et al. 2004). Our results indicate that taurine activates Akt in HT-22 cells exposed to HG. Bad is a member of Bcl-2 proteins. It is a downstream mediator of p-Akt and directly interacts with Bcl-2, forms heterodimers and concomitantly generates Bax homodimers, thus inducing cell death (Datta et al. 1997). Akt phosphorylates Bad in vitro and in vivo (Datta et al. 1997). Phosphorylation of Bad (p-Bad) is suspected to improve cell survival (Steenbergen et al. 2003). The present study showed that Bad was not significantly different among the groups. However, Taurine improved p-Bad in HG indicating phosphorylation of Bad. Thus, in our study, the level of p-Akt was improved by taurine which in turn improved phosphorylation of Bad. This indicates that Akt/Bad signaling pathway may be involved in apoptosis in HT-22 cells induced by HG.

Survival and turnover of the different body cells is based on a critical balance between anti-apoptotic and pro-apoptotic regulators. Disease is caused by disturbance of this balance. Bcl-2 family members are the key factor in apoptosis signals especially Bcl-2 and Bax. These are mostly represented members having structure similarity but have opposite function. The balance between them directly decides the fate of the cells accepting death message. In addition, up-regulation of several anti-apoptotic members of the Bcl-2 family proteins, Bcl-2 is such a protein, has been strongly associated with increased resistance to apoptosis (Garchon et al. 1994; Hanke 2000). It was found that taurine improved Bcl-2 expression in diabetic rats (Das and Sil 2012). It was found that Bax expression increased in PC-12 cells treated with HG and mild reduction was seen for Bcl-2 spinal cord of diabetic rats (Kaeidi et al. 2013). Similar results were found in our study, HG down-regulated Bcl-2 and up-regulated Bax in HT-22 cells. However, taurine reduced expression of Bax and increased expression of Bcl-2 according to dose. These findings indicated that taurine reduced apoptosis in HT-22 cells exposed to HG may be by improving Bax and Bcl-2 balance.

Fig. 4 (continued) Data obtained from three separate analyses are expressed as mean ± SD (n = 3 per group). Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the T1 group. The effect of taurine on expression of Bcl-2 (**d**), Bax (**e**) and ratio of Bax/Bcl-2 (**f**) of HT-22 cells treated with Perifosine. Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the HG + T group

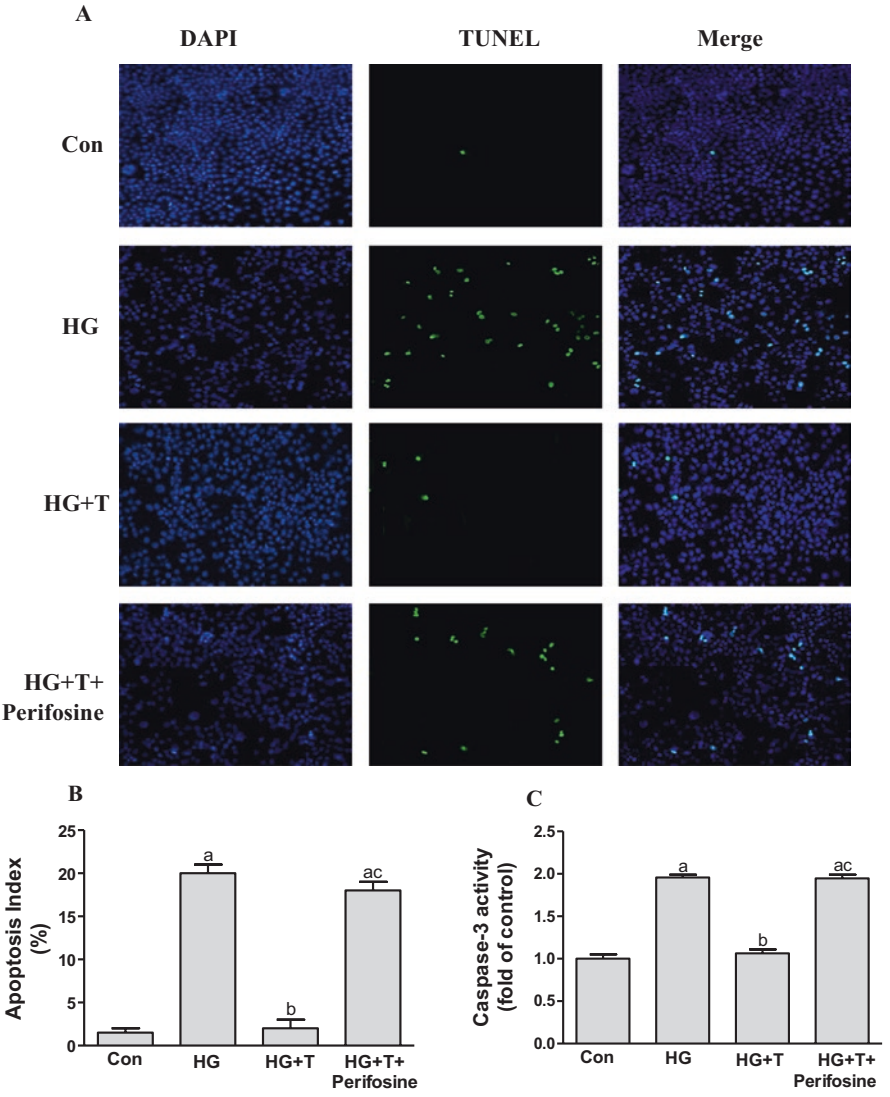

Fig. 5 The effect of taurine on apoptosis in HT-22 cells treated with Perifosine. (**a**) Representative images of TUNEL stained cells are shown for the Con group, HG group and HG + T groups and HG + T + Perifosine. (**b**) Quantify on data of the apoptotic cells. (**c**) Caspase-3 activity. All data obtained from three separate analyses are expressed as mean ± SD (n = 3 per group). Significant statistical difference was indicated by $^{a}p < 0.05$ with respect to the Con group, $^{b}p < 0.05$ with respect to the HG group, $^{c}p < 0.05$ with respect to the HG + T group

In order to confirm this assumption that taurine involved Akt/Bad pathway in its activity, Akt inhibitor (perifosine) was used (Kondapaka et al. 2003). We found that taurine could not perform well in presence of perifosine. Taurine improved p-Bad expression in HT-22 cells under HG condition but in presence of perifosine, it did not give good results. Similarly, taurine increased Bcl-2 expression and reduced Bax expression under HG but in presence of perifosine, taurine had not so good effect. Similarly, TUNEL assay showed that taurine decreased apoptosis in HT-22 cells exposed to HG however, perifosine blocked anti-apoptotic activity of taurine and in this case apoptosis was not ameliorated so better as without perifosine. This result confirmed our assumption that taurine ameliorated apoptosis in HT-22 cells exposed to HG via regulating Akt/Bad pathway.

Based on our results it can be concluded that taurine protected HT-22 cells against HG induced apoptosis. It can predict that taurine may have protective effect against apoptosis in hippocampus induced by hyperglycemia. However, further in vivo studies have to be conducted to confirm this prediction.

5 Conclusion

HG can increase apoptosis in HT-22 cells leading to death of the cells via apoptosis and taurine may control this apoptosis by modifying Akt/Bad pathway. Hence, taurine may be helpful in dealing with memory loss in diabetes.

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Taurine Partially Improves Abnormal Anxiety in Taurine-Deficient Mice

Eunkyue Park, Abdeslem Elidrissi, Georgia Schuller-Levis, and Kathryn K. Chadman

Abstract Taurine is abundant in various tissues including the brain, muscle, heart, spleen, liver and kidney with various physiological functions. Since taurine is produced by cysteine sulfinic acid decarboxylase (CSAD) in the liver and kidney, taurine-deficient mice without CSAD have been investigated for abnormal physiological functions such as retinal development, immune, pancreatic and liver function. In this study, the behavioral effects and abnormal brain development caused by low taurine in the developing brain were examined. In neonatal brains of homozygous CSAD knockout mice (HO), taurine was reduced by 85%, compared to wild-type mice (WT). Taurine was reduced by 35% in the brains of 2 month-old HO, compared to WT. Anxiety, motor coordination and autistic-like behaviors were evaluated at 2 months of age using five behavioral tests: elevated plus maze, open field, social approach, marble burying and accelerating rotarod. Mice were tested from 3 groups including WT, HO and HO with oral treatment of 0.2% taurine in the drinking water (HOT). HOT were born from HO dams treated with taurine from before pregnancy and were continuously treated with taurine in the drinking water after weaning. The taurine levels in the brain and plasma of HOT were restored to WT at 2 months of age. Taurine-deficiency did not lead to changes in autistic-like behaviors as the HO were not significantly different from WT in marble burying and social approach. However, taurine-deficiency increased anxiety-like behavior in HO in the elevated plus maze and open field, compared to WT. Taurine treatment significantly restored the HOT to WT levels of anxiety-like behavior in the elevated plus maze. However, changes in exploratory activity in the open field were not improved with taurine treatment. There was a slight difference in motor ability as the WT mice stayed on the accelerating rotarod longer that the HO and HOT, but the difference was significant in the HOT during the first trial only, compared to WT.

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These data support hypothesis that taurine is essential for the emotional development of the brain. First, taurine is remarkably low in the neonatal brain of HO, compared to the adult brain of HO. Second, taurine treatment in HO partially improves anxiety-like behavior to WT.

Keywords CSAD KO · Taurine · Behavior · Mice · Elevated plus maze · Open field · Social approach · Marble burying · Rotarod

Abbreviations

CSAD	cysteine sulfinic acid decarboxylase
CSAD KO	cysteine sulfinic acid decarboxylase knock-out mice
CDO	cysteine dioxygenase
CDO KO	cysteine dioxygenase knock-out mice
ADO	cysteamine (2-aminoethanethiol) dioxygenase
GADL 1	glutamate decarboxylase-like 1
GADL 1 KO	glutamate decarboxylase-like 1 knock-out mice
TauT	taurine transporter
TauT KO	taurine transporter knock-out mice
WT	wild-type mice (CSAD+/+)
HT	heterozygotic mice (CSAD+/−)
HO	homozygotic mice (CSAD−/−)
HOT	homozygotic mice treated with 0.2% taurine in the drinking water
ASD	autistic spectrum disorder

1 Introduction

Taurine is a sulfur-containing amino acid present in high concentrations in mammalian plasma and tissues. Taurine plays an vital role in various essential biological processes including development of the central nervous system (CNS) and the retina, glucose regulation, calcium modulation, anti-oxidant activity, membrane stabilization, reproduction, and immunity (Sturman 1993; Schuller-Levis and Park 2003, 2006; Schuller-Levis et al. 2009; Schaffer et al. 2009; Park et al. 2014, 2017b; Sidime et al. 2017). Taurine, not incorporated into proteins, is considered to be an essential amino acid for felines and a conditionally essential amino acid for humans and non-human primates. The level of rate-limiting enzymes required for biosynthesis, cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD), is very low in the cat and low in humans and primates (Huxtable 2000; Dela Rosa and Stipanuk 1985; Park et al. 2002; Stipanuk 2004).

The nutritional importance of taurine in human infants is based on the role of taurine in the brain development (Sturman 1993; Imaki et al. 1996; Jenness 1979). In the human placenta, taurine is the most abundant amino acid (100–200-fold that of maternal blood) with its concentration increasing significantly at the end of gestation, where it accumulates in the fetal brain (Roos et al. 2004; Aerts and Van Assche 2002; Lanza and Colombatto 2008). During pregnancy, taurine is released to the fetus via the placenta and is accumulated in the fetal and neonatal brain. For the fetus, taurine is an essential amino acid, derived from maternal circulation via the placenta. Low maternal levels of taurine result in low fetal taurine and can lead to growth retardation and impaired neuronal development (Liu et al. 2014, 2015; Wang et al. 2016). There is evidence that low levels of taurine in neonatal plasma is associated with low scores on the Bayley mental development index at both 18 months and 7 years of age (Wharton et al. 2004).

A study of transsulfuration biomarkers in people with autistic spectrum disorder (ASD) demonstrated significantly decreased plasma taurine levels relative to controls (Geier et al. 2009). Hallmarks of ASD include cerebellar migration abnormalities, Purkinje cell loss and elevation of glial fibrillary acidic protein (Ritvo et al. 1986; Pow et al. 2002; Laurence and Fatemi 2005). Taurine deficiency in the cat is associated with CNS dysfunction including delayed cerebellar cell division and migration in the external granule cell layer, loss of Purkinje cells, and abnormal cortical development (Xu et al. 1992; Lu et al. 1991). In the cat CNS, taurine is concentrated in Purkinje cells as well as glia (Sturman 1993).

It is not known if abnormal neuronal development from taurine-deficiency affects behavior. The previous behavioral studies including anxiety, stereotypical autistic behaviors, learning and memory as well as depression were investigated using taurine supplementation in rats and mice (Franconi et al. 2004; Kong et al. 2006; McCool and Chappell 2004; Chen et al. 2004; Elidrissi et al. 2009a; Neuwirth et al. 2013; Wu et al. 2017). However, the high intrinsic taurine levels found in rodents limit these studies. (Huxtable 2000). Therefore, a taurine-deficient mouse model lacking CSAD was developed (Park et al. 2014). Autistic-like and anxiety-like behaviors as well as motor coordination and balance were evaluated using the taurine deficient mouse to examine whether low taurine in the brain from neonates of HO (Fig. 1) contributes to abnormal brain development.

2 Materials and Methods

2.1 Materials

Chemicals used in this study were purchased from Sigma Chemicals (St. Louise, MO) if not otherwise noted. Taurine was generously provided by Dong-A Pharmaceutical Co (Seoul, Korea). Oligonucleotide primers for PCR were obtained from Eurofins MWG Operon (Huntsville, AL). Primers were designed using Primer

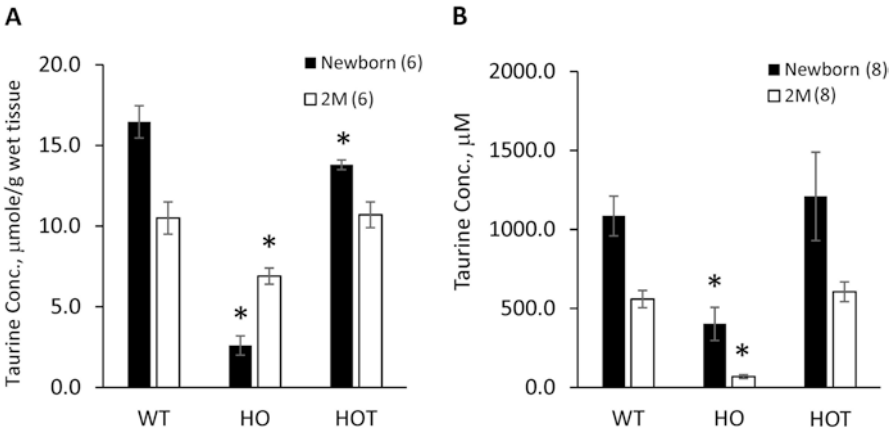

Fig. 1 Taurine concentrations in the brain (**a**) and plasma (**b**) from newborn and 2 month-old adult mice. Taurine was determined using HPLC in three genotypes. Eight mice per each group for plasma and six mice for the brain were used. ∗ Statistical significant difference from WT, $p < 0.05$

Designer 4 (Scientific and Educational Software, Cary, NC). Taq polymerase, deoxynucleotides and DNA standards were obtained from New England Biolabs (Ipswich, MA). Agarose was purchased from Denville Scientific (Metuchen, NJ).

2.2 CSAD KO Mice

Chimeric CSAD KO mice were produced as previously described (Park et al. 2014; Stanford et al. 2006). Briefly, the cells from a gene trap ES cell line (XP0392) were injected into C57BL/6 (B6) blastocysts at the Mouse Mutant Regional Resource Centers (MMRRC, UC Davis, CA) which were implanted into a pseudopregnant B6 mouse. Chimeric mice from MMRRC were mated with B6 (Jackson Laboratories, Bar Harbor, ME) in the animal colony at this institution (NYS Institute for Basic Research in Developmental Disabilities). Using PCR, genotyping of offspring was performed to identify wild-type (WT, CSAD+/+), heterozygotes (HT, CSAD+/−) and homozygotes (HO, CSAD−/−). Due to low reproduction performance, a hybrid strain was made by mating CSAD KO with B6 background and 129P3/J (Jackson Lab, Bar Harbor, ME) which is a similar strain to an original strain (129P2/OlaHsd) of ES cell line. Hybrid HO with the mixed background improved reproduction performance to approximately 80% (data not shown) and the second generation of homozygotes (G2 HO) were used in this study. Taurine levels in HO with the mixed background of B6 and 129P3/J were also confirmed to be low in taurine by HPLC,

Table 1 Sequence of PCR primers

	Sequence (5′–3′)
B-geo-F	TTATCGATGAGCGTGGTGGTTATGC
B-geo-R	GCGCGTACATCGGGCAAATAATATC
VIS-F	GGCCTTGCCACAGGAGATTA
VIS-R	ACCAACCAGAGCTGGAGACA

similar to taurine levels in HO with the background of C57BL/6J. All mice including WT, HT and HO treated with 0.2% taurine in the drinking water (HOT) were fed taurine-free chow (LabDietR, PMI Nutrition International, St. Louis, MO). HOT were HO treated with 0.2% taurine in the drinking water. HOT mice were born from HO dams treated with taurine prior to pregnancy and until weaning. After weaning, HOT were continuously treated with taurine, resulting in these mice maintaining taurine levels similar to WT (Park et al. 2014, 2015, 2017a). All mice were kept under 12-h day/night with free access to food and water. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee at IBR.

2.3 Genotype Determination

Genotyping was done with a modified method previously described (Park et al. 2014). Briefly, template DNA was extracted from a tail and a toe digit samples at 3 weeks of age with DirectPCR (Viogen, Los Angeles, CA) and analyzed with two sets of primers, β-geo and VIS (Table 1), using a PCR kit (New England Biolabs). After an initial 94 °C for 2 min, DNA was denatured at 94 °C for 30 s, annealed at 60 °C for 30 s and extended at 68 ° C for one min for 30 cycles. DNA was extended for an additional 5 min at 68 °C. PCR products were characterized by 1.0% agarose gel electrophoresis. The sizes of PCR products using β-geo and VIS primers were 682 bp and 354 bp, respectively. DNA from CSAD−/− homozygote (HO) and CSAD+/+ wild type (WT) produced one band from either β-geo or VIS, respectively, and CSAD+/− produced bands from both β-geo and VIS.

2.4 High Performance Liquid Chromatography (HPLC)

The levels of taurine were determined using HPLC (Waters, Milford, MA) previously described (Park et al. 2014). Taurine concentrations were determined by comparison to a standard of taurine.

2.5 Behavior Tests

Mice were brought to the testing room at least 1 h before the experiments began to habituate. Data were pooled as an average from no more than two females and two males per litter if mice were available.

2.6 Elevated Plus Maze

Anxiety-like behavior was tested in elevated plus maze as previously described (Chadman 2011; Anchan et al. 2014). The elevated plus maze apparatus consisted of four arms including two open arms and two closed arms at 90o angles to each other with all arm platforms elevated 40 cm from the floor. At the start of a trial, the mouse at the age of 8 weeks was placed in the center with its nose directed toward the an open arm and allowed to explore the maze freely for 5 min. The total time spent, and entries into each arm were digitally recorded analyzed using a video tracking software (ANY-maze, Stoelting Inc. Wood Dale, IL). The number of mice in each group are: WT n = 21, HO n = 21 and HOT n = 21.

2.7 Open Field

General exploratory locomotion in a novel environment was assayed in open field as described previously (Barua et al. 2014; Anchan et al. 2014). Mice at the age of 8 weeks were placed in an open field for a 15-min test session. The open field apparatus was made of a transparent plexiglass apparatus (40 cm × 40 cm × 30.5 cm) with a grey metal floor. A camera was positioned above the apparatus to record the mice, and the videos were scored using a video tracking software (ANY-maze). The testing room was illuminated to 20 lux and kept at a similar temperature as the colony room. Total distance traveled, number of center zone entries and time spent in the center zone were analyzed for 5 min. The data represent WT n = 21, HO n = 21 and HOT n = 21.

2.8 Social Approach

Social approach was measured as described previously (Barua et al. 2014). Social approach behaviors were tested in an apparatus with three chambers in a single session, divided into three 10 min phases. Inverted wire cups were used either to hold a stranger mouse, or to serve as an the novel object. Side chambers were maintained at a lighting level of 20 lux with two desk lamps angled away from the maze. Mice at the age of 8 weeks were placed in the center chamber for 10 min during the first

phase, and then freely moved through doors in all three chambers for 10 min as a second habituation stage. During the third stage, the sociability trial, a stranger mouse (129Sv/ImJ from Jackson Laboratory, Bar Harbor, ME) was held in wire cup placed in the stranger chamber of the apparatus. Test mice were free to moved between the center chamber, object chamber and stranger chamber. A camera was positioned above the apparatus to record the mice, and the videos were scored using a video tracking software (ANY-maze). The sniffing time and time spent in the stranger, center and novel object chambers were determined in three groups including WT n = 21, HO n = 21 and HOT n = 21.

2.9 Marble Burying

Mice at the age of 9 weeks were placed individually in housing cages containing 20 black marbles arranged in a grid on top of 5 cm cobb bedding for 30 min (Weidner et al. 2014). After returning mice to the home cages, the number of buried marbles (more than 2/3) were counted and photographed. The data represent WT n = 21, HO n = 21 and HOT n = 20.

2.10 Accelerating Rotarod

Rotarod was performed as described previously (Chadman et al. 2008). Mice at age of 9 weeks were placed on a rod that accelerated from 4 to 40 rotation per minute over a period of 5 min. The length of time that each animal was able to stay on the rod was recorded as the latency to fall. The data represent mice including WT n = 24, HO n = 24 and HOT n = 23.

2.11 Statistical Analysis

Data are represented as mean + standard error (SE). Statistical significance was determined using Statistica 13 (Dell Inc, Tulsa, OK). For taurine concentrations, significant differences between groups were determined as $p < 0.05$ using LSD and/or Tukey HSD in post-hoc in one way ANOVA. For social approach, repeated measures analysis of variance (ANOVA) was used to compare the time spent in the outer chambers and spent sniffing the stranger mouse or novel object. LSD post-hoc analysis in ANOVA was run for determining the group difference in the repeated measure (stranger mouse or novel object). All other experiments were analyzed using factorial ANOVA, with LSD and/or Tukey HSD post-hoc tests for determining the group difference. Significant differences between groups were determined as $p < 0.05$.

3 Results

3.1 Taurine Levels in the Brain and Plasma from Newborn and 2 Month-Old Adult Mice

Taurine concentrations in the brain of HO were different by developmental stages, with HO newborns having a bigger decrease in taurine levels, compared with WT than 2 month old HO. (Fig. 1a). Specifically, the taurine levels in the brain of 2 month-old HO were decreased by 35%, compared to WT ($p < 0.001$), while taurine levels in the brain of newborn HO were decreased by 85%, compared to WT ($p < 0.001$). Taurine levels in the brain from newborn HOT were also significantly lower by 16% than newborn WT although taurine levels in the brain of newborn HOT were increased ($p < 0.05$). Taurine levels in the brain from 2 M HOT is restored to WT levels.

Newborn HO has 63% lower plasma taurine concentrations, compared to newborn WT ($p < 0.05$) (Fig. 1b). However, By 2 months of age, plasma taurine concentrations were lower in HO by 87%, compared to WT($p < 0.001$). Taurine in the drinking water restored plasma taurine levels to WT in both newborn and 2 month-old HOT.

3.2 Behavior Tests

3.2.1 Elevated Plus Maze

Elevated plus maze was used to examine an anxiety-like behavior. Numbers of open arm entries and the time spent in in the open arms were analyzed between three groups, WT, HO and HOT (Fig. 2a, b, respectively). Both parameters were significantly decreased in HO, compared to WT ($p < 0.05$) and recovered to WT when HO were treated with 0.2% taurine in the drinking water. The overall genotype in both parameters were significant, $F(4,112) = 6.5488$, $p < 0.001$. However, the overall effect of sex was not significant in both time spent in open arm and entries to open arms, $F(2,56) = 0.04$, $p < 0.96$. The HO spent less time and made fewer entries into the open arms, indicating higher anxiety-like behavior. The HO treated with 0.2% taurine in the drinking water recovered to WT levels of open arm time and entries.

3.2.2 Open Field

The mice were placed in an open field test to measure general exploratory behavior and anxiety-like behavior in the three groups. Total distance traveled, number of center zone entries and time spent in the center zone were analyzed (Fig. 3a–c). The overall effect of genotype in all three parameters was significant, $F(6,110) = 13.038$, $p < 0.00001$ but the overall effect of sex was not significant, $F(3,55) = 0.454$, $p < 0.72$.

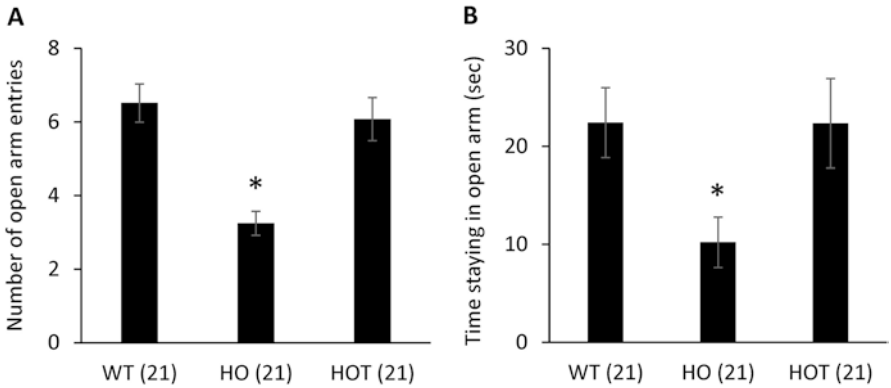

Fig. 2 Elevated plus maze: (**a**) The WT made significantly more entries into the open arms than the HO group but not the HOT. (**b**) The WT spent significantly more time in the open arm than the HO group but not the HOT. N = 21. ∗ Statistical significance is indicated by $p < 0.05$, compared to WT

The HO traveled a shorter distance, made lower entries to the center zone and spent less time in the center zone ($p < 0.05$). Taurine treatment in the water (HOT) did not affect these behaviors ($p < 0.05$).

3.2.3 Social Approach

To examine autistic-like behavior in the taurine-deficient mice, the mice were tested in a three-chambered social approach apparatus where the mice had a choice between a stranger mouse and a novel object. The time spent sniffing to a stranger mouse vs an novel object and time spent in the chambers were analyzed. The sniffing time in HO was no significantly different from WT and HOT (Fig. 4). Mice in all three groups also spent longer times in a stranger chamber, compared to center and object chambers. The overall effect of genotype and the overall effect of sex were not significant, $F(4, 112) = 1.0116$, $p < 0.4046$ and $F(2,56) = 2.5672$, $p < 0.0858$, respectively.

3.2.4 Marble Buyring

Marble burying is a way to measure typical repetitive digging in the mice, similar to the repetitive behaviors which is symptomatic of autism. The number of buried marbles were counted after 30 min. The number of buried marbles did not differ significantly between the three groups, $F(2,56) = 0.64$, NS) (Fig. 5).

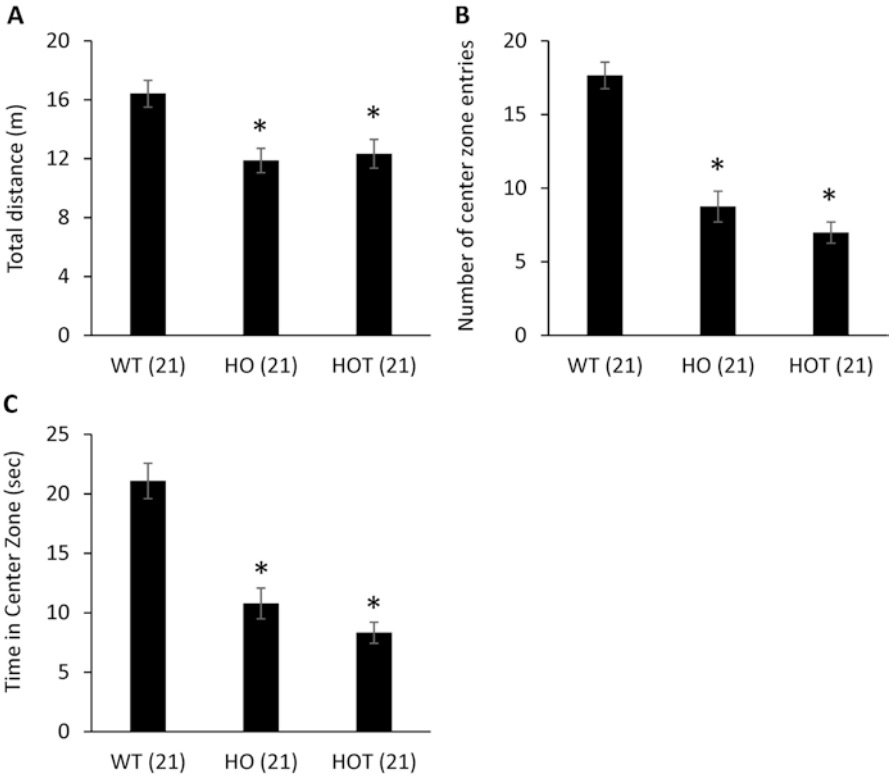

Fig. 3 Open field: (**a**) The total traveled distance in a transparent plexiglass box was significantly lower in the HO and HOT groups, compared to WT. (**b**) The number of center zone entries was significantly lower in the HO and HOT groups. (**c**) The time in center zone was significantly lower in the HO and HOT groups, compared to WT. Twenty-one mice were used in each of the three groups. ∗ Statistical significance is indicated by $p < 0.05$, compared to WT

3.2.5 Rotarod

To examine motor coordination and balance, mice were placed on a accelerating rotarod for 5 min per trial, for 3 trials. Speed and latency to fall were measured. in each session. HO and HOT demonstrated lower motor coordination than WT overall, and HOT showed significantly lower during the first session only. ($p < 0.05$) (Fig. 6). There was no overall effect of genotype, $F(6,126) = .1.364$, NS) or sex, $F(3,63) = 1.126$, NS).

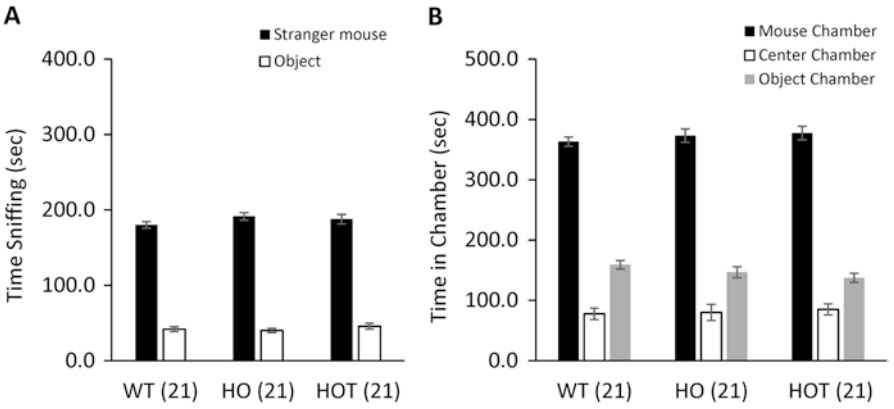

Fig. 4 Social approach. (**a**) Sniff time: All three groups spent more time sniffining the stranger mouse than the novel object, showing no difference in sociability, (**b**) Chamber time: All three groups showed a preference for the stranger mouse chamber over the novel object chamber. n = 21 in all groups

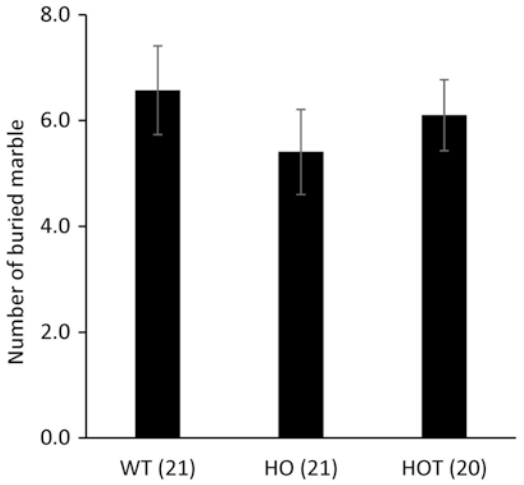

Fig. 5 Marble burying. The number of marbles buried did not significantly differ between the groups, indicating no repetitive digging behavior. WT and HO had n = 21 and HOT had n = 20

4 Discussion

Since taurine is a critical amino acid for neuronal development (Sturman 1993; Aerts and Van Assche 2002; Geier et al. 2009; Liu et al. 2015), the taurine-deficient mice developed in our laboratory were examined for brain development and taurine levels. Behavioral indicative of abnormal brain development include anxiety, ASD and/or motor coordination. These behaviors were evaluated using the behavioral tests including elevated plus maze, open field, social approach, marble burying and accelerating rotarod.

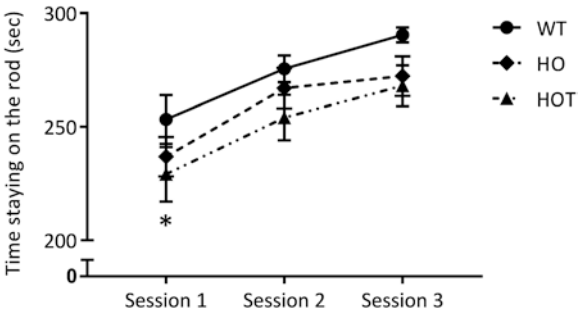

Fig. 6 Accelerating rotarod. HO and HOT demonstrated lower motor coordination than WT overall, and significantly lower for HOT in the 1st session only, comparted to WT. ∗ Statistical significance is indicated by $p < 0.05$). WT n = 24, HO n = 24, and HOT n = 23 in three sessions

The levels of taurine in the brain and plasma were measured and differed by both age and genotype. Newborn HO have little taurine in the brain, compared to newborn WT (Fig. 1a). The 2 month-old adult mice, used here for behavior tests, have a substantial amount of taurine in the brain. The difference in brain levels of taurine between the HO and WT varied by age, with the newborn HO having a remarkable 85% reduction, compared to newborn WT, while 2 month-old HO was reduced by a more modest 35%. The plasma levels of taurine showed the opposite pattern. Plasma taurine in the newborn HO was reduced by 67% compared to newborn WT, whereas plasma taurine in 2 month-old HO was reduced by 87% compared to 2 month-old WT. In addition, taurine levels of the brain in neonatal HOT were significantly lower than neonatal WT although plasma taurine concentrations in neonatal HOT were restored to neonatal WT. These data indicate that taurine in plasma may not be transported to the brain in neonatal HO, possibly due to low levels of the taurine transporter (TAUT). Taurine is transported to the brain by the TAUT, which is present in the blood-brain barrier. TAUT is expressed highly in the cerebellum and hippocampus (Pow et al. 2002; Kang et al. 2002). TAUT is expressed in both Purkinje neurons of the cerebellum and neurons in the hippocampus, suggesting that the supply of taurine is vital to their function (Sturman 1993). Plasma taurine is mainly produced in the liver and kidney (Stipanuk 2004). Very little taurine in the liver was found in both newborn and 2 month-old HO as described previously (Park et al. 2014, 2017a).

Another possibility is that low levels of cysteamine dioxygenase (ADO), an alternative biosynthetic enzyme for taurine, may also contribute to low taurine levels in the newborn HO (Dominy et al. 2007), whereas previous work using RT-qPCR in the brains of 2 month-old HO has shown that there is higher expression of TauT and Ado, compared to 2 month-old WT (Park et al. 2017a). These previous studies suggest that higher taurine levels may be explained by greater taurine transport to the brain and more biosynthesis of taurine in the brain from 2 month-old HO.

Several groups have previously demonstrated that taurine supplementation in rodents modifies hippocampal function. Improved hippocampal function is thought to underlie increased learning and memory (Franconi et al. 2004). Taurine improves

anxiety-like behavior and social interaction (Kong et al. 2006; Elidrissi et al. 2009a), and reduces anxiety by activating the strychnine-sensitive glycine receptor (Zhang and Kim 2007). Taurine has been shown to modulate amygdala-associated anxiety-like behavior through strychnine-sensitive glycine receptor in the basal anxiety state (McCool and Chappell 2004). Both studies indicate that the anxiolytic effects of taurine are mediated through strychnine-sensitive glycine receptor. In this study, CSAD KO, a taurine-deficient mouse, was tested for changes in anxiety-like behavior due to taurine deficiency in fetal and neonatal brain. Anxiety-like behavior in elevated plus maze were increased in the HO (Fig. 2a, b), but were restored to WT levels with administration of 0.2% taurine in the drinking water. This indicates that anxiety-like behavior evaluated by the elevated plus maze may be caused by taurine-deficiency. In the open field test, total distance traveled, entries into the center zone and time in the center zone were less in HO than WT (Fig. 3a–c). However, these activities were not restored with oral taurine treatment. This observation supports that anxiety-like behavior measured by the open field may be due to the deletion of CSAD instead of taurine deficiency. Since a microarray study showed that various genes in the newborn brain from CSAD KO are modulated as described previously (Park et al. 2014), these data indicate that anxiety-like behavior measured with elevated plus maze and open field may be more complicated in their mechanism(s) of action. Future experiments will examine the role of the strychnine-sensitive glycine receptor in the amygdala as a possible mechanism underlying the improvement of anxiety-like behavior with taurine.

Taurine concentrations in children with ASD have been determined in several laboratories. However, the reports are controversial, with some studies showing higher taurine concentrations (Moreno et al. 1992; Moreno-Fuenmayer et al. 1996; Tu et al. 2012), same (Aldred et al. 2003; Arnold et al. 2003) or lower (Geier et al. 2009; Ghanizadeh 2013) in people with ASD, compared to controls. Transsulfuration biomarkers including plasma cysteine, reduced glutathione (GSH), oxidized glutathione (GSSG), total sulfate and free sulfate as well as plasma taurine were decreased in children with ASD, compared to controls(Geier et al. 2009). The data show a 50% reduction in plasma taurine in children with ASD, compared to neurotypical controls. This doesn't seem to be sex-related as there was no significant difference in taurine levels between female and male children with ASD, compared to control siblings (Park et al. 2017). In contrast to these mixed results from human studies, taurine-deficiency in the pregnant cat is more clearly associated with dysfunction of the CNS. The offspring born from taurine-deficient cats show delayed cerebellar cell division and migration in the external granule cell layer, loss of Purkinje cells, astrogliosis, and abnormal cortical development (Sturman 1993). In the CNS, taurine is concentrated in Purkinje cells as well as glia (Lu et al. 1991; Xu et al. 1992). Consistent neurological abnormalities found in people with ASD include marked Purkinje cell loss in the cerebellum and astrogliosis (Ritvo et al. 1986; Laurence and Fatemi 2005). Although it is of great interest that daily supplementation of taurine corrected this condition in the cat (Sturman 1993; Lu et al. 1991), it is difficult to behaviorally assess taurine-deficient cat. Therefore, taurine-deficient mice were tested for ASD-like behaviors, social interaction (Fig. 4a, b) and marble burying

(Fig. 5) as well as motor coordination and balance measured with rotarod (Fig. 6). Taurine deficiency did not affect social interaction, repetitive behavior or motor coordination and balance. These data indicate that taurine-deficiency does not affect behaviors associated with ASD or motor coordination.

Taurine improves cognitive function in a mouse model of fragile X syndrome, characterized by hyperarousal, hypersensitivity to sensory stimuli and an increased prevalence of seizures Elidrissi et al. 2009b). Taurine-supplementation in fragile X mice induced a significant increase in acquisition and retention of a hippocampally -dependent memory task. The taurine enhancing effects are mediated through interaction with the GABAergic system (Elidrissi and L'Amoreaux 2008; Elidrissi et al. 2009a, b). Future studies will examine the effect of taurine on cognitive function in CSAD KO to confirm there is modification of the GABAergic system when there is taurine-deficiency in the fetal and newborn brain.

Although several knockout mice including two TAUT KO, CDO KO and GADL-1 KO are taurine-deficient, these knock-out mice have the limitations. For example, taurine can not enter the brains of TAUT KO because of lack of the TAUT, so it is not possible to rescue the effects of taurine- deficiency (Warsulat et al. 2007; Ito et al. 2008). The HO of CDO KO produces infertile males (Ueki et al. 2011; Asano et al. 2018), making this model not appropriate to investigate all aspects of the role of taurine in HO. GADL-1 KO have less taurine-deficiency because CSAD is intact in this model (Winge et al. 2015). In contrast to these models, CSAD KO allows testing of both taurine deficiency and rescue with taurine supplementation to investigate the mechanism(s) of action at the molecular and behavioral levels.

5 Conclusion

These data indicate that taurine may not be involved in ASD-specific behavior and motor coordination. However, these data show that taurine is important for neuronal development of the brain and affects anxiety-like behaviors. This was shown with the low level of taurine in the newborn brain of HO, compared to the adult brain of HO, and that taurine treatment partially improves anxiety-like behavior. Taurine treatment may be beneficial to women prior to and during pregnancy for intervention of abnormal neuronal development.

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Taurine Promotes Neuritic Growth of Dorsal Root Ganglion Cells Exposed to High Glucose in Vitro

Mengren Zhang, Inam-u-llah, Xiaoxia Shi, Pingan Wu, Kaixin Li, Raheel Suleman, Rana Muhammad Aadil, Muhammad Zubair Saleem, and Fengyuan Piao

Abstract Diabetic neuropathy (DN) is the most common chronic complication of DM and its major pathological changes show axonal dysfunction, atrophy and loss. However, there are few reports that taurine promotes neurite growth of dorsal root ganglion (DRG) cells. In current study, DRG neurons were exposed to high glucose (HG) with or without taurine. The neurite outgrowth of DRG neurons was observed by fluorescent immunohistochemistry method. Expression of Gap-43, Akt, phosphorylated Akt, mTOR and phosphorylated mTOR was determined by Western blot assay. Our results showed that HG significantly decreased the neurite outgrowth and expression of Gap-43 in DRG neurons. Moreover, phosphorylated levels of Akt and mTOR were downregulated in DRG neurons exposed to HG. On the contrary, taurine supplementation significantly reversed the decreased neurite outgrowth and Gap-43 expression, and the downregulated phosphorylated levels of Akt and mTOR. However, the protective effects of taurine were blocked in the presence of PI3K antagonists LY294002 or Akt antagonists Perifosine. These results indicate that taurine promotes neurite outgrowth of DRG neurons exposed to HG via activating Akt/mTOR signal pathway.

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Keywords High glucose · Diabetic neuropathy · Taurine · Dorsal root ganglion cells · Neurite outgrowth

Abbreviations

DRG	Dorsal root ganglion
HG	High glucose
T	Taurine

1 Introduction

Diabetes mellitus (DM) designates various metabolic disorders identified by elevated blood sugar level for an extended period of time as the pathology proceeds (American Diabetes Association 2014). DM is categorized into two major classes including Type 1 (insulin-dependent DM) and type 2 (non-insulin-dependent DM) (Sirdah 2015). The hyperglycemic condition of diabetes gives rise to increased risk of several complications that involve nephropathy, retinopathy, and neuropathy (Sarkar et al. 2017). Diabetic neuropathy (DN) is the most serious long-term complication of DM and its major pathological changes show axonal dysfunction, atrophy, and loss. (Inam-u-llah et al. 2018). DN causes diverse clinical manifestations such as sensory loss, pain and increase the risk of foot ulcers and amputation 5–7 (Kobayashi and Zochodne et al. 2018). About 30% of the diabetic patients have neuropathy while 50% of the patients are expected to develop neuropathy during disease (Asieh Hosseini and Abdollahi 2013), which is associated with considerable mortality and decline in life standard (Bril 2012). Although there are important symptomatic options in pain management, but the treatment that can unequivocally arrest or reverse progressive neuropathy is not still available beyond strict control of glucose levels. Therefore, promoting axonal regeneration and repair may be a good strategy for treating diabetic neuropathy.

Taurine, 2-aminoethylsulphonic acid, is a non-protein amino acid which is present in approximately all animal tissues and most abundant as a free amino acid in human cells (Kim et al. 2012). Especially, Taurine is one of the highly abundant free amino acids in the nervous system and is vital for normal nervous system development and growth. Taurine is considered to exhibit multiple functions, including: osmoregulation, modulation of calcium fluxes, normalization of the electroretinogram, modifying protein phosphorylation, stabilization of cell membranes, antioxidant properties, favoring of migration of cell in brain and retina (Huxtable 1989; Lima 1999). Many studies have revealed the therapeutic effect of taurine and its products against diabetes mellitus (Sarkar et al. 2017). Taurine enhanced glucagon activity, favored glycemic stability, improved glucose levels, addressed hyperglycemia and improved insulin secretion, and insulin resistance. It has also a protective

effect against DN (Inam-u-llah et al. 2018). It was shown that taurine administration reverses deficits of hind limb and improves nerve conduction velocity sciatic motor, threshold in sensory nerves and blood flow in nerves Zucker diabetic fatty rats (Li et al. 2006). It is also known to have neurotrophic actions. It promotes repairing of nerve and regeneration (Oja and Kontro 1990). It was exhibited previously that taurine increased extensions from goldfish ganglions in retina after mild type of crush in optic nerve (Lima et al. 1988) and increased the number of extensions in separated cultured retinal cells (Matus et al. 1997). However, there is small number of reports about that discuss about axonal regeneration by taurine in DN.

In the present study, DRG or dorsal root ganglions cells were subjected to high glucose (HG) with or without taurine. The neurite extensions from DRG neuron was observed by immunofluorescent staining of SMI312. Expression of Gap-43, Akt, phosphorylated Akt, mTOR and phosphorylated mTOR were checked by Western blot assay. Moreover, these parameters were also examined with PI3K antagonists LY294002 or Akt antagonists Perifosine. The results show that taurine promotes neurite development from DRG neuron exposed to HG in vitro via activating Akt/mTOR signal pathway. These results may provide a new therapeutic approach for diabetic neuropathy and a novel insight for explaining protective mechanism of taurine.

2 Methods

2.1 Primary Cultures of Rat DRG Neurons

After 1–2 days of birth SD rats were sacrificed, the vertebrae were exposed under aseptic conditions. The neurons were removed under a microscope and moved to a centrifuge tube containing L15 (Gibco, USA) medium. After washing with PBS, type II collagenase (sigma, USA) was added. After incubation at 37 °C for 45 min, removed and 2 ml of PBS containing 20% FBS was added, and incubated at 37 °C for 10 min, washed with L15 medium containing 1% BSA, and inoculated at a density of 5 × 105 cells/ml. The transwell lower layer of Matrigel was plated. After 24 h, Ara-C was added at final concentration of 5 μg/ml. After 24 h, the normal medium was changed.

2.2 Experimental Grouping

The cells were classified into several groups, Con group: cells were cultured by normal culture medium, HG group: cells were exposed to 50 mM glucose within culture medium, three taurine treatment groups: cells were co-treated at 50 mM glucose with 10 mM, 20 mM and 40 mM taurine. HG + T group: 50 mM glucose

and 40 mM taurine were added in the medium. HG + T + LY294002 group: 20 μM PI3K antagonists LY294002 (Beyotime, China) added into the medium with 50 mM glucose and 40 mM taurine. HG + T + Perifosine group: 50 mM glucose and 40 mM taurine with added 5 μM Akt antagonists Perifosine (Beyotime, China) in the medium.

2.3 Immunofluorescence Staining

The cells were secured in 4% paraformaldehyde at 4 °C for 15 min. These were washed with PBS for 10 min three times. 10% goat serum (10% goat serum, 1% BSA and 0.3% triton 100 dissolved in PBS) closed for 1 h. These were incubated overnight at 4 °C with primary antibody SMI312 (1:400, Biolegend, USA). Three Washings with PBS for 10 min each, Incubation of secondary antibody Alexa Fluor 594 conjugated donkey anti-mouse IgG secondary antibody (1:500, Jackson, USA) at 37 °C while avoiding light, washed with PBS for 10 min three times. Sample was mounted sample by mounting medium, observed by fluorescence microscope (Olympus, Japan) in dark room.

2.4 Western Blot Analysis

Protein was extracted from DRG cell cultures. Equal amounts of protein were separated on 12% SDS – polyacrylamide gels and moved to PVDF membrane (Millipore, USA). The membrane was blocked with TBS containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk, reacted with primary antibodies, include GAP-43 (1:1000, Sigma, USA), Akt (1:1000, Sigma, USA), p-Akt (1:1000, Sigma, USA), mTOR (1:1000, Abcam, USA), p-mTOR (1:1000, Abcam, USA), β-actin (1:1000, Abcam, USA), overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)-conjugated second antibodies about 2 h with wash between each step. The membrane was developed with enhanced chemiluminescence reagent (keyjen, China) and ChemiDoc-It Imaging System UVP Gel Imaging Analysis System Scans PVDF Films. Quantitative analysis was performed Using ImageJ1.4.3.67 software.

2.5 Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) and analyzed by SPSS using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Statistical significance was considered at $p < 0.05$.

3 Results

3.1 *Effect of Taurine on the Axon Growth of DRG Neurons Exposed to HG*

In order to study the consequences of taurine supplementation on axon growth of HG-exposed DRG neurons, the axon outgrowth and the expression of Gap-43 was measured. As shown in Fig. 1a, the axon development of DRG neurons was inhibited in the presence of HG, but taurine promoted axon outgrowth in HG-treated DRG neurons in a dose-dependent manner, as shown in Fig. 2b, the neurite length

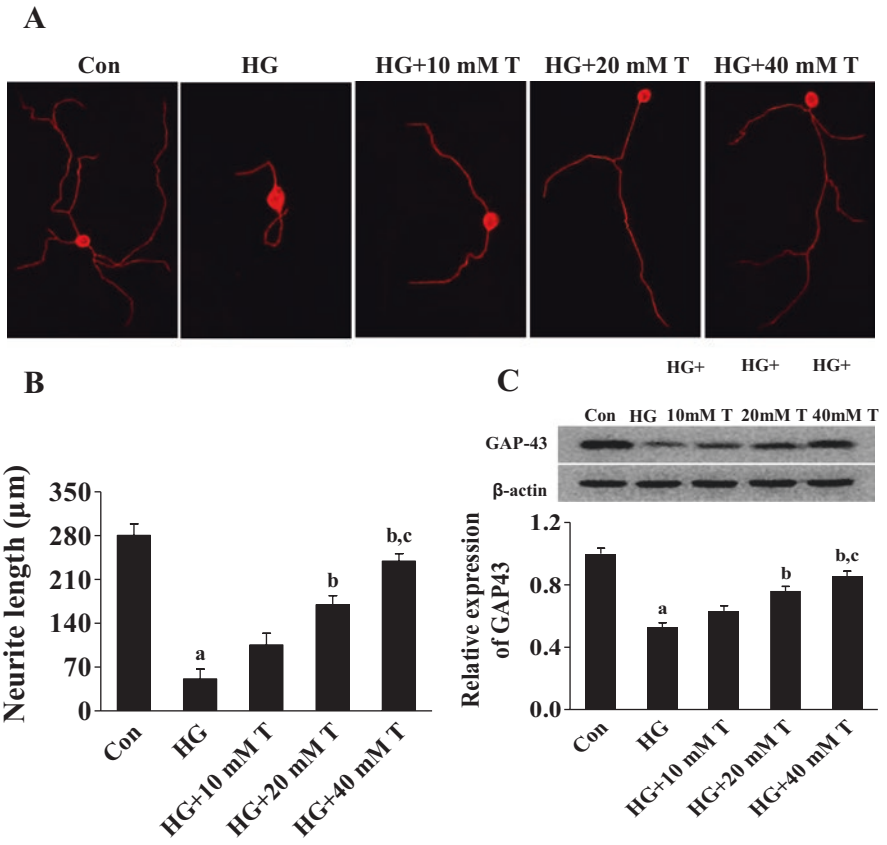

Fig. 1 Neurite outgrowth and the expression of GAP-43 in DRG neurons exposed to high glucose in presence of taurine. (**a**) Immunofluorescent images showing SMI312 immunoreactive DRG neurons. (**b**) Neurite length of DRG neurons. (**c**) Relative expression of GAP-43 in DRG neurons. $^{a}p < 0.05$, compared with Con group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with HG + 10 mM T group; $^{d}p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM

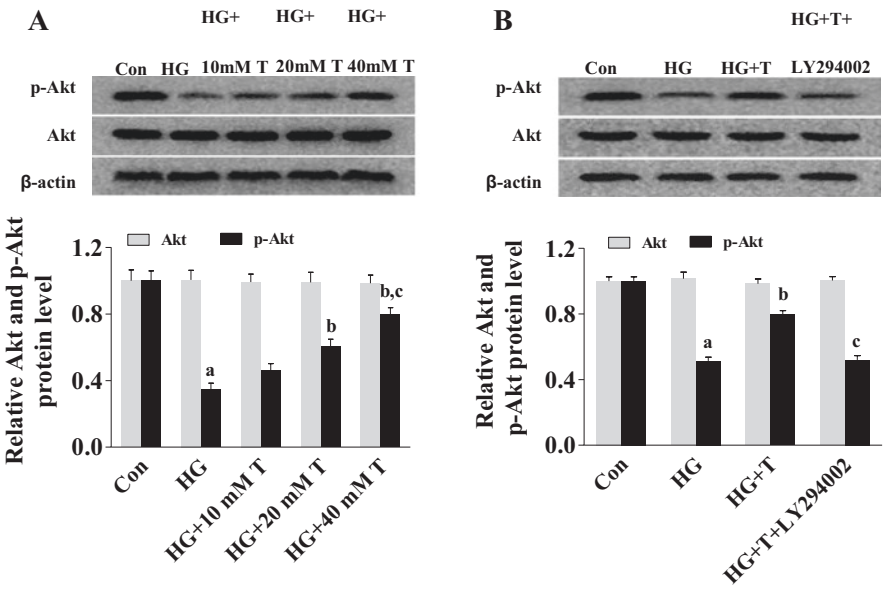

Fig. 2 Akt and p-Akt expression in the treated DRG neurons in presence of taurine. (**a**) Relative Akt and p-Akt protein levels in DRG neurons. (**b**) Relative Akt and p-Akt protein levels in DRG neurons within or without PI3K antagonists LY294002. [a]$p < 0.05$, compared with Con group; [b]$p < 0.05$, compared with HG group; [c]$p < 0.05$, compared with HG + 10 mM T group or HG + T group; [d]$p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM

of DRG cells was significantly decreased in HG group compared with control group ($p < 0.05$). However, the neurite length of DRG neurons were significantly higher in taurine treatment groups than that in HG group ($p < 0.05$), increasing dose-dependently. Axon outgrowth relative protein Gap-43 of DRG neurons was studied by western blot analysis. Then, as shown in Fig. 1c, the Gap-43 expression was evidently decreased in HG group compared with control ($p < 0.05$). However, the expression of Gap-43 were significantly higher in taurine treatment groups than that in HG group ($p < 0.05$), increasing dose-dependently. The results presented that taurine promoted the axon outgrowth in HG-induced DRG neurons.

3.2 Effect of Taurine on Akt (p-Akt) in DRG Cells Exposed to High Glucose

The expression of Akt and p-Akt was studied by Western blot. The results showed as Fig. 2a, it was shown that the expression of Akt was not different among groups ($p > 0.05$). Nevertheless, the p-Akt expression was significantly decreased ($p < 0.05$) in HG group, but increased ($p < 0.05$) after use of taurine in a dose-dependent manner. The expression of Akt and p-Akt in culture DRG neurons were detected at the presence of PI3K antagonists LY294002. As shown in Fig. 2b, the expression of Akt

was not different in different groups ($p > 0.05$). However, It was found that the increase in expression of p-Akt after taurine in HG treated DRG neurons was significantly decreased in the presence of LY294002 ($p < 0.05$). It was implied that taurine increased the phosphorylation of Akt in HG-induced DRG neurons via activation of PI3K.

3.3 Effect of Taurine on mTOR (p-mTOR) in DRG Cells Exposed to High Glucose

The expressions of mTOR and p-mTOR were studied by Western blot. As shown in Fig. 3a, it was found that the expression of mTOR was not different among groups ($p > 0.05$). However, p-mTOR was down-regulated ($p < 0.05$) in HG group, but up-regulated after taurine treatment in a dose-dependent manner ($p < 0.05$). The expressions of mTOR and p-mTOR in culture DRG neuron cells were noticed in the presence of Akt antagonists Perifosine. It was found that the expression of mTOR was not different among groups ($p > 0.05$). However, the increasing expression of p-mTOR after taurine in HG treated DRG neurons was significantly decreased in the presence of Perifosine ($p < 0.05$) (Fig. 3b). The results indicated that taurine promoted the posphorylation of mTOR in DRG neurons via activation of Akt.

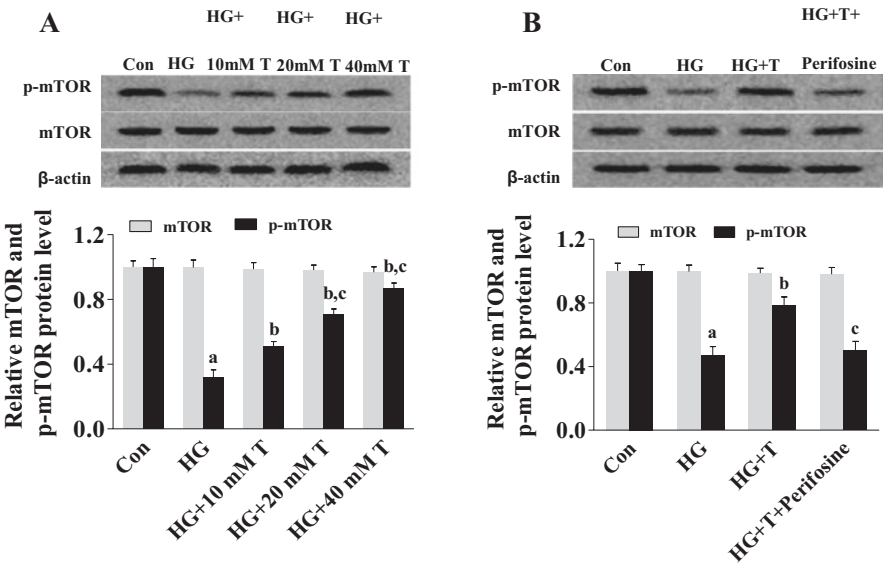

Fig. 3 mTOR and p-mTOR expression in the DRG neurons in presence of taurine. (**a**) Relative mTOR and p-mTOR protein levels in DRG neurons. (**b**) Relative mTOR and p-mTOR protein levels in DRG neurons with added Akt antagonists Perifosine. $^{a}p < 0.05$, compared with Con group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with HG + 10 mM T group or HG + T group; $^{d}p < 0.05$, compared with HG + 20 mM T group. Data are expressed as mean ± SEM

3.4 Promotive Effect of Taurine on Neurite Outgrowth of DRG Neurons Exposed to High Glucose via Akt/mTOR Signalling Pathway

Immunofluorescence was used to detect axon growth of DRG neurons, it was detected by and the expression of Gap-43 by western blotting. It showed that the axonal extensions of DRG neurons were inhibited in the presence of HG, but taurine promoted axonal outgrowth in HG-treated DRG neurons and this effect was reversed in the presence of Perifosine (Fig. 4a). As showed in Fig. 4b, the neurite length of DRG neurons weas significantly higher in taurine treatment group than that in HG cells ($p < 0.05$). However, the increasing neurite length of DRG neurons after taurine in HG treated DRG neurons were significantly decreased in the presence of Perifosine ($p < 0.05$). Figure 4c showed that the increasing expression of GAP-43 after taurine in HG treated DRG neurons was significantly decreased in the presence of Perifosine ($p < 0.05$). It was implied that taurine favored the neurite outgrowth in HG-induced DRG neurons via activation of Akt/mTOR signalling pathway.

4 Discussion

DPN is an extremely lively process with interrelating mechanisms causing progressive abnormalities, the main features of which are impairment of axonal fuctioning, atrophy, and loss. Henceforth, DPN is described as an axonopathy of dying back type (Sima et al. 1988). Jia et al. also reported that HG locally inhibited neurite growth in DRG neurons (Hsu et al. 2013). In the present study, the results indicated a decline in neurite growth of DRG neurons meaningfully in HG group compared with control group. Moreover, the Gap-43 expression also significantly decreased in HG group compared with control group, being in accordance with the neurite's result. However, taurine supplementation significantly reversed the decreased neurite outgrowth and the down-regulated Gap-43 expression in HG-treated DRG neurons in a dose-dependent manner. Taurine is known to have a trophic impact on the nervous system. Moreover, it was shown that taurine stimulated neuritic outgrowth from retinal explants and ganglions (Lima et al. 1989; Matus et al. 1997), being supportive to our results. These results propose that taurine promotes the neurite growth of extensions in DRG neurons exposed to HG. However, the molecular mechanisms behind this are still unclear.

The serine/threonine kinase AKT, recognized as protein kinase B, is the major effector of the phosphati-dylinositol 3-kinase (PI3K) signaling pathway. Overexpression and persistent activation of AKT are caused by oncogenes, growth factors, and cytokines. The activated AKT controls many cellular functions including cell growth and survival (Alessi et al. 1996; Andjelkovic et al. 1997). Moreover, Akt has been shown as an important mediator of various features of neurite outgrowth, including elongation, branching and caliber (Read and Gorman 1997). To

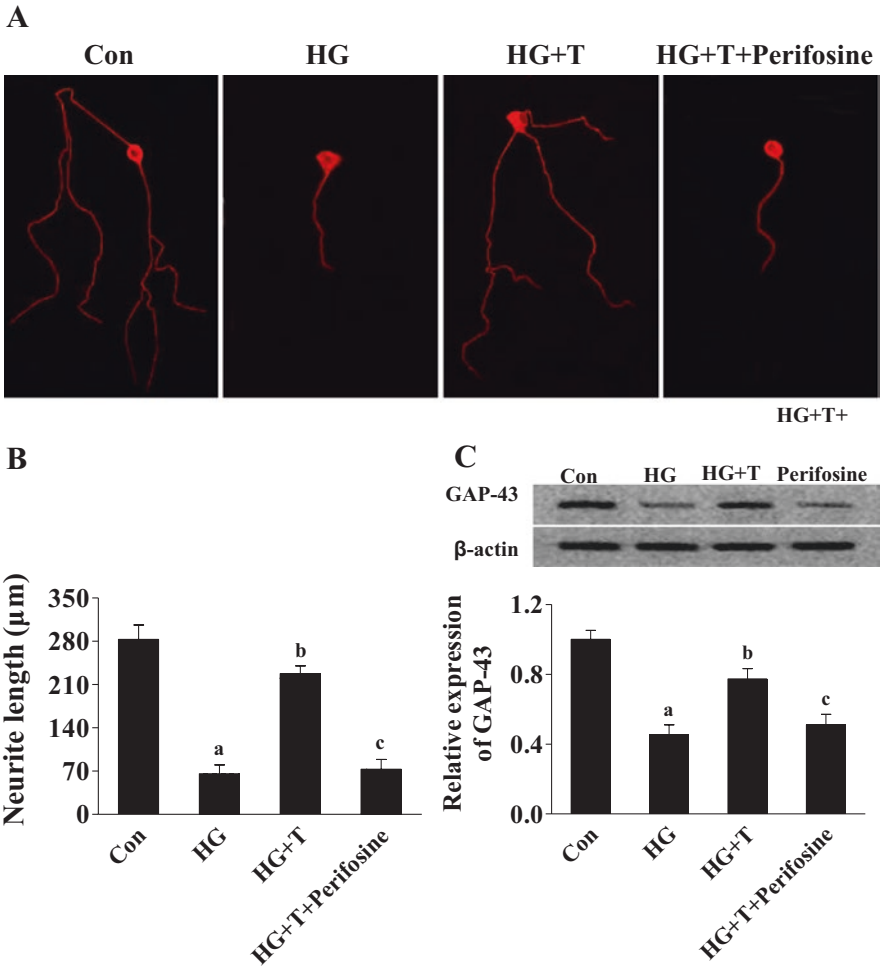

Fig. 4 Neurite outgrowth and the expression of GAP-43 in treated DRG neurons with added Akt antagonists Perifosine. (**a**) Immunofluorescent images showing SMI312 immunoreactive DRG neurons. (**b**) Neurite length of DRG neurons. (**c**) Relative GAP-43 protein levels in DRG cells of four groups with added Akt antagonists Perifosine. $^{a}p < 0.05$, compared with Con group; $^{b}p < 0.05$, compared with HG group; $^{c}p < 0.05$, compared with HG + T group; $^{d}p < 0.05$. Data are expressed as mean ± SEM

investigate whether the promotion of taurine is associated with an activation of Akt, the expressions of Akt and p-Akt in DRG neurons were measured by using Western blotting assay in present study. The results indicated that HG evidently decreased the expression of p-Akt in the DRG neurons.

On the contrary, taurine supplementation significantly reversed the downregulated phosphorylated level of Akt. However, the shielding effects of taurine were hindered in the presence of PI3K antagonists LY294002. Takatani et al. reported

that treatment of taurine significantly reversed the decline in phosphorylation level of Akt in cardiac myocyte cells taken from ischemic rats. It was also shown that acrylamide-induced reduction in the p-Akt expression in the rats was attenuated by taurine administration, being consistent with our findings. These results propose that taurine activates Akt in the DRG neurons exposed to HG (Sun et al. 2018).

Downstream of Akt, various substrates have been recognized which possibly play key roles in Akt-mediated outgrowth of neurites. mTOR is a direct target of Akt. In recent reports, mTOR encouraged axonal renewal in the adult central nervous system and improved the axonal growth in injured peripheral nerves (Park et al. 2008; Abe et al. 2010). In the present research, the results indicated that HG considerably lowered the expression of p-mTOR in the DRG neurons. On the other hand, taurine supplementation significantly reversed the reduction in phosphorylated levels of mTOR. Moreover, the protective effect of taurine was hindered with presence of Akt antagonists Perifosine. Li et al. reported that treatment of taurine significantly reversed decrease in phosphorylation level of mTOR in PC12 cells exposed to Methamphetamine (Li et al. 2012), being in accordance with our findings. Our results show that taurine activates p-mTOR in the DRG neurons HG condition. The PI3K/Akt pathway is known to normalize the mTOR pathway and PI3K/Akt/mTOR signaling promotes growth and branches in hippocampal neurons (Chen et al. 2012). Therefore, it is suggested that taurine may promote neuritic growth in DRG neurons under HG through modulating PI3K/Akt/mTOR signaling pathway. It is necessary to confirm further promotive effect of taurine on damaged axon regeneration in diabetic neuropathy in vivo via the PI3K/Akt/mTOR signaling pathway.

5 Conclusion

In the current study, our results showed that HG significantly reduced the neurite outgrowth and downregulated expression of Gap-43, phosphorylation levels of Akt and mTOR in DRG neurons. On the contrary, taurine supplementation significantly reversed these abnormal changes in DRG neurons under HG condition. However, the presence of PI3K antagonists or Akt antagonists hindered protective effects of taurine. These results suggest that taurine promotes neurite outgrowth in DRG neurons exposed to HG via activating Akt/mTOR signalling pathway. Hence, taurine can be helpful in treating axonal damage in diabetic neuropathy. These findings may provide a new therapeutic approach for diabetic neuropathy and a novel insight for explaining the protective mechanism of taurine.

Conflict of Interest It is declared that there is no conflict of interest among the authors.

Research Subjects In this study no animal was harmed or hurd.

Informed Consent This manuscript is being submitter after consent from all authors and all authors are aware of submission.

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The microRNAs Expression Profile in Sciatic Nerves of Diabetic Neuropathy Rats After Taurine Treatment by Sequencing

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Abstract Taurine protect against diabetic neuropathy. However, the protective mechanism of taurine has been poorly understood. It has been demonstrated that microRNAs (miRNAs) are involved in regulating gene expression. Therefore, it is interested in whether taurine affects miRNAs expression profile in peripheral nerve tissue of diabetic neuropathy. In the present study, rats were treated as three group: (1) control (Con) group, (2) diabetic mellitus (DM) group and (3) taurine treatment (Tau) group. Sciatic nerve tissue was harvested and miRNA expression was determined using sequencing. The results showed that 80 miRNAs showed significant difference in DM group compared to Con group, of which 20 miRNAs showed up-regulating, as well as, 60 miRNAs showed down-regulating. On the other hand, 215 differential miRNAs were found between DM and Tau groups. Moreover, the numbers of up-regulated and down-regulated miRNAs were 1 and 214, respectively. Twelve specific miRNAs were screened out and the target genes were obtained by target analysis software. GO and KEGG enrichment analyses showed that these potential target genes for the miRNAs might be involved in axon guidance, generation of neurons, nervous system development and neurogenesis. Our results provided a miRNA profile for further exploring protective mechanisms of taurine against diabetic peripheral neuropathy.

Xiaoxia Shi and Zewen Qiu have been equally contributed to this chapter.

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Keywords Taurine · MicroRNA · Diabetic neuropathy · Sequencing

Abbreviations

DM	Diabetes mellitus
DPN	diabetic peripheral neuropathy
q-PCR	quantitative real time PCR

1 Introduction

Diabetes mellitus (DM), a chronic metabolic disorder, leads to serious complications (Sima and Sugimoto 1999). One common of complications is diabetic peripheral neuropathy (DPN) that has been defined as with peripheral nerve dysfunction in diabetes (Vinik et al. 2000; Edwards et al. 2008). The characterization of DPN includes neuropathic pain, allodynia, or numbness, sensory loss (Dyck et al. 1993; IDF 2013). It is though that DPN is associated with axonal injury, demyelination, nerve inflammation and progressive loss of peripheral nerve fibers (Sinnreich et al. 2005), which have affected about 50% of diabetic patients with a high morbidity, mortality and quality of life decline. However, in clinical, the therapeutic approach to DPN was mainly targeting good metabolic control, which slows but does not prevent progression (Cameron et al. 2001). Therefore, discovering new potential candidates for protecting against DPN is essential.

Taurine, a conditionally essential amino acid, has ability to cytoprotection in many kinds of tissues (Huxtable 1992). It is mainly synthesized by metabolism of methionine and cysteine in the liver and brain. (Tappaz et al. 1992). It exhibited some key biological functions, like, membrane stabilization, osmoregulation and regulation of calcium signaling neurotransmission which were emphasized by different studies (Sirdah 2015). Recently, the protective effect of taurine on diabetes and diabetic complications has been attention. Some studies have shown that taurine was the potential intervention candidate for preventing the progression of diabetes and its complications. (Chen et al. 2016; Imae et al. 2014). Li et al. reported that taurine supplement attenuated calcium handling and hyperalgesia in neuron of STZ-treated diabetic rats (Li et al. 2005). Moreover, it was also found that taurine was useful to ameliorate the deficits of nerve conduction velocity, nerve blood flow, and sensory thresholds (Li et al. 2006a). These studies implied that taurine had the protective effect against DPN. However, the mechanism of taurine against DPN keeps unknown.

MiRNAs, a small RNA molecules (22 ~ 24 nt), has been interested in the regulatory non-coding RNA, since they were important to regulating many biological processes by binding to the 3′-UTR of target mRNAs. The studies have confirmed miRNAs could regulate the entire set of genes post-transcriptionally, because of the effect of leading to target mRNAs translational inhibition or degradation (Griffin et al. 2010; Sarkar et al. 2017). In addition, the miRNA-specific expression pattern

is responsible for the specific biological functions in a particular system. Typically, this expression specific changes in pathological events suggests an additional regulating role for key signaling pathways in pathology. So, miRNAs constitute the novel targets for exploring pathology and therapeutic intervention to promoting repair. Recently, studies have found that miRNAs were crucial mediator in nerve protection, including promoting nerve regeneration, inhibiting nerve inflammation and apoptosis (Hutchison et al. 2009; Feng et al. 2018; Aghdaei et al. 2017). To date, however, there are no reports on the regulation mechanism of miRNAs on taurine against DPN.

In this study, firstly, we built diabetic rat model and taurine treated diabetic rat model. Then, we identified miRNA profiles in sciatic nerve of differential group rats by sequencing. The miRNAs with significant expression variance were screened out. Lastly, by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, the potential functions of these miRNAs in tanrine against DPN could be predicted. These findings provided the first evidence of a expressional profile of miRNAs in sciatic nerve of taurine-treated diabetic rats. In addition, the findings in this study were also provided new evidences for further exploring protective mechanisms of taurine against DPN.

2 Materials and Methods

2.1 Animal Model and Tissue Preparation

Sprague-Dawley rats were used to performing experiments in this study and were conducted according to the animal feeding requirements. The experimental animal procedures were approved by the Animal Care and Use Committee of Dalian Medical University (approval number: SCXK (liao) 2015-2003, China).

Rats were treated as 3 groups (each containing 4 rats): (1) Control (Con) group: Rats were fed in normal condition. (2) diabetes (DM) group: Rats were fed with high-fat and high-sugar food for 4 weeks and then injected STZ once time with the concentration of 25 mg/kg by intraperitoneal injection. (3) Taurine (Tau) group: Diabetic rats treated with 2% w/v taurine in water orally for 8 weeks after STZ injection. After the rats model being built, each animal was sacrificed and sciatic nerve of the left hind limb was harvested on ice. The sciatic nerve was stored at −80 °C for the following experiments.

2.2 RNA Extraction and Sequencing

Sciatic nerve tissues were prepared and the samples were sent to RiboBio (China). MiRNA libraries constructing and sequencing was performed by RiboBio (China). Briefly, the total RNA of sicatic nerve was extracted and then small RNAs ranging between 18 and 30 nucleotides separating by Tris-borate-EDTA polyacrylamide gel

were used for constructing libraries. After miRNA reverse transcribing and amplifying by PCR, Illumina HiSeq 2500 platform were used to detect the sequences of miRNAs.

2.3 Searching for miRNAs Significant Profiles

All small RNAs expression in each group were investigated via sequencing, the candidate hairpin miRNA corresponding to the reading in the scanned rat genome was calculated and the expression of the miRNAs was identified using comparing sequences of the mature RNA in miRBase (version 21). Moreover, methods for normalizing miRNA expression data were quantified. The correlation was calculated by Pearson coefficient. Then, the differential expression of miRNAs was identified by log (foldchange). The P value was calculated by edgeR.

2.4 Predicating the Functions of miRNA Targets

The target genes of these specific miRNAs were predicated by three miRNA target prediction softwares: TargetScan (http://www.targetscan.org), miRDB (http://www.mirdb.org/) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/). Then, the biological functions of these target genes were analysis by KEGG and GO enrichment analyses. P value was calculated by Fish Exact Test. The pathways that miRNA and targets were involved were analyzed by KEEG enrichment, and the biological functions of the miRNAs and targets were analyzed by GO enrichment.

3 Results

3.1 Construction of Small RNA Libraries by Sequencing

For obtaining the different small RNA libraries in sciatic nerve of diabetic rats with or without taurine treatment, the small RNAs of sciatic nerve were sequenced using Illumina HiSeq 2500 platform. By removing the adaptor at both ends of reads and the low-quality reads, the clean reads were got and analyzed. It was found that and almost half of clean reads has the 22 nucleotides in length, which was consistent with the common length of miRNA (Fig. 1a–c). These small RNAs were further excluded by matching with known miRNA/tRNAs/rRNAs/snRNAs/snoRNAs from the miRBase version 21 (www.mirbase.org), Rfam database (Rfam.xfam.org) and

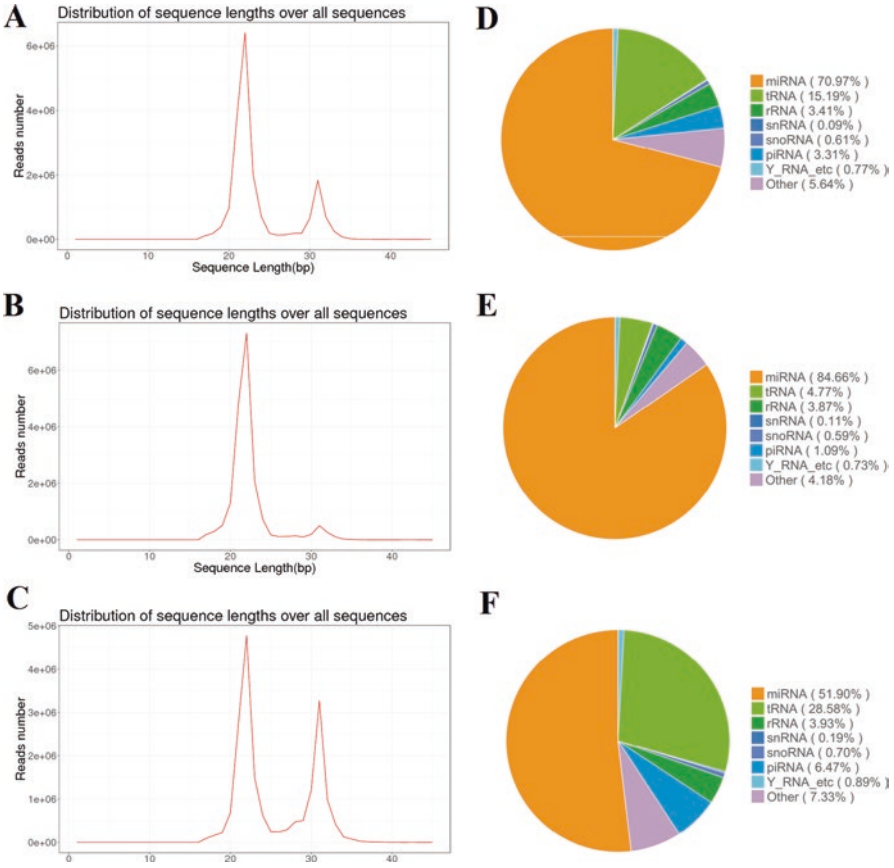

Fig. 1 Lengths distribution of the sequencing reads, and classification of small RNAs in sciatic nerve of diabetic rats with or without taurine treatment. (**a**) Distribution of sequence lengths from sciatic nerve of normal rats. (**b**) Distribution of sequence lengths from sciatic nerve of diabetic rats. (**c**) Distribution of sequence lengths from sciatic nerve of diabetic rats treated by taurine. (**d**) Classification of small RNAs from sciatic nerve of normal rats. (**e**) Classification of small RNAs from sciatic nerve of diabetic rats. (**f**) Classification of small RNAs from sciatic nerve of diabetic rats treated by taurine

pirnabank (pirnabank.ibab.ac.in). It was found that there were 70.97, 84.66 and 51.90 percentage of miRNAs in small RNA library in sciatic nerve of normal rats, diabetic rats and taurine-treated diabetic rats, respectively (Fig. 1d–e). These results implied that there was abundant miRNAs in the small RNA libraries of sciatic nerve tissue of each group rats.

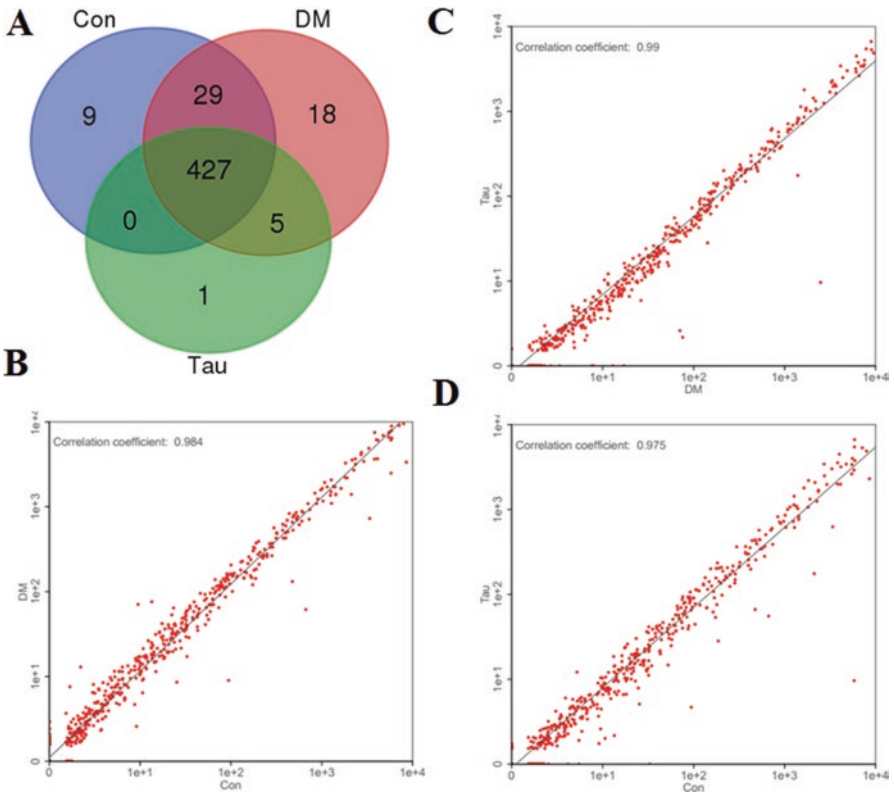

Fig. 2 The expression of miRNAs of in sciatic nerve of diabetic rats with or without taurine treatment. (**a**) The venn map for the miRNA profiles of the three groups. (**b**) The correlation analysis between Con group and DM group. (**c**) The correlation analysis between DM group and Tau group. (**d**) The correlation analysis between Con group and Tau group

3.2 Determining the Expression of miRNAs in Diabetic Rats With or Without Taurine Treatment

By analyzing the expression of known miRNAs, it was found that there were 465, 479 and 433 miRNAs in Con, DM and Tau groups, respectively (data not shown), meanwhile, there was 427 miRNAs in all these three groups (Fig. 2a). Then, the read counts of miRNA and its expression value were calculated, which were used to screening the differential miRNAs in following experiments. Besides, the correlations between the three groups were analyzed. It was showed the correlation coefficient value is more than 0.97 between Con, DM and Tau groups (Fig. 2b–d), which implied the data from sequencing is reliable and reasonable and could be used in the next analysis.

Table 1 The count of differential miRNAs in three groups

Type	Count		
	Con vs DM	Con vs Tau	DM vs Tau
|log2(foldchange)| > =1	80	119	215
0.01 < =P-value<0.05	3	5	14
P-value < 0.01	43	55	41

3.3 Category Analysis of Specific miRNAs in Taurine-Treated Diabetic Rats

The difference of miRNA profiles and expression clustering of differential miRNAs were shown on the Table 1 and Fig. 3 (data not shown). It was found that 80 miRNAs showed significant difference in DM group compared to Con group, of which 20 miRNAs were up-regulated and 60 miRNAs were down-regulated. Moreover, 215 differential miRNAs were found between DM and Tau groups. Moreover, the numbers of up-regulated and down-regulated miRNAs were 1 and 214, respectively. To sum up, it was found 27 miRNAs with up-regulation or down-regulation in DM group compare with Con group, but the effect was attenuated in Tau groups.

For further analyzing the pathways and functions that miRNAs involved, 12 specific miRNAs, has the higher foldchange were screened out and showed on Table 2, which were used for analysis the downstream target genes and pathways.

3.4 Annotating the Function of Specific miRNAs and Targets in Taurine-Treated Diabetic Rats

After the target genes of the above categories of miRNAs being predicted by Targetscan, miRDB and miRWalk, the candidate target gene for miRNAs of the category was obtained. Then, the KEGG pathway and GO function enrichment analyses of miRNAs targets were used to get credible biological functions. As shown in Figs. 4 and 5, the pathway and functions of the categories of gene were listed in KEEG terms and GO terms, respectively. It was found that the most significantly enriched pathways were included axon guidance, PI3K-Akt signaling pathway, MAPK signaling pathway, metabolic pathway, Rap1 signaling pathway, cAMP signaling pathway and apoptosis (Fig. 4). Moreover, the most significant GO functions were included molecular function: enzyme binding; cellular component: neuron part, cell junction; biological process: cell development, nervous system development, neurogenesis and generation of neurons (Fig. 5). The results indicated that the most significant KEEG pathway and GO functions of miRNAs were both related to neuron grow and development.

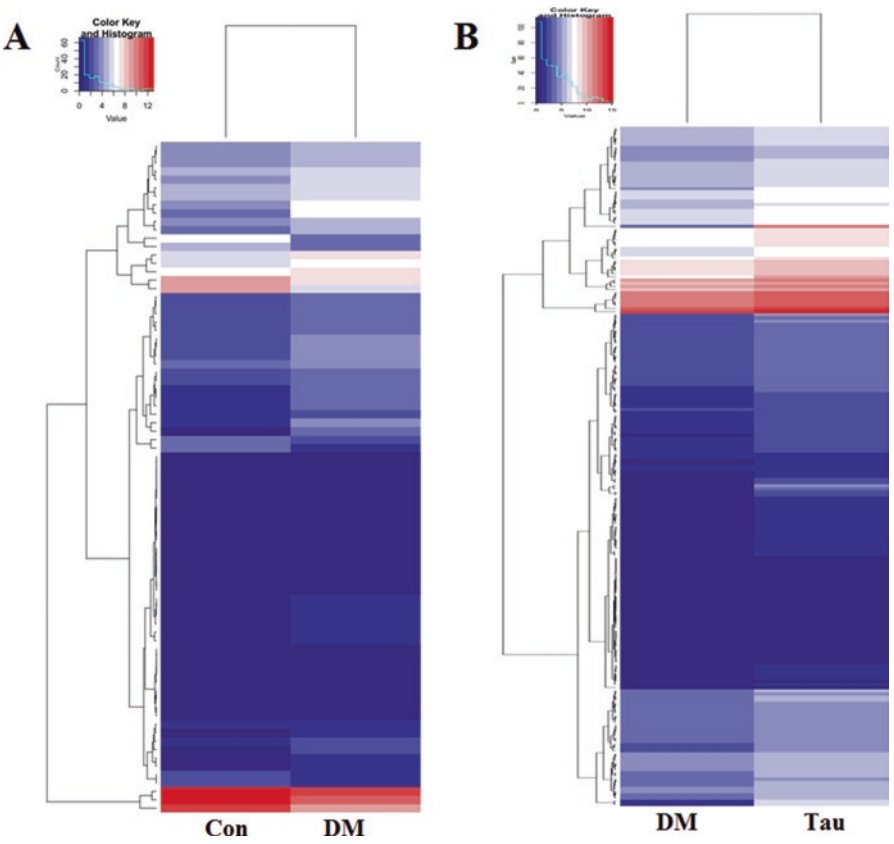

Fig. 3 The heatmap for the miRNA with significant expression variance. (**a**) Heatmap and cluster dendrogram of differential expression of miRNAs from sciatic nerve in DM group rats compared with Con group rats. (**b**) Heatmap and cluster dendrogram of differential expression of miRNAs from sciatic nerve in Tau group rat compared with DM group rats

4 Discussion

Taurine is one of the most abundant amino acids in the body, making up around 0.1% of our body weight. In mammals, taurine is almost ubiquitous in distribution, with high concentration in heart and brain, spinal cord, retina and platelets (Huxtable 1992). The studies have found that taurine was involved in various physiological processes (Sirdah 2015; Kendler 1989) and there was a long list of diseases that were impacted by taurine, a case in point is its role in diabetes. Many studies have confirmed the significant role of taurine in overcoming insulin resistance and other risk factors in animal models of diabetes (Anuradha and Balakrishnan 1999; Franconi et al. 2004; Ito et al. 2012). Not only that, but also the protective effect of taurine in DPN has also found. It was reported that taurine improved electrophysiological parameters, such as nerve glucose, nerve osmolytes and nerve conduction velocity under diabetic conditions (Stevens et al. 1993). Moreover, other study was

Table 2 The expression normalized value of differential miRNAs in each groups

MiRNA	Con group	DM group	Tau group
rno-miR-200b-3p	12.4412	74.8663[a]	20.1756[b]
rno-miR-124-3p	52.1907	21.2976[a]	14.2143[b]
rno-miR-25-5p	0.6947	2.2097[a]	1.3706[b]
rno-miR-433-5p	0.8016	2.4201[a]	1.0543[b]
rno-miR-29a-5p	1.4205	0.6326[a]	0.9878[b]
rno-miR-137-3p	4.8499	10.5223[a]	5.13[b]
rno-miR-129-2-3p	4.2701	8.8914[a]	5.6337[b]
rno-miR-9a-3p	58.4071	118.8496[a]	77.9155[b]
rno-miR-128-3p	687.2584	1232.9007[a]	901.6161[b]
rno-miR-9a-5p	5461.837	7879.5897[a]	5852.0966[b]
rno-let-7i-5p	37813.6428	47901.1962[a]	45685.8909[b]
rno-miR-184	7.6967	3.6828[a]	4.0612[b]

[a]$p < 0.05$, compared with Con group
[b]$p < 0.05$, compared with DM group

also reported taurine supplement in diabetic rats could reverse the lacking of nerve blood flow, nerve conduction velocity and sensory thresholds (Li et al. 2006a). However, the mechanism of taurine against DPN has poorly understood.

Recently, more and more attention has been focused on the roles of miRNAs in regulating molecular mechanism in both physiological and pathological conditions because one miRNA might regulate the hundreds of target mRNAs, moreover, a single mRNA could also be regulated by many kinds of miRNAs (Lewis et al. 2005). It was indicated miRNAs were candidates regulating multiple pathways. The studies have found that miRNAs were involved in regulating the nerve injury and protection (Hutchison et al. 2009; Feng et al. 2018). To further explore the regulating mechanisms of the activation of pathways during nerve protection of taurine against DPN, the investigation of miRNA profiles could be important in finding out the mechanisms of nerve protection meditated by taurine. In the currently miRNA profile analyses, miRNA microarray exhibited low specificity and sensitivity, while, sequencing has significantly increased the specificity and sensitivity, compared with miRNA microarray (Schulte et al. 2010).

In this study, we identified miRNA profiles in sciatic nerve of diabetic rats treated with or without taurine by sequencing and found miRNAs are abundant in sciatic nerve tissues. There were more than 400 miRNAs being found in the sciatic nerve of rats. Moreover, we found there were 427 miRNAs in all three groups, 5 miRNA in both DM and Tau group, as well as, 18 miRNAs unique in DM group and 1 miRNA unique in Tau group. These results indicated the differential expression profiles of miRNAs in Con, DM, and Tau groups. In other words, taurine supplement regulated the expression of miRNAs in sciatic nerve tissues of diabetic rats.

For further finding out specific miRNAs, the expression of miRNAs was normalized by a calculation formula and analyzed the significant expression changes of miRNAs in diabetic rats with or without taurine treatment. The results showed that 80 miRNAs showed significant difference in DM group compared to Con group and 215 differential miRNAs were found between DM and Tau groups. Moreover, we

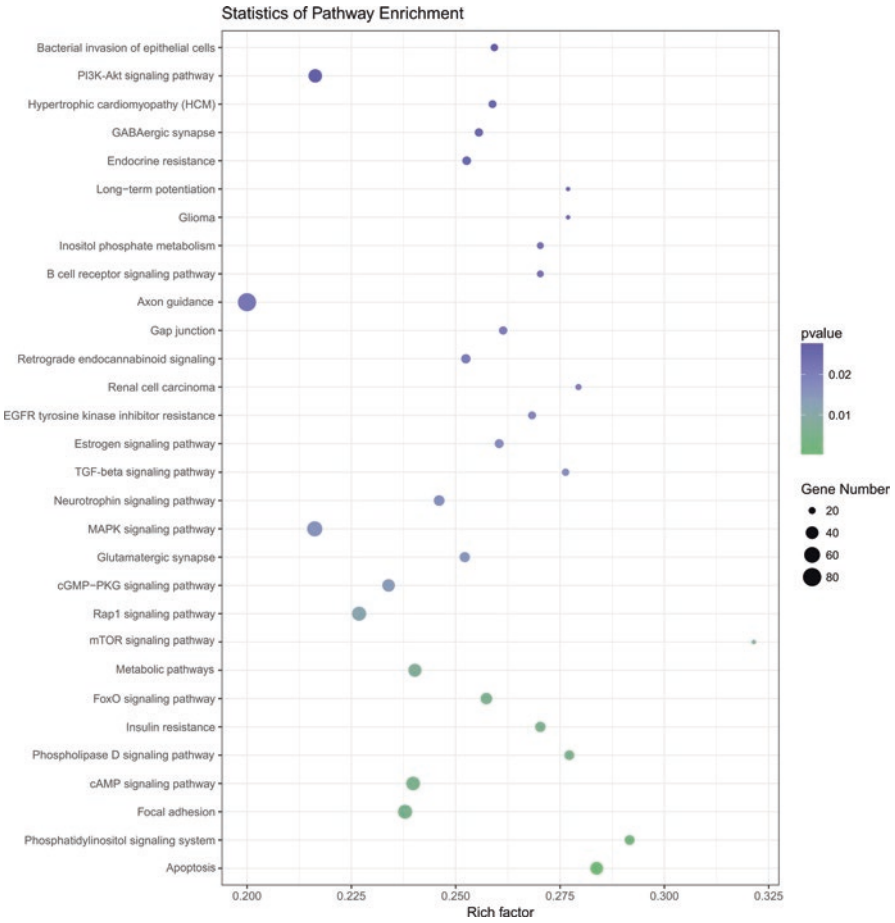

Fig. 4 The most significant KEEG pathways for miRNA targets ($p < 0.05$)

found 27 miRNAs candidates with significantly up-regulated or down-regulated in DM group compare with Con group, but the expression was reversed in Tau groups. Finally, 12 specific miRNAs were screened out by comparing the fold changes of candidate. Among these miRNAs, miR-9a has been reported as a mediator in regulating an early step in neurogenesis (Li et al. 2006b). miR-124 could regulate neuron apoptosis and autophagy process (Wang et al. 2016). It was reported that miR-29a was a regulator of neurite outgrowth via targeting PTEN in PC12 cells (Zou et al. 2015). Our results implied that these specific miRNAs might be the important regulators in neuroprotection of taurine against DPN.

For further exploring the regulating mechanism of miRNAs in taurine against DPN, the bioinformatics analyses were used to enrichment the functions of these specific miRNAs. After candidate target genes of these specific miRNAs being obtained through target predicating softwares, the enrichment analyses of KEGG and GO for miRNAs targets were performed. The results found that the most significant

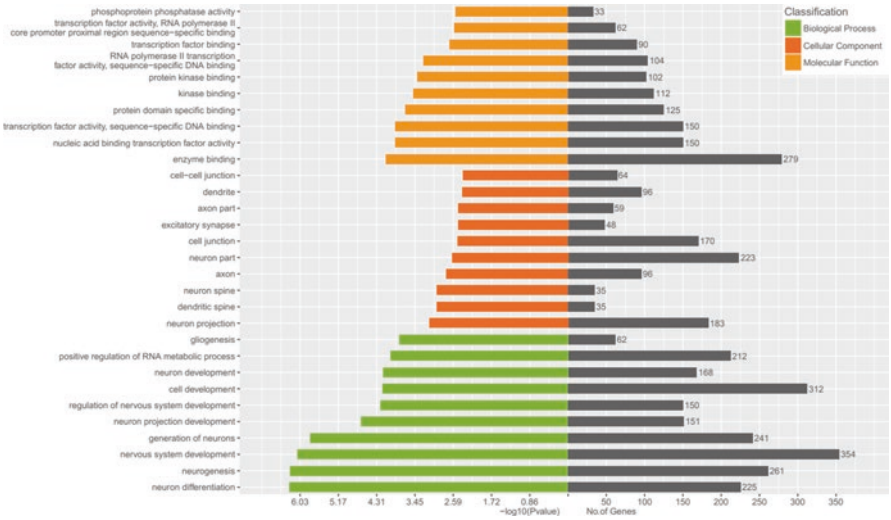

Fig. 5 The most significant GO functions for miRNA targets ($p < 0.05$)

KEEG pathway for miRNA targets were involved in axon guidance, PI3K-Akt signaling pathway, MAPK signaling pathway, metabolic pathway, Rap1 signaling pathway, cAMP signaling pathway and apoptosis, which indicated that these miRNAs were related to nerve regeneration, metabolism, apoptosis and so on. Moreover, the most significant GO functions including: the molecular function in enzyme binding, cellular component in neuron part and cell junction, biological process in cell development, nervous system development, neurogenesis, generation of neurons, which also indicated that these specific miRNAs have responsible for neuron growth, development and homeostasis of nerve. These results provided a clue to study the regulating mechanism of miRNA in taurine against DPN: taurine might protect against DPN by increasing/decreasing the expression of miRNAs and then regulating neuron regeneration, metabolism, and apoptosis.

5 Conclusion

In the present study, we firstly found the dynamic expression changes of miRNA profiles in the sciatic nerve of diabetic rats and taurine treated diabetic rats by sequencing. The KEEG and GO enrichment analysis indicated that the specific miRNAs might regulate the key processes in neuron growth, development and neurogenesis. Our results provide a specific expression profile of miRNA in sciatic nerve of DPN rats in the presence of taurine. Our findings also provided a novel clue for further exploring protective mechanisms of taurine against DPN. In the future study, we need to find the miRNA-target gene pairs and further clarify their regulating mechanism in taurine against DPN.

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The Protection of Taurine on Abnormal Expression of Deiodinase 3 Induced by BDE 209 in JEG Cells Under the Normal Culture Conditions

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Abstract Taurine is an important amino acid for the growth and development of the central nervous system and plays an important role in the development of the nervous system. Many studies have shown that taurine can prevent and repair neurodevelopmental damage, and its mechanism has also become a research hotspot. While most studies focus on nerve cells, less on placental cells that are closely related to early neurodevelopment (developmental neurotoxicity) by modulating fetal circulation level of thyroid hormones. Studies have shown that exposure of placental cells to the common environmental endocrine disruptor BDE 209 during early pregnancy may lead to developmental neurotoxicity due to thyroid hormone interference caused by abnormal expression of deiodinases. Therefore, in this study, the placenta-derived JEG cells cultured at 95% air/5% CO2 was used as a in vitro model, and the potential protection from taurine on BDE 209-mediated cytotoxicity was examined. When BDE 209 was found to cause a decrease in cell viability and disturbance in the gene and protein expressions of placental deiodinase 3, pretreatment of the JEG cells with taurine can moderately reduce the BDE 209-meditated

Chang Qin and Xiaoji Hao are contributed equally to this work.

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cytotoxicity, and restore gene and protein expressions of placental deiodinase, so that thyroid hormone levels tend to be normal in cell culture medium. Our data suggest that taurine may have some protection on the developmental neurotoxicity caused by BDE 209.

Keywords JEG cells (Human trophoblastic JEG-3 cells) · Polybrominated diphenyl ethers (PBDEs) · Taurine · Placental deiodinases · Developmental neurotoxicity

Abbreviations

BDE 209	Decabromodiphenyl ether
Dio	Deiodinase
JEG cells	Human trophoblastic JEG-3 cells
MTT	4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

1 Introduction

Polybrominated diphenyl ether (PBDEs) are brominated flame retardants (BFRs) widely incorporated in household electrical appliances and other indoor products, such as sofa, mattress and carpet made with textiles, foams and fabrics (Alaee et al. 2003). PBDEs have 209 congeners, and decabromodiphenyl ether (BDE 209) is one of the most widely used types. PBDEs have become a global concern for its persistent presence in the natural environment and large environmental accumulation and potential multi-organ and multi-system toxicity, especially the developmental neurotoxicity and thyroid hormone interference (Birnbaum and Staskal. 2004). Currently, PBDEs have been detected in human biological samples such as maternal blood, milk, placenta, and the level of accumulation shows an increasing trend (Kun Ni et al. 2013). Animal and population studies have shown that exposure to PBDEs during pregnancy can cause developmental neurotoxicity in offspring (Hongmei Zhang et al. 2017; Joseph M. Braun et al. 2017). Some studies have suggested that intrauterine thyroid hormone disorders may be one of the mechanisms of developmental nerve injury, and placenta deiodinase (Dio) is the key regulation factor of intrauterine thyroid hormone balance.

Dio is divided into three types (Dio 1, 2, 3), Dio 3 is highly expressed in the placenta, and is a thyroid hormone inactivating enzyme that converts highly active triiodothyronine (T3) into inactive reverse triiodothyronine (rT3), thereby preventing intrauterine high T3 levels and protecting the normal development of the early fetuses (Valerie Anne Galton et al. 1999). Taurine is an essential bio-molecule for the nervous system development. Studies have shown that it has preventive protection and repairing effects on neurodevelopmental damage. Therefore, we

hypothesize that PBDEs may affect the expression of Dio 3 in placental cells to produce developmental neurotoxicity, but whether taurine would exert its protective effect on early neurodevelopmental damage by attenuating this toxic effect would be the central interest of our present study.

Placental cells are inhabited in a hypoxic environment in pysiological conditions, but as a parallel study to the in vitro hypoxic study, the present study was looking at how the placenta-derived JEG cells cultured under the normoxia (95% air/5% CO2) would respond to the BDE 209 toxicity on cell proliferation and Dio 3 gene and protein expression, and whether taurine would exert protective effect against BDE 209 toxicity.

2 Methods

2.1 Chemicals

BDE 209 (purity >98%) was bought from the Sigma, USA. Anti-Dio3 antibody was bought from the Abcam, UK. Mouse anti-β actin mAb was bought from the ZSGB-Bio, Beijing China. Anti-Rabbit antiboby and anti-Mouse antiboby were bought from the Proteintech, Chicago USA. The primers for GAPDH and Dio 3 were designed and synthesized at the TaKaRa, Japan. PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) and SYBR® Premix Ex Taq™(Tli RNaseH Plus) were also bought from the TaKaRa, Japan. All the other reagents were analytical grade.

2.2 Cell Culture

The human placenta-derived JEG cell line was purchased from the BNCC, China. The cells were maintained in MEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (NQBB, Australia). Cells were passaged when they grew to about 80% confluency by trypsinizing with 0.25% Trypsin (Solarbio, China), and grown in the 37 °C humid incubator at 5% CO2 for about 5 min.

2.3 Cell Viability

We applied the MTT assay to determine the impact of BDE 209 on JEG cell viability, the experiment was based on a literature method (Tang et al. 2004) with slight modifications. JEG cells in good condition were seeded at a density of 8 × 103 cells per well in a 96-well plate and incubated at 37 °C, 5% CO2 incubator for 24 h, then treated with BDE 209 or the vehicle control for 24 h. The supernatant was removed

and 100 μl of MTT application solution (0.5 mg/ml) was added in serum-free medium to each well. After incubating at 37 °C for 4 h, the MTT application solution was gently discarded and replaced with 100 μl DMSO in each well. The cells were incubated without light for 1 h to dissolve crystallization, and analyzed on the Multiskan Ascent (Thermo Fisher Scientifc, MA USA) for the OD value of each well at the wavelength of 595 nm.

2.4 Western Blot Analysis of Dio 3 Protein

JEG cells were seeded at a density of 2 × 106 cells/dish in a 10 cm dish, placed in a 37 °C, 5% CO2 incubator for 24 h, and exposed to BDE 209 or vehicle control for 24 h. The RIPA (Beyotime, China) of 1 mM PMSF was used to extract total cellular protein, and after adding 5 × loading buffer, the protein was denatured for 5 min in the boiling water. Protein was separated by SDS-PAGE (12% gel, 100 V, 70 min), and then proteins were transferred to the activated PDVF membrane (220 mA, 120 min). After blocking the membrane for 2 h in 5% skim milk, PDVF blots were incubated with rabbit-derived anti-Dio3 antibody or mouse-derived anti-β actin antibody as primary antibody, Overnight (4 °C). After washing with the Tween 20 in TBS (0.1%, v/v), incubate for 2 h with anti-rabbit antibody or anti-mouse antibody as secondary antibody at room temperature. Western blot images were captured with a UVP chemiluminescent gel imaging system (USA). Image bands were analyzed with the Image J software.

2.5 Dio 3 mRNA Expression Measured by qRT-PCR

Treatment of JEG cells was the same as mentioned in the methods 2.4. Total RNAs were extracted with RNAiso Plus per the manufacturer's instructions (Takara, Japan). And 800 ng of total RNA from each sample was reversed transcribed into 20 μl cDNA with PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time). Two μl cDNA was amplified on a Thermal Cycler Dice Real Time System with SYBR Premix EX Taq. GAPDH was used to normalize Dio 3 mRNA expression. The primers for qPCR were as follows: GAPDH-F: 5'-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-R: 5'-TGGTGAAGACGC CAGTGGA-3′; Dio3-F: 5'-CTTCGAGCGTCTCTATGTCATCCA-3′, Dio3-R: 5'-TGCAGTTGCTCATCATAGCGTTC-3′. The PCR conditions: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of PCR reaction (95 °C for 5 s and 60 °C for 30 s). We calculated the CT value of each group and derived the relative expression of Dio 3 mRNA using 2-ΔΔct method.

2.6 Statistical Analysis

Statistical analysis was conducted by the GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The data were typically expressed as mean ± (SEM). The differences were evaluated by the one-way analysis of variance (ANOVA) followed by the Dunnett's Test and the Newman-Keuls Test, and the significance was detected as treatment-related at a p value <0.05.

3 Results

3.1 Toxic Effect of BDE 209 on JEG Cells Cultured Under the Normal Condition

3.1.1 Effect of BDE 209 on the Proliferation of the JEG Cells

The proliferation of JEG cells is shown in Fig. 1. Compared to the medium-control group (0), no change was found in the DMSO-control group (1‰ DMSO, v/v), then the cell viability of the BDE 209-treated group gradually decreased, which was reduced by about 40% at 3 μM BDE 209 ($p < 0.05$).

3.1.2 Effect of BDE 209 on Dio 3 Protein Expression in the JEG Cells

BDE 209 can affect the expression of Dio 3 protein in JEG cells, as shown in Fig. 2. The results were consistent with the cell viability results. Basically, the expression of Dio 3 protein was dose-related to BDE 209 exposure, and significantly decreased in the 3 μM-dose group ($p < 0.01$). However, it is worth noting that Dio 3 protein expression was down-regulated by nearly 40% in the 1 μM-dose group.

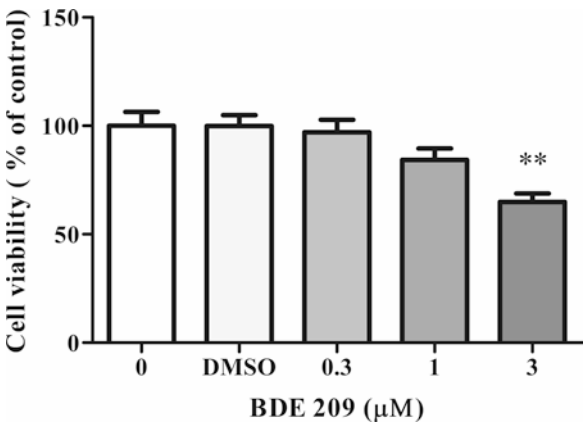

Fig. 1 BDE 209-induced cytotoxicity in JEG cells. ∗p < 0.05, compared to the control (0)

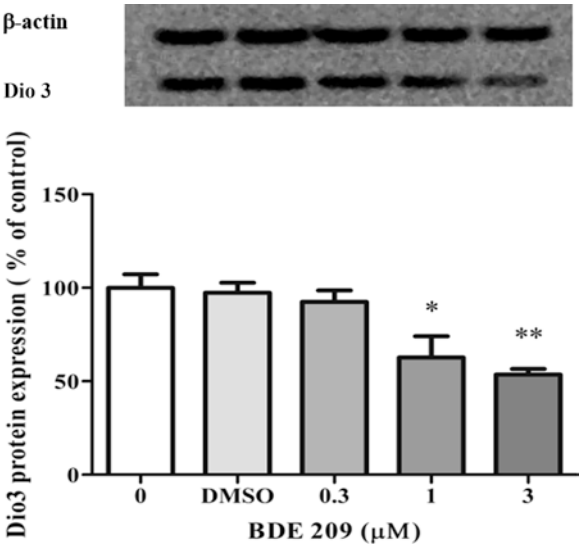

Fig. 2 Effect of BDE 209 on Dio3 protein expression in JEG cells. $*p < 0.05$, $**p < 0.01$, compared to the control (0)

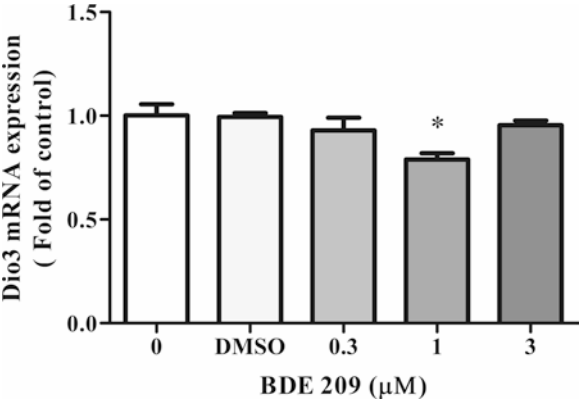

Fig. 3 Effect of BDE 209 on Dio3 mRNA expression in JEG cells. $*p < 0.05$, compared to the control (0)

3.1.3 Effect of BDE 209 on the Dio 3 mRNA Expression in the JEG Cells

As can be seen from Fig. 3, the results of Dio 3 mRNA expression were slightly different from those of the protein. The expression of Dio 3 mRNA was down-regulated in each group, but the decrease was only dose-related in the lower dose group (0.3 μM and 1 μM) of BDE 209. And it was significantly decreased in the 1 μM BDE 209 exposed group ($p < 0.05$).

3.2 Protection of Taurine on JEG Cells Exposed to BDE 209

Combining all the results of JEG exposure to BDE 209 in vitro (3.1), we selected a more stable BDE 209-exposed dose (1 μM) for subsequent taurine protection studies to investigate the improvement of JEG toxicity induced by taurine on BDE 209 exposure. JEG cells were pretreated with 1, 3 and 9 mM of taurine for 24 h prior to 1 μM BDE 209 for 24 h.

3.2.1 Effect of Taurine on Cytotoxicity in JEG Cells Exposed to BDE 209

From Fig. 4 we can see that taurine intervention can protect the proliferative activity of BDE 209-exposed cells at a dose-dependency, with 9 mM taurine that essentially restored the viability to the DMSO-control groups' level ($p < 0.001$).

3.2.2 Effect of Taurine on Dio 3 Protein Expression of JEG Cells Exposed to BDE 209

Figure 5 suggested that Taurine can reverse the inhibition of cell Dio 3 protein expression by BDE 209. it is showed as the dose of taurine increased, Dio 3 protein gradually recovered, and there is a significant increasing with 9 mM taurine intervention ($p < 0.05$).

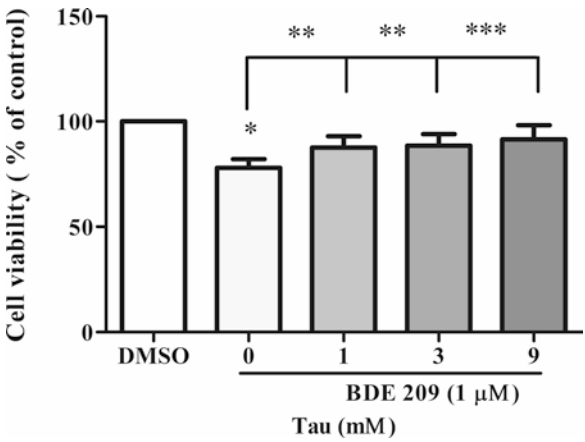

Fig. 4 Protection of taurine on the viability of JEG cells following BDE 209 treatment. ∗$p < 0.05$, ∗∗$p < 0.01$ and ∗∗∗$p < 0.001$, with respect to the DMSO control cells or the BDE 209 exposed-control cells (0)

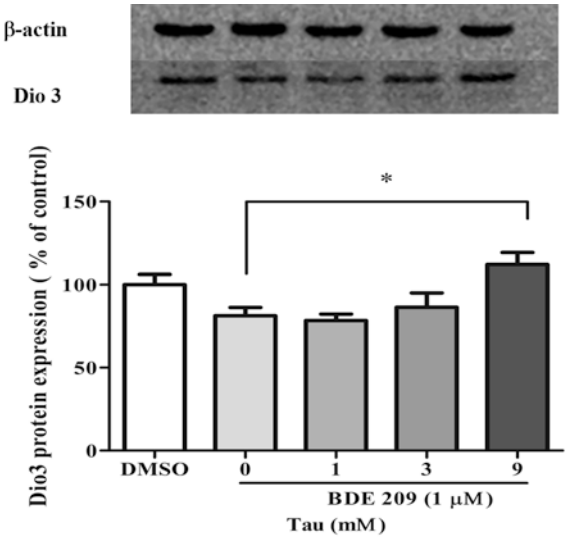

Fig. 5 Protection of taurine on Dio3 protein expression of JEG cells following BDE 209 treatment. $*p < 0.05$, with respect to the BDE 209 exposed-control cells (0)

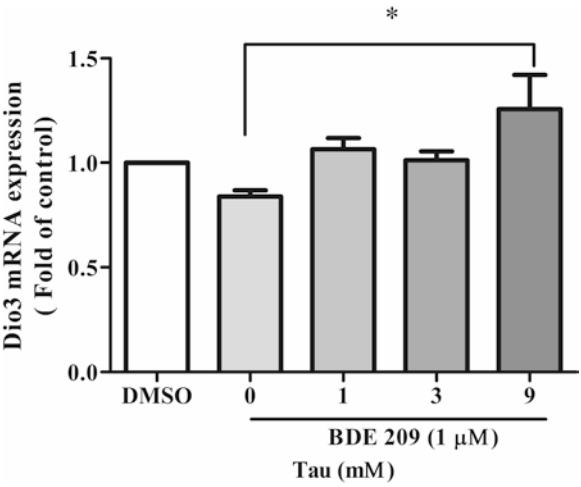

Fig. 6 Protection of taurine on Dio3 mRNA expression of JEG cells following BDE 209 treatment. $*p < 0.05$, with respect to the BDE 209 exposed-control cells (0)

3.2.3 Effect of Taurine on Dio 3 mRNA Expression of JEG Cells Exposed to BDE 209

The regulation of Dio 3 mRNA expression by taurine is consistent with the protein. 1 mM and 3 mM taurine intervention allowing it to reach control levels, while 9 mM caused Dio 3 mRNA expression to exceed the control ($p < 0.05$) (Fig. 6).

4 Discussion

PBDEs are a class of additive flame retardants that have been used extensively since the 1970s. They are easily released from the finished product and are abundant in the environment. It is of great concern because of their potential toxicity such as liver toxicity, embryo toxicity, and thyroid dysfunction. It was listed as a persistent organic pollutant by the Stockholm Convention in 2009, and many regions have also restricted the production and use of various homologues of PBDE. However, PBDEs will continue to exist in the environment for decades because they have a long half-life and are not easily degraded in the environment. A number of epidemiological investigations have shown that prenatal exposure to PBDE may be one of the factors in the developmental abnormality of offspring. The placenta is the important hub for connecting mother and child during pregnancy. Therefore, it is extremely important to investigate the cytotoxicity of PBDEs on placental cells in early pregnancy (early neurodevelopment).

In the present study, a reduction in the viability of placenta-derived JEG cells exposed to BDE 209 indicates that PBDEs has a cytotoxic effect on placental cells at low concentrations, it is consistent with a study: DE-71 can cause the apoptotic cell death at 7 μM (Vuong AM et al. 2018). Many research findings have indicated that PBDEs may cause damage to the intrauterine thyroid hormone balance environment required for early embryonic neural development by affecting placental deiodinase activity, and the results of BDE 209 inhibition Dio 3 expression of JEG cell in this study seem to make this hypothesis more Persuasive. Oxidative stress is the main pathway for PBDEs to induce cytotoxicity in hepatocytes and neurons in vitro. Recently, it was found that BDE 47 can induce Oxygen (ROS) produced and the expression of IL-6 in human placental trophoblast cells, and deiodinase activity requires thiol groups associated with oxidation, so redox homeostasis may be an important factor in maintaining and regulating cell deiodinase activity.

Being an essential amino acid in the nervous system development, taurine also participates in many important physiological functions, including bile salt conjugation, osmoregulation, membrane stabilization, calcium modulation, anti-oxidation, and immunomodulation (Shigeru Murakami 2015). Among them, we are most concerned about antioxidants. It has been reported that taurine can exert immunomodulatory effects by inhibiting ROS production (Schuller-Levis GB and Park E. 2004), Kenneth N. Maclean et al. (Kenneth N. Maclean et al. 2018) found that taurine can regulate the balance of thiol and sulfinic acid metabolism to prevent the disorder of liver γ-glutamyl cycle and methylglyoxal metabolism observed in the classical homocystinuria mouse model. Taurine may improve the biological toxicity of BDE 209 by reducing ROS production and regulating thiol metabolism. This study observed that taurine pretreatment can improve the cell viability caused by exposure to BDE 209 in JEG cells and up-regulate the expression of cell Dio 3 inhibited by BDE 209. This study only made a preliminary exploration of the possible protection of taurine, and the precise mechanism needs further exploration.

5 Conclusion

In this study, we observed the phenomenon that taurine showed corresponding protection in BDE 209-induced cell proliferation damage and abnormal expression of Dio 3. Whether BDE 209 and taurine affect the expression of deiodinase by regulating various oxidative damage factors, this question needs further exploration.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81773389/H2601 and 81273031/H2601 to JS; Dalian Tech Stars Programme (2016RQ044 to XL) and China's Post-doctoral Science Fund (2016M591438 to XL).

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The Effect of Drug Pre-treatment on Taurine Transport at the Inner Blood-Retinal Barrier Under Variable Conditions

Asmita Gyawali and Young-Sook Kang

Abstract Taurine is essential for the development and function of the central nervous system, retina, and cardiovascular system. It is a naturally occurring amino acid, abundantly found in the retina. It has been shown to exhibit antioxidant, neuroprotective, and osmoregulatory functions in the retina. We used conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB), *in vitro*, to investigate the effects of oxidative stress, high glucose (HG) and hypertonic conditions on taurine transport. TR-iBRB cells pre-treated with tumor necrosis factor alpha (TNF-α) showed a significant increase in [^{3}H]taurine uptake rate, which, however, decreased when treated with taurine (50 mM). Addition of paeonol and propranolol to TNF-α pre-treated cells had no significant effect on [^{3}H]taurine uptake, but the addition of 10 mM taurine caused a reduction. The uptake rate decreased under HG conditions, in contrast to that under hypertonic conditions. [^{3}H]Taurine uptake increased with pre-incubation time. Additionally, uptake of [^{3}H]taurine and mRNA expression of taurine transporter (TauT) decreased significantly under hypertonic and HG conditions, following pre-incubation with 10 mM taurine, 1 mM paeonol, and 0.1 mM propranolol. [^{3}H]Taurine uptake was significantly inhibited in the presence of taurine transporters such as taurine and β-alanine. Results indicate that oxidative stress and hypertonic conditions increased taurine uptake in iBRB cell lines, whereas HG conditions reduced the uptake rate. Taurine may be useful in stabilizing the microenvironment in cells affected by oxidative stress as well as hypertonic and HG conditions. Moreover, taurine may play a key role in maintaining taurine concentrations in the taurine transporter system of retinal cells.

Keywords Taurine · Inner blood-retinal barrier · Hypertonic · High glucose · TNF-alpha · Paeonol · Propranolol

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Abbreviations

iBRB	Inner blood retinal barrier
RT-PCR	Real time polymerase chain reaction
HG	High glucose
Taut	Taurine transporter
TNF-α	tumor necrosis factor alpha

1 Introduction

Taurine protects photoreceptor cells and regulates calcium transport and signal transduction, by inhibiting protein phosphorylation (Lombardini 1991). Taurine concentration in the retina is 100 times more than that in the serum, suggesting a role for taurine in the retina. Insulin resistance and failure of pancreatic β cells characterize type 2 diabetes mellitus (T2D). Taurine regulates glucose and lipid homeostasis under normal, pre-diabetic, and diabetic conditions (Borck et al. 2018). Diabetes is associated with activation of aldose reductase (AR), hyperglycemia, intracellular osmotic stress, oxidative stress, and tissue damage (Nakashima et al. 2005). In addition, taurine may be used as a therapeutic supplement to prevent type-I and II diabetes, and enhance osmoregulation in the retina. Taurine significantly decreased blood glucose levels and related mortality rates, as well as symptoms of heart failure, whereas commonly used anti-diabetic drugs did not reduce mortality rates related to diabetes (Chen et al. 2016). Similarly, taurine may play a key role in the regulation of cell volume, osmoregulation, and membrane stabilization also act as a neurotransmitter, neuromodulator and an anti-oxidant (Olson and Martinho 2006). It has been identified as a strong osmolyte with hypertonicity, and a signal inducer for the taurine transporter system in the glial cells, renal cells and epithelial cells (L'Amoreaux 2012).

The retina, which is composed of neural tissue, has a blood retinal barrier (BRB) which is essential for vision. BRB plays a key role in maintaining retinal functions and homeostatic regulation of the nutrient supply to the retina from blood (Kubo et al. 2016). Based on structural and functional properties, taurine transporter (TauT) is categorized as a member of the solute carrier (slc6a) family and functions as a Na^+ and Cl^- dependent neurotransmitter transporter (Chen et al. 2004). The influx and efflux of solutes such as taurine is mediated by TauT. Hypertonicity upregulates and HG down regulates TauT in several cell types such as astrocytes, hepatocytes and retinal cells (El-Sherbeny et al. 2004).

In previous study, already investigated the change of taurine transport in variable conditions (Kang et al. 2009). And in the present study investigated characteristics of the taurine transport system under hypertonic, HG and oxidative stress conditions following pre-treatment with the drugs: taurine, paeonol, and propranolol, using (TR-iBRB) cell lines, *in vitro*. Furthermore, mRNA expression under those conditions was also used to elucidate regulating mechanisms. Our results indicated that the taurine transporter was upregulated under hypertonic and oxidative stress conditions and down regulated under HG condition in TR-iBRB cells.

2 Methods

2.1 Cell Culture

TR-iBRB cells (provided previously from Prof. T. Terasaki) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA); supplemented with 10% fetal bovine serum (FBS); (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Grand Island, NY, USA). Cells were seeded on type I collagen coated culture dishes (Biocoat, Kennebunk, ME, USA) incubated at 33 °C, and cultured in a humidified atmosphere of 5% CO_2 and 95% air as described previously (Kang et al. 2002).

2.2 In Vitro *[^{3}H]Taurine Uptake Study in TR-iBRB Cells*

[^{3}H]Taurine uptake at the inner blood retinal barrier cell lines was evaluated, as previously reported (Kang et al. 2009; Jung et al. 2013). In addition, iBRB cells (1×10^5 cells/well) were seeded on type I collagen coated 24 well plates (Biocoat, Kennebunk, ME, USA) and the uptake was carried out by washing with extracellular fluid (ECF) buffer (pH 7.4) at 37 °C. In addition, 200 μL ECF buffer containing [^{3}H]taurine (5.2 nM) at 37 °C, was used both in the presence and absence of inhibitors (1 mM except β-alanine). Following the designated time period (5 min), uptake was terminated by removing the solution and washed with 1 mL ice-cold ECF buffer (Tamai et al. 1995). Next, the cells were dissolved in 1 N NaOH overnight at room temperature. The following day, measurement of radioactivity and protein assay was conducted using a liquid scintillation counter (LS6500, Beckman Instruments Inc., Fullerton, OH, USA), and DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard (Kang et al. 2002). To investigate the effect on [^{3}H]taurine under normal, hypertonic, HG and oxidative stressed conditions by iBRB cells under pre-treatment conditions for 24 h and 4 h respectively. The pre-treatment was done with normal media (280 mOsm/kg) which was added to 100 mM of sucrose to produce hypertonic conditions (390 mOsm/kg) and to 25 mM of glucose to produce HG conditions, were used for pre-incubation (Kang et al. 2009). iBRB cells were pre-incubated with cold taurine (10 mM), paeonol (1 mM), and propranolol (100 μM) as necessary, to evaluate the effects of these compounds under hypertonic, HG and oxidative stressed conditions.

In addition, the cell-to-medium ratio (μl/mg protein) was calculated by using the following equation:

$$\text{Cell / medium ratio} = \frac{\left[3\text{H}\right]\text{dpm per cell protein}\left(\text{mg}\right)}{\left[3\text{H}\right]\text{dpm per}\,\mu L\,\text{medium}} \quad (1)$$

2.3 Real-Time Reverse Transcription Polymerase Chain Reaction

For gene silencing purposes, TR-iBRB cell lines were seeded on type-I collagen coated 6 well plates (3×10^5cells/well) and incubated for 24 h at 33 °C. Total cellular RNA was extracted by washing the cells with phosphate-buffered saline (PBS). The cells were pre-treated for 24 h under isotonic, hypertonic and HG conditions with or without inhibitors for 4 h under oxidative stress (TNF-α) conditions. Isolation of total RNA from cultured cells was carried out using the RNeasy kit (Qiagen, Velencia, CA, USA) according to manufacturer's guidelines. Single-stranded cDNA was prepared from 1.0 μg of total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using rat TauT or GAPDH-specific primers in the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocols. Polymerase chain reactions were investigated using a gene amplification system (MyCycler, BioRad Inc., Hercules, CA, USA). (Lee and Kang 2015).

2.4 Statistical Analysis

Unless otherwise indicated, all data are presented as mean ± SE. Statistically significant differences were calculated using the unpaired two-tailed student's t-test. A Significance level of $p < 0.05$ was used.

3 Results

3.1 Inhibitory Effect of Various Compounds on [^{3}H]Taurine Uptake by TR-iBRB Cells

To characterize the taurine transport system of iBRB cells, compounds listed in Table 1 were used to examine their inhibitory effect on [^{3}H]taurine uptake, *in vitro*. Taurine transporter inhibitors such as, 1 mM taurine and 0.5 mM B-alanine strictly inhibited [^{3}H]taurine uptake by 97% and 89% respectively. Verapamil, paeonol, clonidine, and gamma amino butyric acid (GABA) also decreased [^{3}H]taurine uptake by 85%, 58%, 40% and 53% respectively. In contrast, other compounds such as imperatorin, propranolol, acetyl L-carnitine (ALC), citrulline, L-leucine, probenecid, para-aminohippuric acid (PAH) and tetraethyl ammonium (TEA) made no marked difference to [^{3}H]taurine uptake (Table 1).

Table 1 Effects of various compounds on [^{3}H]taurine uptake by TR-iBRB cells

Compounds	Conc. (mM)	Uptake (% of control)
Control		100 ± 3
+Taurine	1	3.2 ± 0.1***
+β-alanine	0.5	10.9 ± 0.9***
+Paeonol	1	42.6 ± 4.0***
+Clonidine	1	60.0 ± 2.4***
+Verapamil	1	14.8 ± 9.0***
+GABA	1	46.7 ± 2.6***
+Imperatorin	1	91.0 ± 3.9
+Propranolol	1	94.0 ± 0.9
+ALC	1	90.3 ± 6.5
+Citrulline	1	85.0 ± 10
+L-leucine	1	93.2 ± 7.1
+Probenecid	1	95.7 ± 8.2
+PAH	1	112 ± 6
+TEA	1	111 ± 5

[^{3}H]Taurine uptake was performed in the absence (control) or presence of 1 mM of inhibitor for 5 min at 37 °C. Each value represents the mean ± SE (n = 3–4)
***$p < 0.001$, significantly different from the control

3.2 Time Course Effect of Taurine and TNF-α on [^{3}H]Taurine Uptake at the TR-iBRB Cells

Taurine transport activity in TR-iBRB cells was examined *in vitro*. Unlabeled taurine at a concentration of 50 mM and 20 ng/mL TNF-α were used for pre-treatment for 24 h. [^{3}H]Uptake rate decreased significantly with increasing pre-treatment time in the presence of taurine. However, although the uptake rate increased until and up to 4 h after pre-treatment with TNF-α, there was no increase in the uptake rate after 4 h (Fig. 1).

3.3 Time Course Effect of Hypertonicity and HG on [^{3}H] Taurine Uptake in TR-iBRB Cells

To investigate the effect of hypertonic (390 mOsm/kg) and HG (25 mM) conditions, taurine uptake in iBRB cells was examined *in vitro*. [^{3}H]Taurine uptake in TR-iBRB cells was significantly increased and decreased during 24 h of incubation with hypertonic media and HG media respectively (Fig. 2).

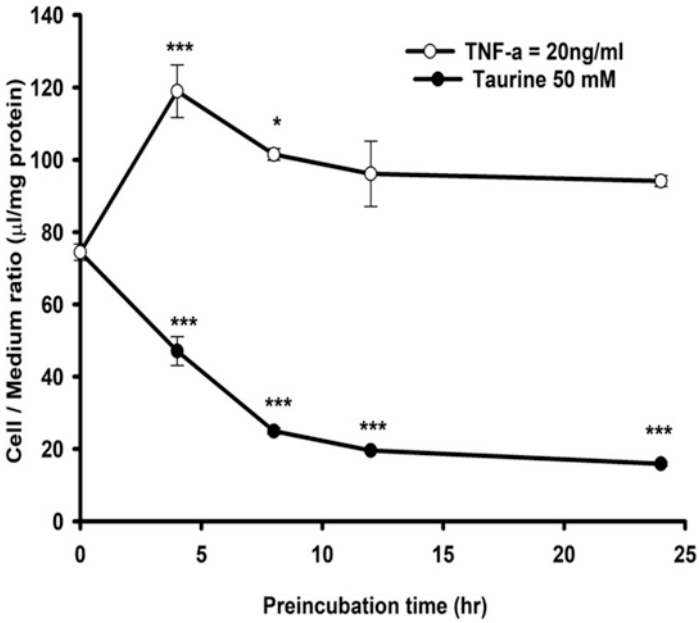

Fig. 1 Time-course effect of TNF-α or unlabeled taurine pre-treatment on [^{3}H]taurine uptake by TR-iBRB cells at 37 °C for 5 min. Cells were pre-treated with 20 ng/mL TNF- α (open circles), or 50 mM unlabeled taurine (closed circles) for the time periods indicated in the figure. Each time point represents the mean ± SE (n = 3–4). $^*p < 0.05$, $^{**}p < 0.001$ significantly different from time = 0

3.4 Cold Inhibition Effect of Taurine on the Hypertonicity, High Glucose and TNF-α Conditions

[^{3}H]Taurine uptake in iBRB cells was examined under various conditions after pre-treatment. Cells were pre-incubated under hypertonic, high glucose and oxidative stress conditions for 24 h and 4 h respectively. After pre-incubation, [^{3}H]taurine was evaluated following pre-treatment with unlabeled 10 mM taurine as a cold inhibitor. Cold inhibition by 10 mM taurine caused [^{3}H]taurine uptake to significantly drop by more than 95% in 5 min under all conditions (Fig. 3).

3.5 The Pre-treatment Effect of Taurine, Paeonol and Propranolol of Taurine Transport Function at Normal Condition

[^{3}H]Taurine uptake in TR-iBRB cells was monitored to investigate the effect of unlabeled taurine (10 mM), paeonol (1 mM) and propranolol (100 μM) under normal conditions. Similarly, to clarify the transport system of taurine under normal

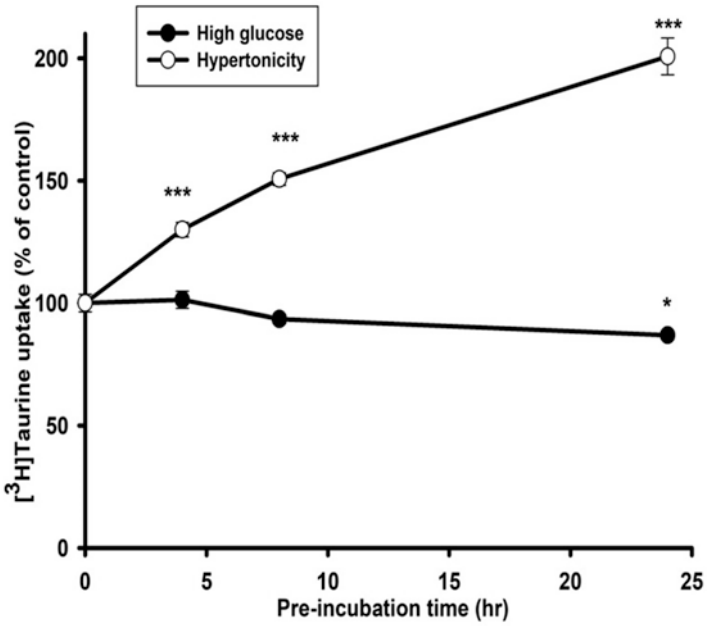

Fig. 2 [^{3}H]Taurine uptake by TR-iBRB2 cells. Uptake was performed for 5 min at 37 °C after pre-incubation in hypertonic culture medium (open circles) or high glucose culture medium (closed circles). Each point represents the mean ± SE (n = 4). $^{***}p < 0.001$ and $^{*}p < 0.05$, significantly different from control

conditions, mRNA expression level was evaluated during 24 h after pre-treatment. The uptake of [^{3}H]taurine was reduced by 67%, 30% and 20% following pre-treatment with unlabeled taurine, paeonol and propranolol for 24 h under normal conditions (Fig. 4a). The expression of TauT mRNA was reduced by 43%, 59% and 19% under normal conditions for 24 h following pre-treatment with taurine, paeonol and propranolol respectively (Fig. 4b).

3.6 Effect of Taurine Uptake and mRNA Expression Level at TNF-α Condition in TR-iBRB Cells

The pre-treatment effect of TNF-α in the presence of 10 mM taurine, 1 mM paeonol, and 0.1 mM propranolol on the rate of [^{3}H]taurine uptake and mRNA expression in TR-iBRB cells was investigated. Both mRNA expression and [^{3}H]taurine uptake showed a significant increase of more than 120% in the TNF-α pre-treated cells 4 h after treatment. Similarly, following the addition of taurine, paeonol and propranolol to the TNF-α containing media, a significant decrease was noticed only in taurine in the TNF-α containing media. Propranolol and paeonol did not show

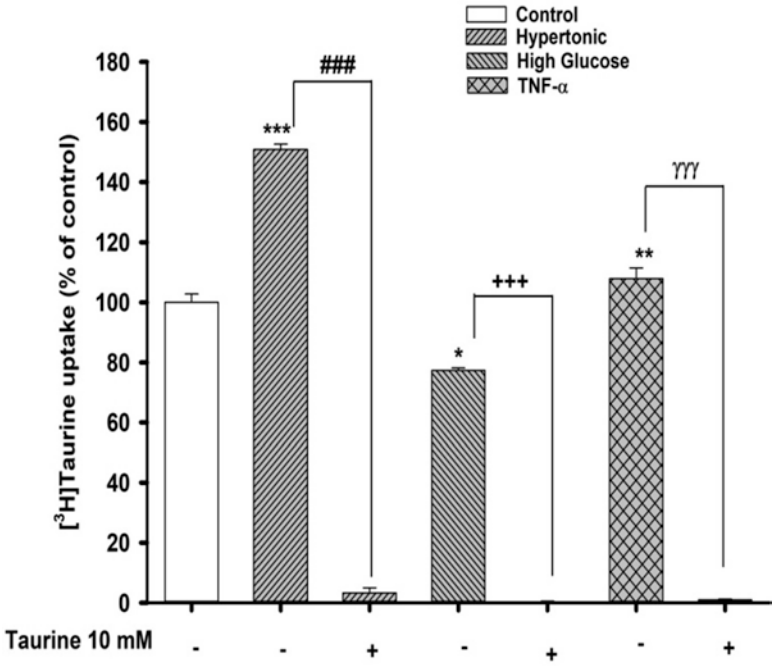

Fig. 3 TR-iBRB cells were pre-incubated with hypertonic media, high glucose media, or TNF- α containing media for 24 h and 4 h respectively. After a designated pre-incubation time, the uptake of [^{3}H]taurine was performed in the presence of unlabeled taurine (10 mM) for 5 min at 37 °C. Each column represents the mean ± SE (n = 3–4). $^{*}p < 0.05$ and $^{***}p < 0.001$, significantly different from the control; $^{\#\#\#}p < 0.01$, significantly different from the hypertonic media; $^{+++}p < 0.001$, significantly different from the high glucose media; $^{\gamma\gamma\gamma}p < 0.001$, significantly different from the TNF- α media

any significant effect. However, mRNA expression and [^{3}H]taurine uptake value was markedly reduced in the pre-treated cells for 4 h in the presence of TNF-α together with taurine, paeonol and propranolol (Fig. 5).

3.7 The Pre-treatment Effect of Taurine, Paeonol and Propranolol of Taurine Transport Function at Hypertonic Condition

To clarify the transport system of taurine under hypertonic conditions, the [^{3}H]taurine uptake and mRNA expression were monitored for 24 h following pre-treatment. [^{3}H]taurine uptake and mRNA expression under hypertonic condition (390 mOsm/

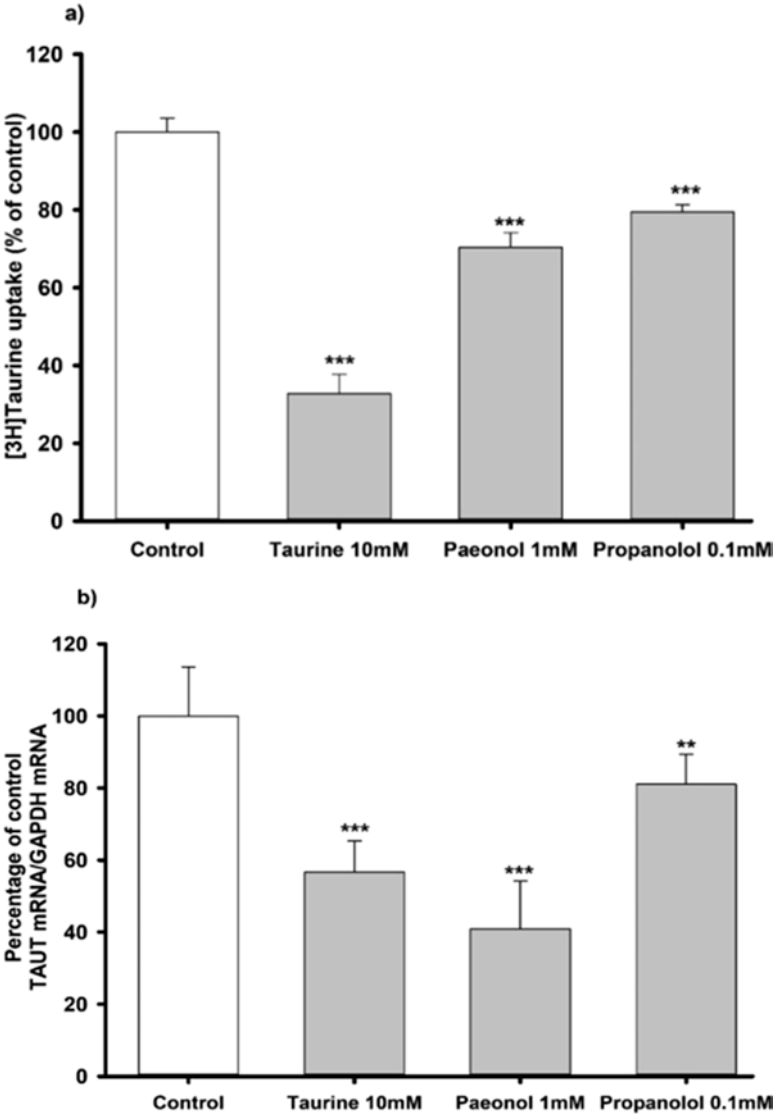

Fig. 4 (**a**) Regulation of taurine transport and (**b**) TAUT mRNA expression in TR-iBRB cells. Cells were cultured with a pre-treatment of unlabeled 10 mM taurine, 1 mM paeonol, and 0.1 mM of propranolol for 24 h. [^{3}H]Taurine uptake was performed for 5 min at 37 °C. Each column represents the mean ± SE (n = 3–4). $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different from the control

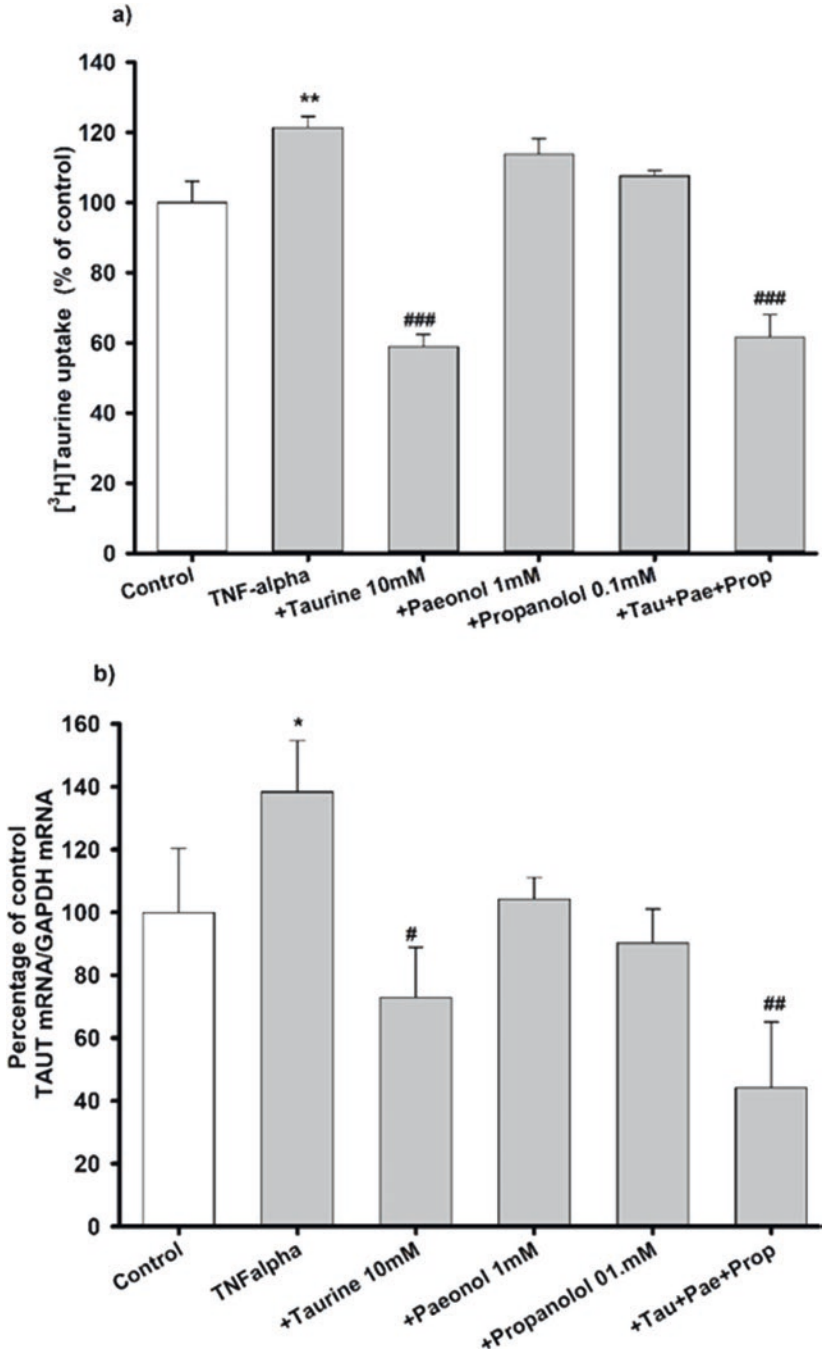

Fig. 5 (**a**) The rate of [^{3}H]taurine uptake and (**b**) mRNA expression level in TR-iBRB cells. Cell were pre-treated with 20 ng/ml TNF-α, 10 mM of taurine, 1 mM of paeonol, and 0.1 mM propranolol for 4 h. [^{3}H]taurine uptake was performed for 5 min at 37 °C. Each column represents the mean ± SE (n = 4–5). $^{**}p < 0.01$, significantly different from the control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ significantly different from TNF-α

kg), showed a significant increase compared to that under isotonic conditions (280 mOsm/kg). Exposure of TR-iBRB cells to the hypertonic condition, for 24 h, following treatment with 10 mM taurine, 1 mM paeonol, 0.1 mM propranolol, caused taurine uptake and mRNA expression level to decrease significantly, compared to the hypertonic conditions (Fig. 6).

3.8 The Pre-treatment Effect of Taurine, Paeonol and Propranolol of Taurine Transport Function at High Glucose Condition

[^{3}H]Taurine uptake and mRNA expression under HG conditions was investigated. The cells pre-treated with HG conditions were treated with taurine, paeonol, and propranolol and evaluated for 24 h for both mRNA and [^{3}H]taurine uptake. Studies demonstrated that the taurine uptake rate was decreased by 87% and mRNA expression level reduced by 84% under the high glucose condition. HG condition combined with taurine, paeonol and propranolol pre-treated cells showed a significant reduction in the [^{3}H]taurine uptake rate by 71%, 33%, and 21% and, in mRNA expression level by 43%, 34% and 35% respectively, compared to the HG condition alone (Fig. 7).

4 Discussion

The present *in vitro* study demonstrated that taurine transport in the inner blood-retinal barrier (iBRB), under oxidative stress, hypertonic and HG conditions, is regulated by the taurine transporter (TauT) system. TR-iBRB cells play a crucial role as a tool for screening the transport of compounds to the retina. TauT is described as a Na^+ and Cl^- dependent transporter. [^{3}H]Taurine uptake was time and concentration dependent with a K_m value of 22.2 μM, and is regulated by a low affinity (22.2 μM) transporter in iBRB cells (Kang et al. 2009; Tomi et al. 2006).

Uptake of [^{3}H]taurine was inhibited by more than 90% in the presence of typical taurine substrates, taurine, and *β*-alanine. These observations suggest that taurine uptake by retinal endothelial cells may occur via a carrier mediated process (Tamai et al. 1995). Similarly, in the presence of verapamil, a novel organic cationic substrate/calcium channel blocker, [^{3}H]taurine uptake was reduced by more than 85%. Taurine transport was affected by extracellular Ca^{2+} and Ca^{2+} calcium channel blocker. Paeonol and clonidine, which are novel organic cationic substrates, also reduced the rate of [^{3}H]taurine uptake in iBRB cells. Furthermore, GABA is a substrate of TauT, and GABA 1 mM inhibited [^{3}H]taurine uptake by 53%. (Tomi et al. 2008). However, novel organic cationic substrates such as imperatorin, propranolol, acetyl L-carnitine (ALC) and other compounds probenecid, L-leucine and citrulline had no effect on [^{3}H]taurine uptake by TR-iBRB cells (Table 1).

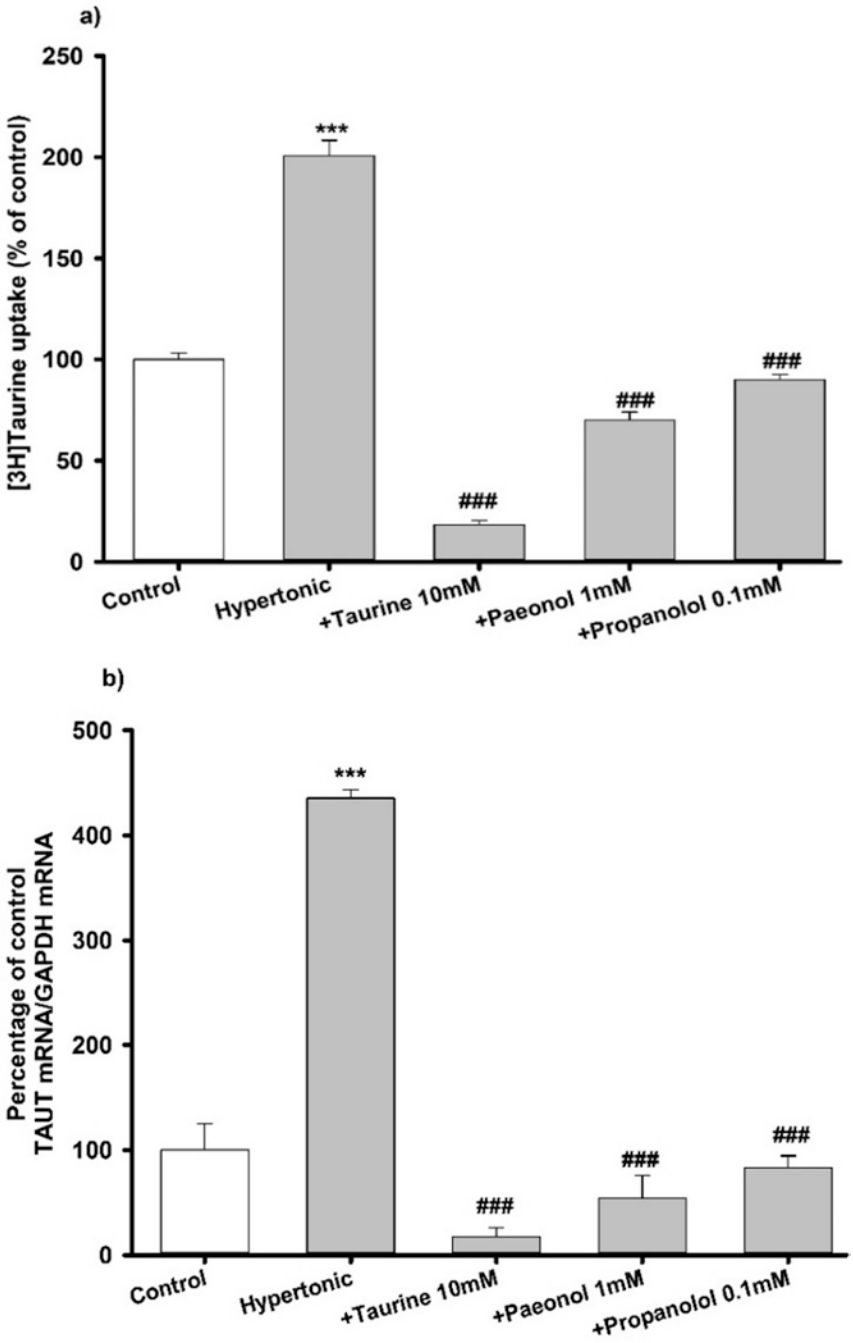

Fig. 6 (**a**) Regulation of taurine transport and (**b**) TAUT mRNA expression in TR-iBRB cells. Cells were cultured under hypertonic conditions and pre-treated with 10 mM taurine, 1 mM paeonol, and 0.1 mM of propranolol for 24 h. [^{3}H]Taurine uptake was performed for 5 min at 37 °C. Each column represents the mean ± SE (n = 3–4). $^{***}p < 0.001$, significantly different from the control; $^{###}p < 0.001$ significantly different from hypertonic condition

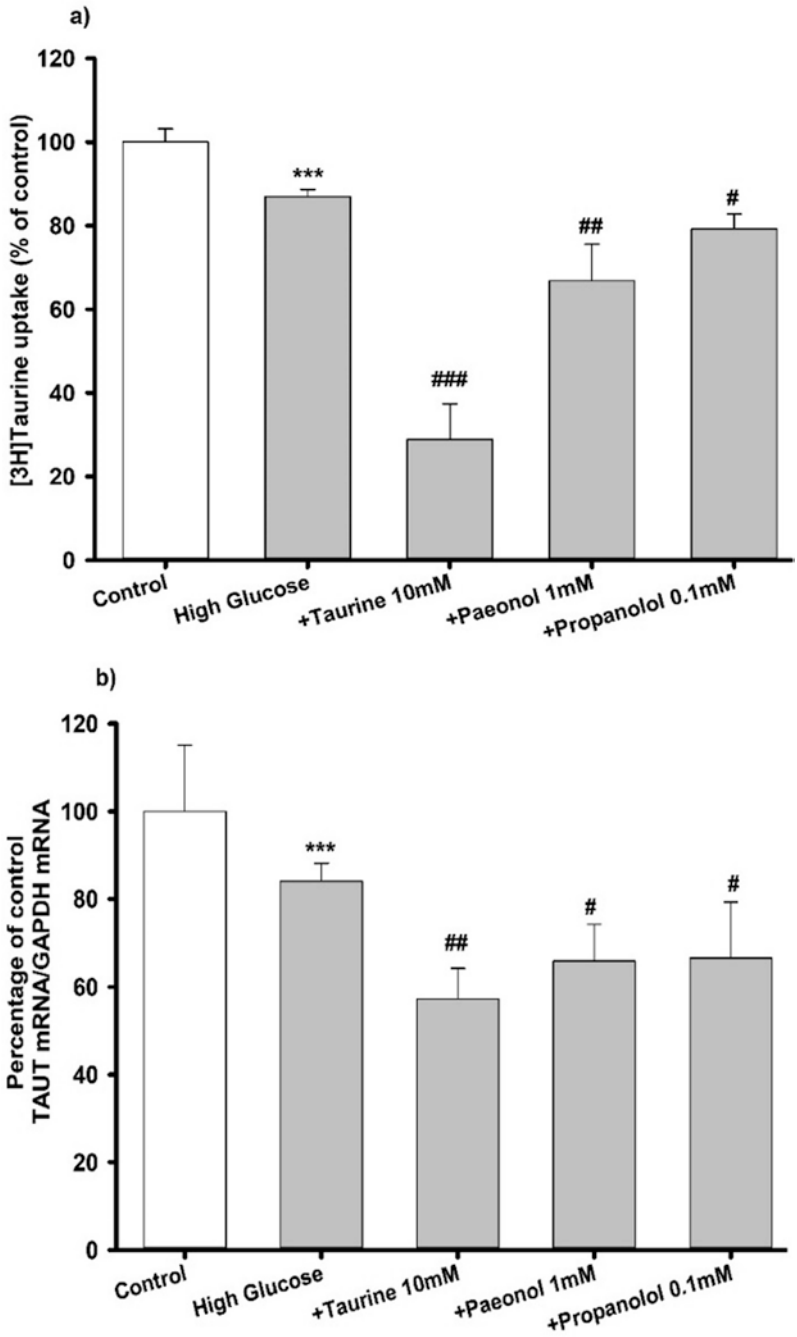

Fig. 7 (**a**) Regulation of taurine transport and (**b**) TAUT mRNA expression in TR-iBRB cells. Cells, cultured under high glucose conditions, were pre-treated with unlabeled 10 mM taurine, 1 mM paeonol, and 0.1 mM of propranolol for 24 h. [^{3}H]Taurine uptake was performed for 5 min at 37 °C. Each column represents the mean ± SE (n = 3–4). $^{***}p < 0.001$, significantly different from the control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, significantly different from high glucose conditions

[^{3}H]Taurine uptake was evaluated for 24 h, using cells pre-treated with unlabeled taurine 50 mM and TNF-α 20 ng/mL. The uptake rate was markedly reduced in the taurine 50 mM pre-treated cells but in the TNF-α 20 ng/mL pre-treated cells the uptake rate remained unchanged after 4 h of incubation (Fig. 1). TNF-α is a pro-inflammatory cytokine induced by cell damage. Therefore, results indicate that oxidative stress inducing agents such as TNF-α may increase taurine uptake. However, 4 h after pre-treatment with cold taurine 50 mM, taurine uptake rate was markedly suppressed by 39% in the iBRB cell line. This may be due to an increase in the concentration of taurine in the retina being suppressed by [^{3}H]taurine transport activity (Lee and Kang 2004; Kang et al. 2002). Time course effect of hypertonic and HG conditions on [^{3}H]taurine uptake by TR-iBRB cells resulted in an increase and a decrease in the taurine uptake rate, respectively, with pre-treatment time. Taurine acts as an antioxidant and osmolyte, and the taurine transport system may likely be involved in osmoregulation. Therefore, the induction effect may follow osmolality, where, under hypertonic stress conditions, cells initially shrink and the volume subsequently recovers during uptake (Fig. 2) (Yahara et al. 2010; Lee and Kang 2013). TR-iBRB cells were pre-incubated with hypertonic, HG and TNF-α containing media for 24 h and 4 h, after which pre-incubation 10 mM taurine was used for the uptake study. The results indicated a marked inhibition of more than 95% in taurine uptake, because the presence of taurine suppressed the uptake rate (Fig. 3). The TNF-α signal activates NF-KB transcriptional activity by nuclear translocation and therefore the TNF-α-NF-KB pathways may be involved in TauT induction. However, taurine uptake level was increased by other factors such as TNF-α and hypertonicity. High concentration of taurine suppressed the taurine uptake rate in iBRB cells (Kang 2006).

Taurine is physiologically important as an antioxidant as well as in osmoregulation and stress responses. In isotonic media, unlabeled 10 mM taurine, 1 mM paeonol, and 0.1 mM propranolol were used for pre-treatment for 24 h after which [^{3}H] taurine uptake and TauT mRNA expression were observed. In the presence of these compounds, both uptake and mRNA expression level decreased significantly (Fig. 4a, b). The transport system of taurine is subject to adaptive regulation, and was down regulated during the pre-incubation time in the culture system, as reported in a previous report on human placental choriocarcinoma cells (JAR) (Jayanthi et al. 1995). In addition, this transport system is dependent on incubation time and concentration of taurine in TR-iBRB cells (Satsu et al. 1997). Based on the same mechanism, compounds such as paeonol and propranolol (substrates of the organic cationic transporter system) may also downregulate the taurine transport system in iBRB cells. The mRNA expression and taurine uptake rate was increased in the cells pre-treated with TNF-α for 4 h. However, in the presence of 10 mM taurine, 1 mM paeonol, and 0.1 mM propranolol with TNF-α pre-treatment for 4 h, there was a significant decrease in the uptake of [^{3}H]taurine and mRNA expression (Fig. 5a, b). The expression level of taurine transporter (TauT) in the rat retina was

downregulated with pre-treatment of organic cationic drugs such as paeonol and propranolol. The uptake of these drugs through the iBRB follows the cationic transporter system but the taurine follows the TauT. So, these drugs can affect the uptake of taurine and expression level of TauT in the iBRB. In addition, the level of taurine in the cell depends on the TauT and the taurine synthesizing enzyme CSAD/CAD (Wu 1982).

When hypertonic media was pre-incubated for 24 h with taurine, paeonol and propranolol, [^{3}H]taurine uptake and TauT mRNA expression level decreased significantly in iBRB cells. However, presence of only hypertonic media induced both taurine uptake and mRNA level in TR-iBRB cells (Fig. 6a, b). Previous reports also indicate that the activity of taurine transport was upregulated by hypertonic conditions and that such upregulation was associated with increased gene expression of TauT (Satsu et al. 1999). In regard to diabetic conditions, elevated levels of glucose may evidently disturb the cellular osmoregulation mechanism. Disturbance in osmoregulation may lead to cellular dysfunction. Our previous results also reveal that [^{3}H]taurine uptake may decrease in under 24 h in cells pre-incubated with HG media (Lee and Kang 2013; Lee and Kang 2015). Presumably, under HG conditions, pre-treatment with taurine, paeonol and propranolol, suppressed the [^{3}H]taurine uptake rate and mRNA expression of TauT in TR-iBRB cells (Fig. 7a, b). Therefore, we contend that taurine may be useful in preventing retinal diseases such as diabetic retinopathy. An imbalance in cell volume may cause retinal diseases such as diabetic retinopathy, neurodegeneration, ischemia and macular edema, but taurine may be able to regulate cell volume in the retina (Tomi et al. 2007). Taurine is highly concentrated in the retina where it may act as an organic osmolyte. Our study demonstrates the [^{3}H]taurine uptake as well as TauT mRNA expression may decrease during pre-incubation with various compounds. Therefore, these results indicate that taurine may play an important role in maintaining a healthy retina.

5 Conclusion

Our study demonstrates that oxidative stress and hypertonic conditions may increase taurine uptake in iBRB cell lines, whereas HG conditions may decrease such uptake. Pre-treatment with paeonol and propranolol under hypertonic and HG conditions, decreased taurine transport function, whereas these compounds had no effect on taurine transport function under the TNF-α condition. Pre-treatment effect of taurine was shown in carrier mediated transport function under all conditions. Taurine transport system in the presence of organic cationic compounds is regulated under various conditions by TauT, and may play a key role in maintaining taurine concentration in retinal cells.

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Taurine Regulation of Neuroendocrine Function

Abdeslem El Idrissi

Abstract Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid. It is one of the most abundant free amino acids in many excitable tissues, including the brain, skeletal and cardiac muscles. Physiological actions of taurine are widespread and include regulation of plasma glucose levels, bile acid conjugation, detoxification, membrane stabilization, blood pressure regulation, osmoregulation, neurotransmission, and modulation of mitochondria function and cellular calcium levels. Taurine plays an important role in modulating glutamate and GABA neurotransmission and prevents excitotoxicity in vitro primarily through modulation of intracellular calcium homeostasis. Taurine supplementation prevents age-dependent decline of cognitive functions. Because of the wide spread actions of taurine, its levels are highly regulated through enzymatic biosynthesis or dietary intake. Furthermore, depletion of endogenous or dietary supplementation of exogenous taurine have been shown to induce wide spread actions on multiple organs. Cysteine sulfonic acid decarboxylase (CSAD) was first identified in the liver and is thought to be the rate-limiting enzyme in taurine biosynthesis. CSAD mRNA is expressed in the brain in astrocytes. Homozygous knockout mice lacking CSAD (CSAD-KO) have very reduced taurine content and show severe functional histopathology in the visual system, skeletal system, heart, pancreas and brain. Conversely, dietary supplementation of taurine results in significant health benefits acting through the same organ systems. Fluctuation of taurine bioavailability lead to changes in the expression levels of taurine transporters in neuronal plasma membranes, endothelial cells forming the blood-brain barrier and proximal cells of the kidneys. Suggesting a highly regulated mechanism for maintaining taurine homeostasis and organ systems function. Here we show how alterations in taurine levels directly affect the function of one organ system and through functional interaction and compensatory adaptation; these effects extend to another organ systems with focus on the nervous system.

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Abbreviations

CSAD KO	cysteine sulfinic acid decarboxylase knock-out mice
CSAD	cysteine sulfinic acid decarboxylase
Glut4	glucose transporter 4
IR	insulin receptor
TAU	taurine
TauT	taurine transporter
VSCC	voltage sensitive calcium channels

1 Introduction

Taurine, a sulfur-containing amino acid, is present in high concentrations in mammalian plasma and tissues, plays a vital role in various essential biological processes such as development of the central nervous system (CNS) and the retina, glucose regulation, calcium modulation, anti-oxidant activity, membrane stabilization, reproduction, and immunity (Huxtable 1992; Sturman 1993; Schuller-Levis and Park 2003, 2006; Schuller-Levis et al. 2009; Schaffer et al. 2010). It is clear that taurine induces a multitude of cellular and physiological actions directly or indirectly through compensatory mechnisms. Furthermore, alterations in the function of one organ system can have functional effects on another one. A good illustration of this phenomenon is how the modulatory effects of taurine on the endocrine pancreas induce adaptive changes in the brain. As a potent anti-oxidant, taurine has been shown to have a protective effect on the pancreas by preventing or scavenging free radicals (Arany et al. 2004). Previous reports show that the islets from taurine treated mice had almost double the number of cells positive for proliferating cell nuclear antigen (PCNA) (El Idrissi et al. 2010). This increase proliferation was accompanied by a reduction in the incidence of apoptosis in islet cells, and also a significant increase in the number of islet cells immunopositive for IGF-II (Arany et al. 2004). IGF-II was shown to function as an islet survival factor in vitro (Petrik et al. 1998). The induction of islet cell apoptosis in vivo may involve an increased expression of inducible nitric oxide synthase (iNOS) within β cells (Liu et al. 1998). Interestingly, taurine is a potent inhibitor of iNOS (Liu et al. 1998). These data show that the endocrine pancreas undergoes significant modification during early postnatal life and that apoptosis is an important mechanism in this remodeling (Scaglia et al. 1997). Consistent with this, taurine induces histological, biochemical and

endocrine changes in the pancreas. These positive alterations in pancreatic physiology are beneficial for islets function and confer tolerance to glucose and resistance to pharmacologically-induced diabetes. Plasma glucose levels are inversely proportional to the plasma insulin levels, which in turn, are determined by insulin levels present in the islets. Insulin is primarily a metabolic hormone functioning on muscle, fat and liver via activation of insulin receptor (IR). Insulin also functions on other non-metabolic tissues such as the brain. Once insulin is secreted, it crosses the blood-brain barrier by a transporter-mediated saturable mechanism. The IR is widely expressed in the brain at various levels and the regional specify implicates insulin, through activation of its receptor, in various brain function (Unger et al. 1991). There are numerous studies demonstrating that IR signaling plays a role in both excitatory and inhibitory neurotransmission and that the expression of IR in the brain is activity-dependent (Plum et al. 2005). The expression of potassium ion channel Kv1.3 in the olfactory bulb is increased in response to intranasal insulin delivery to mice (Marks et al. 2009). These changes led to increased cognitive function as measured by short- and long-term object recognition, suggesting the insulin modulates neuronal activity and improves memory through changes in Kv1.3 expression levels (Marks et al. 2009). Furthermore, insulin was shown to promote neuronal survival in the brain and prevent hippocampal cell death in response to glucose deprivation in vitro (Mielke et al. 2006). The finding that taurine supplementation regulates the expression of both insulin and its receptor suggests a role for taurine in both peripheral regulation of glucose homeostasis and central neuronal excitability (El Idrissi et al. 2010). Interestingly, chronic dietary intake of taurine resulted in long-lasting biochemical and behavioral changes characterized by increased neuronal excitability. Taurine-fed mice for example, showed increased susceptibility to KA-induced seizures (El Idrissi et al. 2003). Associated with this increased state of neuronal excitability, there are biochemical changes in the GABAergic system. Chronic supplementation of taurine in drinking water causes an increase in the levels of glutamate and GABA as well as the enzyme responsible for GABA synthesis, glutamic acid decarboxylase (GAD). Additionally, there was an upregulation in the expression levels of the NR1 subunit of the NMDA receptors and a reduced expression of the β3 subunit of $GABA_A$ receptors (El Idrissi et al. 2013). Here, we show that concomitant with increased IR expression in the taurine-fed mice there was an increase in the expression of glucose transporter (Glut 4). Furthermore, we found that taurine supplementation lead to a decrease in the expression of β3 subunit of the voltage sensitive calcium channel (VSCC) and taurine transporter (TauT) in the hippocampus. On the other hand, Calbinding and Na-K-ATPase were upregulated in the brain of taurine-fed mice. Thus, taurine affects neuronal function directly through activation of GABAA and glycine receptors and indirectly though the biochemical modifications and the alteration in endocrine function.

2 Materials and Methods

2.1 Animals

All mice used in this study were two-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 0.05%, and this so-lution was made available to the mice in place of drinking water for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 hr light/dark cy-cle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Immunohistochemistry

Frozen sections were made as previously described (Levinskaya et al. 2006 and placed onto gelatin-subbed slides. Non-specific binding sites were blocked using 4% bovine serum albumin (BSA), 2% normal goat serum (NGS), and 0.05% Triton X-100 in 0.01M phosphate-buffered saline (PBS; pH 7.2). Following the blocking step, the slides were rinsed in an antibody dilution cocktail (ABD) consisting of 2% BSA and 1% NGS in 0.01M PBS. Primary antibodies (Chemicon In-ternational) employed were directed against insulin receptor (mouse host) diluted 1:500 in ABD. The primary antibody was incubated overnight at 40C and then unbound antibodies rinsed with ABD. Sec-ondary antibodies were all raised in goat and directed against appropriate primary antibody type. The anti-mouse IgG was conjugated to Alexa Fluor 488 (Invitrogen/Molecular probes). Images were obtained by confocal microscopy (Leica SP2 AOBS). Nuclei were counter-stained with SlowFade with DAPI (Invitrogen). To determine relative changes in protein expression, the gain and offset was identical for all comparisons. The intensity ratios of immunoreactivity was deter-mined by importing the data from the Leica confocal software into Imaris X64 (Bitplane). For each Z stack, the threshold values for insulin receptor immunoreactivity were set for the untreated tissues. When the Z stacks for the taurine-treated tissues were imported, the Z stack were treated the same as the control. Coupling these manipulations with the consistent imaging parameters (same lens, gain and offset for each laser), the data changes are treatment-related. The mean pixel in-tensity values for each thresholded channel were obtained from the Imaris software and those data imported into InStat statistical software (GraphPad Software Inc.)

2.3 Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statis-tically significant when the calculated P value was less than 0.05.

3 Results

3.1 Taurine-fed Mice Have Increased Expression of Insulin Receptors in the Pancreas and Brain

To investigate the functional significance of the histological changes occurring in the pancreas and the increased insulin production and secretion in response to glucose challenge, we examined the expression of the insulin receptor in the pancreas and brain. Insulin is primarily a metabolic hormone functioning on muscle, fat and liver via activation of its cognate receptor, though it also functions on tissues that are not considered classically metabolic, such as the vasculature and the brain. Once insulin is secreted it crosses the blood-brain barrier by a transporter-mediated saturable mechanism. The IR is widely expressed in the brain but demonstrates denser expression in certain regions (Unger et al. 1991). A higher level of expression is found in the ol-factory regions, amygdaloid complex, hippocampus, pyriform cortex and thalamus. This regional specify implicates insulin, through activation of its receptor, in various brain function that are mediated by these brain structures. We found that taurine supplemented mice have elevated expression of IR in the pancreas (Fig. 1) and brain (Fig. 2).

3.2 Excitability-Induced Changes in Hippocampal Biochemistry

To further investigate the functional significance increased insulin receptor expression in the brain and the resulting neuronal hyper-excitability, we examined the expression of key proteins relevants to synaptic transmission and regulation of membrane excitability. We found that taurine-fed mice have elevated expression of glucose transporter (Glut4) compared to controls (Fig. 3a and d). we also found an increased expression of calbindin (Fig. 3e) and Na-K-APTase (Fig. 3f) and a reduced expression of the voltage-sensitive calcium channels (VSCC) (Fig. 3e) and Taurine transporter (TauT, Fig. 3f) compared to controls (Fig. 3b, c). Interestingly, the

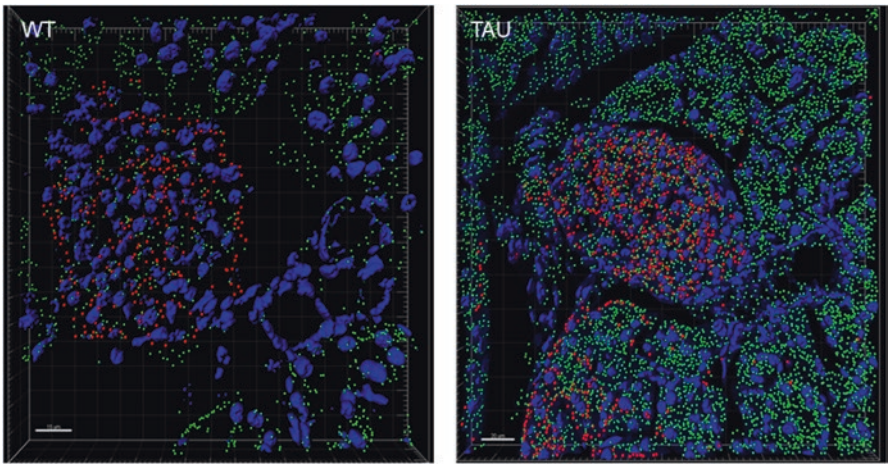

Fig. 1 Effect of taurine supplementation on insulin (red) and insulin receptor (green) immunoreactivity in the pancreas. Images depict Imaris reconstructions of the z-stacks obtained with a confocal microscope. Pancreas from taurine-fed mice show a significant increase in immunoreactivity for inulin and insulin receptor. Scale bar = 20 μm

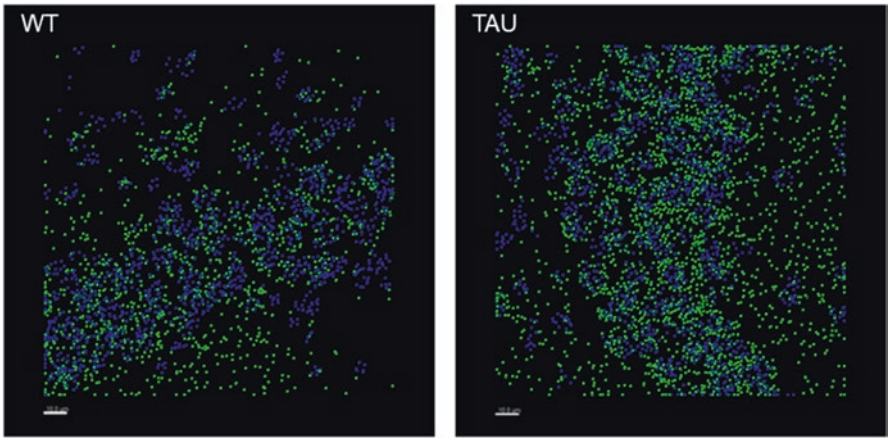

Fig. 2 Effect of taurine supplementation on insulin receptor expression in the hippocampus. Representative Imaris reconstruction of the z-stacks images obtained with a confocal microscope showing immunoreactivity for insulin receptor (green) in CA3 region of the hippocampus from control and taurine-fed mouse, respectively. Scale bar = 10 μm

hyperexcitability induced by taurine supplementation resulted in functional adaptation of the hippocampus through changes in protein expression to cope with the elevated state of excitability. For example, the calcium binding protein calbindin and the NA-K-ATPase were upregulated in taurine-fed mice to increase calcium buffering and ionic movement.

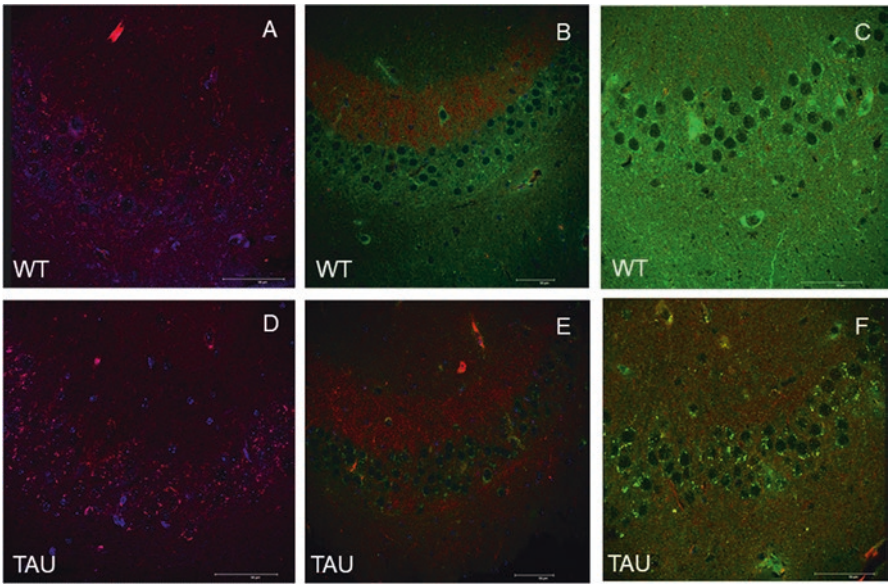

Fig. 3 Taurine-induced biochemical alterations in the hippocampus. Representative maximum projection images obtained from a z stack with a confocal microscope showing immunoreactivity for Glut4 (**a**, **d**), calbindin (red) and VSCC (green) (**b**, **e**), TauT (green) and Na-K-ATPase (red) (**c**, **f**)

4 Discussion

Interestingly, the increase excitability observed with taurine supplementation lead to biochemical changes that are functionally consistent with modulating neuronal excitability. If left unchecked, such hyperexcitability may lead to excitotoxicity. Calbindin, a calcium-binding protein, was upregulated in the hippocampus of taurine-fed mice. This would prevent glutamate-induced excitotoxicity in the most succeptible region of the hippocampus (CA3) (El Idrissi et al. 2003). Concomitant with increase calcium buffering capacity, the Na-K-ATPase was significantly upregulated in taurine-fed mice. This upregulation in the sodium potassium pump is to increase the ability of hippocampal cells to rapidly establish the ionic gradient across plasma membranes. Interestingly, the VSCCs were down-regulated in taurine-fed mice. This would prevent excessive calcium accumulation in hippocampal neurons and the resulting calcium mediated neuronal damage.

The role of insulin receptor in fundamental biological processes (e.g., development, brain function, metabolism, etc.), along with more recent data linking brain insulin function to the etiology of a number of neurodegenerative diseases will, undoubtedly, translate into more clinically-oriented research in the near future.

5 Conclusion

Taurine wide spread tissue distribution makes its physiological roles equally wide spread. More importantly, alterations in the function of one organ system are reflected in the function of another organ system. A good example that typifies this interaction and cross organ reactivity is the neuro-endocrine cross talk between the brain and the pancreas. Taurine enhanced the endocrine function of the pancreas by increasing insulin production and secretion as well as an overall enhancement of pancreatic function. These increases in insulin production and release by the pancreas in response to glucose challenges induced an increase in the insulin receptor expression in the hippocampus which lead to increase excitability. Furthermore, increase in IR expression were accompanied by changes in the expression of key protein releveand to the maintemance of neuronal excitability. In summary, taurine is responsible for a vast array of neurochemical changes in the brain. The culmination of these factors leads to an enhanced cognitive functions and neuroprotection.

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Part VIII
Taurine and Anti-inflammatory

Anti-inflammatory Action of Glucose-Taurine Reduced by Inhibiting NF-κB Activation in LPS-Activated RAW264.7 Macrophages

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Abstract In the present study, we investigated the regulation of inflammatory effects by glucose-taurine reduced (G-T-R), a taurine-carbohydrate derivative, on lipopolysaccharide (LPS)-induced RAW264.7 macrophages. The anti-inflammatory action of G-T-R revealed that this derivative markedly inhibited the nitric oxide (NO) and prostaglandin E2 (PGE2) production in RAW264.7 macrophages induced by LPS. Suppression of NO and PGE2 production was involved in the inhibitory action by G-T-R on the inducible nitric oxide synthase and cyclooxygenase-2 proteins expression. G-T-R decreased the production of a variety of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and interleukin-6. Moreover, G-T-R effectively suppressed the nuclear factor-kappa B (NF-κB) activation in LPS-stimulated RAW264.7 macrophages according to evaluation of the molecular inflammatory mechanisms. Thus, we suggest that G-T-R modulates several inflammatory pathways mediated by NF-κB activation, demonstrating its potential or preventing and treating inflammatory conditions.

Keywords Glucose-taurine reduced · Lipopolysaccharide · Anti-inflammatory · Nuclear factor-κB activation · RAW264.7 macrophages

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Abbreviations

G-T-R	glucose-taurine reduced
LPS	lipopolysaccharide
iNOS	inducible nitric oxide synthase
NO	nitric oxide
COX-2	cyclooxygenase-2
PGE_2	prostaglandin E2
IL-6	interleukin-6
IL-1β	interleukin-1β
TNF-α	necrosis factor-α
NF-κB	nuclear factor-kappa B

1 Introduction

Macrophages plays many significant role in development of immune regulation and host defense systems. Lipopolysaccharide (LPS) is well-known as a glycolipid existent in the cell wall of gram-negative bacteria. Among the various intrinsic or extrinsic stimuli, LPS can stimulate the production of inflammatory mediators and cytokines, including nitric oxide (NO) synthesized by inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF-α), interleukin 1β (IL-1β), and prostaglandins (PGs) in macrophages (Bian and Murad 2001; Fujihara et al. 2003). Inflammatory cytokines activate I-kappa-B kinase by ubiquitinating kappa B inhibitors, which releases NF-κB to the nucleus. In the nucleus, NF-κB promotes transcription of the TNF-α, IL-1β, and IL-6 genes (Schmid and Birbach 2008). Together, these factors contribute to the inflammatory response.

Taurine (2-aminoethanesulfonic acid, TAU) is the most abundant sulfur-containing amino acid in animal tissues, including the brain, skeletal muscle, and cardiac muscle (Ijiri et al. 2013). Taurine play key role in many biological processes including osmoregulation, bile acid conjugation, calcium homeostasis, membrane stabilization, and protection against hypertension, oxidative injury-related diseases, and cardiovascular diseases (Silva et al. 2011; Ijiri et al. 2013; Marcinkiewicz and Kontny 2014). Although taurine has various beneficial effects, it also has some disadvantages including a high-concentration requirement, poor absorption, and rapid release into the urine. Thus, researchers have synthesized various functional taurine derivatives, some of which are commercially available (Cho et al. 2014). Although several studies of the physicochemical characteristics of synthetic taurine derivatives have been reported, few have described the regulation of inflammatory activity and mechanisms of taurine-carbohydrate derivatives. In this study, we examined the anti-inflammatory effects of glucose-taurine reduced (G-T-R), a synthetic taurine-carbohydrate derivative, by inhibiting NF-κB signaling in LPS-related inflammation in RAW264.7 macrophages.

2 Methods

2.1 Reagents and Devices

Synthesized G-T-R was provided by Prof. Sung-Hoon Kim (Kunkuk University). RPMI 1640 medium, fetal bovine serum, and antibiotics were purchased from Gibco (Grand Island, NY, USA). LPS and 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Ninety-six-well tissue culture plates and other tissue culture dishes were from SPL Life Sciences (Pocheon, Korea).

2.2 RAW264.7 Macrophages Culture

The cells, RAW264.7 macrophages, were purchased frozen at ATCC (Manassas, VA, USA). RAW264.7 macrophages were cultured in RPMI-1640 medium containing fetal bovine serum (10%) with anti-biotics (1%) at 37 °C in a 5% CO_2 incubator under humid conditions.

2.3 Cytotoxicity

The MTT assay was conducted to determine cytotoxicity and concentration of G-T-R in RAW264.7 macrophages. RAW264.7 macrophages were seeded to 24-well plates at 1 × 104 cells/well. And then, cells were incubated for 24 h. After removing the existing medium and adding fresh medium, the samples dissolved in dimethyl sulfoxide (DMSO) were diluted in RPMI 1640 medium at various concentrations. The treatment concentration of DMSO was adjusted to ≤0.1% of the medium. After 24 h, the medium were removed, MTT reagent (5 mg/mL) were added, and the supernatant were removed after standing for 4 h. After added DMSO, the formazan were dissolved by shaking on an orbital shaker. The absorbance were measured at 540 nm for 30 min. Experiments were repeated three times and mean values were obtained. Cell viability was determined based on the control absorbance values.

2.4 Western Blot Analysis

Cells were cultured in a 6well plate at a density of 3 × 105 cells/well for 24 h, and the samples were treated at each concentration. RIPA buffer was added to the cells, followed by centrifugation at 14,000 × g for 25 min at 4 °C. Protein levels were

quantified using a BSA protein kit. Each sample was separated by 12% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose (NC) membrane. The membrane was blocked with fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline), and then iNOS and COX-2 antibodies diluted 1:1000 were added and reacted for 1 h. The membrane was blocked with fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline) containing 5% fat-free milk and incubated for 1 h at 1:1000 with iNOS and COX-2 antibodies. The secondary antibody (anti-mouse IgG) was diluted 1:1000 and reacted for 1 h. ECL solution was mixed with each sample in a 1:1 ratio, and the mixture was added to the NC membrane for electrochemiluminescence. An actin antibody was used as a control.

2.5 Isolation of Nuclear and Cytoplasmic Fractions

Cells were homogenized by adding PER-mammalian protein extraction buffer containing protease inhibitor cocktail I and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 15,000 × g at 4 °C for 10 min. The supernatant was stored at −75 °C for use as the cytoplasmic fraction. The remaining precipitates were washed with phosphate-buffered saline, and then RIPA buffer was added. The mixture was mixed at 4 °C for 15 min and centrifuged at 16,000 × g for 15 min at 4 °C.

2.6 Nitrite Assay

The cells were cultured in a 96-well plate at a density of 5 × 105 cells/well (100 μL) for 24 h. The medium was removed and LPS (1 μg/mL) was added. The amount of NO released from the cells in the medium after 24 h was measured using Griess reagent (0.1% w/v N-(1-naphathyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid). Absorbance was measured at 570 nm using an ELISA microplate reader (BioRad Laboratories Inc., Hercules, CA, USA, Model 550).

2.7 PGE_2, TNF-α, IL-1β, and IL-6 Measurement and NF-κB DNA-Binding Activity Assays by Kit

The culture medium was collected and PGE2, TNF-α, IL- 1β, and IL-6 levels were determined using a commercially available kit from R&D Systems, Inc. (Minneapolis, MN, USA). Additionally, NF-κB DNA-binding activity in the nucleus was measured using the Trans AM kit (Active Motif, Carlsbad, CA, USA). These assays were performed according to the manufacturer's instructions.

2.8 Statistical Analysis

Statistical analysis were executed using Newman-Keuls post hoc test method. Data were indicated as the mean ± S.D. of 3 independent experiments. Statistical analysis were performed with GraphPad Prism software, version 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

3 Results

3.1 Effects of G-T-R the Production of NO and PGE_2 in LPS-Induced RAW264.7 Macrophages

In the present study, we conducted the MTT assay to exclude the possibility that G-T-R is cytotoxic towards RAW264.7 macrophages. As shown in Fig. 1, cell viability was not affected by incubation with 0.05–2 mg/mL of G-T-R for 48 h (Fig. 1a). Therefore, we selected the concentrations of 0.1–1 mg/mL for further studies. In our

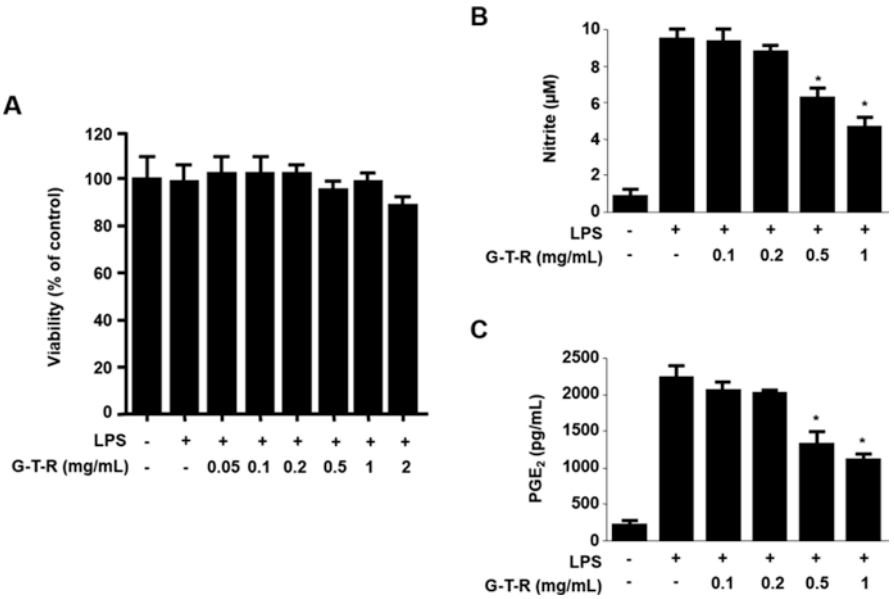

Fig. 1 The effects of G-T-R on cell viability by MTT assay (**a**) and nitrite (**b**) and PGE_2 (**c**) production in RAW264.7 macrophages. Cells were incubated for 48 h with various concentrations of G-T-R (0.05–2 mg/mL) (**a**). Based on the cell viability results, the cells were pretreated with G-T-R at four concentrations (0.1–1 mg/mL) except for the lowest and highest concentrations for 3 h and stimulated with LPS (1 μg/mL) for 24 h (**b** and **c**). Data expressed as mean ± S.D. values of three independent experiments. $*p < 0.05$ compared to the group treated with LPS

study, we also evaluated the anti-inflammatory effects of G-T-R on LPS-stimulated RAW264.7 macrophages. RAW264.7 macrophages were pretreated with G-T-R for 3 h, followed by stimulation with LPS (1 μg/mL) for 24 h. As shown in Fig. 1b, LPS treatment dramatically triggered the NO concentration, compared to that of the untreated group. However, pre-treated RAW264.7 cells with G-T-R significantly decreased the production of NO at the concentrations of 0.5 and 1 mg/mL (Fig. 1b). Similarly, G-T-R also markedly suppressed PGE2 production in a dose-dependent manner in the range of 0.5–1 mg/mL (Fig. 1c). Thus, G-T-R inhibited the production of pro-inflammatory mediators in LPS-stimulated RAW264.7 macrophages.

3.2 Effects of G-T-R on Pro-inflammatory Enzymes the Expression in LPS-Stimulated RAW264.7 Macrophages

As shown in Fig. 2, we also evaluated the effects of G-T-R on LPS-induced iNOS and COX-2 expression by western blots. RAW264.7 macrophages were challenged with LPS (1 μg/mL) in the absence or presence of G-T-R at non-cytotoxic

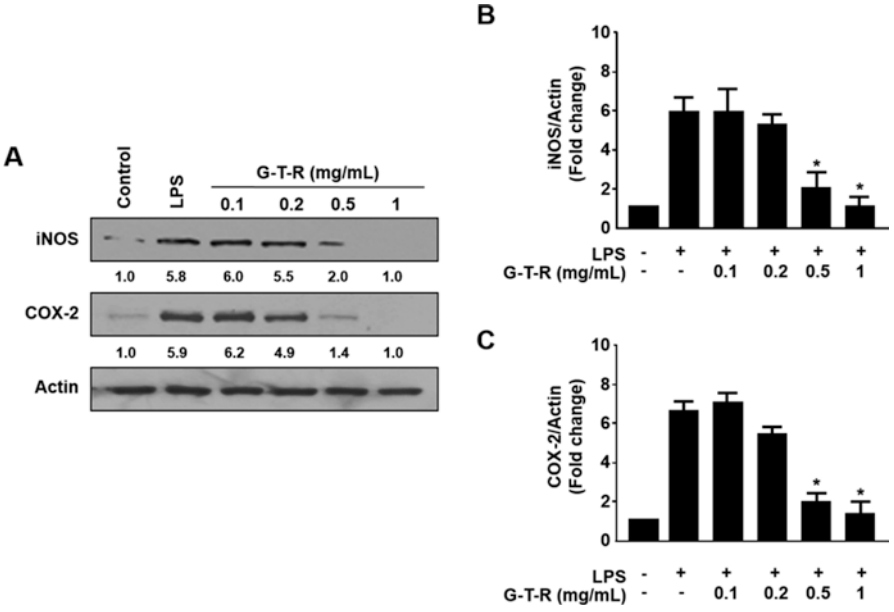

Fig. 2 The effects of G-T-R on iNOS and COX-2 protein levels in RAW264.7 macrophages. Based on the cell viability results, the cells were pretreated with G-T-R at four concentrations (0.1–1 mg/mL) except for the lowest and highest concentrations for 3 h and stimulated with LPS (1 μg/mL) for 24 h. The representative blots of three independent experiments are shown. Data represent the mean values of three experiments ± SD. $*p < 0.05$ compared to the group treated with LPS

concentrations ranging from 0.1 to 1 mg/mL. Pre-treatment of G-T-R significantly suppressed the rise in the LPS-induced iNOS and COX-2 expression in a dose-dependent manner at the concentrations of 0.5 and 1 mg/mL. These results indicate that G-T-R inhibits pro-inflammatory mediators and cytokines by reducing iNOS and COX-2 protein expression in LPS-stimulated RAW264.7 macrophages.

3.3 Effects of G-T-R on Pro-inflammatory Cytokine Production in LPS-Stimulated RAW264.7 Macrophages

To examine the effects of G-T-R on LPS-stimulated proinflammatory cytokine production, RAW264.7 macrophages were pretreated with G-T-R at a variety of the concentrations for 3 h and then stimulated with 1 μg/mL of LPS for 24 h. As shown in Fig. 3, the production of TNF-ῦFC;, IL-1β, and IL-6 were markedly increased by LPS stimulation. On the other hand, pretreatment with G-T-R significantly and dose-dependently suppressed the rise in the production of these pro-inflammatory cytokines.

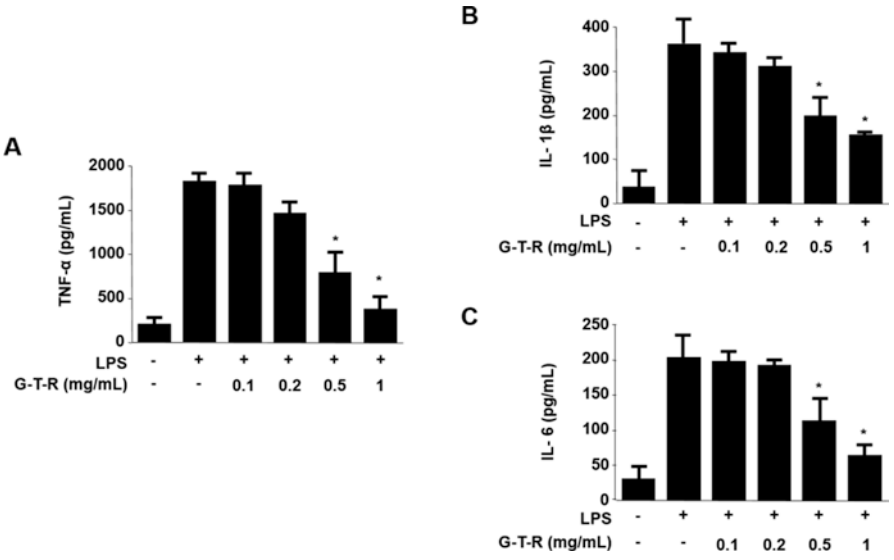

Fig. 3 Effects of G-T-R on TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) levels in RAW264.7 macrophages. Based on the cell viability results, the cells were pretreated with G-T-R at four concentrations (0.1–1 mg/mL) for 3 h and stimulated with LPS (1 μg/mL) for 24 h. The concentrations of TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) were determined as described in the Materials and Methods. Data represent the mean values of three experiments ± SD. ∗$p < 0.05$ compared to the group treated with LPS

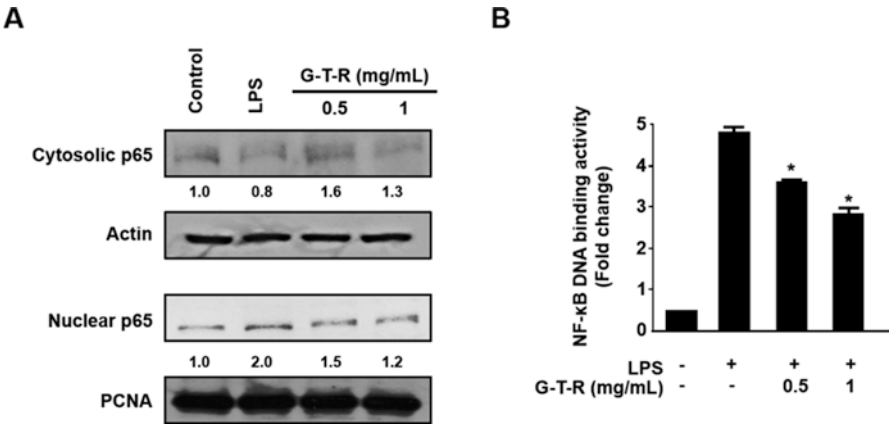

Fig. 4 Effects of G-T-R on nuclear translocation of NF-κB p65 (**a**) and NF-κB DNA binding activity (**b**) in RAW264.7 macrophages. Cells were pretreated with two concentrations (0.5 and 1 mg/mL) of G-T-R for 3 h and stimulated with LPS (1 μg/mL) for 1 h. Representative diagrams of three independent experiments are shown in (**a**). Nuclear extracts were tested using a commercially available NF-κB ELISA kit and the degree of NF-κB DNA binding (**b**) was determined. The data are the mean values of three independent experiments ± SD. $*p < 0.05$ compared to the group treated with LPS

3.4 Effects of G-T-R on NF-κB Activation in LPS-Stimulated RAW264.7 Macrophages

Next, we further investigated the effects of G-T-R on the NF- B signaling pathway. As shown in Fig. 4, pre-treatment with G-T-R for 3 h, particularly at concentrations of 0.5–1 mg/mL, significantly inhibited the translocation of p65, an NF-κB heterodimer subunit, to the nucleus. Additionally, to determine whether the reductions in iNOS and COX-2 expression after treatment with G-T-R were related with the suppression of NF-κB binding activity in the nucleus, RAW264.7 cells were pretreated with G-T-R for 3 h, and then the nuclear extracts were analyzed in a NF-κB DNA-binding assay. RAW264.7 cells stimulated by LPS showed markedly higher NF-κB DNA-binding activity than controls. However, G-T-R significantly suppressed the increase in the NF- B binding to DNA in a dose-dependent manner. Our findings suggest that the anti-inflammatory action of G-T-R is related to the suppression of NF-κB activation in LPS-induced RAW264.7 macrophages.

4 Discussion

Taurine is the most plentiful non proteinogenic sulfur containing amino acids present at high concentrations in all mammalian tissues (Niu et al. 2008; Oliveira et al. 2010). Several biological functions have been attributed to taurine including

anti-oxidant, anti-inflammatory, anti-apoptotic, and vasodilatory effects (Learn et al. 1990; Huxtable 1992). A previous in vivo study demonstrated that taurine reacts with hypochlorous acid, a powerful oxidant, to produce taurine chloramine (TauCl) which in turn inhibits the production of proinflammatory cytokines (Kontny et al. 2000). It has also been shown that taurine derivatives including taurine-xylose, taurine-ribose, and taurine-lyxose have good anti-adipogenesis properties in human preadipocytes (Cho et al. 2014). Although numerous studies of the beneficial effects of taurine and taurine derivatives have been reported, the underlying mechanisms of the anti-inflammatory action of G-T-R, a synthetic taurine-carbohydrate derivative, remain unclear. Therefore, to determine the effects of G-T-R on NF-κB-mediated pro-inflammatory actions, RAW264.7 macrophages were treated using LPS with non-cytotoxic concentrations of G-T-R. G-T-R treatment did not affect cell viability. However, G-T-R markedly suppressed NO and PGE2 production in a dose-dependent manner. G-T-R effectively suppressed the production of LPS-stimulated inflammatory mediators, and we further investigated the regulatory action by G-T-R on the expression of iNOS and COX-2 proteins as well as production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Our findings demonstrated that up to 0.5 mg/mL G-T-R effectively suppressed the increases in iNOS and COX-2 protein expression, thereby inhibiting iNOS-derived NO, TNF-α, IL-6, and IL-1β production and COX-2-derived PGE2. Similarly, other study reported that taurine haloamines, TauCl, and TauBr suppressed the inflammatory cytokines by reducing the synthesis of COX-2 and iNOS (Marcinkiewicz and Kontny 2014). Sapronov et al. (2001) reported that TAU-15 and TAU-60, which are new taurine derivatives, markedly inhibited TNF-α and IL-6 production in cultured splenocytes from rats. Another study also demonstrated that taurine administration significantly reduced serum IL-6 and TNF-α levels following CCl4-induced liver damage of rats (Abdel-Moneim et al. 2015). More recently, we already confirmed that another taurine-carbohydrate derivative, xylose-taurine reduced, significantly inhibited the pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α through NF-κB pathway (Lee et al. 2017). NF-κB in the cytoplasm binds to IκB under normal physiological conditions. When the cell is stimulated by pro-inflammatory cytokines including TNF-α, NF-κB was uncoupled with IκB and then released to the nucleus. In the nucleus, NF-κB activates iNOS, COX-2, inflammatory mediators and cytokines, and then mediates inflammatory responses (Li and Verma 2002). We also clearly observed that LPS-induced NF-κB binding activity and nuclear translocation of the NF-κB subunit, p65 were significantly reduced after pre-treatment with G-T-R. These findings suggest that G-T-R exerts its anti-inflammatory activities by decreasing the pro-inflammatory cytokines and enzymes by inhibiting NF-κB activation. Several researchers demonstrated that both taurine and TauCl are involved in inflammatory processes by inhibiting the migration of NF-κB to the nucleus (Barua et al. 2001; Caetano et al. 2017). Similarly, Kim et al. (2006) reported that TauCl and 5-aminosalicyltaurine (5-ASA-Tau) suppressed TNF-related NF-κB signaling by blocking DNA binding and phosphorylation of p65 in human colon epithelial cells. Additionally, it was well-known that taurine reduced NF-κB mediated pro-inflammatory gene expression in both in vitro and in vivo (Das et al. 2009, 2011).

Based on our findings, G-T-R effectively inhibited NF-κB-mediated production of NO, PGE2, TNF-α, IL-1β, and IL-6 in LPS-induced RAW 264.7 macrophages. These results demonstrate the beneficial role of G-T-R in preventing inflammation-related disorders.

5 Conclusion

Our results demonstrate that G-T-R treatment exerts inhibitory activity against LPS-stimulated inflammation in RAW264.7 macrophages. G-T-R markedly suppressed the NF- B-mediated production of NO, pro-inflammatory mediators, and cytokines as well as iNOS and COX-2 protein expression. Taken together, this study indicate that G-T-R is beneficial for ameliorating LPS-induced inflammation.

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Anti-inflammatory Effects of *Batillaria multiformis* Water Extracts via NF-κB and MAPK Signaling Pathways in LPS-Induced RAW 264.7 Cells

Woen-Bin Shin, Xin Dong, Yon-Suk Kim, Jin-Su Park, Su-Jin Kim, Eun-Ae Go, Eun-Kyung Kim, and Pyo-Jam Park

Abstract *Batillaria multiformis* (*B. multiformis*) belong to gastropods. They live generally in the sandpit of the lagoons and the estuaries of the intertidal zone. Most of them are distributed in Korea, Japan and China. In this study, we investigated the anti-inflammatory potential of *B. multiformis* water extracts (BMW). The results showed that the extracts significantly decreased the production of nitric oxide (NO) and pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS-induced RAW 264.7 macrophages. In addition, the extracts suppressed the protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a dose dependent manner. Further investigation indicated that BMW suppressed phosphorylated c-Jun N-terminal kinase (JNK), extracellular regulated protein kinase (ERK) and p38 through the MAPK signaling pathway and influenced the NF-κB signaling pathway by suppressing the IκBα degradation in LPS-induced RAW 264.7 macrophages.

Keywords Taurine · *Batillaria multiformis* · RAW 264.7 cells · Anti-inflammation

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1 Introduction

Inflammatory reactions are characterized by pain, swelling, redness and dysfunction of organs (Conese and Assael 2001). For staying healthy, a well-functioning immune system is needed. Therefore, to strengthen immune system, natural substances and other artificial chemicals have been explored for a long time. However, many artificial immune-stimulators are found to have side effects. Hence immune-stimulators from natural substances would be alternative for those synthetic compounds (Kim et al. 2007).

NO and Prostaglandin E_2 (PGE_2) play an important inflammatory mediator. NO is synthesized from L-arginine by nitric oxide synthase (NOS). But, under pathological condition, inducible NOS (iNOS) increased NO production. PGE_2 is synthesized from arachidonic acid stimulated with COX-2. Therefore, reduction of NO and COX-2 may be an effective strategy for the prevention of inflammatory diseases (Kim et al. 1999; Ahmad et al. 2002).

Activation of the NF-κB/Rel transcription family, by nuclear translocation of cytoplasmic complexes, plays an important role on inflammation through its ability to induce transcription of pro-inflammatory genes (Baldwin 1996). In most unstimulated cells, NF-κB exists in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κB (IκB), of which the most important may be IκBα, IκBβ, and IκBε genes (Tak and Firestein 2001). IκBα binds to the p50 and p65 heterodimer and the p50 homodimer (Phelps et al. 2000). Phosphorylated IκBα is then ubiquitinated, which targets it for degradation by the 26S proteasome, thereby releasing NF-κB dimers from the cytoplasmic NF-κB–IκB complex and allowing them to translocate to the nucleus (Chen et al. 1995).

Taurine which is also known as 2-aminoethanesulfonic acid is a sulphur containing amino acid which is largely found in different animal tissues (Sochor et al. 2014). The studies have reported that taurine has a neuroprotective function by inhibiting extracellular calcium influx and the outflow of calcium from intracellular pools, basically the balance of neurotransmitters. In addition, taurine is reported to possess anti-inflammatory properties (Chen et al. 2001). Taurine is supplied to the body from diet, even though it can be synthesized endogenously. It plays a role of bile acid formation which helps in fat digestion. In addition, it has effects of proliferation, differentiation and scavenging of free radicals and regulating membrane excitability (Chen et al. 2001; Godfrey et al. 2000; Hanna et al. 2004). Also it has protective effect of heart disease (Oktawia et al. 2010).

There is a report that fish and shellfish are rich in taurine (Kibayashi et al. 2000). Therefore, we analyzed amino acid composition of some shellfish. Above all, *B. multiformis* has much taurine contents than any other test samples.

Snails in the genus *Batillaria* dominate to the gastropods. Three species of this genus*, B. multiformis, B. cumingi and B. zonalis* are one of the shellfish that has been used by coastal people for a long time. They live generally in the sandpit of the

lagoons and the estuaries of the intertidal zone. Most of them are distributed in Korea, Japan and China (An and Koh 1992). In addition, there is not much study about *B. multiformis* and has not reported about anti-inflammatory effect of the *B. multiformis*. In present study, we performed whether *B. multiformis* water extract (BMW) has anti-inflammatory for the first time. Results of this study provide basic research data for developing beneficial effect on the treatment for inflammatory diseases.

2 Materials and Methods

2.1 Reagent

Dulbecco's modified eagle's medium (DMEM), penicillin and streptomycin, fetal bovine serum (FBS) were purchased from Hyclone (Thermo Scientific, Waltham, MA, USA). Phosphorylated-ERK antibody was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Phosphorylated-JNK, Phosphorylated-p38, Phosphorylated-IκBα, iNOS, COX-2, NF-κB and β-actin antibodies were purchased from Cell Signaling Technology Inc. (Denvers, MA, USA). LPS isolated from Escherichia coli 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polymyxin B (PMB) was purchased from InvivoGen (San Diego, CA, USA). TNF-α and IL-6 ELISA kits were bought from R&D Systems (Minneapolis, MN, USA). All other reagents were of the highest grade commercially available.

2.2 Cell Culture

The murine macrophage RAW 264.7 cells were obtained from the Korean cell line bank (Seoul, Korea) and maintained in DMEM supplemented with heat-inactivated 10% FBS and antibiotics (100 U/mL penicillin, and 100 μg/mL streptomycin) at 37 °C in an incubator containing humidified atmosphere with 5% CO_2. In all experiments, RAW 264.7 cells were incubated in the presence or absence of different BMW concentrations 1 h prior to LPS (0.1 μg/mL) stimulation.

2.3 Amino Acid Composition

Amino acid composition was analyzed using following steps. First, BMW was mixed with 10 mL of 6 N HCl. After that, N_2 gas was used to purge the samples in the test tube and then the samples were hydrolysed in a dry oven at 110 °C for 24 h.

The hydrolysed samples were then evaporated and sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and amino Acid composition was determined by measuring the absorbance at 440 and 570 nm.

2.4 Cell Viability

The effect of various concentration of BMW on murine macrophage RAW 264.7 cells viability was evaluated using MTT assay.

Briefly, RAW 264.7 macrophages were plated into 96-well plates at a density of 2×10^4 cells/well. After 24 h, these cells were pretreated with various BMW concentrations (50, 100 and 200 μg/mL) and PMB (50 μg/mL) for 1 h and then exposed to LPS (0.1 μg/mL) for 24 h. After LPS treated for 24 h, the media were replaced by 100 μL of DMEM media contatining MTT (500 μg/mL) in each well and incubation for 2 h. After removing MTT solution, the resultant formazan crystals in each well were dissolved in 150 μL of DMSO. Absorbance at 540 nm was measured by multiscanGO (Thermo Scientific, Waltham, MA, USA). Values were calculated in comparison with those of control cells.

2.5 Measurement of NO Production

NO production levels in the RAw264.7 cell culture supernatant after different treatments were analyzed by measuring the accumulation of nitrite (NO_2^-) in the cell culture media via reaction with Griess reagent (containing 0.1% N-(1-naphthyl)-ethylene diamine, 1% sulfanilamide, and 5% phosphoric acid) using sodium nitrite ($NaNO_2$) as standard. The cell culture media supernatant was collected and mixed with an equal volume of Griess reagent and shaken lightly for 10 min at room temperature. After that, 540 nm absorbance was measured by multiscanGO (Thermo Scientific, Waltham, MA, USA).

2.6 Measurement of TNF-α and IL-6

The murine macrophage RAW 264.7 cells were plated in 6-well plates at a density of 1×10^6 cells/well and incubated for 24 h. These cells were pretreated with different BMW concentrations (50, 100 and 200 μg/mL) and PMB (50 μg/mL) for 1 h and then exposed to LPS (0.1 μg/mL) for 18 h. After incubation, the cell-free supernatants were used in the pro-inflammatory cytokine contents determination assays, which were performed with a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.7 Western Blot Analysis

RAW 264.7 cells were seeded in 6-well plate and incubated at 37 °C for 24 h prior to treatment with different concentrations of BMW (50, 100 and 200 μg/mL) and PMB (50 μg/mL). After 1 h, cells were treated with LPS (0.1 μg/mL) and incubated at 37 °C in an incubator containing humidified atmosphere with 5% CO_2.

Cells were collected and suspended with PRO-PREP lysis buffer. After incubating in the ice for 30 min, cell lysates were centrifuged at 12,000 rpm for 30 min. The protein concentration was analyzed by Bradford assay method (Bio-Rad) using bovine serum albumin (BSA) as a standard. Proteins (20 μg of RAW 264.7 lysates) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins in the gel were transferred to Polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK). After then, these membranes were blocked by 5% (w/v) skim milk (BD, New Jersey, USA) in Tris-buffered saline-Tween 20 solutions (TBS-T) for 1 h at room temperature. Next, these membranes were incubated with specific primary antibodies at 4 °C overnight. After primary antibody incubation, these membranes were washed with TBS-T and incubated with specific appropriate HRP-conjugated secondary anti-bodies (1:1000 diluted) for 1 h at room temperature. Protein bands were developed by ECL chemiluminescence detection reagent and blots were visualized using Davinch-western imaging system (CAS400SM; YoungWha science, Seoul, Korea).

2.8 Statistical Analysis

The data were expressed as the mean ± standard deviation for triplicate determinations. Analysis of variance (ANOVA), together with Tukey's test and Dunnett's test (GraphPad Prism 5), were conducted to identify the significant differences between the samples.

3 Results

3.1 Cytotoxicity and Production of NO

The cytotoxicity of BMW was analyzed by MTT assay. The cultured RAW 264.7 cells were incubated with various concentrations of BMW (50, 100 and 200 μg/mL) and PMB (50 μg/mL). The results showed that BMW were not toxic at any of the concentrations tested (Fig. 1).

The LPS (0.1 μg/mL) used as positive control and it markedly increased in NO release as compared to the non-treated cells. BMW remarkably inhibited the levels of NO in a dose-dependent manner (Fig. 2).

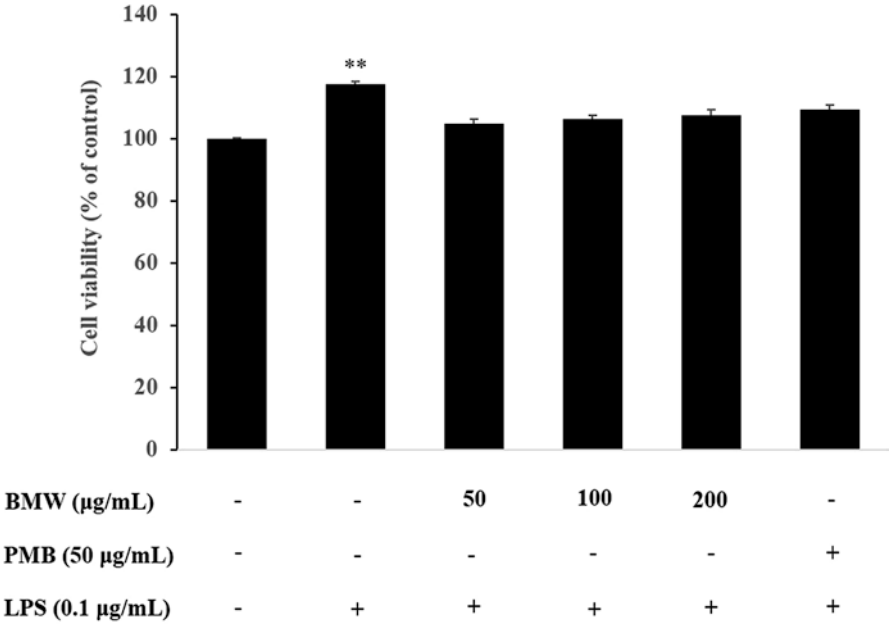

Fig. 1 Effect of *B. multiformis* water extracts on the cell viability to murine macrophage RAW 264.7 cells using MTT assay. Data are represented as mean ± SD (n = 3). $**p < 0.01$ compared to control group

3.2 Amino Acid Composition

Test samples were water extract of *Patinopecten yessoensis* (PY), *Pollicipes mitella* (PM), *B. multiformis* (BMW), *Notoacmea schrenckii* (NS), *Solen corneus* (SC) and *Semisulcospira libertine* (SL). The analysis of chemical and amino acid composition results showed that *B. multiformis* contains 4.34 mg of taurine in 1 g of BMW (Table 1).

3.3 Inhibitory Effect of BMW on LPS-Induced Production of Pro-inflammatory Cytokines, TNF-α and IL-6

TNF-α and IL-6, pro-inflammatory cytokines, are mainly produced by activated macrophage cells and known to be potent immune modulators in activated macrophages (Kim et al. 2007). We treated RAW264.7 cells with various concentrations of BMW (50, 100 and 200 μg/mL) and PMB (50 μg/mL) in the absence or presence of LPS (0.1 μg/mL). As seen in Fig. 3, the TNF-α contents increased remarkably

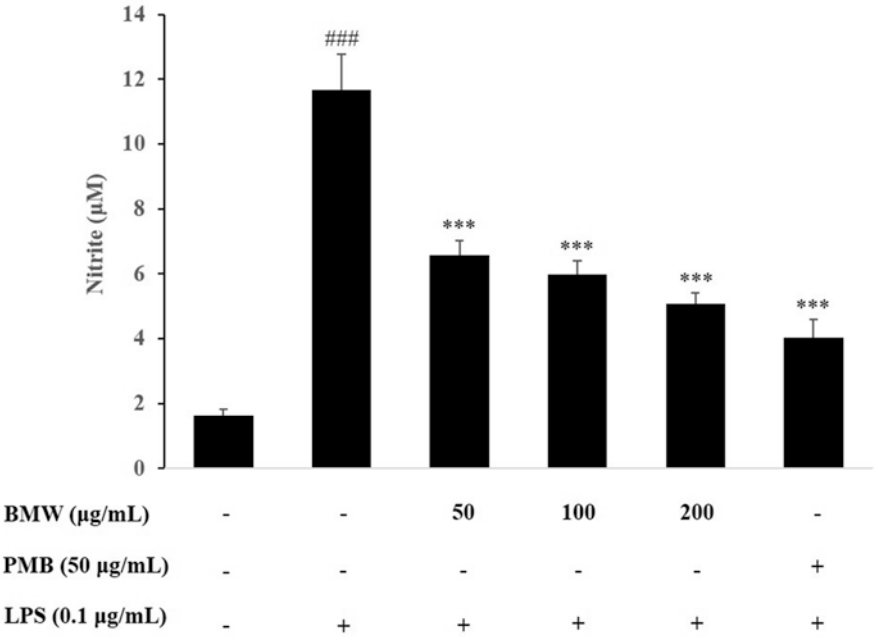

Fig. 2 *B. multiformis* water extracts (BMW) reduced the production of NO. Data are represented as mean ± SD (n = 3). $^{###}p < 0.001$ compared to control group. ∗∗∗$p < 0.001$ compared to LPS group

Table 1 Amino acid contents of some shellfish. Unit of the result is mg of taurine in the sample/g of sample

	Taurine contents (mg/g)					
Group	BMW	PY	PM	NS	SC	SL
Taurine	4.34	4.22	0.55	1.97	2.29	0.06
Asp	0.54	0.30	0.10	0.13	0.04	0.15
Thr	0.17	0.19	0.50	0.22	0.37	0.21
Ser	0.24	0.29	0.10	0.28	1.38	0.28
Glu	1.65	0.84	0.85	0.56	0.74	0.87
Gly	0.81	15.03	0.97	1.63	0.83	0.36
Ala	2.45	0.71	0.71	0.08	10.08	1.59
Val	0.27	0.07	1.34	0.21	0.18	0.33
Cys	–	–	–	0.10	0.06	–
Met	0.11	0.03	0.52	0.13	0.12	0.18

when RAW 264.7 cells were induced with LPS only. But treated LPS with BMW groups secretion of TNF-α (Fig. 3) and IL-6 (Fig. 4) contents were significantly decreased in concentration-dependent manner.

3.4 *Effect of BMW on the Expression of iNOS and COX-2*

Inducible nitric oxide synthase (iNOS) is responsible for synthesizing NO from L-arginine and expressed in a variety of cell types including macrophages. Cyclooxygenase-2 (COX-2) is participated in the conversion of arachidonic acid to prostaglandin E_2 (Murakami and Ohigashi 2007).

The iNOS and COX-2 protein expression levels were analyzed with western blot after BMW treatments. As seen in Fig. 5, treatments with various concentrations of BMW (50, 100 and 200 μg/mL) for 18 h after LPS (0.1 μg/mL) treatment could remarkably decrease the levels of iNOS and COX-2 protein expression compared to LPS group. Detection of β-actin was used for the same blot as an internal control. These results demonstrate that BMW can downregulate the expression of pro-inflammatory mediator enzymes, iNOS and COX-2.

3.5 *Effect of BMW on IκBα Degradation and NF-κB Activation*

Degradation of IκBα is known to be the key step in NF-κB activation. NF-κB plays as transcription factor to regulate pro-inflammatory mediators iNOS and COX-2 (Gloire et al. 2006). To investigate whether BMW could affect IκBα degradation and Nf-κB activation, RAW 264.7 cells were pre-treated with various concentrations of BMW for 1 h before exposure to LPS for 15 min. And these protein levels were estimated using western blot analysis. As shown in Figs. 6 and 7, various concentrations of BMW (50, 100 and 200 μg/mL) with LPS (0.1 μg/mL) for 15 min decreased phosphorylation level of IκBα and NF-κB p65 subunit in the nucleus.

3.6 *BMW Inhibited Activation of the MAPK Signaling Pathway in RAW 264.7 Macrophages*

The MAPK family such as p38, JNK and ERK1/2 signaling pathway shows that MAPKs do important role in regulating the production and secretion of inflammatory mediators in LPS-induced RAW 264.7 macrophages (Coskun et al. 2011). As seen in Fig. 8, LPS increased phosphorylation of JNK, ERK and p38. However, BMW treated group shows the levels of phosphorylated JNK, ERK and p38 decreased significantly.

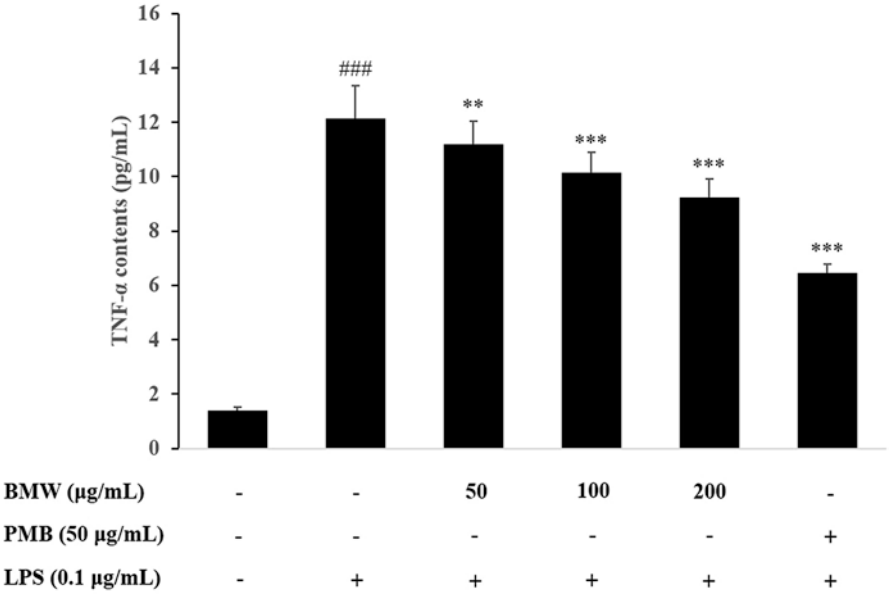

Fig. 3 *B. multiformis* reduced the release of TNF-α. ###p < 0.001 compared to control group. ***p < 0.001, **p < 0.01 compared to LPS group

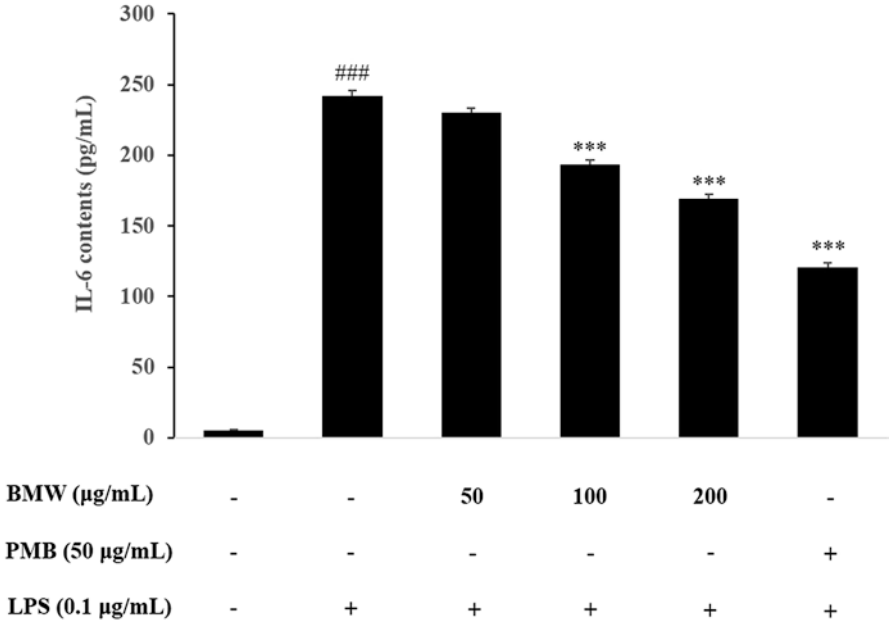

Fig. 4 *B. multiformis* reduced the release of IL-6. ###p < 0.001 compared to control group. ***p < 0.001 compared to LPS group

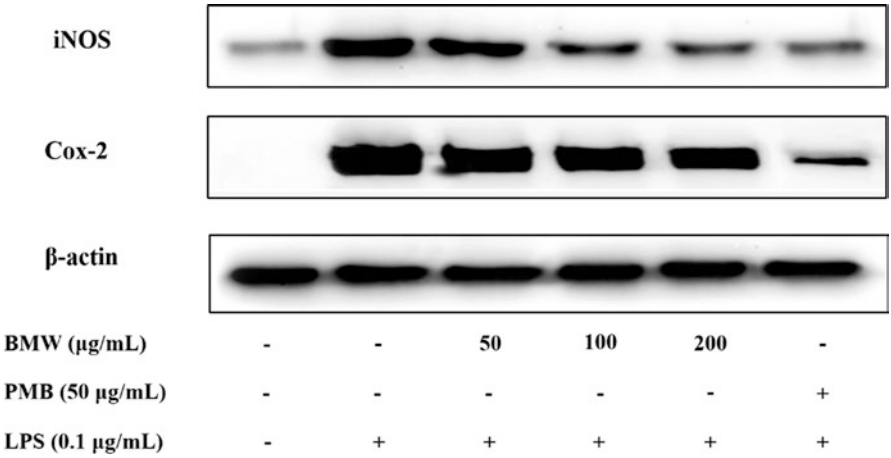

Fig. 5 *B. multiformis* reduced iNOS and COX-2 protein expression. Cells were treated with BMW or LPS for 18 h and protein expression levels were determined by western blot analysis with specific antibodies

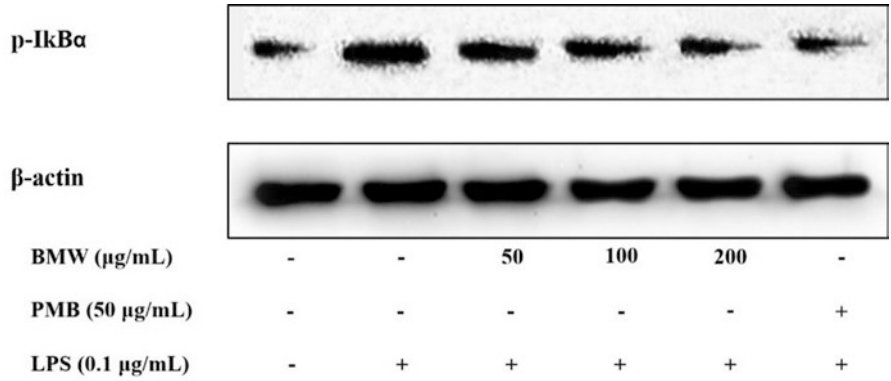

Fig. 6 Effect of BMW on IκBα degradation RAW 264.7 macrophages were measured using western blot analysis with specific antibodies

4 Discussion

Inflammatory reactions are characterized by pain, swelling, redness and dysfunction of organs (Conese and Assael 2001). For being healthy, a well-functioning immune system is needed. Therefore, to strengthen immune system, natural substances and other artificial chemicals have been explored for a long time. But, many artificial immune-stimulators are found to have side effects. Hence immune-stimulators from natural substances would be alternative for those synthetic compounds (Kim et al. 2007).

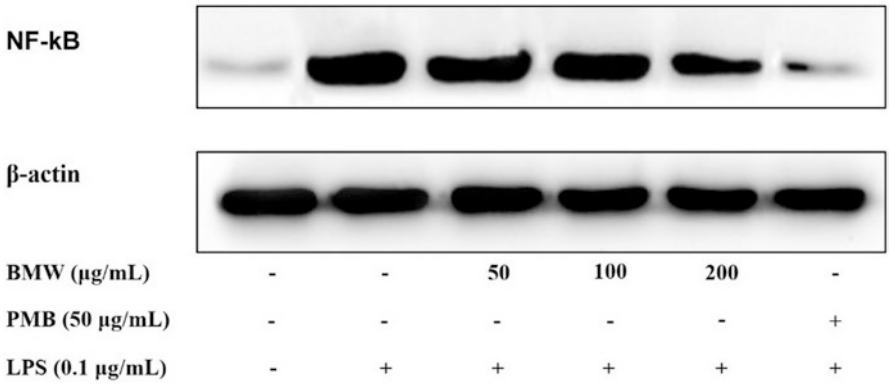

Fig. 7 Effect of BMW on NF-κB activity in RAW 264.7 macrophages. Total cellular proteins were prepared and subjected to western blot analysis for determination of NF-κB p65 subunits in nucleus with specific antibodies

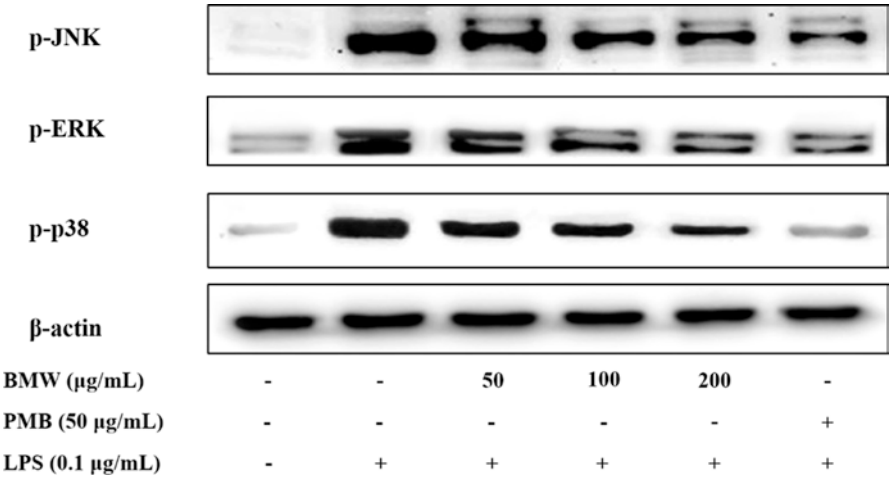

Fig. 8 Effect of BMW on MAPK signaling in RAW 264.7 macrophages. Cells were treated with BMW or LPS (0.1 μg/mL) for 30 min and MAPK protein expression levels were determined by western blot analysis with specific antibodies

Results of this study showed that BMW possessed anti-inflammatory effect in a concentration-dependent manner without exerting significant cytotoxicity to RAW 264.7 macrophages. In the present study, we found that inflammation activity in RAW 264.7 macrophages were decreased by BMW treatment. It has been reported that LPS can elevate expression of iNOS, production of NO and pro-inflammatory cytokines (Kim et al. 2007; Kim et al. 2017).

NO and Prostaglandin E_2 (PGE_2) play an important inflammatory mediator. NO is synthesized from L-arginine by nitric oxide synthase (NOS). But, under pathological condition, inducible NOS (iNOS) increased NO production. PGE_2 is synthe-

sized from arachidonic acid stimulated with COX-2. Therefore, reduction of NO and COX-2 may be an effective strategy for the prevention of inflammatory diseases (Kim et al. 1999; Ahmad et al. 2002). Our results demonstrate BMW has capacity to reduced LPS-induced NO, iNOS and COX-2 production in a concentration-dependent manner in RAW 264.7 macrophages.

Previous studies have shown that LPS stimulates the inflammatory signaling pathways in RAW 264.7 macrophages, such as MAPK and NF-κB signaling pathway (Fang et al. 2014). For those reasons we investigated BMW has regulatory effect on RAW 264.7 cells.

NF- κB is regulated by IκBα and p65 in the cytoplasm. After IκBα is phosphorylated and cleaved, the subunits of NF-κB heterodimer (p65 and p50) are released. As a result, NF-κB p65, believed to play a crucial role in inflammation, enters nucleus and the genes encoding various cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6 are transcribed (Youn et al. 2008). Our results show after BMW added to the culture medium, the phosphorylation of IκBα in cytosol and NF-κB p65 subunits in nucleus is reduced. It demonstrates BMW suppresses phosphorylation of IκBα. For this reason, the level of NF-κB p65 subunits in the nucleus is reduced.

Activation of MAPK family, such as ERK, JNK and p38, is generally related with the immune-stimulatory activity of macrophages (Lee et al. 2016). Phosphorylation of ERK is involved in macrophages activity such as production of pro-inflammatory cytokines and JNK could be activated by environmental stress and some pro-inflammatory cytokines. In addition, activation of p38 by LPS has been thought to play a crucial role in TNF-α gene expression (Bhat et al. 1998; Dong et al. 2002; Karin and Gallagher 2002). Our results show that BMW could suppress MAPK signaling pathway downregulation through suppressing of the phosphorylation of ERK, JNK and p38 proteins in RAW 264.7 macrophages.

In conclusion, we provided a mechanism to explain that BMW could have anti-inflammatory activity by suppressing NO production and iNOS expression, which may be involved with the attenuation of TNF-α formation. And suppresses down-regulation of MAPK and NF-κB signaling pathway in LPS-induced RAW 264.7 macrophages. Therefore, these results suggest that BMW possesses potential anti-inflammatory activity and might have a beneficial effect on the treatment for inflammatory diseases.

Acknowledgement This chapter was modified from the paper published by our group in Food Science and Biotechnology (Kim et al. 2017; 26(6):1633–1640). The related contents are re-used with the permission.

Conflict of Interest The authors declare that there are no conflicts of interest.

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Combined Biological Effects of N-Bromotaurine Analogs and Ibuprofen. Part I: Influence on Inflammatory Properties of Macrophages

Maria Walczewska, Marta Ciszek-Lenda, Angelika Peruń, Aneta Kiecka, Katarzyna Nazimek, Anthony Kyriakopoulos, Markus Nagl, Waldemar Gottardi, and Janusz Marcinkiewicz

Abstract Taurine haloamines (N-chlorotaurine, N-bromotaurine) due to their strong antiseptic and anti-inflammatory properties are good candidates for topical application in treatment of skin inflammatory/infectious disorders. Recently, we have demonstrated that more stable N-bromotaurine analogs (N-dibromo-dimethyl taurine, N-monobromo-dimethyl taurine) and bromamine T show strong microbicidal and anti-inflammatory properties at concentrations well tolerated by human cells and tissue. Non-steroidal anti-inflammatory drugs (NSAIDs) with cyclooxygenase (COX) inhibitory activity are commonly used in various inflammatory diseases. However, systemic administration of NSAIDs may result in adverse side effects. For example, the use of ibuprofen in children with varicella is associated with enhanced serum levels of TNF-α and with increased risk of necrotizing soft tissue infections and secondary skin infections caused by invasive streptococci. The aim of this study was to examine combined immunomodulatory effects of bromamines and ibuprofen on J774.A1 macrophages. We have shown that the primary activity of ibuprofen, the inhibition of PGE2 production by activated macrophages was intensified in the presence of bromamines. Most importantly, the stimulatory effect of ibuprofen on production of inflammatory cytokines (TNF-α, IL-6) was inhibited by all tested bromamines. These observations indicate that bromamines may neutralize massive production of TNF-α at sites of inflammation, a side effect of ibuprofen. Therefore, we suggest that systemic administration of ibuprofen

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(NSAIDs) in treatment of inflammatory/infectious skin diseases should be supported by topical application of bromamines as an adjunctive therapy.

Keywords Taurine · N-bromotaurine analogs · Bromamine T · Ibuprofen · Inflammation · soft-tissue infections · S. pyogenes

Abbreviations

Tau	taurine
HOBr	hypobromous acid
Tau-NHBr	N-bromotaurine, taurine bromamine
DM-NBrT	(Br-622) N-monobromo-dimethyltaurine
DM-NBr_2T	(Br-422) N-dibromo-dimethyltaurine
BAT	bromamine T, N-bromo-N-sodio-p-toluenesulfonamide
LPS	lipopolysaccharide
NSAIDs	non-steroidal anti-inflammatory drugs
COX-2	cyclooxygenase-2
PGE_2	prostaglandin E_2
TNF-α	tumor necrosis factor
J774.A1	murine macrophage cell line
S. pyogenes	*Streptococcus pyogenes*
IC50	half maximal inhibitory concentration

1 Introduction

Taurine haloamines, N-chlorotaurine (taurine chloramine) and N-bromotaurine (Tau-NHBr, taurine bromamine), are generated at a site of inflammation by activated neutrophils and eosinophils (Weiss et al. 1982; Klebanoff 2005; Thomas et al. 1995). Both haloamines exert similar anti-inflammatory properties (Marcinkiewicz et al. 2005; Schuller-Levis and Park 2003; Walczewska et al. 2013). On the other hand, N-bromotaurine shows stronger microbicidal activity against bacteria, viruses and fungi than N-chlorotaurine (Nagl et al. 2003; Gottardi and Nagl 2010, 2014; Marcinkiewicz et al. 2005). Due to its dual anti-inflammatory and microbicidal properties, TauNHBr has been tested as a local antiseptic in a number of skin inflammatory/infectious disorders (Marcinkiewicz et al. 2008; Marcinkiewicz 2009, 2010; Kyriakopoulos et al. 2016). However, in spite of a high efficacy of TauNHBr, its therapeutic applicability has been limited for years due to its poor stability. To overcome this disadvantage, we have recently examined the anti-inflammatory and microbicidal properties of DM-NBrT (N-monobromo-dimethyltaurine) and

DM-NBr2T (N-dibromo-dimethyltaurine), the novel stable analogs of N-bromotaurine. Moreover, we have compared their properties with that of bromamine T (BAT) (Walczewska et al. 2017). We have demonstrated that all stable bromamines show strong microbicidal and anti-inflammatory properties at concentrations well tolerated by human cells and tissues (Gottardi et al. 2014; Walczewska et al. 2013; Walczewska et al. 2017). The results from these studies suggest that N-bromotaurine analogs and BAT are better candidates for a local treatment of skin/mucosa inflammatory diseases associated with infections such as acne vulgaris and herpes zoster than native (TauNHBr) (Marcinkiewicz et al. 2008; Kyriakopoulos et al. 2016).

Ibuprofen (C13H18O2), one of the most popular non-steroidal anti-inflammatory drug (NSAID) is widely used for its analgesic, anti-inflammatory, and antipyretic properties. The main mechanism of ibuprofen action is a non-selective, reversible inhibition of cyclooxygenase COX-1 (IC50 = 20 μM) and COX-2 (IC50 ~400 μM) (Rainsford 2009). COX-1 and COX-2 enzymes catalyze the first step in the synthesis of prostanoids (prostaglandins – PGE2, PGD2, PGI and thromboxane A2) (Martin et al. 2006). PGE2 is the major prostaglandin of inflammatory response responsible for edema formation, increase in vascular permeability, leukocyte infiltration, and for triggering inflammatory pain, swelling and fever (Ricciotti and FitzGerald 2011).

Many beneficial therapeutic effects of ibuprofen are directly linked to the inhibition of PGE2 biological activity. Nevertheless, like other NSAIDs, ibuprofen can cause various gastrointestinal and cardiovascular adverse events, especially when used at high doses (Solomon et al. 2018). Moreover, it has been described in some case reports that the use of ibuprofen was associated with an increased risk of necrotizing soft tissue infections and infections with invasive group A beta-hemolytic streptococci (GAS) (Stevens 1995; Weng et al. 2011).

Several mechanisms have been proposed to explain how ibuprofen may promote bacterial (GAS) skin complications: (i) enhancement of TNF-α production, a major mediator of hyper-inflammation, septic shock and tissue failure; (ii) impairment of the immune response; (iii) masking the symptoms of infections through antipyretic and analgesic effect leading to delayed diagnosis and treatment (Stevens 1995; Bryant et al. 2015).

In this study we have asked the question whether the combined action of bromamines and ibuprofen will neutralize the adverse effect of ibuprofen on the production of pro-inflammatory mediators. For that purpose we have examined a combined immunomodulatory effects of bromamines and ibuprofen on activated macrophages, the major source of TNF-α at a site of inflammation (Dahlén et al. 1998).

2 Methods

2.1 Tested Agents

Bromamines: N-bromotaurine (taurine bromamine, Tau-NHBr), N-dibromo-dimethyltaurine (DM-NBr2T), bromamine T (N-bromo-N-sodio-p-toluenesulfonamide, BAT). Dimethyltaurine was kindly provided by D. Debabov and R. Najafi (NovaBay Pharmaceuticals, Inc.) and brominated to DM-NBrT and DM-NBr2T. BAT was synthesized from dibromamine T as published (Nair et al. 1978). Ibuprofen (IBU), the selected NSAIDs drug, was purchased in Sigma-Aldrich.

2.1.1 Tau-NHBr (N-bromotaurine) Preparation

Tau-NHBr was prepared in a two-step procedure (Marcinkiewicz et al. 2006). First, NaOBr was synthesized in reaction between equimolar amounts of NaOCl and NaBr (POCH, Poland) in PBS solution. In such conditions virtually all the OCl^- present reacts with Br^- to form OBr^- and Cl^-. The presence and concentration of OBr^- was confirmed by UV spectra ($\lambda = 200$ to 400 nm). In the second step, 20 mM NaOBr was added dropwise to equal volume of 400 mM taurine. UV absorption spectrum was checked to exclude the formation of taurine dibromamine or chloramines and to estimate the concentration of Tau-NHBr (molar extinction coefficient – 430 M-1 cm^{-1} at A288). Stock solution of Tau-NHBr was kept at 4 °C for a maximum period of 3 days before use.

2.2 Bacteria

Streptococcus pyogenes ATCC 19615 was kindly provided by the Center of Microbiological Research and Autovaccines Ltd. (Krakow, Poland). Bacteria were propagated by 48 h incubation at 37 °C on Mueller Hinton agar with 5% horse blood (bioMérieux). Bacteria were suspended at 1×109 ml^{-1} density in PBS and were heat killed by 60 min. Incubation at 65 °C.

2.3 Cell Culture

J774.A1 murine macrophages were cultured in 24-well flat-bottom cell culture plates at 5×105 cells/well in DMEM medium supplemented with 5% FBS, at 37 °C in an atmosphere of 5% CO_2. In all experimental models macrophages were

pre-incubated with the tested agents (DM-NBr2T, BAT, Tau-NHBr at concentrations of 100–300 μM and IBU at concentrations of 600 μM) in DMEM without FBS, after 1.5 h medium was removed and fresh DMEM +5% FBS with LPS (100 ng/ml) or S. pyogenes to MOI of ~ 30:1 was added for additional 24 h. After 24 h, culture supernatants and/or cell lysates were collected for further analysis.

2.4 Cell Viability

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan. For MTT reduction experiments, cells in 96-well plates were incubated at 37 °C with 0.2 mg/ml MTT for 60 min. (Promega). Culture medium was removed by aspiration, and cells were solubilized in 200 μl of DMSO. Extent of reduction of MTT to formazan within cells was quantified by measurement of absorbance at 550 nm. In all experimental groups, the J774.A1 cell viability measured after 24 h was similar to that of the control, non-treated cells (~90%).

2.5 Determination of PGE_2 and Cytokines (IL-6, IL-10, TNF-α)

2.5.1 PGE_2

PGE2 concentration in cell supernatants was determined by a PGE2 high sensitivity ELISA kit (Enzo Life Sciences), according to the manufacturer's protocol.

2.5.2 Cytokines

Cytokine levels in cell culture supernatants were measured by sandwich ELISA. Microtiter plates (Costar EIA/RIA plates, Corning Inc.) were coated with a cytokine-specific antibody. Expression levels of IL-6 and IL-10 were measured according to the manufacturer's instructions (OptEIA Sets, BD Biosciences). TNF-α level was measured according to the manufacturer's instructions (ELISA Ready-Set-Go, eBioscience). In all cases, 10% FBS in PBS was used as a blocking solution. TMB substrate solution (BioLegend) was used to develop a colorimetric reaction, which was stopped with 2 M sulfuric acid. Optical density was measured at 450 (570) nm using a microtiter plate reader (PowerWaveX, Bio-Tek Instruments).

2.6 Western Blot Analysis of Cyclooxygenase-2 (COX-2) and Nitric Oxide Synthase (iNOS) Expression

24 h after in vitro stimulation of macrophages, expression levels of COX-2 and iNOS proteins in cell cytosol were determined by Western blot analysis. Cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS in PBS) containing protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations in lysates were determined by using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Samples containing equal amounts of total protein were mixed with gel loading buffer (0.125 M Tris, 4% SDS, 20% glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue) at a 2:1 ratio (v/v) and boiled for 4 min. Samples of 20 μg of total protein per lane were separated on 10% SDS-polyacrylamide gels (Mighty Small II, Amersham Biosciences) using the Laemmli buffer system. Proteins were transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked overnight at 4 °C with 3% nonfat dried milk. Membranes were incubated for 2 h at room temperature (RT) with rabbit polyclonal antibodies to COX-2 (1:200, Cayman) or rabbit polyclonal antibodies to iNOS (1:2000, Enzo Life Sciences). Bands were detected with alkaline phosphatase-conjugated secondary goat antibody to the rabbit IgG whole molecule (1 h, RT, 1:3000, Sigma–Aldrich) or alkaline phosphatase-conjugated secondary goat antibody to the mouse IgG whole molecule (1 h, RT, 1:3000, Sigma-Aldrich) and developed with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich). Membranes were re-probed with monoclonal mouse anti-beta-actin antibody (1 h, RT, 1:3000, Sigma-Aldrich). Pre-stained SDS-PAGE standards (low and high range; Bio-Rad) were used for molecular weight determinations. Protein bands were scanned and analyzed with the Scion Image freeware (Scion Corp). Data were normalized to the constitutive expression level of beta-actin protein.

2.7 Nitrite (NO_2^-) Determination

Nitric oxide, quantified by the accumulation of nitrite as a stable end product, was determined by a microplate assay (Ding et al. 1988). Briefly, 100 μl of sample supernatants were incubated with an equal volume of Griess reagent [1% sulphanilamide in 2 M HCl (Sigma-Aldrich) and 0.1%N-1-naphthylenediamine dihydrochloride in deionized water (POCH)] at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve.

2.8 Statistical Analysis

Statistical significance between groups was tested using one-way ANOVA comparison and Tukey's post hoc test. Results are expressed as a mean ± SEM values. A p value <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism version 5.01 program (GraphPad Software).

3 Results

3.1 Combined Effect of Ibuprofen and Bromamines on the Viability of Macrophages

Previously we have demonstrated that DM-NBr2T and BAT, the stable analogs of Tau-NHBr, when used at concentrations of up to 300 μM, were non-cytotoxic against J774.A1 macrophages, the representative inflammatory cells (Walczewska et al. 2017). Importantly, viability of the cells exposed to bromamines was not altered in the presence of 600 μM IBU (Fig. 1). This concentration was chosen due to its high IC50 value for COX-2 (~400 μM), the primary target of NSAIDs (Rainsford 2009).

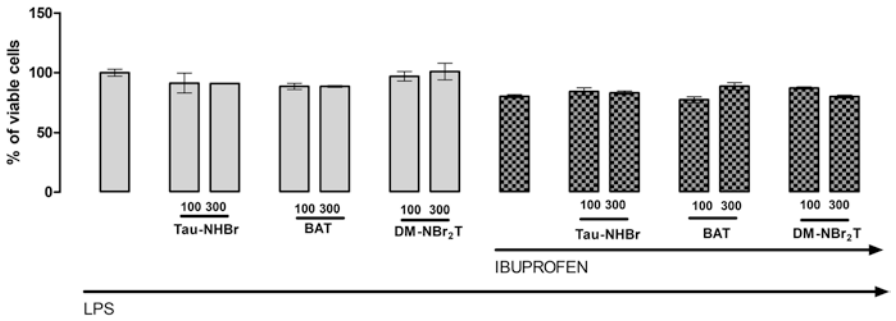

Fig. 1 Influence of ibuprofen (600 μM) and bromamines (100 and 300 μM) on cell viability. J774.A1 macrophages were pre-incubated with the tested agents in DMEM without FBS. After 1.5 h, the medium was removed and fresh DMEM +5% FBS with LPS (100 ng/ml) was added for additional 24 h. Cell viability was tested using MTT. Data were calculated from three separate experiments

3.2 *Combined Effect of Ibuprofen and Bromamines on the COX-2/PGE2 Pathway*

The next step of our study was to determine if the bromamine compounds with their anti-inflammatory potential affect the inhibition of COX-2 activity by IBU. As shown in Fig. 2, neither IBU alone nor IBU combined with bromamines altered the expression of COX-2 in macrophages stimulated either with LPS (Fig. 2a) or S.

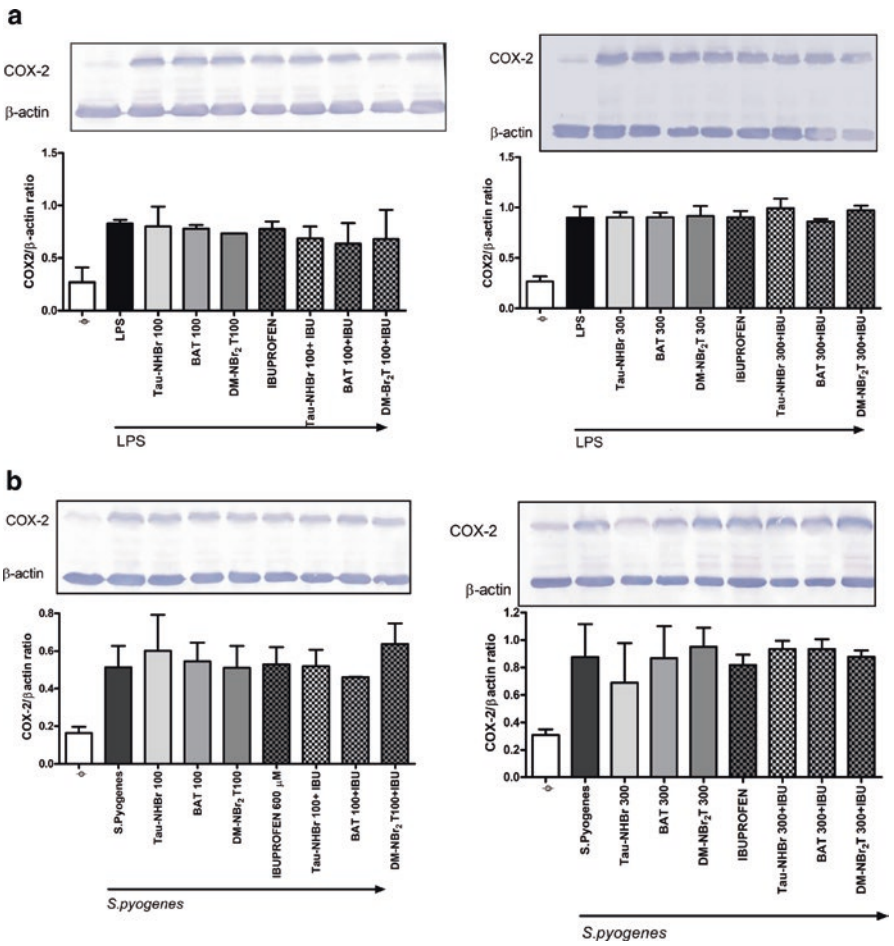

Fig. 2 Combined effect of bromamines (100 and 300 μM) and ibuprofen (600 μM) on the COX-2/PGE_2 pathway in LPS or *S. pyogenes* stimulated macrophages. Cells were pre-incubated with tested agents in DMEM without FBS. After 1.5 h, the medium was removed and fresh DMEM+5% FBS with LPS (100 ng/ml) or *S. pyogenes* (30:1) was added for additional 24 h. After 24 h supernatants and cell lysates were collected. In cell lysates, (**a** and **b**) COX-2 expression was analyzed using western blot. **(c** and **d**) PGE_2 production was determined in culture supernatants as described in Methods. Data were calculated from three separate experiments. $*p < 0.05$; $***p < 0.001$ versus LPS

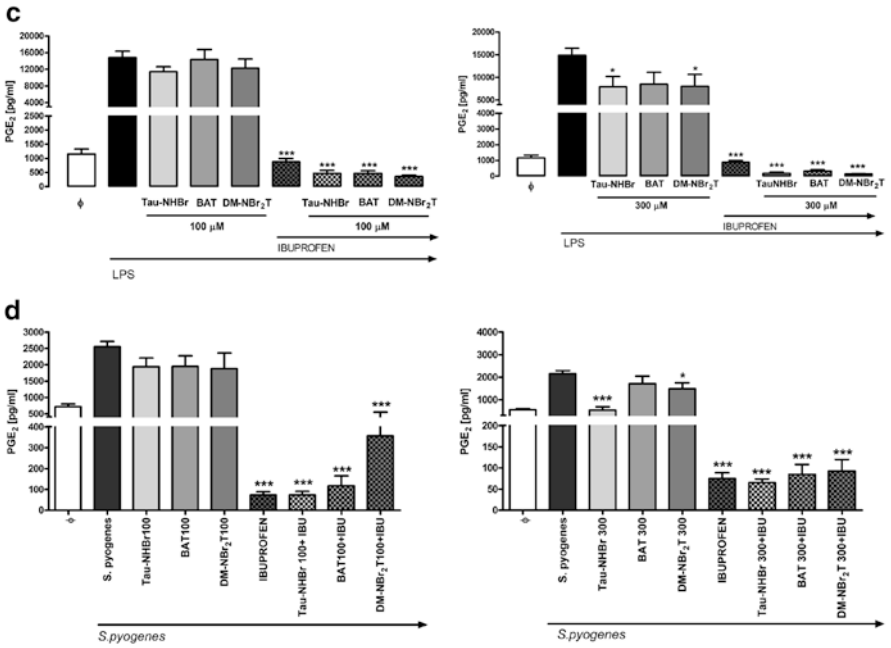

Fig. 2 (continued)

pyogenes (Fig. 2b). On the other hand, IBU at a concentration of 600 μM markedly decreased secretion of PGE2 (>90%). Importantly, the presence of bromamines (100 and 300 μM) amplified the inhibition of PGE2 production by IBU in J774.A1 cells stimulated with LPS (Fig. 2c). On the other hand, the combined inhibitory effect of bromamines and IBU was similar to that of IBU alone in the cells stimulated with S. pyogenes (Fig. 2d).

3.3 Combined Effect of Ibuprofen and Bromamines on Cytokine Production by Activated Macrophages

It has been reported previously that both Tau-NHBR and the stable forms of bromamines suppress the production of inflammatory cytokines by activated macrophages in a similar manner (Walczewska et al. 2017). In this study, we have observed that all the tested bromamines (Tau-NHBr, DM-NBr2T and BAT) used at a concentration of 300 μM significantly supressed the production of TNF-α, IL-6 and IL-10 by macrophages stimulated with LPS (Fig. 3a, b) and the production of pro-inflammatory cytokines (TNF-α, IL-6) in the cells stimulated with S. pyogenes (Fig. 4a, b). By contrast, the production of all inflammatory cytokines was significantly enhanced in macrophages pre-treated with IBU. Importantly, the bromine

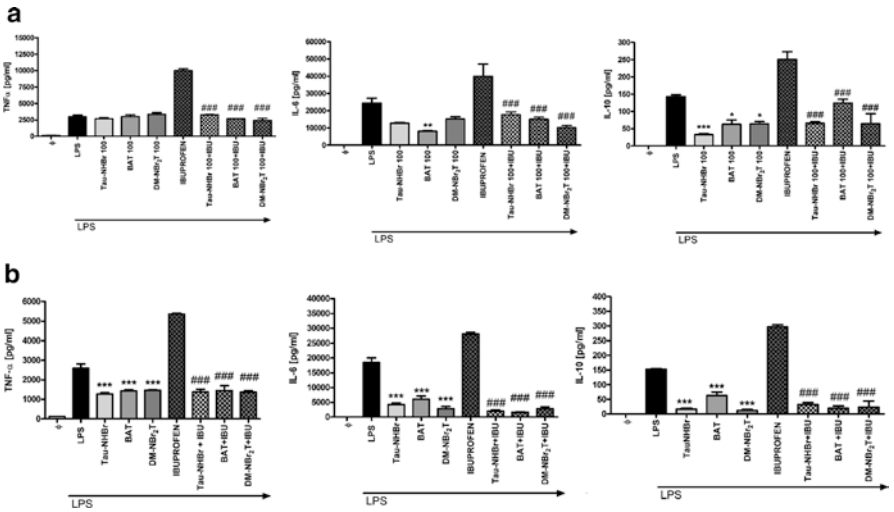

Fig. 3 Combined effect of bromamines (**a**-100 μM, **b**-300 μM) and ibuprofen (600 μM) on cytokines production in LPS stimulated macrophages. Macrophages were pre-incubated with tested agents in DMEM without FBS. After 1.5 h, the medium was removed and fresh DMEM+5% FBS with LPS (100 ng/ml) was added for additional 24 h. Medium content of cytokines was measured by ELISA. Data were calculated from three separate experiments. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$ versus LPS, ### $p < 0.001$ versus IBU

compounds neutralized this effect of IBU activity, especially when macrophages were stimulated with LPS. Interestingly, S. pyogenes did not induce the synthesis of IL-10.

3.4 Combined Effect of Ibuprofen and Bromamines on the iNOS/NO/NO_2^- Pathway in LPS Stimulated Macrophages

J774.A1 macrophages stimulated with LPS showed the expression of iNOS and produced substantial amount of nitric oxide as measured by the level of nitrites in the culture supernatants (Fig. 5a, b). On the contrary, S. pyogenes stimulated macrophages did not express iNOS and did not produce detectable amounts of nitrites (data not shown). As shown in Fig. 5b, IBU did not affect the production of NO/NO_2^- and did not alter the suppression of nitrites production by the cells exposed to the bromine compounds.

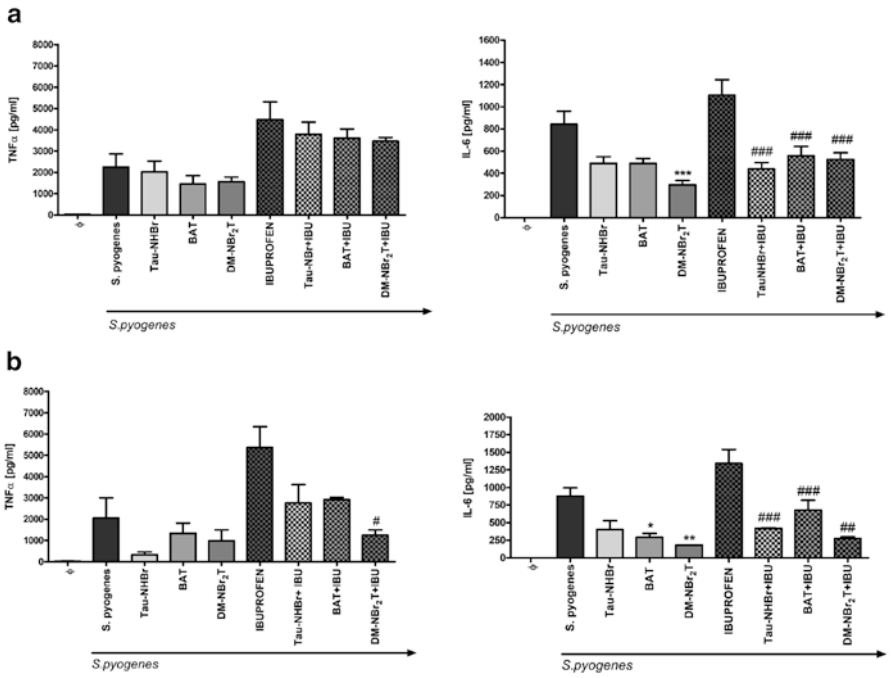

Fig. 4 Combined effect of bromamines (**a**-100 μM, **b**-300 μM) and ibuprofen (600 μM) on cytokines production in *S. pyogenes* stimulated macrophages. Macrophages were pre-incubated with tested agents in DMEM without FBS. After 1.5 h, the medium was removed and fresh DMEM+5% FBS with *S. pyogenes* (30:1) was added for additional 24 h. Medium content of cytokines was measured by ELISA. Data were calculated from three separate experiments. ∗$p < 0.05$; ∗∗$p < 0.005$; ∗∗∗$p < 0.001$ versus *S. pyogenes*, # $p < 0.05$; ## $p < 0.005$; ### $p < 0.001$ versus IBU

4 Discussion

NSAIDs, including ibuprofen, are important non-opioid analgesic and anti-inflammatory drugs in modern medicine. However, increasing body of clinical and basic evidence confirms the association between NSAIDs administration and exacerbation of group A streptococcal (GAS) soft tissue infections. These findings suggest that NSAIDs do more than simply mask the signs and symptoms of GAS infection resulting in delayed adequate antibiotic therapy (Bryant et al. 2015; Le Turnier et al. 2017). Namely, it has been suggested that NSAIDs administration may interrupt COX-2/PGE2 pathway that limits overproduction of TNF-α – a key mediator of hyperinflammatory response, septic shock and tissue injury (Pettipher and Wimberly 1994; Norrby-Teglund et al. 1995).

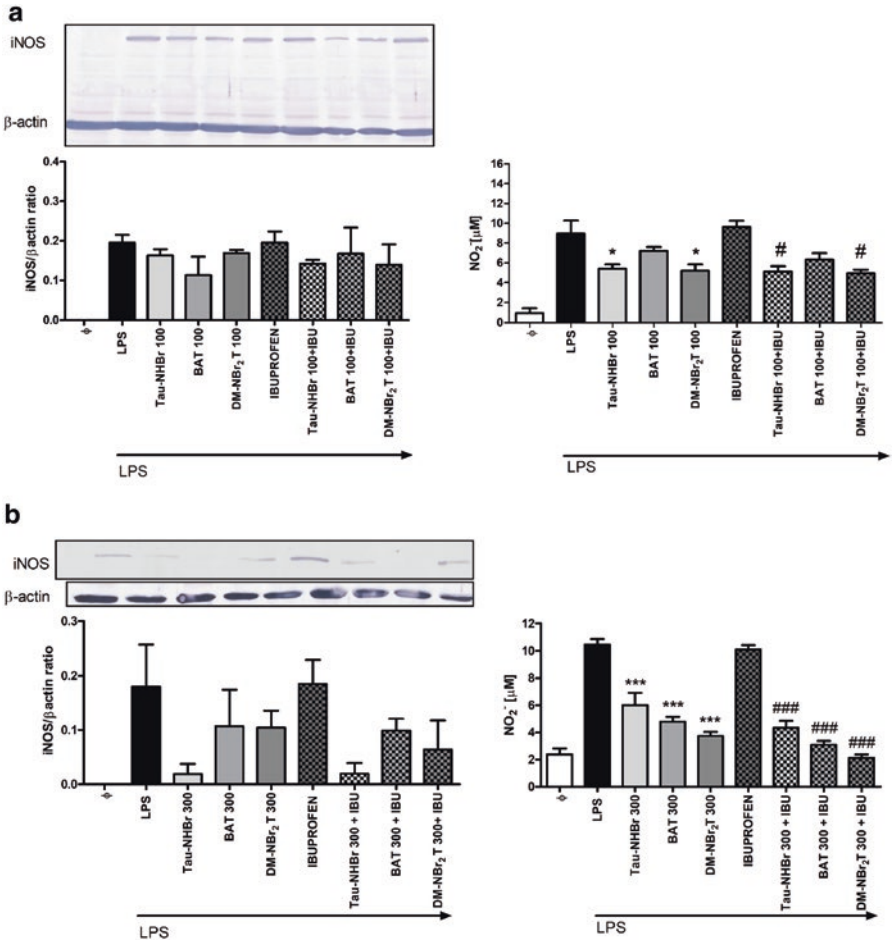

Fig. 5 Effect of ibuprofen (600 μM) and bromamines (**a**-100 μM, **b**-300 μM) on iNOS expression and NO/NO_2^- production in LPS stimulated macrophages. Cells were pre-incubated with tested agents in DMEM without FBS. After 1.5 h, the medium was removed and fresh DMEM+5% FBS with LPS (100 ng/ml) was added for additional 24 h. After 24 h, supernatants and cell lysates were collected. In cell lysates, iNOS expression was analyzed using western blot. NO_2^- production was determined in culture supernatants as described in Methods. Data were calculated from three separate experiments. $*p < 0.05$; $***p < 0.001$ versus LPS, # $p < 0.05$; ### $p < 0.001$ versus IBU

It is well documented that exogenous PGE2 added into activated macrophages down-regulates the synthesis of TNF-α (Wiliams and Shacter 1997). Since macrophages are a major source of PGE2 and TNF-α during a course of inflammation/infection, the PGE2 generated by macrophages regulates cytokine synthesis in an autocrine fashion. Indeed, it is a number of studies that demonstrated the association between administration of COX-2 inhibitors (ibuprofen, NSAIDs) and enhancement of systemic production of TNF-α (Cagiltay et al. 2015; Pettipher and Wimberly

1994). Importantly, enhanced serum levels of TNF-α associated with exacerbation of GAS infections and septic shock were observed in humans and experimental animals. For example, in GAS-infected mice, tissue levels of TNF-α and IL-6 were significantly higher in IBU-treated mice than in the control group. Moreover, IBU-treated mice exhibited more evident macrophage tissue infiltration, wound necrosis and the increased mortality rate (Weng et al. 2011). In agreement with experimental studies, there are concerns that the use of NSAIDs (IBU) is associated with an increased risk of necrotizing soft tissue infections and infections with GAS (Lesko et al. 2001; Norrby-Teglund et al. 1995). Such risk concerns primarily children with varicella and concomitant bacterial (GAS) skin infections (Hay et al. 2008; Zerr et al. 1999).

All these data support the idea that ibuprofen may promote bacterial (GAS) skin complications through the enhancement of TNF-α production at a site of inflammation. Independently, it has been well documented that N-bromotaurine (Tau-NHBr), N-dibromo-dimethyltaurine (DM-NBr2T), its more stable analogue, and bromamine T (BAT), suppress the production of TNF-α by various cells of inflammatory response, including macrophages (Marcinkiewicz et al. 2005; Walczewska et al. 2017).

The present study was undertaken to investigate the combined biological effect of ibuprofen and bromamines. We have hypothesised that bromamines are able to neutralize IBU-dependent overproduction of TNF-α by activated macrophages without affecting IBU impact on the COX-2/PGE2 pathway. To prove this hypothesis, J774.A1 macrophages were exposed to IBU and bromamines before stimulation with LPS or S. pyogenes (GAS).

In our experimental model, we observed that both LPS and GAS markedly induced expression of COX-2 enzymes with concomitant synthesis of PGE2. As expected, IBU significantly inhibited the synthesis of PGE2 if used at a concentration higher than IC50 for COX-2. A similar effect was observed when macrophages were exposed to the tested bromamines. Importantly, the joint action of IBU and bromamines caused an additive inhibitory effect on the synthesis of PGE2. These results clearly indicate that combined administration of IBU and bromamines amplifies inhibition of prostaglandin synthesis by activated macrophages, the primary pharmacological effect of IBU and other NSAIDs.

S. pyogenes (GAS) is a common human pathogen that normally causes mild skin and mucosa infections, but it can also cause severe diseases, such as necrotizing fasciitis and toxic shock. It has been reported that PGE2 is one of the major inflammatory mediators responsible for the severity and outcome of GAS infection (Goldman et al. 2010). Thus, the synergistic inhibition of the COX-2/PGE2 pathway by ibuprofen and bromamines indicates the positive effect of such joint therapy.

This beneficial combined immunomodulatory effect of IBU and bromamines is supported by their capacity to suppress the production of nitric oxide, as shown in the present study. Although as nitric oxide does not contribute to the defense mechanisms against GAS infections, it may contribute to the induction and outcome of sepsis and toxic shock (Titheradge 1999). As expected, in our experimental set-up

S. pyogenes did not induce the iNOS/NO pathway. Importantly, all tested agents markedly reduced the production of NO by LPS stimulated macrophages eliminating its impact in the induction of anticipated toxic shock (Diao et al. 2002).

Together, one may conclude that suppression of synthesis of both PGE2 and NO during GAS infections by IBU (NSAIDs) is a positive therapeutic effect. In spite of these facts, association of IBU (NSAIDs) with severe GAS soft tissue infections and an increased risk of sepsis and toxic shock it has been reported (Bernard et al. 1997; Le Turnier et al. 2017). For example, it has been documented that ibuprofen worsens GAS soft tissue infections in mice (Weng et al. 2011) and causes soft tissue GAS-complications in patients with varicella and zoster diseases (Mikaeloff et al. 2008).

Indeed, new experimental data suggest that nonselective NSAIDs (ibuprofen) do more than simply mask the signs and symptoms of developing GAS infection (Bryant et al. 2015). One of the proposed mechanisms, responsible for the detrimental adverse effect of ibuprofen, is its potential to enhance TNF-α synthesis in the course of inflammatory/infectious diseases (Cagiltay et al. 2015; Bessler et al. 2017). The enhanced systemic level of TNF-α during NSAIDs therapy seems to be effect of lack of PGE2 and an uncontrolled production of cytokines by activated inflammatory cells (Kudo and Murakami 2005; Goldman et al. 2010). A large body of evidence points to TNF-α as a key factor in pathogenesis of severe GAS infections, including necrotizing fasciitis and toxic shock syndrome (Norrby-Teglund et al. 1995; Stevens 2000).

Therefore, the most important data in this study concern the combined effect of ibuprofen and bromamines on cytokine production by activated macrophages. We have shown that all tested bromamines (Tau-NHBr, DM-NBr2T and BAT) supress the overproduction of TNF-α, IL-6 and IL-10 in spite of the absence of PGE2. Together, these data suggest that the detrimental side effect of ibuprofen on the induction and outcome of skin GAS infections may be neutralized by a joint topical application of N-bromotaurine analogues or bromamine T. However, topical application of N-bromine compounds should be limited to the infected skin surfaces as their bactericidal activity in the presence of body fluid proteins is reduced (Gottardi et al. 2014).

5 Conclusion

The present study suggests that topical application of N-bromotaurine (Tau-NHBr), N-dibromo-dimethyltaurine (DM-NBr2T) or bromamine T (BAT) on infected skin may decrease the risk of severe GAS soft tissue infections associated with NSAIDs (ibuprofen) therapy. This opinion is supported by the following data:

Combined application of ibuprofen and bromamines retains primary therapeutic activities of both partners, the suppression of the PGE2 synthesis and anti-inflammatory properties, respectively.

The suppression of the COX-2/PGE2 pathway by ibuprofen is even amplified by bromamines.

Ibuprofen does not enhance the production of TNF-α in the presence of bromamines.

All tested bromamines, in our experimental set-up, affect the immunomodulatory properties of IBU to a similar extent.

To confirm these data, further studies using animal models of GAS infections are necessary.

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Combined Biological Effects of N-Bromotaurine Analogs and Ibuprofen. Part II: Influence on a Local Defense System

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Abstract The stable N-bromotaurine analogs (N-dibromo-dimethyl taurine, N-monobromo-dimethyl taurine), and bromamine T (BAT) show anti-inflammatory and microbicidal properties. These bromamines are good candidates for a treatment of skin infectious/inflammatory diseases as local antiseptics. Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), is commonly used in various infectious/inflammatory diseases due to its analgesic and antipyretic therapeutic effects. However, systemic administration of ibuprofen may also result in adverse side effects. It has been reported that ibuprofen enhances serum levels of TNF-α and worsens secondary skin infections caused by invasive streptococci (S. pyogenes). Recently we have demonstrated that bromamines inhibit the stimulatory effect of ibuprofen on the production of inflammatory cytokines (TNF-α, IL-6). The aim of this study was to examine the combined antibacterial actions of ibuprofen and bromamines against S. pyogenes and their joint effect on the generation of reactive oxygen species (ROS) by activated neutrophils and macrophages. We have shown that the microbicidal activity of bromamines against S. pyogenes was not altered by ibuprofen. On the other hand, co-administration of ibuprofen and bromamines markedly decreased the generation of ROS by activated neutrophils and macrophages. Finally, we discuss how the antioxidant combined effect of bromamines and ibuprofen may affect a local defense system.

Keywords Taurine · N-bromotaurine analogs · Antiseptics · Ibuprofen · ROS · Phagocytes · Infectious skin diseases · S. pyogenes

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Abbreviations

Tau	taurine
HOBr	hypobromous acid
Tau-NHBr	N-bromotaurine, taurine bromamine
DM-NBr_2T (Br-422)	N-dibromo-dimethyltaurine
BAT	bromamine T, N-bromo-N-sodio-p-toluenesulfonamide
LCL	luminol-dependent chemiluminescence
OZ	opsonized zymosan
ROS	reactive oxygen species
NO	nitric oxide
PMN	polymorphonuclear cells, murine peritoneal neutrophils
Mϕ	murine peritoneal macrophages
S. pyogenes	*Streptococcus pyogenes*
MBC	minimal bactericidal concentration

1 Introduction

Phagocyting neutrophils generate at a site of inflammation highly microbicidal hypohalous acids (HOCl and HOBr), the products of the myeloperoxidase (MPO) – halide system (Klebanoff 2005; Weiss et al. 1982). Taurine, the most abundant non-protein amino acid in neutrophil cytosol reacts with HOCl and HOBr to produce N-chlorotaurine (NCT) and N-bromotaurine (Tau-NHBr), respectively (Zgliczyński and Stelmaczyńska 1975; Tomas et al. 1995). Both haloamines exert antimicrobial and anti-inflammatory properties and have been used as topical antiseptics in various infectious/inflammatory diseases (Park et al. 1997; Nagl et al. 2003, Gottardi and Nagl 2010; Marcinkiewicz et al. 2008; Marcinkiewicz 2009, 2010). Recently, bromamines arouse interest because of their activity against all classes of microorganisms and because of invention of new stable analogs of Tau-NHBr, such as N-monobromo-dimethyltaurine (DM-NBrT) and N-dibromo-dimethyltaurine (DM-NBr2T) (Gottardi et al. 2014). We have previously demonstrated that all tested bromamines (Tau-NHBr, DM-NBrT and DM-NBr2T) show similar anti-inflammatory properties. Importantly, the stable N-bromotaurine analogues exerted even stronger microbicidal activity than Tau-NHBr (Walczewska et al. 2017). Ibuprofen is among the most widely prescribed non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of pain, fever, and inflammation (Rainsford, 2009). It is well documented that the primary mechanism of anti-inflammatory properties of IBU is associated with the inhibition of the COX-2/PGE2 pathway (Rainsford 2009). Therefore, its impact on the onset and outcome of infectious/inflammatory diseases is associated with the reduction of PGE2, one of the major proinflammatory mediators (Kalinski 2012). While low doses of ibuprofen (NSAIDs) markedly inhibit the synthesis of prostaglandins, high doses of IBU, depart from inhibition of COX-1/COX2 activity, are considered to be associated

with anti-oxidant activity and inhibition of neutrophil functions (Nilsen and Webster 1987; Diaz-Rodriguez et al. 2012; Maderazzo et al. 1984). Nevertheless, conflicting data have been published showing differences of anti-oxidant activities between various NSAIDs (Costa et al. 2006; Wilkinson et al. 2012). Moreover, it has been demonstrated that ibuprofen either enhanced or inhibited the generation of ROS by activated neutrophils and macrophages, the major cells of innate immunity and inflammatory response (Parij et al. 1998; Benbarek et al. 2012). The aim of this study was to examine the combined antibacterial actions of ibuprofen and bromamines and their joint effect on the generation of reactive oxygen/nitrogen species (ROS/RNS) by activated neutrophils and macrophages. We address this issue to explain whether bromamines affect anti-oxidant potential of high doses of ibuprofen and whether their bactericidal properties against Streptococcus pyogenes are not diminished by ibuprofen itself. It is well documented that administration of ibuprofen is associated with exacerbation of invasive group A beta-hemolytic streptococci (GAS) infections (Stevens 1995; Le Turnier et al. 2017; Weng et al. 2011). Therefore, we have chosen S. pyogenes (GAS bacteria) as a target pathogen and stimulant of the respiratory burst in phagocytes.

2 Methods

2.1 Tested Agents

Bromamines: N-bromotaurine (taurine bromamine, Tau-NHBr), N-dibromo-dimethyltaurine (DM-NBr2T), bromamine T (N-bromo-N-sodio-p-toluenesulfonamide, BAT). Dimethyltaurine was kindly provided by D. Debabov and R. Najafi (NovaBay Pharmaceuticals, Inc.) and brominated to DM-NBrT and DM-NBr2T. BAT was synthesized from dibromamine T as published (Nair et al. 1978). Ibuprofen (IBU), the selected NSAIDs drug, was purchased in Sigma-Aldrich.

2.1.1 Tau-NHBr (N-Bromotaurine) Preparation

Tau-NHBr was prepared in a two-step procedure (Marcinkiewicz et al. 2008). First, NaOBr was synthesized in reaction between equimolar amounts of NaOCl and NaBr (POCH, Poland) in PBS solution. In such conditions virtually all the OCl- present reacts with Br- to form OBr- and Cl-. The presence and concentration of OBr- was confirmed by UV spectra (λ = 200 to 400 nm). In the second step, 20 mM NaOBr was added dropwise to equal volume of 400 mM taurine. UV absorption spectrum was checked to exclude the formation of taurine dibromamine or chloramines and to estimate the concentration of Tau-NHBr (molar extinction

coefficient – 430 M^{-1} cm^{-1} at A288). Stock solution of Tau-NHBr was kept at 4 °C for a maximum period of 3 days before use.

2.2 Animals

Inbred CBA mice (8–12 weeks of age, 18–22 g) were maintained at the Animal Breeding Unit of the Department of Immunology of Jagiellonian University Medical College. All mice were held in standard caging conditions with water and standard diet ad libitum. This study was carried out in strict accordance with recommendations from the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Information of Poland. The protocol was approved by the I Local Committee on the Ethics of Animal Experiments of Jagiellonian University. All surgeries were performed under isoflurane (Abbott Laboratories) anesthesia. Every effort was made to minimize animal suffering. Mice were used as donors for peritoneal exudate cells.

2.3 Cells

Neutrophils (PMNs) and macrophages (Mϕ) isolated from CBA mice were induced by intraperitoneal injection of 1.5 ml of 3% thioglycolate (Difco Laboratories). PMNs were collected 18 h later, whereas macrophages were collected 4 days later by washing out the peritoneal cavity with 5 ml of PBS containing 5 U heparin/ml. Then cells were centrifuged, and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by addition of 2× concentrated PBS.

2.4 Cell Viability

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan. For MTT reduction experiments, cells in 96-well plates were incubated at 37 °C with 0.2 mg/ml MTT for 60 min (Promega). Culture medium was removed by aspiration, and cells were solubilized in 200 μl of DMSO. Extent of reduction of MTT to formazan within cells was quantified by measurement of absorbance at 550 nm. The minimal cytotoxic concentration (MCC) of bromamines and IBU has been estimated and the non-cytotoxic concentrations of the agents were used in further experiments.

2.5 Bacteria

Streptococcus pyogenes ATCC 19615, the selected strain of GAS bacteria, was kindly provided by the Center of Microbiological Research and Autovaccines Ltd. (Krakow, Poland). Briefly, bacteria were grown on Mueller Hinton agar with 5% horse blood (bioMérieux) at 37 °C for 48 h. For the luminol-dependent chemiluminescence assay (LCL), bacteria were suspended at 1×10^9/ml density in HBSS and then heat killed by 60 min incubation at 65 °C, before use. To examine microbicidal activity of tested agents, bacteria were grown on Mueller Hinton agar with 5% horse blood (bioMérieux) and incubated at 37 °C for 48 h (see below).

2.6 Microbicidal Activity of Tested Compounds (Tau-NHBr, DM-NBr_2T, BAT and IBU)

S. pyogenes was diluted to the concentration of 105 CFU/ml in 0.9% NaCl (pH 7.4) and then 100 µl of bacteria suspension was incubated with tested agents (7×10^{-5}-10 mM) at 37 °C for 1 h. After that, 100 µl of aliquots were plated on agar plates and incubated at 37 °C for 48 h. Then, CFU was counted considering dilution and compared to the control (bacteria incubated without tested compounds). A detection limit was 100 CFU/ml. The minimal concentration of tested agents that led to complete killing of bacteria to the detection limit (zero CFU on the agar plates) was determined as minimal bactericidal concentration (MBC).

2.7 Luminol-Dependent Chemiluminescence Assay

Effect of tested agents on generation of reactive oxygen species (ROS) by PMNs and Mϕ was evaluated in vitro using luminol-dependent chemiluminescence (LCL). LCL was counted at 37 °C in temperature-stabilized luminometer Lucy 1 (Anthos). Briefly, cells (5×10^5/cells/well) were mixed with luminol (0.8 mg/ml) at 1:1 volume ratio (both Sigma-Aldrich) and incubated at 37 °C for 30 min, then cells were incubated with tested agents in Hank's balanced salt solution (10 min at 37 °C in an atmosphere of 5% CO2) on a 96-well flat-bottom black plate (Nunc). After incubation, the cells were immediately stimulated with opsonized zymosan 0.2 mg/ml (Sigma-Aldrich) or S. pyogenes (MOI of ~ 100:1). Photon emission over 85 min with 4 min intervals was measured. Results are expressed as relative light units (RLU) where photons were counted every 5 seconds. Each type of experiment was performed in a duplicate.

2.8 Nitrite (NO_2^-) Determination

Nitric oxide, quantified by the accumulation of nitrite as a stable end product, was determined by a microplate assay (Ding et al. 1988). Briefly, 100 µl of sample supernatants were incubated with an equal volume of Griess reagent [1% sulphanilamide in 2 M HCl (Sigma-Aldrich) and 0.1%N-1-naphthylenediamine dihydrochloride in deionized water (POCH)] at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve.

2.9 Statistical Analysis

Statistical significance between groups was tested using one-way ANOVA comparison and Tukey's post hoc test. Results are expressed as a mean ± SEM values. A p value <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism version 5.01 program (GraphPad Software).

3 Results

3.1 Combined Microbicidal Effect of Ibuprofen and Bromamines Against S. pyogenes

The first aim of this study was to test antibacterial properties of bromamines and ibuprofen against S. pyogenes, the major pathogen responsible for secondary soft tissue bacterial infections (Stevens 1995). In our experimental conditions ibuprofen did not show any antibacterial activity at concentrations up to 600 µM. As expected, all bromamines confirmed their strong antibacterial properties and killed S. pyogenes at concentrations below 30 µM. Importantly, ibuprofen did not affect the microbicidal potential of the tested bromamines (Fig. 1). Moreover, for comparison of tolerability of bromamines and their microbicidal activity, the biocompatibility index (BI) was calculated. As shown in Table 1, the positive BI of all bromamines was not changed by IBU.

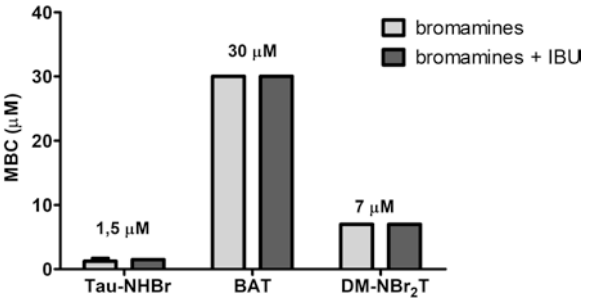

Fig. 1 Combined microbicidal effect of bromamines and ibuprofen against *S. pyogenes* ATCC 19615. Bacteria (5×10^5/ml) were incubat-ed with either bromamines alone or with the mixture of bromamines and ibuprofen. Bactericidal activity of the tested agents was deter-mined as described in Methods. Values are expressed as MBC (minimal bactericidal concentration) of 3 independent experiments. No antibacterial activity of IBU was found up to 600 μM

Table 1 Biocompatibility index of bromamines

Bromamines ± IBU	MCC (μM)	MBC (μM) *S. pyogenes*	BI MCC/MBC
Tau-NHBr + IBU	500	1,5	>300
	500	1,5	>300
BAT + IBU	500	30	~16
	500	30	~16
DM-NBr_2T + IBU	300	7	>40
	300	7	>40

Biocompatibility index, BI = MCC/MBC
MBC of Tau-NHBr, DM-NBr_2T, and BAT with or without IBU against *S. pyogenes* ATCC 19615 was defined as the minimal concentration of the agent (μM) that reduced the CFU count to the detection limit. For details see Methods. MCC (minimal cytotoxic concentration, μM) of the bromamines +/–IBU against neutrophils and macrophages was determined as described in Methods

3.2 *Effect of Ibuprofen on the Respiratory Burst of Neutrophils and Macrophages*

To detect ROS generation, products of the respiratory burst of neutrophils and macrophages, we used luminol-dependent chemiluminescence (LCL) (Wang et al. 1993: Castro et al. 1996). Upon addition of opsonized zymosan (OZ) LCL-dependent light emission was markedly increased reaching a maximum (RLU) about 15 min after application of the stimulus (Fig. 2b). S. pyogenes was weaker stimulus of LCL and the maximum level of RLU was observed 40 min after neutrophil and ~60 min after macrophage stimulation, respectively (Fig. 2a, c). When ibuprofen was added to the reaction mixture, a dose dependent decrease of LCL was

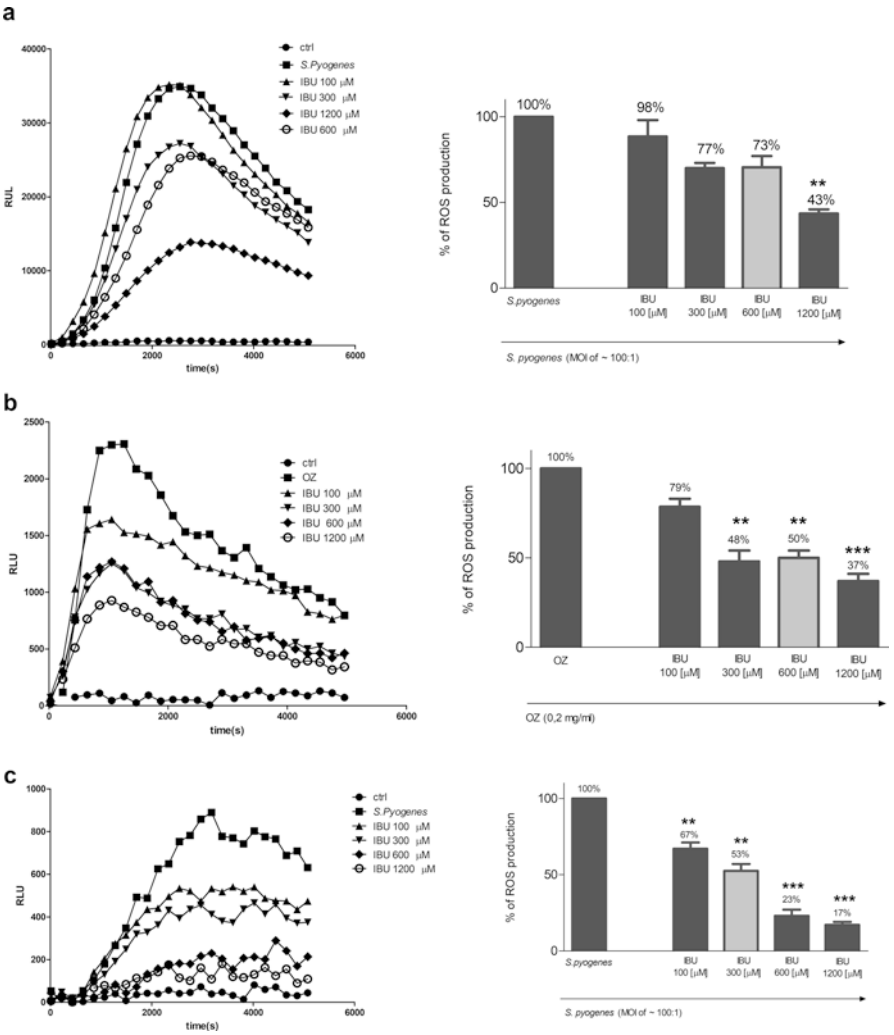

Fig. 2 Effect of ibuprofen on LCL emitted from: (**a**) Neutrophils stimulated with *S. pyogenes* ATCC 19615; (**b**) Macrophages stimulated with OZ; (**c**) Macrophages stimulated with *S. pyogenes* ATCC 19615. The cells (5×10^5/well), were preincubated with different doses of IBU for 10 min. Then, LCL was performed as described in Methods. Ctrl – LCL of non-stimulated cells. OZ – or *S. pyogenes*- stimulated cells, positive control, LCL = 100%. The left-charts show the time-trace of photon emission from one representative experiment. The right-charts (histograms) show the percentage of total photon count and represent the mean ± SD of two experiments. $**p < 0.005$; $***p < 0.001$ versus positive control

observed (Fig. 2). IBU, at a concentration of 600 μM (the concentration > IC50 for COX-2) only slightly inhibited (~30%) ROS production by S. pyogenes stimulated neutrophils (Fig. 2a). On the contrary, IBU markedly inhibited LCL in macrophages stimulated with OZ (maximal inhibition 50%) or with S. pyogenes (about 80%) (Fig. 2b, c).

3.3 Combined Effect of Ibuprofen and Bromamines on the Respiratory Burst of Neutrophils and Macrophages.

The next task of this study was to examine a joint effect of IBU and bromines on ROS generation by neutrophils and macrophages activated with either opsonized zymosan (OZ) or S. pyogenes. The tested agents were used at maximal non-cytotoxic concentrations. Namely, IBU was used at a concentration of 600 μM and Tau-NHBr, DM-NBr2T, and BAT at concentrations of 100 and 300 μM. As shown in Fig. 3, all bromines at the concentration of 300 μM completely inhibited LCL in neutrophils stimulated with OZ. The inhibitory effect of bromamines was not altered in the presence of IBU. Bromamines, used at a concentration of 100 μM, markedly inhibited LCL in activated neutrophils (~60–70%). This inhibitory effect of bromamines was more pronounced (>80%) in the presence of IBU.

Distinct combined effect of IBU and bromamines on LCL in neutrophils stimulated with S. pyogenes was observed. As shown in Fig. 4, bromamines markedly decreased LCL (100 μM ~60–80%; 300 μM – >90% the inhibitory effect). In

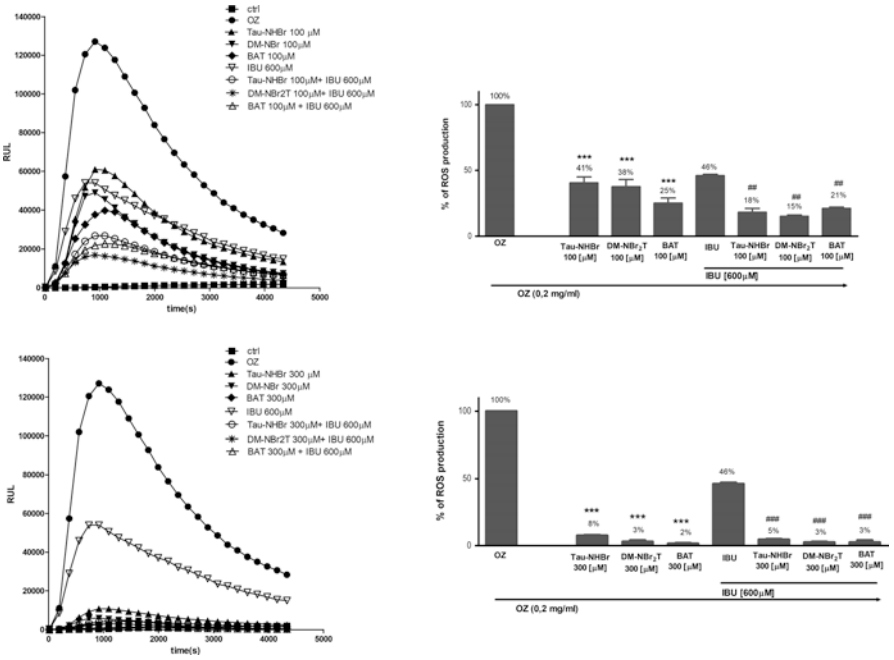

Fig. 3 Effect of Tau-NHBr, DM-NBr$_2$T, and BAT with or without IBU on LCL emitted from PMNs stimulated with OZ. PMNs (5×10^5/well), were preincubated with the tested agents for 10 min. Then, LCL was performed as described in Methods. Ctrl – non-stimulated cells. OZ-stimulated cells, positive control, LCL = 100%. The left-charts show the time-trace of photon emission from one representative experiment. The right-charts (histograms) show the percentage of total photon count and represent the mean ± SD of two experiments. ***$p < 0.001$ versus OZ; ##$p < 0.005$, ###$p < 0.001$ versus IBU

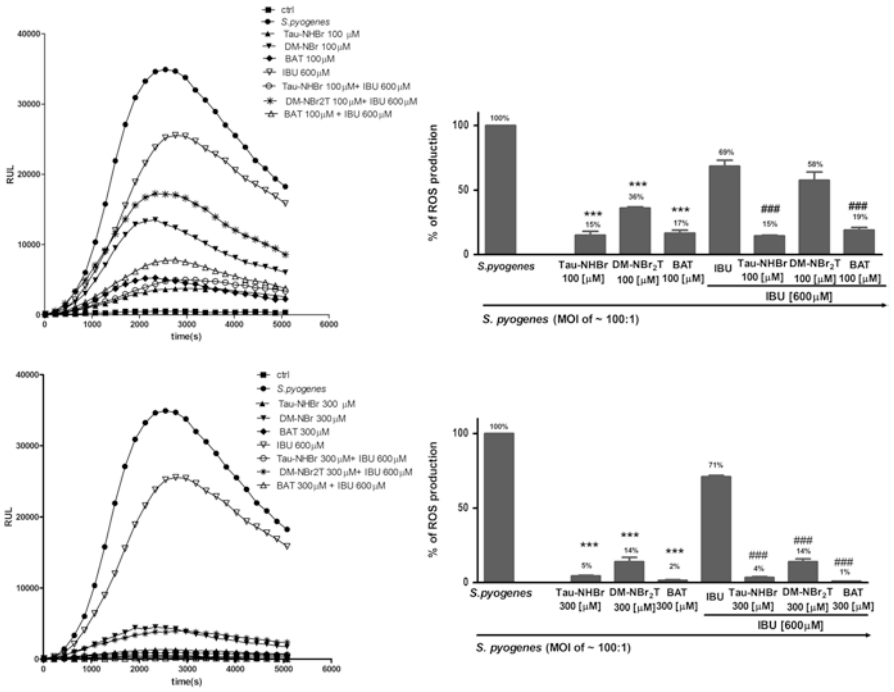

Fig. 4 Effect of Tau-NHBr, DM-NBr$_2$T, and BAT with or without IBU on LCL emitted from PMNs stimulated with *S. pyogenes* ATCC 19615. PMNs (5×10^5/well), were preincubated with the tested agents for 10 min. Then, LCL was performed as described in Methods. Ctrl – non-stimulated cells. *S. pyogenes*-stimulated cells, positive control, LCL = 100%. The left-charts show the time-trace of photon emission from one representative experiment. The right-charts (histograms) show the percentage of total photon count and represent the mean ± SD of two experiments. ***p < 0.001 versus *S. pyogenes*; ###p < 0.001 versus IBU

contrast to the amplified combined inhibitory effects observed in OZ-induced LCL, IBU did not affect the reduction of LCL caused by bromamines in S. pyogenes stimulated cells.

The influence of bromamines on LCL in macrophages stimulated with OZ or S. pyogenes was similar to that observed in activated neutrophils. Tau-NHBr, DM-NBr2T and BAT, used at a concentration of 100 or 300 μM, without significant differences between the agents, fairly inhibited LCL by about 70% or 90%, respectively. These inhibitory effects were not changed in the presence of IBU (Fig. 5). In general, the inhibition of LCL by all tested agents was more pronounced in macrophages stimulated with S. pyogenes (Fig. 6).

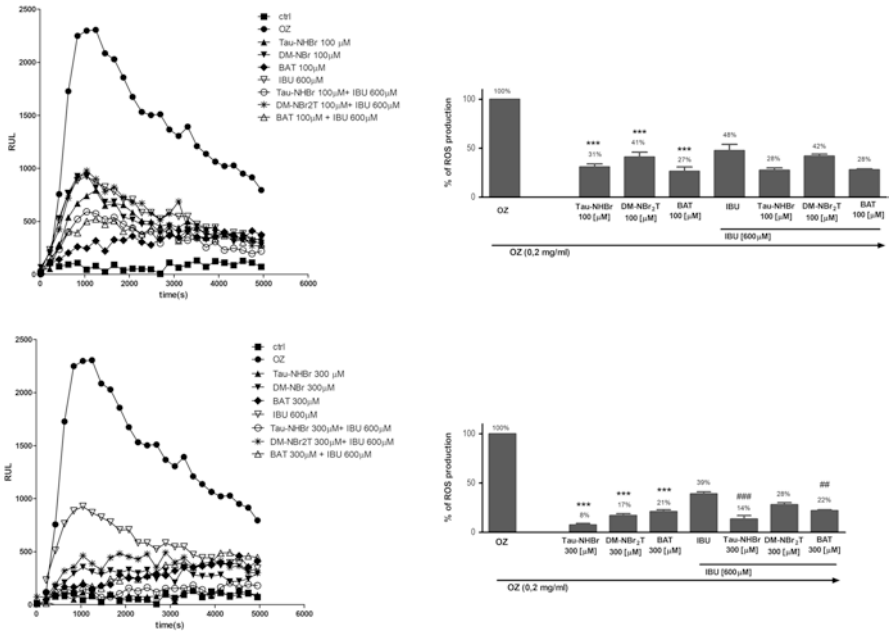

Fig. 5 Effect of Tau-NHBr, DM-NBr_2T, and BAT with or without IBU on LCL emitted from macrophages stimulated with OZ. Macrophages (5×10^5/well), were preincubated with the tested agents for 10 min. Then, LCL was performed as described in Methods. Ctrl – non-stimulated cells. OZ-stimulated cells, positive control, LCL = 100%. The left-charts show the time-trace of photon emission from one representative experiment. The right-charts (histograms) show the percentage of total photon count and represent the mean ± SD of two experiments. ∗∗∗p < 0.001 versus OZ; ##p < 0.005, ###p < 0.001 versus IBU

3.4 Combined Effect of Ibuprofen and Bromamines on theiNOS/NO/NO2- Pathway in S. Pyogenes Stimulated Neutrophils and Macrophages.

After stimulation of neutrophils and macrophages with S. pyogenes no detectable amounts of nitrites (the end products of the iNOS/NO/NO2- pathway), were found (data not shown). We also did not observe the expression of iNOS in cytosol of the stimulated cells.

4 Discussion

A number of clinical and basic science evidence points to the association between ibuprofen (NSAIDs) use and progression of severe group A streptococcal (GAS) soft tissue infections (Stevens 1995; Weng et al. 2011; Hay et al. 2008; Zerr et al.

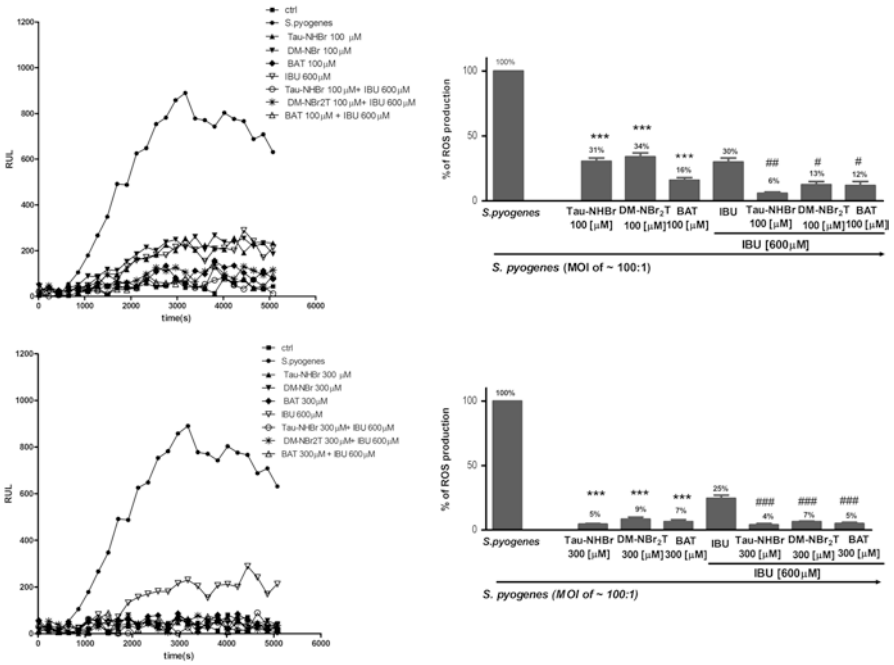

Fig. 6 Effect of Tau-NHBr, DM-NBr$_2$T, and BAT with or without IBU on LCL emitted from macrophages stimulated with *S. pyogenes* ATCC 19615. Macrophages (5×10^5/well), were preincubated with the tested agents for 10 min. Then, LCL was performed as described in Methods. Ctrl – non-stimulated cells. *S. pyogenes*-stimulated cells, positive control, LCL = 100%. The left-charts show the time-trace of photon emission from one representative experiment. The right-charts (histograms) show the percentage of total photon count and represent the mean +/− SD of two experiments. ∗∗∗p < 0.001 versus *S. pyogenes*; #p < 0.05, ##p < 0.005, ##p < 0.001 versus IBU

1999). However, direct impact of ibuprofen on accelerations of such infections still needs elucidation.

Several mechanisms have been proposed to explain how ibuprofen may promote bacterial (GAS) skin complications: (i) enhancement of TNF-α production, a major mediator of hyper-inflammation, septic shock and tissue failure; (ii) impairment of the immune response; (iii) masking the symptoms of infections through antipyretic and analgesic effect leading to delayed diagnosis and treatment (Bryant et al. 2015).

Current data indicate that ibuprofen administration enhances the systemic production of TNF-α and is associated with an exacerbation of cryptogenic GAS infections (Cagiltay et al. 2015).

On the other hand, there are conflicting data supporting the idea that ibuprofen may promote bacterial (GAS) skin complications through impairment of host defence. For instance, it has been demonstrated that ibuprofen may decrease neutrophil functions such as chemotaxis and phagocytosis (Nielsen and Webster 1987; Diaz-Rodriguez et al. 2012). On the other hand, it has been suggested that ibuprofen

may protect infected tissues from oxidative damage through a decreased neutrophil respiratory burst, independently of the COX-2/PGE2 pathway inhibition (Carey et al. 1992; Wilkinson et al. 2012).

Recently, we have demonstrated that co-administration of stable forms of N-bromotaurine and bromamine T with ibuprofen neutralizes its adverse effect on the production of TNF-α with concomitant enhancement of PGE2 suppression and maintaining anti-inflammatory properties of bromamines (Walczewska et al. submitted).

In this study we have asked the question whether co-administration of bromamines and ibuprofen will maintain microbicidal potential of bromamines and how this combined action will affect respiratory burst of activated phagocytes. For that purpose we have examined a combined microbicidal effect of bromamines and IBU against S. pyogens, the selected strains of GAS species. Moreover, luminol-dependent chemiluminescence (LCL) was used to examine the generation of ROS/RNI species by neutrophils and macrophages exposed to bromamines and IBU. Luminol reacts with various ROS and NO, but mainly with HOCl, the product of neutrophil myeloperoxidase (MPO)-halide system (Hasegawa et al. 1997).

In our experimental conditions, all tested bromamines (Tau-NHBr, DM-NBr2T, BAT) showed similarly strong bactericidal properties against S. pyogens with the MBC much below their cytotoxic activity (Walczewska et al. 2017). Importantly, ibuprofen did not affect the antibacterial properties of bromamines. Thus, co-administration of these agents does not change very positive biocompatibility index (BI) of bromamines against S. pyogens. The BI index, calculated by the ratio between the minimal cytotoxic concentration against macrophages and MBC against S. pyogens, ranges between 15 for BAT and even >200 for Tau-NHBr. This may indicate a very good tolerability of bromamines by human tissues since BI slightly above 1 has been found satisfactory for many antiseptics (Muller and Kramer 2008). However, significant consumption of oxidation capacity of bromamines by high protein load suggests that bromamines seem to be particularly suited for application on the skin or mucous membranes with low exudate (Gottardi and Nagl 2010; Gottardi et al. 2014). For instance, they can be used as topical antiseptics in bacterial (GAS) skin infections.

Apart from the beneficial combined antibacterial effect of IBU and bromamines their capacity to suppress the production of nitric oxide by LPS stimulated macrophages has been shown recently (Walczewska et al. submitted). Importantly, NO may contribute to the pathogenesis of sepsis and toxic shock but does not contribute to the defence mechanisms against GAS (S. pyogens) infections (Titheradge 1991). It was confirmed by the present study as S. pyogenes did not induce the iNOS/NO pathway in neutrophils and macrophages, the major phagocytes of antibacterial defence system.

In contrast to S. pyogenes incapability to induce the generation of NO, both neutrophils and macrophages incubated with these bacteria produced a significant amount of ROS, as measured by LCL. Opsonized zymosan (OZ) was used as a reference stimulus of the respiratory burst of phagocytes. The present data confirmed our previous reports showing the reduction of LCL in activated phagocytes

by N-bromotaurine analogues and BAT (Marcinkiewicz et al. 2005; Walczewska et al. 2017). Moreover, our data indicate that co-administration of IBU (600 μM) and bromamines (100, 300 μM) significantly decreased the generation of ROS produced by OZ and S. pyogens stimulated phagocytes. However, the impact of IBU on inhibition of LCL in neutrophils was much lower than that in macrophages. It may suggest that IBU does not inhibit the MPO/HOCl system, but it supresses the production of NO. This conclusion is based on results demonstrating that NO largely contributes to LCL in macrophages, in which HOCl formation does not occur. On the other hand, HOCl is the major ROS responsible for LCL in neutrophils, where NO is formed only in minor quantities, if at all (Wang et al. 1991, 1993).

Since the co-administration of IBU and bromamines inhibits the respiratory burst in major cells of innate immunity, one may suggest a negative therapeutic effect in the treatment of bacterial (GAS) skin infections. Indeed, the inhibition of ROS/NO production reduces the killing potential of phagocytes. However, the strong antiseptic properties of bromamines will compensate for this deficit. On the other hand, reduction of excess of ROS and protection of infected tissue from the oxidative stress is a positive therapeutic effect. Such anti-oxidant effects of ibuprofen (NSAIDs) therapy were reported by others (Carey et al. 1992; Wilkinson et al. 2012). Carrey et al. demonstrated the protective effect of ibuprofen in acute lung injury in swine resulting from a decreased neutrophil respiratory burst. Wilkinson's study has shown that ibuprofen attenuates superoxide production by activated microglia and monocytes. The authors suggest that ibuprofen may prevent oxidative damage in the brain. Finally, one may conclude that the co-administration of IBU and bromamines for treatment S. pyogenes (GAS) skin infections should neutralize the side effects of IBU and give a positive effect.

5 Conclusion

The present results along with the recent report (Walczewska et al. submitted) indicate that topical application of bromamines (Tau-NHBr, DM-NBr2T or BAT) on infected skin may alleviate the symptoms of severe S. pyogens (GAS) soft tissue infections associated with NSAIDs (ibuprofen) therapy. This opinion is supported by the following data:

Combined application of ibuprofen and bromamines retains primary therapeutic activities of both partners, the suppression of the PGE2 synthesis and anti-inflammatory/anti-bacterial properties, respectively.

Ibuprofen does not affect very high biocompatibility index of bromamines used as topical antiseptics. MBC of all bromamines against S. pyogenes was not altered by ibuprofen.

Co-administration of bromamines and ibuprofen decreases the risk of oxidative stress through the inhibition of neutrophil and macrophage respiratory burst.

Local application of bromamines, the strong antiseptics, should compensate for the likely deficit of ROS/RNI species at a site of inflammation.

To confirm these data further studies using animal models of GAS infections are necessary.

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Effects of Taurine on Bowel Inflammatory Factor of Small Intestinal Mucosa Impaired by Heat Stress in Broilers

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Abstract This study investigated the effects of taurine on bowel inflammation resulting from heat stress in broilers, with the intent of providing insight into potential improvement of the condition of broilers. A total of 300 healthy 1 day AA broilers were selected, fed normally until day 7, and allocated randomly to 5 treatment groups, namely, the control group(C), the heat stress group(HS), the low Tau (LTau) group, the middle Tau (MTau) group and the high Tau (HTau) group, which represent low, medium and high concentrations of taurine respectively. In the study, various concentrations of taurine were added to the drinking water. The Heat Stress model was produced by maintaining Broilers in a room at 34 °C.Heat stress persisted for 6 h, 12 h, 7 days, and 14 days. The results showed that the expression levels of TNF-α, IFN-γ, and IL-1β of the HTau group were significantly lower than that of the HS group at all time points examined (6 h, 12 h, 7 days, and 14 days) ($P < 0.05$). Compared with the HS group subjected to 6 h, 12 h and 14 days of heat stress, the MTau group exhibited significantly lower degrees of TNF-α and IL-1β expression. Moreover, the expression of IFN-γ was higher in the HS group after 6 h, 12 h and 7 days of heat stress than that of the MTau group subjected to similar times of heat stress ($P < 0.05$).There were no significant difference among the groups at other periods of heat stress ($P > 0.05$).

Keywords Taurine · Bowel inflammation · Heat stress · Broilers

Authors Qunhui Yang, Zhenyong Wang and Yiqing Cui have been equally contributed to this chapter.

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1 Introduction

Taurine, also known as beta-aminoethanesulfonic acid, was originally isolated from bull bile. Taurine, which is a white crystal, without taste, is chemically stable and soluble in water. (Bai Xiaoqiong and Kong Deyi 2011). It is widely distributed in animal tissues and organs as a free amino acid; it has several physiological functions. Although it is a non-essential amino acid in humans, some animals are incapable of synthesizing it. When fed a taurine deficient diet, these animals develop retinal pathology that can lead to blindness although recent studies suggest that taurine may also be an essential nutrient for function of the optic nerve. Presently, taurine is added to infant formula in several countries (Yuzhen Chen 1994). Marine molluscs are rich in taurine.

Recent studies have shown that taurine exerts several actions, such as anti-stress, anti-inflammatory, anti-oxidation and anti-apoptosis. Heat stress increases serum corticosterone levels, but reduces food intake, daily gain and the feed conversion rate in broiler chickens, conditions that may result in acute polynesic enteronitis gastroenteritis (Quinteiro-Filho et al. 2012). RT-PCR was used to examine the jejunum tissue of heat-stressed rats, focusing on mRNA levels of tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) (Shasha He 2016). It was concluded that heat stress may cause inflammation in the small intestines.

Few studies have focused on the effects of taurine on intestinal inflammation induced by heat stress. Our experiment established a broiler's heat stress-induced model which can be used to examine the effects of taurine on heat stress-induced inflammation. These studies have potential application, as they provide information on favorable conditions for poultry breeding.

2 Methods

2.1 Materials

The PCR and iQ5 fluorescence quantitative PCR machines were purchased from Bio-Rad, USA. The RNA isolater total RNA extract (100 ml) and the reverse transcription kit R223-01 were purchased from Nanjing Nuweizan Biotechnology Co., Ltd.fluorescence quantitative SYBR Premix Ex TaqII was purchased from Dalian Bao Biological Reagent Co., Ltd.

2.2 Animals

Caged 1-day AA broilers were randomly divided into five groups on 7 days, including normal control group (C), heat stress group (HS) and three taurine groups (Tau), known as LTau (low level of taurine and heat stress), MTau (medium levels of

taurine and heat stress) and HTau (high level of taurine and heat stress). The Tau groups were administered different amounts of taurine in drinking water beginning on 8 days, while the other two groups were given tap water. All groups were given water during the test period. On 15 days, a hot environment was created artificially, in which the cage temperature was continuously increased to 34 ± 2 °C and the C cage was maintained at 23–26 °C. Heat stress lasted up until 30 days. During the experiment, each group was maintained under standard ventilation, humidity and light and was given standard feed.

2.3 Sample Collection

The broilers were fasted but had access to drinking water for 12 h before the start of each test period. After heat stresses of 6 h, 12 h, 7 days, and 14 days, three broilers were randomly selected from each group. The peritoneal cavity was opened with a sterilized surgical instrument, and a longitudinal section of the jejunum was taken and washed with PBS. The samples were cut into small pieces of 100 mg–150 mg, placed in marked de-enzyme cryopreservationtubes, and frozen in liquid nitrogen. Then they were kept at −80 °C until use. HS groups of 6 h and 12 h were classified as the short-term heat stress group, while the 7 days and 14 days HS group was considered the long-term stress group.

2.4 Isolation of Total RNA and Preparation of cDNA

Jejunal RNA was extracted according to the instructions of the RNA isolater kit, the concentration and purity of RNA were determined; cDNA was prepared based on the procedure of reverse transcriptase of the reverse transcription reaction (HiScriptR II Reverse Transcriptase) (R223-01).

2.5 Real-Time Fluorescence Quantitative PCR Detection

Primers were designed using Primer Premier 5.0, the GenBank for accession sequence and GAPDH mRNA for reference. The results are shown in Table 1. The synthesis of primers was completed by Shanghai Shenggong Biological Engineering Co., Ltd.

2.6 Statistical Analysis

Statistical analysis was performed using SPSS17.0 statistical software. All thedata were expressed as means ± standard deviation.

Table 1 Design of primer

Gene	Accession number	Primer sequence	Product length	Annealing temperature
GAPDH	NM_204305.1	F:GAGGGTAGTGAAGGCTGCTG	116 bp	59.8 °C
		R:CGCATCAAAGGTGGAGGAAT		
HSP70	NC_006092.4	F:CATCGAGACAGCTGGTGGAG	120 bp	59.9 °C
		R:ACCTGGACGAGGACACTGCT		
IL-1β	NC_006109.4	F:TCTTCTACCGCCTGGACAGC	145 bp	58.7 °C
		R:TAGGTGGCGATGTTGACCTG		
IFN-γ	NC_006088.4	F:TGCTAGGAGACCAGCTGCAA	128 bp	59.0 °C
		R:CTGCCAGAAGCTCACCACTG		
TNF-α	NC_006101.4	F:GCACTCCGTTCAGACATCCA	112 bp	58.3 °C
		R:CGCACCTGTCCTGTATCTGC		
Occludin	NM_205128.1	F:TCAACGACCGCCTCAATCAG	122 bp	58.9 °C
		R:GTAGTCTGGGCTCCGCTTCA		
ZO-1	XM_015278981.1	F:TATGAAGATCGTGCGCCTCC	208 bp	57.8 °C
		R:GAGGTCTGCCATCGTAGCTC		
Claudin-1	NM_001013611.2	F:TTTGTTGCTGTGACGGGCAT	116 bp	58.0 °C
		R:AGTGCTGACAGACCTGCAATG		

SYBR Green II was used for fluorescence quantitative PCR reaction. According to kit instructions as ice configuration reaction and cDNA as a template, each sample was set for 3 replicates, and negative controls were also set

The final result is represented by $2^{-\Delta\Delta Ct}$, where ΔCt target gene = Ct target gene – Ct housekeeping gene

3 Results

3.1 The Result of TNF-α Gene mRNA Expression

As shown in Fig. 1, TNF-α expression of the jejunum of the HS group was 89.11%, 80.19%, 61.95%, 63.64% higher than that of the C group after heat stress of 6 h, 12 h, 7 days, and 14 days, respectively ($P < 0.05$); the HTau and MTau groups were 11.52% and 9.95%lower, respectively, than that of the HS group ($P < 0.05$) and there was no significant difference between the LTau group and the HS group after heat stress of 6 h. After heat stress of 12 h, the HTau and MTau groups were significantly lower (8.38% and 6.81%, respectively) than that of the HS group ($P < 0.05$), and there was no significant difference between the values of the LTau and HS groups. After heat stress for 7 days, the values of the HTau groups were significantly lower than that of the HS group, but there was no significant difference between the values of the MTau, LTau and HS groups ($P < 0.05$). After heat stress for 6 h, 12 h, 7 days and 14 days, there was no significant difference between the HTau and MTau groups, both of which showed reductions in the expression of TNF-α relative to that of the LTau group.

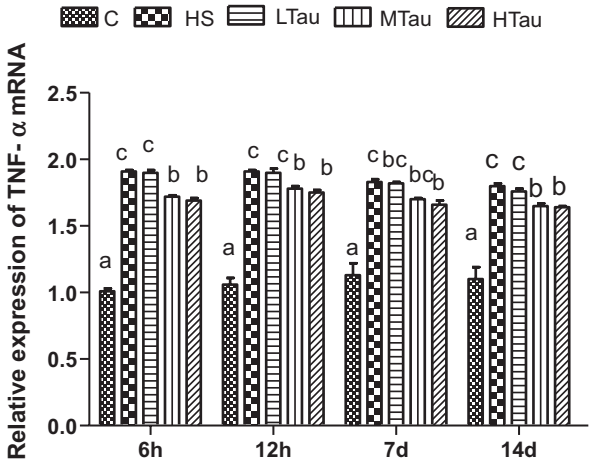

Fig. 1 Relative mRNA expression of TNF-α in the jejunum of broilers. C: the control group HS: the heat stress group LTau: the low Tau group MTau: the middle Tau group HTau: the high Tau group

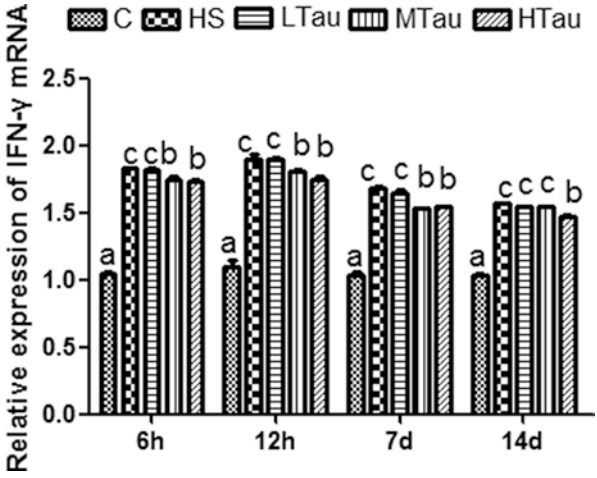

Fig. 2 Relative mRNA levels of IFN-γ in the Jejunum of Broilers. C: the control group HS: the heat stress group LTau: the low Tau group MTau: the middle Tau group HTau: the high Tau group. Note: Different small letters indicate significant differences ($P < 0.05$)

3.2 The Result of IFN-γ Gene mRNA Expression

As shown in Fig. 2, the expression of IFN-γ in the jejunum of broilers in the HS group was 75.96%, 74.31%, 63.11%, and 52.43% higher than in that of the group C after heat stress of 6 h, 12 h, 7 days, and 14 days, respectively ($P < 0.05$). However, the expression of IFN-γ for the HTau group was 5.46%, 7.89%, and 8.33% lower than that of the HS group ($P < 0.05$) after heat stress of 6 h, 12 h and 7 days, respectively. Similarly, the MTau group showed expression values that were 4.37%, 4.74%, and 8.93% lower after 6 h, 12 h and 7 days of heat stress, respectively, than those of the HS group ($P < 0.05$). Nonetheless, there was no significant difference in the mRNA levels of IFN-γ between the LTau and HS groups after heat stress of

6 h, 12 h, and 7 days. The levels of IFN-γ in the HTau group was 8.89%lower than that of the HS group ($P < 0.05$) and there was no significant difference between the expression values of the MTau, LTau and HS groups after heat stress for 14 days. In most conditions, the high and medium levels of taurine were more effective in reducing IL-1β mRNA in heat stressed broilers than those of the lower taurine group.

3.3 The Result of IL-1β Gene mRNA Expression

As shown in Fig. 3, the expression of IL-1βin the jejunum of the HS group was 76.92%, 76.27%, 84.16%, and 92.39% higher than in that of the group C after a period of heat stress of 6 h, 12 h, 7 days, and 14 days, respectively ($P < 0.05$). The levels of IL-1β mRNA in the HTau group were 9.18%, 6.99%, and 10.17% lower than of those in the HS group after a period of heat stress of 6 h, 12 h and 7 days, respectively ($P < 0.05$). The MTau group also exhibited declines in IL-1β expression relative to those of the HS group, with mRNA levels lower by 9.66%, 5.91%, and 9.60% after 6 h, 12 h and 7 days, respectively ($P < 0.05$). There was no significant difference in IL-1β mRNA levels between the LTau and HS groups after heat stress of 6 h, 7 days, and 14 days. After heat stress of 12 h, the IL-1β mRNA content of the HTau group was 10.10% lower than that of the HS group ($P < 0.05$). There were no significant differences in IL-1β mRNA levels between the MTau, LTau and HS groups. It was suggested that high and medium levels of taurine were more effective in lowering IL-1β mRNA levels of HS jejunum than the lowest taurine concentration examined.

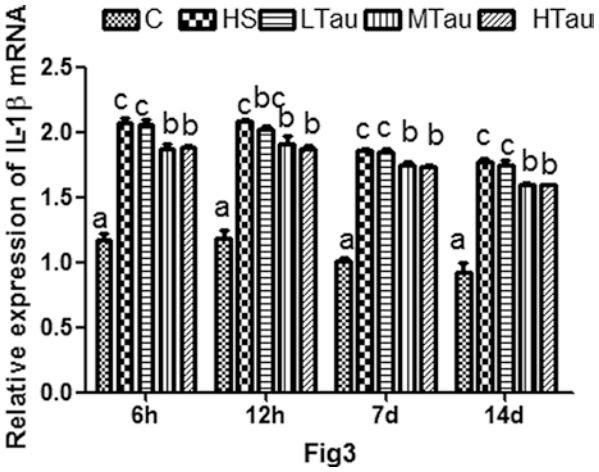

Fig. 3 Relative expression of IL-1β mRNA in the jejunum of Broilers. C: the control group HS: the heat stress group LTau: the low Tau group MTau: the middle Tau group HTau: the high Tau group. Note: Different small letters indicate significant differences ($P < 0.05$)

4 Discussion

Heat stress mediated the over expression of pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-1β, in broiler intestine tissues, indicating that the intestines were undergoing inflammatory reactions. TNF-α was considered as a marker of intestinal inflammatory diseases, but the mechanism of its damage to intestinal tissues has not been elucidated. Some scholars believe that TNF-α is unlikely to act on the intestines. Indeed, the incidence of enteritis is often related to the synergistic effects of TNF-α, IFN-γ and IL-1β (Xing LIU 2011; Juan LI 2014).

Significant accumulation of TNF-α in the intestinal mucosa can cause massive epithelial cell shedding and disruption of the mechanical barrier of the intestinal mucosa (Lu Ping and Gao Shirong 2015). Taurine, an important nutrient, can regulate ion transportation, inflammation and protein phosphorylation and finally inhibit or ameliorate pathological injury of cells.

Inflammation is unavoidable when sufficient TNF-α accumulates in the intestines. As an activating factor of the NF-κB signaling pathway, TNF-α regulates multiple stages of the inflammatory response. Once activated, the NF-κB signaling pathway regulates the production of more inflammatory factors, further promoting the inflammatory response (Juan LI 2014). It has been reported that taurine has antibacterial and anti-inflammatory effects through the formation of chloramine taurine (TauCl) which was formed by taurine and hypochloric acid from neutrophil myeloperoxidase (MPO)-halide system in the inflammatory reaction (Marcinkiewicz and Kontny 2014). IFN-γ, which is produced by activated T cells and NK cells, elevates neutrophils and NK cells and regulates the immune response and anti-tumor actions, etc. Also, it acts in synergy with TNF-α-induced intestinal inflammation, and upregulates intestinal inflammatory markers. Some scholars have found that IFN-γ regulates the expression of intestinal epithelial tight junction protein via the NF-κB/HIF-1α pathway, thereby affecting the function of the intestinal mucosa as a mechanical barrier (Songwei YANG 2014).

IL-1β, a cytokine produced mainly by activated mononuclear phagocytes, can be an immunomodulator at low concentrations, inducing neutrophil infiltration and the release of inflammatory mediators. It also generates body heat when produced in large quantities. This potent heat modulator effects may be the main reason that heat stress leas to an increase in body temperature (Wu Xiaolan and Zhao Shufen 2004).

Some scholars believe that taurine reduces the expressions of IL-1β, TNF-α, and IFN-γ by inhibiting the activation of NF-κB and blocking several key steps in the inflammatory response, thereby alleviating injury of the animal's intestines by heat stress (Barua et al. 2001; Roy et al. 2009; Ling XU et al. 2015). Not only are the anti-inflammatory properties of taurine manifest in the intestine, but are also apparent in other parts of the body, such as heart, blood vessels, brain tissue, muscles and so on (Sun Ruiyuan and Lu Qiufeng 2016; Wu Zhenming 2017; Huang Jing et al. 2016).

In our study, heat stress induced pro-inflammatory cytokines in the jejunum, elevating them above that of the control group. Our study indicates that the jejunum contains lymphocytes and macrophages and causes damage by releasing pro-inflammatory factors through autocrine or paracrine mechanisms.

5 Conclusion

The supplementation of high and medium levels of taurine reduces the expression of TNF-α, IFN-γ and IL-1β, pro-inflammatory cytokines in the jejunum of broilers induced by heat stress, reflecting the anti-inflammatory properties of taurine.

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Ribose-Taurine Suppresses Inflammation Through NF-κB Regulation in Activated RAW 264.7 Macrophages

Hwan Lee, Dong-Sung Lee, Kyung Ja Chang, Sung Hoon Kim, and Sun Hee Cheong

Abstract Ribose-taurine (Rib-T) suppressed the generation of inflammatory mediators and cytokines, such as nitric oxide (NO) and prostaglandin E2 (PGE2) through the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β induced by LPS was effectively blocked by Rib-T. Moreover, the anti-inflammatory actions of Rib-T were involved in its inhibitory effects against the nuclear translocation of nuclear factor-kappa B (NF-κB) p65, and NF-κB DNA-binding activity. These results suggest that the anti-inflammatory action of Rib-T is associated with NF-κB regulation.

Keywords Ribose-taurine · Inflammatory · NF-κB activation · RAW 264.7 macrophage

Abbreviations

Rib-T	ribose-taurine
LPS	lipopolysaccharide
iNOS	inducible nitric oxide synthase

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NO	nitric oxide
COX-2	cyclooxygenase-2
PGE_2	prostaglandin E2
IL-6	interleukin-6
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor-α
NF-κB	nuclear factor-kappa B

1 Introduction

Acute and chronic inflammation can cause many different kinds of diseases, including cancer, diabetes, rheumatoid arthritis, and neurodegenerative disorders (Heller et al. 1997). Under normal physiological conditions, NO generated by inducible nitric oxide synthase (iNOS) plays a major action in host-defense responses, cytoprotection, inflammation, and neurotransmission. However, the overproduction of NO can lead to inflammatory and auto-immune diseases (Maeda and Akaike 1998; Pautz et al. 2010). Activation of macrophages stimulated by lipopolysaccharide (LPS) contributes to inflammatory processes by increasing signal transduction, which induces several inflammatory mediators, cytokines, and enzymes, such as such as NO, iNOS, cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), interleukin 1β (IL-1β), and tumor necrosis factor-α (TNF-α) (Karpurapu et al. 2011; Laskin et al. 2011). In the inactive state, nuclear factor-κB (NF-κB), one of the most common transcription factors, presents in the cytosol and complexes with its inhibitory factor, inhibitor of kappa-B (IκB). When the cell is stimulated by various stimuli, IκB phosphorylation occurs, leading to NF-κB translocation into the nucleus (Surh et al. 2001). The activation of NF-κB is involved in acute-phase, and inflammatory responses by regulating the gene expression of several inflammatory mediators and cytokines (Otterbein et al. 2000). For this reason, several therapeutic interventions against the activation of NF-κB has been used for the prevention of inflammatory diseases. Taurine is a non-essential sulfur-containing amino acid endogenously synthesized from methionine or cysteine or obtained from the diet in foods such as seafood and meat (Wojcik et al. 2010). Taurine has several physiological actions including anti-inflammatory, anti-oxidative, and anti-apoptotic properties, and regulates blood pressure and cholesterol levels (Huxtable 1992; Zhang et al. 2004). Taurine derivatives have been shown to possess anti-hypoxic and anti-adipogenic effects (Sapronov et al. 2001; Cho et al. 2014). Although these beneficial effects of taurine and its derivatives have been investigated, anti-inflammatory actions and the direct mechanism of ribose-taurine (Rib-T), a synthetic taurine-carbohydrate derivative, remain unclear. In this study, therefore, we aimed to examine the anti-inflammatory effect of Rib-T and its effects on NF-κB regulation in LPS-stimulated RAW 264.7 macrophages.

2 Methods

2.1 Reagents and Devices

Synthesized Rib-T, a taurine-carbohydrate derivative, was gifted from Prof. Sung Hoon Kim (Kunkuk University, South Korea). 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI-1640 medium, trypsin, ethylenediaminetetraacetic acid (EDTA), and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Absorbance was measured using a Molecular Devices microplate reader.

2.2 Cell Culture

RAW 264.7 macrophages were purchased as frozen stocks from ATCC (Manassas, VA, USA) and were cultured in RPMI-1640 medium containing 10% FBS, streptomycin sulfate (100 μg/mL), and penicillin (100 U/mL) at 37 °C and 5% CO_2 in an incubator.

2.3 Cytotoxicity

An MTT assay was used to examine the cytotoxicity of Rib-T at different concentrations in RAW 264.7 macrophages (Mosmann 1983). To determine cytotoxicity, the cells were plated in 24-well plates (1 × 104 cells/well) and cultured in an incubator for 24 h. Rib-T was dissolved in DMSO and subsequently diluted to various concentrations in RPMI-1640 medium. The concentration of DMSO was adjusted to ≤0.1%. After 24 h, the medium was removed, 5 mg/mL MTT reagent was added to the plate and then incubated for 4 h. The supernatant was removed from each well, and 200 μL DMSO was added followed by shaking using an orbital shaker. The formazan product absorbance was measured at 540 nm for 30 min. The experiment was repeated three times to obtain an average absorbance value. Cell viability was determined by comparing the absorbance of samples with that of the control.

2.4 Nitrite Assay

A nitrite assay was used to measure the amount of NO produced according to the method described by Titheradge (1998). The cells were cultured in a 24-well plate at a density of 4 × 105 cells/well. After incubation for 24 h, the medium was removed

and LPS (1 μg/mL) was added followed by Griess reagent [N-(1-naphathyl)-ethylenediamine (0.1%, w/v), and sulfanilamide (1%, w/v) in phosphoric acid (5%, v/v)]. The absorbance was determined at 540 nm using an ELISA microplate reader (Molecular Devices).

2.5 Western Blot Analysis

Western blotting was performed according to the method described by Lee and Jeong (2016). RAW 264.7 macrophages were cultured in 6-well tissue culture plates (5 × 105 cells/well) for 24 h. Radioimmunoprecipitation assay (RIPA) buffer was added to RAW 264.7 macrophages, followed by centrifugation at 16,000 × g for 25 min at 4 °C. The supernatant was removed, then added to separate tubes. Protein quantification was performed using a bovine serum albumin (BSA) protein kit. Samples were resolved via 7.5% SDS-PAGE and transferred to a nitrocellulose (NC) membrane. The membrane was blocked using blocking buffer (0.1% Tween 20 in Tris-buffered saline) containing 5% skim milk, which was then diluted (1:1000) with iNOS and COX-2 antibodies and incubated for 1 h. After this, the secondary antibody (anti-rabbit immunoglobulin G, IgG), also diluted to 1:1000, and the enhanced chemiluminescence (ECL) solution were added to the NC membrane and the solutions were mixed well (1:1; v:v). The process was repeated to measure actin using the actin antibody in the same manner.

2.6 TNF-α, PGE_2, IL-6, and IL- 1β Measurement and NF-κB DNA-Binding Activity Assays

PGE2 production was measured using the method described by Lee and Jeong (2016). The culture medium was collected and the levels of PGE2, TNF-α, IL-6, and IL- 1β were determined by a commercially available kit from R&D Systems, Inc. (Minneapolis, MN, USA). NF-κB DNA-binding activity was determined using the TransAM kit (Active Motif, Carlsbad, CA, USA).

2.7 Isolation of Nuclear and Cytoplasmic Fractions

Mammalian protein extraction reagent (M-PER) containing protease inhibitor cocktail I and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to homogenize RAW 264.7 macrophages followed by centrifugation at 15,000 × g at 4 °C for 10 min. Following centrifugation, the supernatants were removed and stored at −75 °C for use as cytoplasmic fractions. The remaining precipitate was washed with PBS and RIPA buffer containing 0.5% sodium deoxycholate, 50 mM Tris-HCl

(pH 7.4), 150 mM NaCl, 20 mM ethylene glycol tetraacetic acid, 1% NP-40, 0.1% SDS, 1 mM dithiothreitol (DTT), 20 mM NaF, 50 mM glycerophosphate, 1 mM Na3VO4, and protease inhibitors, was added. The mixture was mixed at 4 °C for 15 min and centrifuged at 16,000 × g for 15 min at 4 °C. These samples were then subjected to western blotting according to the method described in 1.2.5.

2.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA). Multi-group comparisons were carried out by the Newman-Keuls post-hoc test. Data are represented as means ± standard deviations (SD) of three independent experiments.

3 Results

3.1 Inhibitory Effects of Rib-T on the Formation of Pro-inflammatory Mediators in LPS-Stimulated RAW 264.7 Macrophages

To determine whether Rib-T affected cell viability, RAW 264.7 macrophages were incubated for 48 h with different concentrations of Rib-T and cell viability was evaluated using MTT assay. Rib-T did not show significant cytotoxic effects on RAW 264.7 macrophages (Fig. 1a). NO and PGE2 formation was measured to examine the anti-inflammatory action of Rib-T treatment. RAW 264.7 macrophages were pretreated with different concentrations of Rib-T for 3 h and stimulated with LPS (1 μg/mL) for 24 h. In RAW 264.7 macrophages, LPS treatment markedly and dose-dependently increased NO (Fig. 1b) and PGE2 (Fig. 1c) production. However, pretreatment with Rib-T significantly suppressed the rise in the formation of NO and PGE2 stimulated by LPS.

3.2 Effects of Rib-T on the iNOS and COX-2 Expression in LPS-Stimulated RAW 264.7 Macrophages

We also investigated the effects of Rib-T on LPS-stimulated iNOS and COX-2 upregulation. The cells were pretreated with various concentrations of Rib-T for 3 h prior to 1 μg/mL LPS stimulation for 24 h followed by the measurement of iNOS and COX-2 protein expression. As shown in Fig. 2, Rib-T dose-dependently and effectively inhibited iNOS and COX-2 expression.

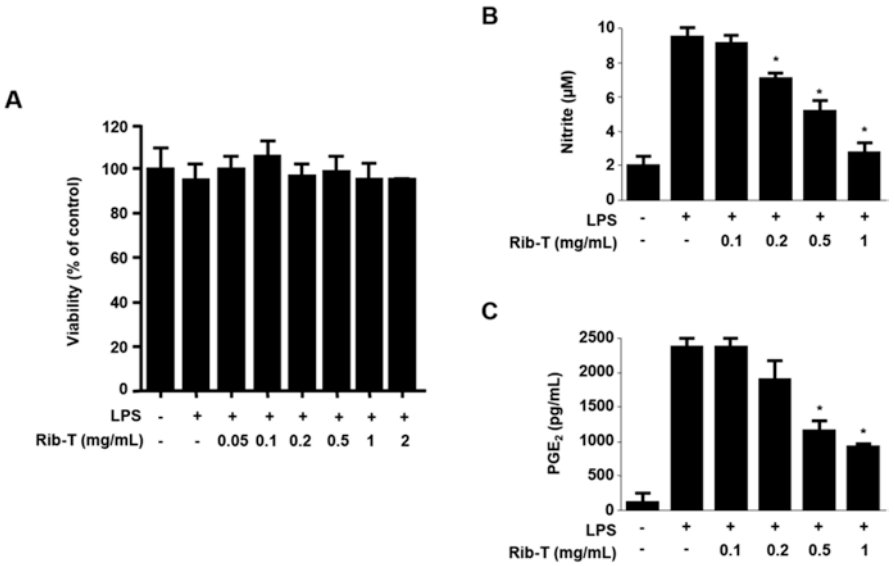

Fig. 1 The effects of Rib-T on (**a**) cell viability, (**b**) nitrite, and (**c**) PGE_2 production in RAW 264.7 macrophages. Cells were incubated for 48 h with different concentrations of Rib-T (0.05–2 mg/mL) (**a**). Based on the results of cell viability, the cells were pretreated with Rib-T at four concentrations (0.1–1 mg/mL) for 3 h and induced by LPS (1 μg/mL) for 24 h (**b** and **c**). Data represent the mean values ± SD. $*p < 0.05$ compared to the LPS treated group

3.3 Effects of Rib-T on LPS-Stimulated Pro-inflammatory Cytokine Production in RAW 264.7 Macrophages

To examine the effects of Rib-T on LPS-induced TNF-α, IL-1β, and IL-6, cells were induced by 1 μg/mL LPS for 24 h with non-cytotoxic levels of Rib-T. As shown in Fig. 3, Rib-T dose-dependently and markedly suppressed the pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β productions in LPS-induced RAW 264.7 macrophages.

3.4 Effects of Rib-T on DNA-Binding Activity and NF-κB Nuclear Translocation in LPS-Stimulated RAW 264.7 Macrophages

Finally, we aimed to explain the mechanisms by which Rib-T diminished the production of LPS-stimulated pro-inflammatory cytokines and mediators by inhibiting the NF-κB nuclear translocation. As shown in Fig. 4, Rib-T markedly suppressed the nuclear translocation of p65, one of the subunits of NF- B. In addition, as shown in Fig. 4c, we determined NF- B DNA-binding ability in the nuclear fractions. LPS

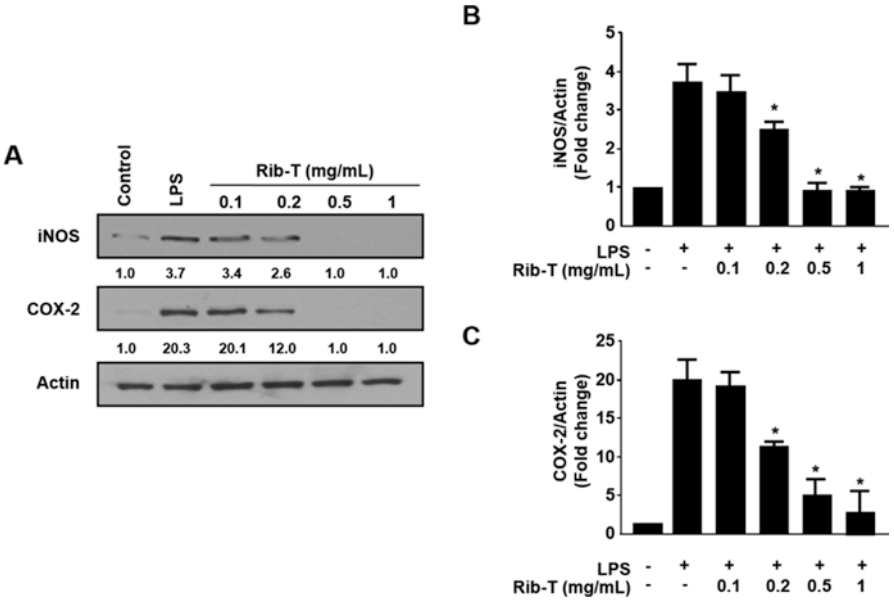

Fig. 2 The effects of Rib-T on (**a**) iNOS and COX-2 protein levels in RAW 264.7 macrophages. The cells were pretreated with Rib-T at four concentrations (0.1–1 mg/mL) for 3 h and induced by LPS (1 μg/mL) for 24 h. Band intensity of iNOS (**b**) and COX-2 (**c**) was quantified by densitometry and normalized to β-actin. Data represent the mean values ± SD of three independent experiments. $*p < 0.05$ compared to the LPS treated group

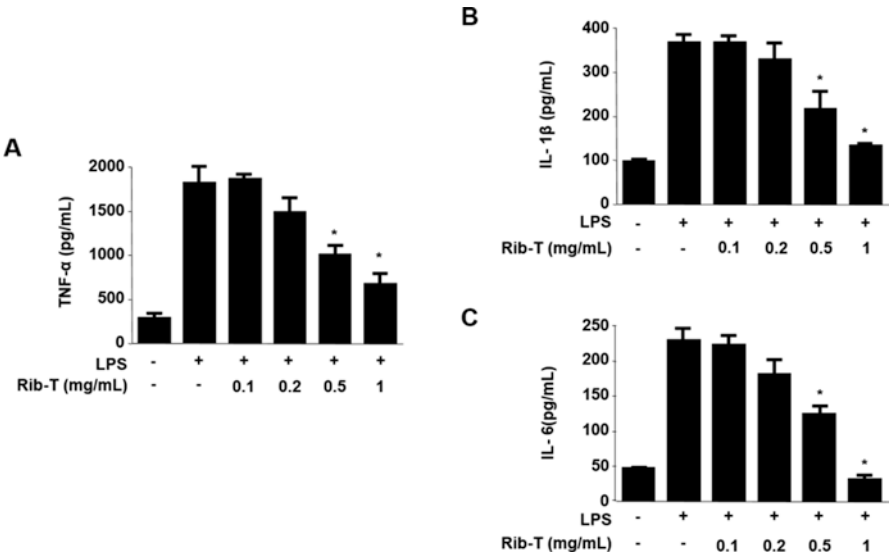

Fig. 3 The effects of Rib-T on (**a**) TNF-α, (**b**) IL-1β, and (**c**) IL-6 levels in RAW264.7 macrophages. The cells were pretreated with Rib-T at four concentrations (0.1–1 mg/mL) for 3 h and induced by LPS (1 μg/mL) for 24 h. Data represent the mean values ± SD. $*p < 0.05$ compared to the LPS treated group

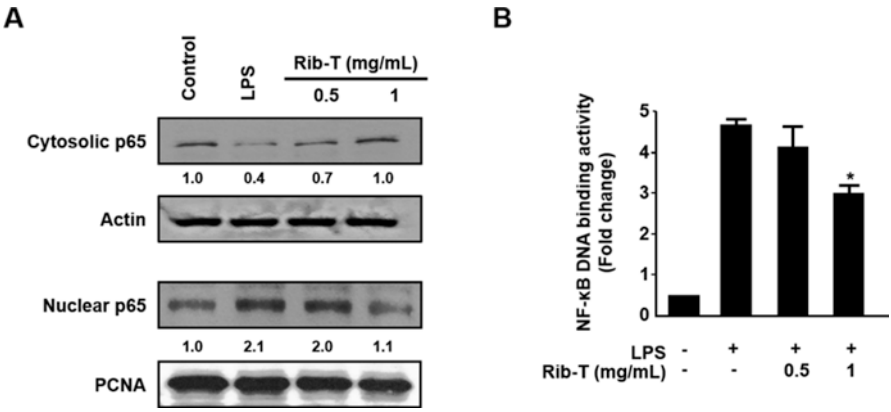

Fig. 4 The effects of Rib-T on the (**a**) nuclear translocation of NF-κB p65, and (**b**) DNA-binding activity in RAW264.7 macrophages. Cells were pretreated with two concentrations (0.5 and 1 mg/mL) of Rib-T for 3 h and induced by LPS (1 μg/mL) for 1 h. The data are represented as mean values ± SD. $*p < 0.05$ compared to the LPS treated group

stimulation significantly increased the degree of NF- B bound to DNA as compared to that in the controls. Rib-T significantly suppressed NF- B DNA-binding activity at a concentration of 1 mg/mL.

4 Discussion

Taurine, a sulfur-containing free amino acid, is well known for its beneficial actions including detoxification, conjugation with bile acids, cell membrane stabilization, anti-inflammatory, anti-oxidant, and anti-apoptotic properties (Friedewald et al. 1972; Wójcik et al. 2010; Chowdhury et al. 2016). Taurine reacts with hypochlorous acid in activated neutrophils forming the more stable and less cytotoxic taurine chloramine (TauCl) (Thomas et al. 1985). TauCl, a taurine derivative, suppresses the release of NO and TNF-α from activated cells in several different tissues (Marcinkiewicz et al. 1995; Kontny et al. 1999). However, the effects and mechanism of action of the new taurine-carbohydrate derivative, Rib-T on activated macrophages have not been reported. To the best of our knowledge, this is the first report of the anti-inflammatory actions of Rib-T in LPS-stimulated RAW 264.7 macrophages.

NO, which is produced by iNOS plays a key role in the inflammatory response particularly in cases where, excess NO generation results in the exacerbation of several inflammatory diseases (Szabó et al. 1994). Many pro-inflammatory mediators and cytokines also play important roles in triggering inflammatory responses in macrophages (Galli et al. 2008). Therefore, suppression of these pro-inflammatory mediators and cytokines is very important for controlling immune responses. In the

present study, pretreatment with Rib-T markedly suppressed the rise in the NO and PGE2 generation in LPS-stimulated RAW 264.7 macrophages with no reported cytotoxicity. In a previous study, it has been demonstrated that both TauBr and TauCl significantly decreased the accumulation of NO production due to the inhibition of nitric oxide synthase 2 activity in LPS-stimulated J774.2 macrophages (Olszanecki and Marcinkiewicz 2004). Similarly, Ward et al. (2011) reported that taurine and the taurine analogue, ethan-β-sultam, inhibited NO and IL-6 expression via changes in the NF-κB activation in LPS-induced alveolar macrophages. In the present study, Rib-T markedly suppressed the pro-inflammatory cytokines formation, including TNF-α, IL-1β, and IL-6, mediated by inhibition of iNOS and COX-2 protein expression. These results demonstrate that Rib-T led to anti-inflammatory effects by decreasing formation of NO and PGE2 through inhibiting the protein expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells. Numerous studies containing in vitro and in vivo have demonstrated that TauCl impedes the transcription and translation of the iNOS gene and downregulates the pro-inflammatory mediators (Kim et al. 1998; Marcinkiewicz et al. 1999). NF- B activation results from the degradation and phosphorylation of I B, thereby leading to the nuclear translocation of NF- B dimers from the cytoplasm, and activating the transcription of various NF- B-dependent target genes (Chae et al. 2006). In this study, we evaluated the mechanisms by which Rib-T suppressed the release of LPS-stimulated pro-inflammatory mediators and cytokines via the inhibition of NF-κB activation. Our findings demonstrated that Rib-T decreased the nuclear translocation of NF-κB and DNA-binding activity in LPS-induced RAW 264.7 macrophages. Similarly, Kim et al. (2006) reported that 5-aminosalicyltaurine inhibited TNF-dependent NF-κB activation in human colon epithelial cells. More recently, our previous results showed that galactose-taurine (Gal-T) effectively regulated the expressions of inflammatory cytokines and mediators stimulated by LPS in zebrafish model (Kim et al. 2017). We have also reported that another taurine derivative, xylose-taurine reduced, diminished the LPS-stimulated inflammatory response by inhibition of pro-inflammatory cytokines and mediators expression via decreased the activation of NF-κB in RAW 264.7 cells (Lee et al. 2017). Our findings confirmed that Rib-T effectively suppressed the formation of NO, PGE2, TNF-α, IL-6, and IL-1β mediated by the NF-κB activation in LPS-stimulated RAW 264.7 macrophages. These results suggest that Rib-T may be a beneficial candidate for the prevention and treatment of several inflammatory diseases.

5 Conclusion

In this study, Rib-T, a taurine derivative, exerted anti-inflammatory properties by suppressing the formation of NO and release of several pro-inflammatory mediators and cytokines such as PGE2, IL-6, IL-1β, and TNF-α via the inhibition of NF-κB pathways. To our knowledge, this study is the first demonstration that the mechanism of the anti-inflammatory properties of Rib-T in LPS-induced RAW 264.7

macrophages. Therefore, Rib-T may be an effective candidate for the management of inflammatory disorders.

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Scallop Extracts Inhibited LPS-Induced Inflammation by Suppressing MAPK and NF-κB Activation in RAW264.7 Macrophages

Xin Dong, Yon-Suk Kim, Eun-Kyung Kim, Woen-Bin Shin, Jin-Su Park, Su-Jin Kim, Eun-Ae Go, Pyo-Jam Park, and Sang-Chul Kwon

Abstract Scallops belong to cosmopolitan family of bivalves which are found in any oceans. They are one of the most important marine fishery resources in the world. The shell, meat and pearl layer have a high utilization value and a lot of scallops are eaten as food. In this study, we established anti-inflammatory effect of Scallops water extract in lipopolysaccharide (LPS) stimulated RAW 264.7 mononuclear macrophage. Our results indicated that Scallop water extract effectively reduced the synthesis of nitric oxide (NO). In addition, Scallop water extract suppressed the reactive oxygen species (ROS) generation and the expression of IL-6 and TNF-α. Further investigation indicated that anti-inflammatory effect of Scallop water extract via suppressing downregulation of MAPK (JNK, p38 and ERK) and NF-κB signaling.

Keywords Scallop extracts · Inflammation · MAPK and NF-κB signaling · Macrophages

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1 Introduction

Inflammation is generally considered as a main defensive response to injury or infection. Continuous recruitment and activation of macrophages in inflammation is a major feature of chronic inflammation. Macrophages play an important role in inflammatory responses through, antigen presentation, phagocytosis and immunomodulation (Fujiwara and Kobayashi 2005). In macrophages, excessive release of pro-inflammatory mediators may be important factors for chronic inflammation and severe tissue damages and eventually lead to the occurrence of many diseases. LPS which derived from Gram negative bacteria, has been known to be a potent inducer of inflammatory cytokines (Yoon et al. 2010).

A high concentration levels of ROS has been proven to elevate the expression of NF-κB (Chen et al. 2008). Exposing LPS to the cells leads to cellular ROS production increasing, and excess ROS contents is associated with the expression of various inflammatory pathways. Therefore, interaction exists between anti-oxidative and anti-inflammatory activity, and anti-oxidative assays might be able to be a marker for anti-inflammatory activity.

Activation of RAW 264.7 macrophages with LPS are used to evaluate the anti-inflammatory activity of different agents. Activated macrophages can produce pro-inflammatory mediators, such as TNF-α and IL-6 or many other cytokines and nitric oxide (Möller and Villiger 2006). TNF-α and IL-6 are among the most important cytokines released by activated macrophages. NF-κB is an important transcription factor playing important roles in the inflammatory response by regulating the gene expression of pro-inflammatory cytokines TNF-α and IL-6, inducible enzymes (COX-2 and iNOS), some acute phase proteins and immune receptors (Calixto et al. 2003).

It has been reported that MAPKs and NF-κB play a key role of regulating cellular responses to cytokines. Expression of phosphorylated JNK, p38 and ERK1/2 (MAPKs family) in RAW264.7 macrophages is increased after stimulating by LPS. The inhibition of any of these three is sufficient to prevent the induction of pro-inflammatory mediators and cytokines, which shows that inhibition of MAPKs signaling pathways may improve inflammatory diseases (Bhat et al. 1998).

In this study, we suggested that Scallop water extracts (SW) significantly suppressed the release of NO from LPS-induced RAW 264.7 cells and regulate intracellular ROS production. We also explored the relevant mechanism through NF-κB and MAPKs signaling pathway to prove the anti-inflammatory effects of SW. Our findings strongly suggested that the potential application of SW as anti-inflammatory agents.

2 Materials and Methods

2.1 Reagent

Dulbecco's modified eagle's medium (DMEM), antibiotics (penicillin and streptomycin) and fetal bovine serum (FBS) were purchased from Hyclone (Thermo Scientific, Waltham, MA, USA). Lipopolysaccharide (LPS), 2′,7-dichlorofluorescein diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kits and Mouse TNF-α ELISA kits were purchased from BD Biosciences (San Diego, CA, USA). The detection agents and polyvinylidine fluoride (PVDF) membrane were purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK). Antibodies for phosphorylated-ERK, −p38, -JNK, iNOS and COX-2 were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Antibodies for phosphorylated-IκBα, and β-actin were purchased from Cell Signaling Technology Inc. (Denvers, MA, USA). All of the other reagents were of the highest commercially available grade.

2.2 Preparation of the Extracts

The extracts were prepared by hot water extraction of dried SW (100 g) for 90 min in 1 L of water at 90 °C. Then filtered using Whatman No. 41 filter at room temperature (RT). The filtrates were evaporated using an evaporator (EYELA, Tokyo, Japan) at 50 °C. After evaporation, the water extracts were freeze dried and stored at −20 °C until use.

2.3 Cell Culture

The murine macrophage RAW 264.7 cells were supplied by Korea Cell line Bank (Seoul, Korea), were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 μg/mL of streptomycin and 100 units/mL of penicillin at 37 °C in a humidified incubator containing with 5% CO_2.

2.4 MTT Assay for Cell Viability

The cell viability effect of SW was evaluated using the MTT colorimetric assay. The RAW 264.7 macrophages were plated at a density of 1×10^4 cells/well into 96-well plates before the treatment. The cells were treated with various concentrations (50, 100 and 200 μg/mL) of SW and PMB (50 μg/mL) as positive control. Cells were stimulated with or without medium containing LPS (100 ng/mL) for 18 h. After treatment, the media were replaced by 100 μL of the DMEM medium containing MTT (200 μg/mL) and followed by incubation at 37 °C for 2 h. The MTT solution was then discarded and the intracellular formazan product was dissolved in 200 μL DMSO with shaken for 5 min. The absorbance was measured at 540 nm using a microplate reader (Tecan, Grödig, Austria) and values were calculated in comparison to the control cells.

2.5 Determination of Nitric Oxide (NO) Production

The Nitric oxide (NO) in the RAW 264.7 cell culture supernatant was determined via reaction with Griess reagent. Cells were plated at a density of 1×10^4 cells/well in 96-well plates and pre-treated with various concentrations (50, 100 and 200 μg/mL) of SW and exposed with medium containing LPS (100 ng/mL) at 37 °C for 18 h. The culture supernatant (100 μL) was mixed with Griess reagent (100 μL, 1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine) and shaken lightly for 10 min at room temperature. The absorbance of the mixture was determined at 540 nm using a microplate reader and nitrite concentration was determined using a dilution of sodium nitrite as the standard.

2.6 Determination of Intracellular ROS

The LPS-induced intracellular ROS generation by RAW264.7 cells was determined using the ROS sensitive fluorescent dye DCFH-DA. Cells plated at a density of 1×10^6 cells/well in 6-well plates were pre-treated with SW (50, 100, and 200 μg/mL) and N-acetyl-L-cysteine (NAC, 20 mM) for 1 h and then incubated with LPS (100 ng/mL) for 18 h, followed with DCFH-DA (1 mM) for 30 min. Cells were washed with PBS and gently scraped. The fluorescent intensity was analyzed at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a FACS Calibur flow cytometer (Becton & Dickinson Co., Franklin Lakes, NJ, USA).

2.7 Measurement of Cytokine (TNF-α and IL-6)

The RAW 264.7 macrophages were plated in 6-well plates at a density of 1×10^6 cells/well and incubated for 24 h. The cells were pretreated with different SW concentrations (50, 100 and 200 μg/mL) and PMB (50 μg/mL) for 1 h and then exposed to LPS (0.1 μg/mL) for 18 h. After incubation, the cell-free supernatants were used in the pro-inflammatory cytokine contents determination assays, which were performed with a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8 Western Blot Analysis

The RAW 264.7 cells were plated at a density of 1×10^6 cells in 6-well plates. After relevant treatments, the cells were subsequently washed with PBS, collected and suspended in a lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (1 μg/mL leupeptin and 100 μg/mL PMSF). After incubating in the ice for 30 min and centrifuged at 12,000 rpm for 20 min at 4 °C. The protein concentration was analyzed by Bradford assay method (Bio-Rad) using bovine serum albumin (BSA) as a standard. The proteins (30 μg of RAW 264.7 lysates) were separated with 12% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was washed with Tris-buffered saline-Tween (TBS-T, 20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20). Non-specific sites on the membrane were blocked by incubating the membrane in blocking solution containing 5% non-fat dry milk in TBS-T for 1 h at room temperature. Then, these membranes were incubated with specific primary antibodies at 4 °C overnight. After primary anti-body incubation, these membranes were washed with TBS-T and incubated with specific appropriate HRP-conjugated secondary anti-bodies (1:1000 diluted) for 1 h at room temperature. Protein bands were developed by ECL chemiluminescence detection reagent and blots were visualized using Davinch-western imaging system (CAS400SM; YoungWha science, Seoul, Korea).

2.9 Statistical Analysis

The data were expressed as the mean ± standard deviation for triplicate determinations. Analysis of variance (ANOVA), together with Tukey's test and Dunnett's test (GraphPad Prism 5), were conducted to identify the significant differences between the samples.

3 Results

3.1 Cell Viability and NO Assay

We evaluated the effect of SW on LPS-induced NO production. As shown in Fig. 1, the cells treated with LPS alone enhanced NO production remarkably and SW inhibited the levels of NO generation in a dose-dependent manner. RAW 264.7 macrophages were pretreated with SW (50, 100 and 200 μg/mL) for 1 h before stimulation with LPS (100 ng/mL) for 18 h. The results of the MTT assay showed that SW was not cytotoxic to RAW 264.7 cells (Fig. 2).

3.2 Measurement of Intracellular ROS

The LPS-induced intracellular reactive oxygen species (ROS) generated in RAW264.7 cells were determined using the ROS sensitive fluorescent dye DCFH-DA. As shown in Fig. 3, the LPS-induced intracellular ROS production compared to the control. When the pretreatment of SW, the ROS production was

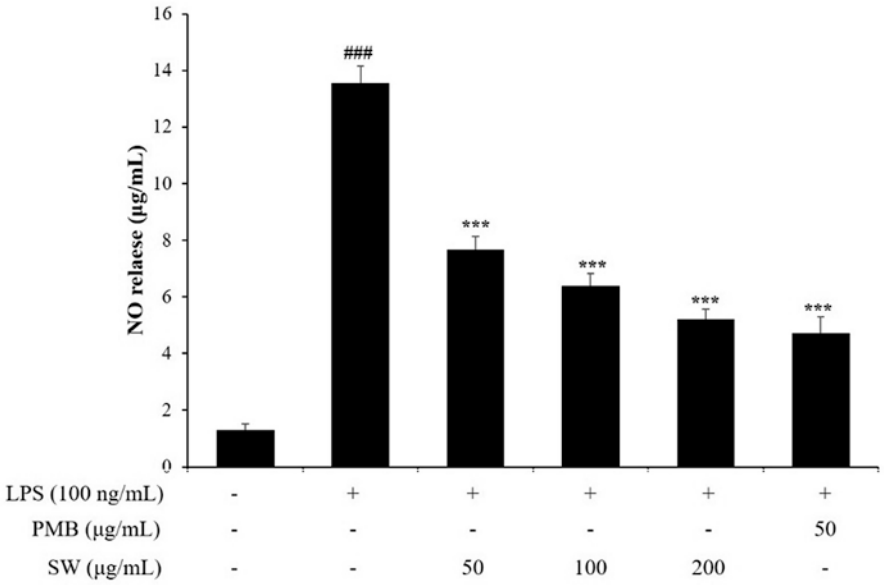

Fig. 1 Inhibition of NO production. Cells were pretreated for 1 h with SW and then stimulated to LPS (100 ng/mL) for 18 h. The concentration of NO was determined by using the Griess reagent. Data were represented as mean ± SD (n = 4) analyzed by one-way ANOVA followed by the Tukey's test. $^{###}p < 0.001$ compared to control, and $^{***}p < 0.001$ compared to LPS

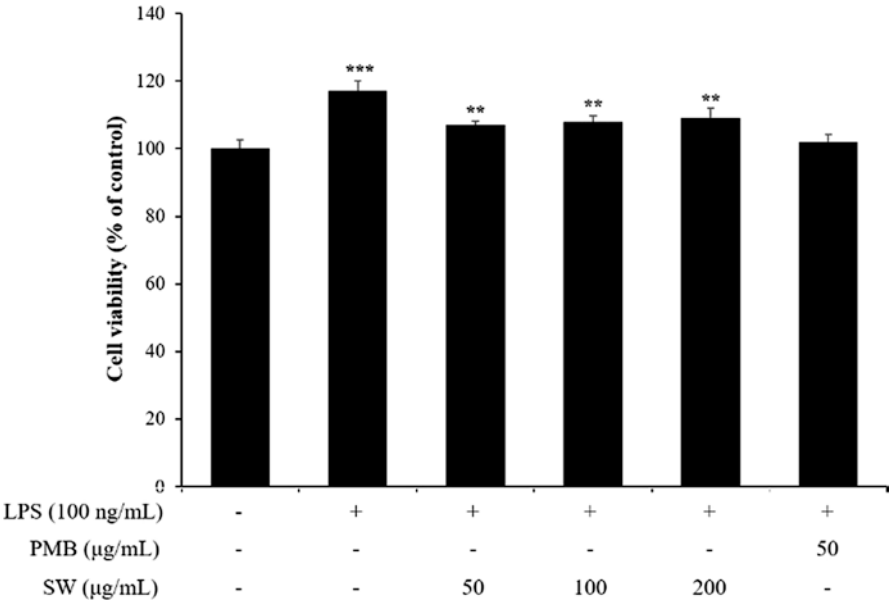

Fig. 2 Cell viability. RAW 264.7 cells were pretreated with SW for 1 h (50, 100, 200 μg/mL), and then treated with LPS (100 ng/mL) for 18 h. Cell viability was measured with the MTT assay. Data are represented as mean ± SD ($n = 4$) analyzed by one-way ANOVA followed by the Dunnett's test. $^{**}p < 0.01$, $^{***}p < 0.01$ vs. Control

decreased in a concentration-dependent manner. These results indicate that SW effectively suppressed LPS-induced intracellular ROS generation.

3.3 Cytokine assay

TNF-α and IL-6 are representative pro-inflammatory cytokines, produced by activated macrophage cells. To investigate the anti-inflammatory activity of SW, RAW264.7 cells were stimulated with LPS in the presence of SW. ELISA was performed to quantify secreted pro-inflammatory cytokines TNF-α and IL-6. As shown in Fig. 4, LPS significantly induced TNF-α and IL-6 release. Treatment with SW inhibited LPS-induced release of TNF-α and IL-6.

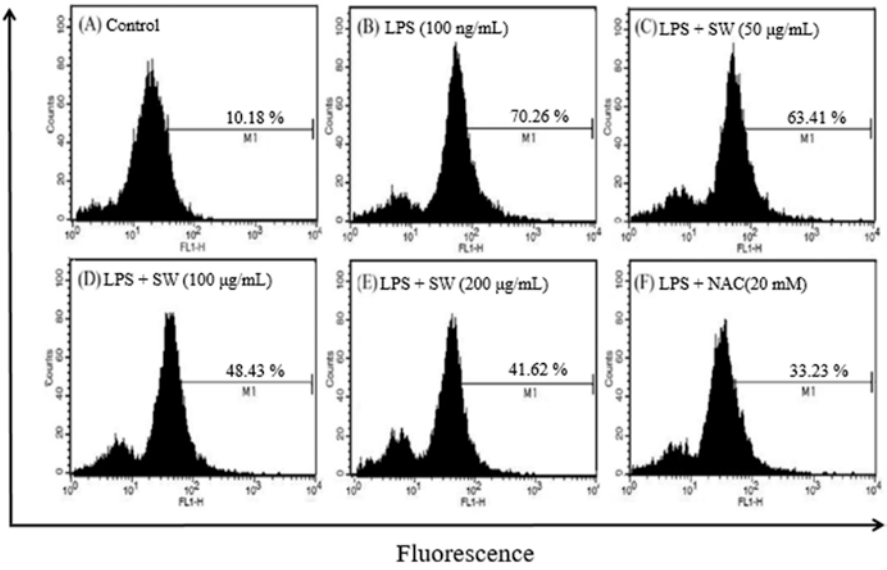

Fig. 3 Intracellular ROS determination using DCFH-DA on the LPS-induced RAW264.7 macrophages. Cells in 6-well plates were pretreated with SW (50, 100 and 200 μg/mL) for 1 h and then incubated with LPS (100 ng/mL) for 18 h, flowed by DCFH-DA incubation for 30 min. Cells were washed twice with PBS, and the intracellular levels of ROS were analyzed by flow cytometry

3.4 iNOS and COX-2 Expression

It is well reported that LPS strongly up-regulates iNOS and COX-2 levels in RAW264.7 macrophages. The levels of iNOS and COX-2 were determined by Western blot analysis. As shown in Fig. 5, western blot analysis showed that protein levels of iNOS and COX-2 were markedly increased after exposure to LPS, however the increased iNOS and COX-2 levels were significantly reduced by SW in a dose-dependent manner.

3.5 Inhibition of MAPKs Phosphorylation

It is known that the MAPKs pathway is critical for the inflammatory response stimulated by LPS in RAW 264.7 cells. To demonstrate the effects of SW on the MAPKs pathway, we investigated the effects of SW on the phosphorylation of ERK1/2, JNK, and p38 MAPKs in LPS-stimulated RAW 264.7 macrophages. As shown in Fig. 6, LPS significantly promoted the phosphorylation of ERK1/2, JNK, and p38 MAPKs. However, SW extracts strongly suppressed LPS-induced phosphorylation.

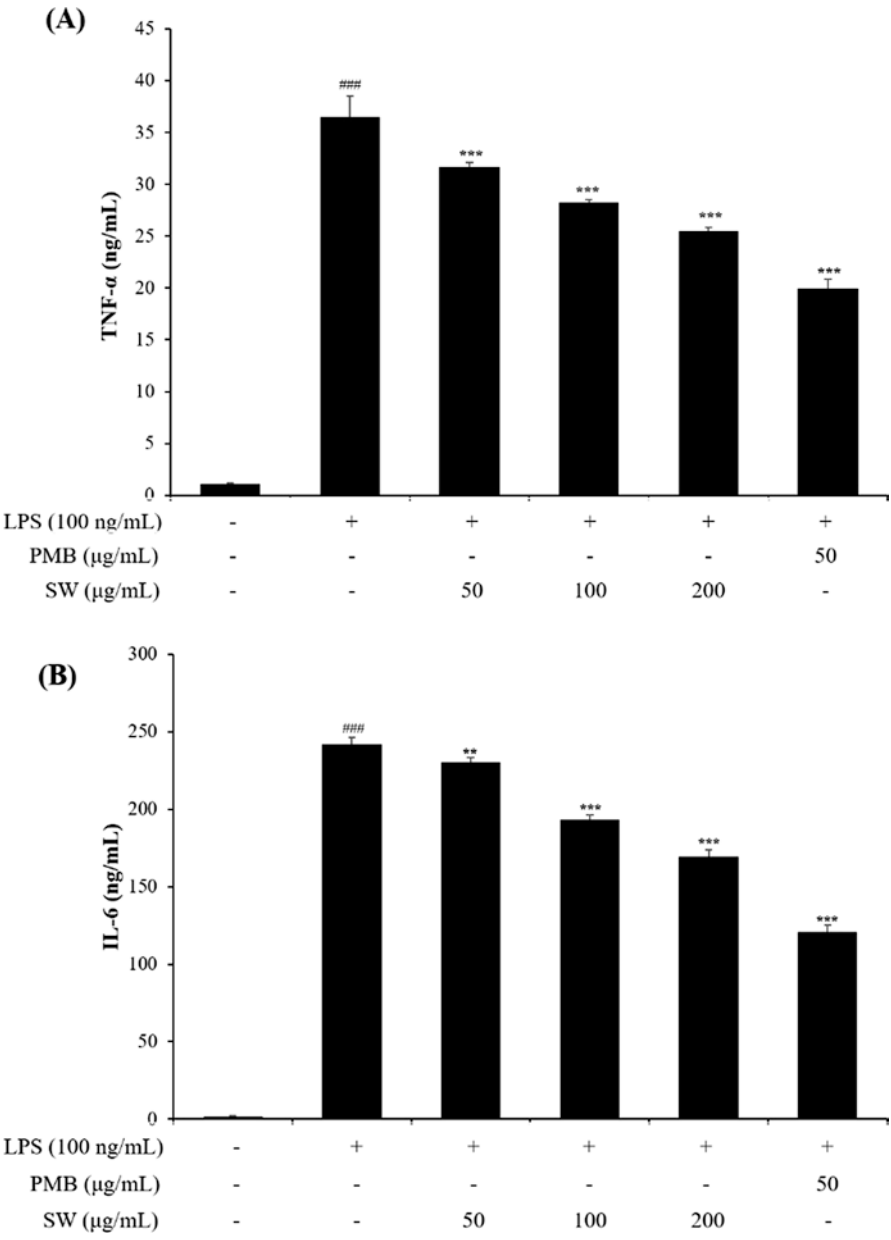

Fig. 4 Pro-inflammatory cytokine (TNF-α (**a**) and IL-6 (**b**)) inhibitory effects of SW in LPS-stimulated RAW264.7 macrophages. Cells were pretreated with SW (50, 100 and 200 μg/mL) for 1 h before exposure to LPS (100 ng/mL) for 18 h. The levels were detected using ELISA. Results were expressed as mean ± SD from three independent experiments. $^{###}p < 0.001$ compared to control, and $^{***}p < 0.001$ compared to LPS

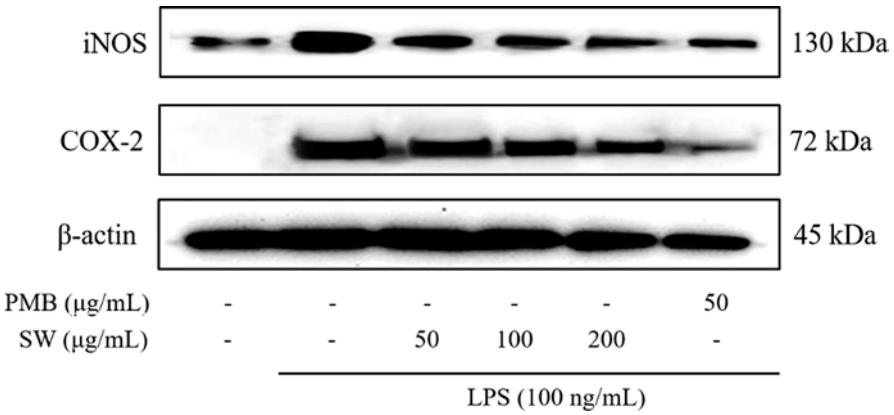

Fig. 5 iNOS and COX-2 expression in LPS-induced RAW264.7 macrophages. Cells were pre-treated with SW (50, 100 and 200 μg/mL) and PMB (50 μg/mL) for 1 h before exposure to LPS (100 ng/mL) for 18 h. The results were detected using western blot

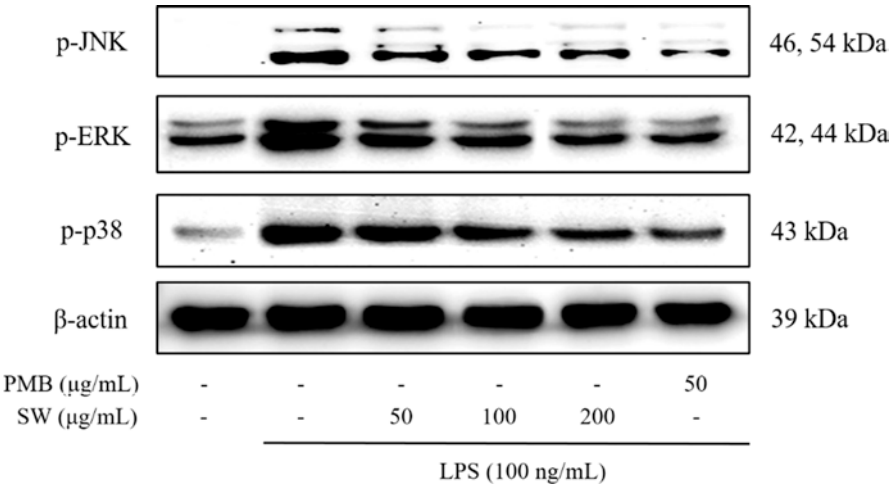

Fig. 6 Inhibitory effect of SW on LPS-induced activation of MAPKs. Effects of SW on LPS-induced activation of MAPKs, including ERK, p38, and JNK, in RAW264.7 were examined after cell treatments with different SW concentrations (50, 100 and 200 μg/mL) in the presence or absence of LPS (100 ng/mL) for 30 min using Western blot analysis

3.6 Phosphorylation of NF-κB and IκB-α Degradation

The NF-κB pathway plays a critical role in regulating the expression of many inflammatory mediators, we examined whether the inhibitory effects of SW were mediated through the modulation of the NF-κB pathway in RAW 264.7 cells. IκBα was rapidly phosphorylated and degraded by pro-inflammatory stimulation, which

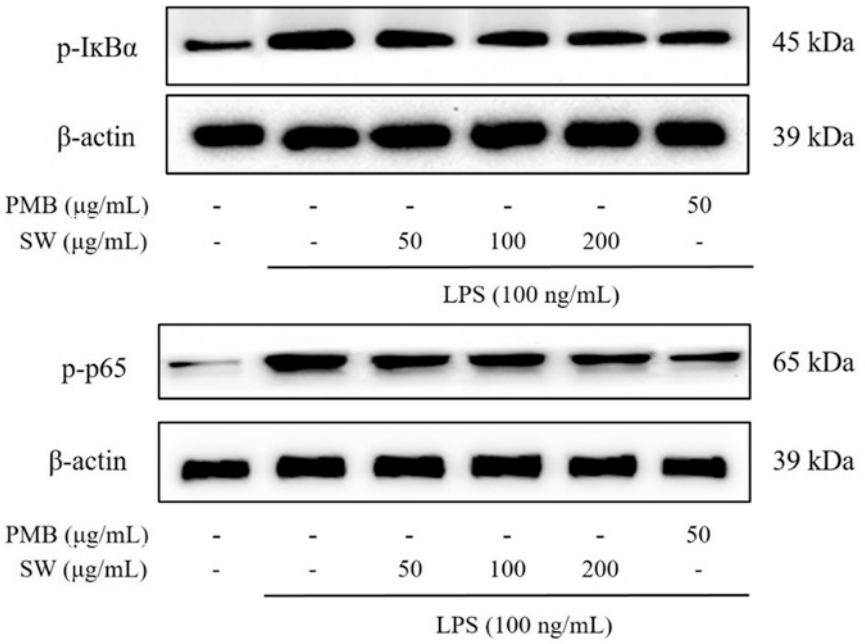

Fig. 7 Effect of SW on IκBα degradation and p65 translocation. RAW264.7 macrophages were pretreated with SW (50, 100 and 200 μg/mL) for 1 h and then stimulated with LPS (100 ng/mL) for 15 min

results in the release of NF-κB. As shown in Fig. 7, LPS stimulation strongly induced the phosphorylation of IκBα. Pretreatment with SW for 30 min markedly reduced phosphorylation of IκBα in a concentration-dependent manner. Next, we investigated whether SW could suppress the p65 NF-κB activation. Pretreatment of SW results in a decrease in p65 NF-κB activation in a concentration-dependent manner.

4 Discussion

ROS induces oxidative damage to biomolecules such as deoxyribo-nucleic acid, proteins, lipids and nucleic acids. It causes aging, cancer and other many diseases (Kehrer 1993). Exposing the cells to LPS occurs an increased production of cellular ROS contents and excess ROS levels are associated with the expression of various inflammations (Chanput et al. 2016). In short, there might be an interaction between oxidative stress and inflammation. Also, between anti-oxidative and anti-inflammatory activity. As shown in Fig. 3, the LPS-induced intracellular ROS production were significantly elevated compared to those in control. Due to the treatment with SW, the ROS production was decreased in a dose-dependent manner.

These results indicate that SW can regulate intracellular ROS production. Therefore, we believe that SW may be used as an anti-inflammatory agent.

NO as one of the pro-inflammatory molecules, it can mediate the functions of many types of cells at the site of inflammation, including lymphocytes, macrophages and endothelial cells (An et al. 2004). In addition, overproduction of NO may promote the production of cytokines, thereby leading to apoptosis and promoting the development of inflammation. Therefore, regulation of NO is considered to be one of the main strategies against inflammatory diseases. The cause of NO production is mainly due to the expression of iNOS protein (Hoesel and Schmid 2013). Long-term expression of pro-inflammatory genes, such as iNOS and COX-2 lead to chronic inflammation that is responsible for various diseases such as cardiovascular disease and cancer (Aggarwal et al. 2006). Our results proved that the inhibitory effects of the SW on NO production by LPS-stimulated RAW264.7 macrophages. As shown in Fig. 5, We demonstrated that SW decreased the expression of iNOS and COX-2 in a concentration-dependent manner.

It is well known that macrophages release the stimulation of pro-inflammatory cytokines by LPS. TNF-α and IL-6 cytokines mediate and regulate inflammatory diseases (Gabay 2006). TNF-α and IL-6 are implicated in the pathophysiological changes that occur during different disease states, such as vascular disease, multiple sclerosis and stroke (Zhang and An 2007). In this study, we found that the release of TNF-α and IL-6 was significantly and concentration-dependently inhibited by SW treatment in RAW 264.7 macrophages.

The MAPK pathway is one of the intracellular signaling pathways involved in the regulation of inflammatory responses in LPS-stimulated macrophages and it is involved in the activation of the transcription factor NF-κB (Xie et al. 1994). MAP kinases are a group of serine/threonine protein kinases comprising three subfamilies: the ERK, JNK, and the p38. Activated MAPK by various extracellular molecules, can induce phosphorylation of many key signaling molecules related to cell proliferation, inflammation and apoptosis (Craig et al. 2000). Our results showed that SW attenuated the phosphorylation of JNK, ERK and p38.

The transcription factor NF-κB is involved in the regulation of inflammation-related gene expression and has been reported (Tak and Firestein 2001). LPS induces phosphorylation and degradation of the inactive inhibitor IκBα, resulting in the inability to bind to NF-κB. In our study showed that SW inhibited the activation of NF-κB protein through suppressing IκBα degradation.

In this study, our findings suggest that the scallop water extracts inhibited the production of LPS induced intracellular ROS in RAW264.7 macrophages and reduced the production of NO through the inhibition of iNOS and COX-2 protein expression. Further research demonstrates that SW suppressed the production of pro-inflammatory cytokines, such as TNF-α and IL-6. SW was also found to inhibit the phosphorylation of MAPKs and NF-κB activation.

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Conflict of Interest The authors declare that there are no conflicts of interest.

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Correction to: Taurine Ameliorates High Glucose Induced Apoptosis in HT-22 Cells

Pingan Wu, Xiaochi Chen, Inam-u-llah, Xiaoxia Shi, Mengren Zhang, Kaixin Li, Raheel Suleman, Muhammad Shahbaz, Shahid Alam, and Fengyuan Piao

Correction to:
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Affiliations of authors Muhammad Shahbaz and Shahid Alam were incorrect in the published book. This has now been corrected as below:

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