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Taurine 12

A Conditionally Essential Amino Acid

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Preface

The global spread of COVID-19 has had a major impact on people's activities, including research activities and the holding of academic societies. The 22nd International Taurine Meeting, which was scheduled to be held in Greece in the spring of 2020, was postponed due to COVID-19. Because taurine exerts anti-inflammatory actions that could diminish the severity of COVID-19, the cancelling of the meeting was an ironic turn of events. Therefore, the officers of the International Taurine Society decided to publish a Taurine Book on recent advances in taurine research. We are grateful that many researchers have submitted their papers to Taurine Book 12. The Taurine Book will be evidence of the fact that many researchers around the world are working every day to unravel the effects of taurine and its derivatives.

Despite its simple chemical structure, taurine has a wide variety of actions, which include osmoregulation, neuromodulation, anti-inflammation, antioxidation, protein stabilization, membrane stabilization, and modulation of calcium ion mobilization. These actions may be associated with cytoprotective and homeostatic effects of taurine. This makes taurine an attractive research target. From many research results so far, the physiological and pharmacological actions of taurine have been elucidated. According to Pubmed, more than 700 papers on taurine have recently been published each year, and the number is increasing year by year. However, the essential role of taurine in living organisms has not yet been fully clarified. Elucidation of the action of taurine will also open new avenues for drug development and taurine-based therapies. We hope that you will continue to promote and contribute to the progress of taurine research around the world for a better elucidation of taurine's action and its health benefits.

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Covid 19 and Anti-Inflammatory Actions of Taurine

The Disease-Modifying Role of Taurine and Its Therapeutic Potential in Coronavirus Disease 2019 (COVID-19)

Larissa E. van Eijk, Annette K. Offringa, Maria-Elena Bernal, Arno R. Bourgonje, Harry van Goor, and Jan-Luuk Hillebrands

Keywords

Taurine · Coronavirus disease 2019 (COVID-19) · Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) · Oxidative stress · Inflammation · Treatment

Abbreviations

ACE2	angiotensin-converting enzyme 2
ADAM17	a disintegrin and metalloproteinase 17
Ang(1-7)	angiotensin 1-7
Ang II	angiotensin II
ARBs	AT_1R blockers
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
AT_1R	angiotensin II type 1 receptor
BH4	tetrahydrobiopterin
COVID-19	coronavirus disease 2019
CD147	cluster of differentiation 147
CRP	c-reactive protein
DAD	diffuse alveolar damage
EFSA	The European Food Safety Agency
EMMPRIN	extracellular matrix metalloproteinase inducer
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ET-1	endothelin-1
GSH	glutathione
HOCl	hypochlorous acid
H_2S	hydrogen sulfide
H_2S_n	hydrogen polysulfides
ICU	intensive care unit
IFN	interferon
IL	interleukin
mACE2	membrane-bound ACE2
MasR	Mas receptor
MAVS	mitochondrial antiviral-signaling proteins
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MOF	multi-organ failure

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NLRP3	NLR family pyrin domain containing 3
NO	nitric oxide
nsp	nonstructural protein
NETs	neutrophil extracellular traps
$O_2^{\cdot-}$	superoxide
OTD	1.4.5-oxathiazinane-4,4-dioxide
PAI-1	plasminogen activator inhibitor 1
PDH	pyruvate dehydrogenase
PKG	protein kinase G
RAAS	renin-angiotensin-aldosterone system
RdRp	RNA-dependent RNA polymerase
ROS	reactive oxygen species
S	spike
sACE2	soluble ACE2
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TLRs	Toll-like receptors
TMPRSS2	transmembrane serine protease type 2
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
TRD	taurolidine
TRPC3	transient receptor potential channel 3

1 Introduction

Taurine, or 2-aminoethanesulfonic acid, is a semi-essential compound that is produced endogenously in the human body but is also taken up by diet since it is abundantly present in seafood and meat. Although not incorporated into proteins, taurine is classified as an amino acid, where it can be distinguished from "true" amino acids by the presence of a sulfonic acid moiety, instead of a carboxyl one. Taurine is widely distributed in human tissues, residing both intracellularly (especially in leukocytes, platelets, heart, retina, and brain) and extracellularly (Wójcik et al. 2010). In human plasma, concentrations normally range between 65 and 179 mmol/L (8–22 µg/mL) (Ghandforoush-Sattari et al. 2009). Taurine has many physiological functions, including involvement in the conjugation of bile acids, regulation of oxidative stress, mitochondrial membrane stabilization, and osmoregulation, as well as modulation of cardiovascular and neurological functions (Qaradakhi et al. 2020; Wójcik et al. 2010). Considering its versatile biological roles, taurine has been shown to exert promising effects on improving overall health and for treatment purposes in several disease states, such as hypertension and diabetes (Maleki et al. 2020a, b; Schaffer and Kim 2018; Sun et al. 2016). The therapeutic potential of taurine has also recently been implicated for coronavirus disease 2019 (COVID-19), the infectious respiratory disease induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that emerged in December 2019 and rapidly spread worldwide causing millions of deaths (Iwegbulem et al. 2021). Decreased serum taurine levels have been reported in SARS-CoV-2-infected subjects, where taurine levels were half the level compared to that of healthy controls, most notably in patients with moderate-to-high pro-inflammatory interleukin (IL)-6, with higher levels of IL-6 predictive of a severe disease course (Sabaka et al. 2021; Thomas et al. 2020). Furthermore, lower hypotaurine levels, the precursor of taurine, have been associated with an unfavorable prognosis of COVID-19 in an interventional metabolomics study (Danlos et al. 2021). These results have attracted our attention to the potential use of taurine as a beneficial supplement in taurine-depleted SARS-CoV-2-infected patients.

COVID-19 symptomatology ranges from asymptomatic or mild, self-limiting respiratory disease to severe disease, including (typical and atypical) acute respiratory distress syndrome (ARDS) and multi-organ failure (MOF) (Wang et al. 2021a). Some individuals are particularly at risk of developing severe disease, including the elderly (with associated "inflammaging" and "immunosenescence"), males, and those with comorbidities (e.g., diabetes, cardiovascular disease, obesity, chronic respiratory disease, immunodeficiency disorders) (van Eijk et al. 2021; Wang et al. 2021a). Progression of COVID-19 is assumed to result from multiple intertwined pathophysiological mechanisms, including (1)

direct cytopathic effects of SARS-CoV-2 invasion, (2) a hyperactive immune response with hypercytokinemia, (3) an inflammation-driven "oxidative storm," and (4) vascular-related effects, such as thrombotic microangiopathy and endothelial dysfunction (Cumpstey et al. 2021; van Eijk et al. 2021). We propose that the immune-modulating effects of taurine may be beneficial to each of these mechanisms and limit progression to severe disease considering its previously reported antiviral, (indirect) antioxidant, anti-inflammatory, and vascular-related effects.

Although COVID-19 was originally being considered a purely respiratory disease, we currently know that the virus also induces inflammation in and damage to other organ systems (Bourgonje et al. 2020a; van Eijk et al. 2021). Thus, the versatile effects of taurine may be widespread throughout the body. In addition, considering that a substantial subset of patients develop long-term symptoms after SARS-CoV-2 infection – so-called "chronic COVID syndrome," "long-COVID" or "long-haulers" – taurine may also be beneficial in the postinfectious phase (Baig 2021). Herein we aim to clarify the potential role of taurine in COVID-19 by dissecting the various pathophysiological mechanisms of this disease. Finally, we explore the possibilities of taurine as a putative supplementary therapy for COVID-19.

2 SARS-COV-2 Life Cycle and the Antiviral Actions of Taurine

2.1 Animals

SARS-CoV-2 is thought to be primarily transmitted between people through respiratory droplets and aerosols (van Eijk et al. 2021). As shown in Fig. 1, the binding of SARS-CoV-2 to angiotensin-converting enzyme 2 (ACE2) at the cell membrane was found to be essential for viral cell entry (Hoffmann et al. 2020). In addition, transmembrane serine protease type 2 (TMPRSS2) was found to facilitate viral entry via endocytosis and be essential for direct fusion of the viral envelope to the cell membrane (Heurich et al. 2014; Hoffmann et al. 2020; Mahmoud et al. 2020). This route of viral entry matches that of SARS-CoV, the virus that caused an outbreak of severe acute respiratory syndrome in 2002−2004 (Heurich et al. 2014).

ACE2 is expressed in many tissues and cells throughout the human body, including a small population of lung cells – mainly type II alveolar epithelial cells (Wang et al. 2020b; Zou et al. 2020). Comparative studies investigating mRNA levels and protein expression of ACE2 demonstrated that other tissues besides the lung express high levels of ACE2, including the nasopharyngeal mucosa, suggesting that the main entry route of viral invasion is the nasal epithelium (Soni et al. 2021; Wang et al. 2020b). ACE2 was also found to be expressed on the enterocytes of the small intestine, as well as the vascular endothelium and arterial smooth muscle cells in various organs, supporting potential oral and blood-borne infectious routes, respectively (Hamming et al. 2004). In line with this wide tissue distribution of ACE2, the presence of SARS-CoV-2 has been demonstrated in various organs other than the lungs (Bourgonje et al. 2021b; Schurink et al. 2020; Song et al. 2021). For example, a cohort autopsy study examined the systemic SARS-CoV-2 distribution in postmortem organs from 26 COVID-19 patients and found SARS-CoV-2 in, among others, the lungs (92%), hilar lymph nodes (76%), small intestine (31%), colon (23%), heart (19%), and kidneys (15%) (Yao et al. 2021). Moreover, co-localization analysis demonstrated the co-expression of ACE2 and SARS-CoV-2 antigen in multiple organs (including the lungs, trachea, small intestine, heart, kidney, and pancreas), suggesting a potential correlation between membrane-bound ACE2 (mACE2) expression and SARS-CoV-2 tissue tropism (Liu et al. 2021). Alternative viral entry mechanisms include the presence of other viral entry receptors, such as furin, neuropilin-1, and cluster of differentiation 147 (CD147) – also known as extracellular matrix metalloproteinase inducer (EMMPRIN) or basigin – as well as the shedding of ACE2 into a soluble form, which provides a possible mechanism of dissemination of infection from the pri-

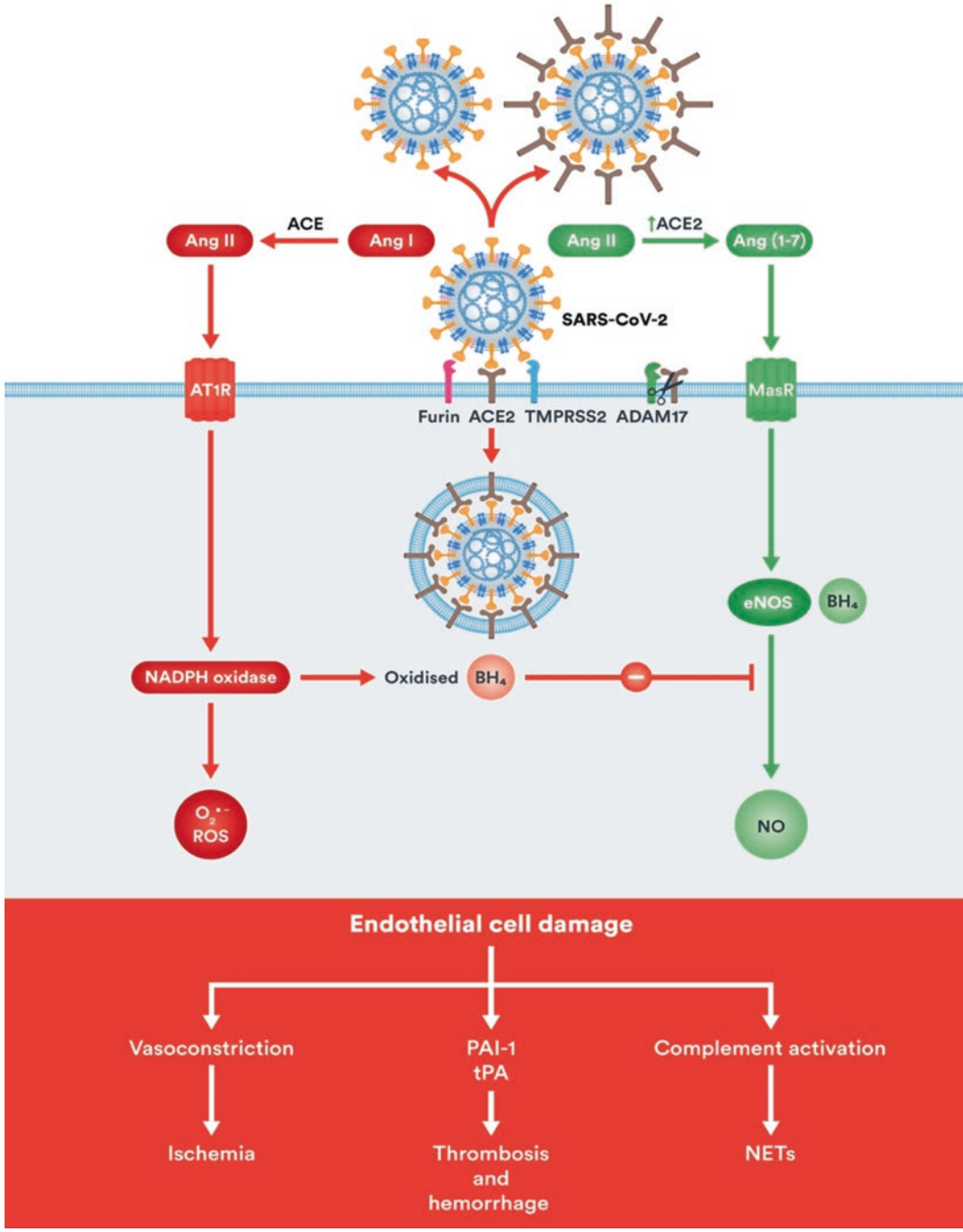

Fig. 1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry and the involvement of angiotensin II (AngII) signaling in oxidative stress and inflammation in coronavirus disease 2019 (COVID-19). SARS-CoV-2 binding to ACE2 leads to viral entry into the host cell, which may be facilitated by transmembrane serine protease type 2 (TMPRSS2) and furin, among others. ADAM17 is a major sheddase of ACE2, resulting in soluble ACE2 that may bind to viral particles and potentially facilitate viral cell entry. Taurine stimulates ACE2, which functions as converter of Ang II into angiotensin 1-7 (Ang(1-7)) that in turn activates the Mas receptor (MasR). Ang(1-7)/MasR signaling stimulates endothelial nitric oxide synthase (eNOS) – in conjunction with its essential cofactor tetrahydrobiopterin (BH4) – to produce anti-inflammatory nitric oxide (NO). However, angiotensin II – converted from angiotensin I by angiotensin-converting enzyme (ACE) – activates the angiotensin II type 1 receptor (AT_1R), which stimulates NADPH oxidase to oxidize BH4, thereby inducing eNOS to produce superoxide instead of NO. The production of reactive oxygen species (ROS) may cause endothelial dysfunction that contributes to COVID-19-related vascular pathology in various ways, including vasoconstriction-induced ischemia, dysregulated fibrinolysis, and the formation of NETs via complement activation

mary site of infection to peripheral locations (Kyrou et al. 2021; Yeung et al. 2021). Although CD147's definite involvement in SARS-CoV-2 cell entry has not been established (Shilts et al. 2021), it was found to play a potential role in SARS-CoV-2 infection, either as a viral entry receptor (specifically in immune cells that do not express ACE2) or by reducing the expression of ACE2, which underscores a potential two-way contradictory effect of CD147 (Fenizia et al. 2021; Wang et al. 2020a).

A major sheddase of ACE2 is a disintegrin and metalloproteinase 17 (ADAM17), previously found to be activated when TMPRSS2 was not (Heurich et al. 2014). Notably, soluble ACE2 (sACE2) retains an intact interaction site for binding to SARS-CoV-2, which has recently been shown to form complexes with the SARS-CoV-2 spike protein (S) in a human kidney cell line (Yeung et al. 2021). In this study, the sACE2-S complex (either with or without interaction with vasopressin) facilitated cell entry via binding to angiotensin II type 1 receptor (AT_1R) (or AVPR1B in case of vasopressin interaction), resulting in receptor-mediated endocytosis. These findings indicate a potential role for sACE2 as a co-receptor required for viral entry in cells lacking mACE2 and additionally highlight the involvement of the renin-angiotensin-aldosterone system (RAAS) in this cell entry mechanism.

SARS-CoV-2 infection is assumed to downregulate mACE2 either via shedding or endocytosis of the virus/ACE2 complex (Vieira et al. 2021). Normally, at the cell membrane, ACE2 converts angiotensin II (Ang II) – the main effector of RAAS causing blood pressure elevation – into angiotensin 1-7 (Ang(1-7)), which has opposing effects through binding to the Mas receptor (MasR), inducing the production of nitric oxide (NO) which lowers blood pressure. SARS-CoV-2-induced downregulation of ACE2 shifts this balance toward Ang II, which exerts its effects via AT_1R. Apart from its main function as a regulator of blood pressure, Ang II also exerts pro-oxidative, pro-fibrotic, and pro-inflammatory effects (Benicky et al. 2009; Bourgonje et al. 2020a; Ruiz-Ortega et al. 2002). Interestingly, a study on neurogenic hypertension showed that Ang II-AT_1R signaling promotes ACE2 internalization into the cell and subsequent lysosomal degradation (Deshotels et al. 2014). This finding supports the hypothesis that the endocytosis of the SARS-CoV-2/mACE2 complex is AT_1R-mediated too, also considering the common occurrence of systemic hyperinflammation and the potential positive effects of AT_1R blockers (ARBs) on the prognosis of COVID-19 – although still under debate (Baral et al. 2021; Duarte et al. 2021; Jarcho et al. 2020; Singh et al. 2021a). In this case, viral cell entry would not solely depend on surface ACE2 expression, but also on its endocytosis induced by Ang II-AT_1R signaling. Taurine has previously been shown to upregulate mACE2 – albeit its magnitude still unknown – and attenuate the actions of Ang II (Lv et al. 2017; Schaffer et al. 2000). Taurine may therefore, in theory, impede viral cell entry by attenuating AT_1R-mediated endocytosis of the SARS-CoV-2/ACE2 complex, thereby preserving ACE2 protective functions, primarily by converting Ang II to Ang1-7. High-risk groups for severe COVID-19 (e.g., the elderly, cardiovascular disease, diabetes, etc.) could especially benefit from these protective effects of taurine, as they tend to show decreased ACE2 expression and activity (Bourgonje et al. 2020a; van Eijk et al. 2021). However, the question remains whether taurine may also affect mACE2-independent viral cell entry. Albeit an inhibitory effect of taurine on CD147 has been reported, and its effect on other potential viral entry receptors or sACE2 requires further examination (Jin et al. 2018).

2.2 Viral Replication

Next to potentially limiting host cell entry of SARS-CoV-2, taurine may also exert antiviral actions during the process of viral replication. Upon entering the cell, viral uncoating follows, in which SARS-CoV-2 is disassembled to release its genomic RNA. After primary translation, the process of transcription and replication is initiated, mediated by the RNA-dependent RNA polymerase (RdRp, or nonstructural protein [nsp] 12). This is followed by translation of structural proteins, which assemble and encapsulate the newly formed genomic RNA, thereby generating

new viral particles that are released from the host cell by exocytosis (V'Kovski et al. 2021). RdRp forms a complex with two nonstructural proteins, nsp7 and nsp8, which catalyze RNA synthesis, together forming a potential therapeutic target to inhibit SARS-CoV-2 replication. For instance, the antiviral actions of RdRp inhibitors (e.g., remdesivir) and hydrogen sulfide (H_2S) donors (e.g., N-acetylcysteine) are thought to be mediated by acting on the RdRp/nsp7/nsp8 complex (Bourgonje et al. 2021a). Taurine has previously been shown to increase endogenous H_2S levels and may therefore indirectly inhibit viral replication by inhibiting RdRp (Sun et al. 2016; Zhao et al. 2018).

When viruses exploit the hosts' cellular machinery for their own replication, stress in cellular organelles such as the endoplasmic reticulum (ER) and mitochondria is induced, while host response mechanisms are inhibited (Ajaz et al. 2021; Li et al. 2015). Viral replication also requires large quantities of ER-produced proteins and lipids, which accumulate and induce ER stress whenever the amount exceeds the post-translational process of protein folding (Li et al. 2015). ER stress was found to promote viral replication as was previously demonstrated for reovirus and hepatitis B virus, which potentially also relates to SARS-CoV-2 (Choi and Song 2019; Li et al. 2015). In fact, research on coronavirus-induced ER stress has been suggested to provide new targets for treating COVID-19 (Sureda et al. 2020). Previous studies have demonstrated that taurine inhibits ER-stress and its downstream effects, including apoptosis and activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome (Bian et al. 2018; Liu et al. 2019; Men et al. 2010).

Furthermore, taurine enhances mitochondrial function that may result in decreased viral replication due to its stimulating effect on interferon (IFN) production – which is initially compromised in COVID-19 (vide infra) (Bender et al. 2015; van Eijk et al. 2021). Mitochondrial antiviral-signaling proteins (MAVS), located on the outer mitochondrial membrane, peroxisomes, and the mitochondria-associated membrane of the ER, lead to the production of IFNs (Bender et al. 2015). A recent study on COVID-19 demonstrated that SARS-CoV-2 evades the antiviral type I IFN response by targeting MAVS (Wang et al. 2021b). These literature-based hypotheses on the antiviral effects of taurine provide a rationale for conducting future research (e.g., in SARS-CoV-2-infected cell lines) to test the actual effects of taurine on SARS-CoV-2 uptake and replication.

Finally, modulation of lipid metabolism by taurine could potentially interfere with the viral life cycle, considering lipids are the essential components of the SARS-CoV-2 envelope and double-membrane vesicles in the host cell involved in viral cell entry, viral replication, and viral propagation (Abu-Farha et al. 2020; Caterino et al. 2021). Taurine may modify the structure of the phospholipid bilayer through inhibition of phospholipid N-methyltransferase – an enzyme that regulates the ratio of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Schaffer et al. 2010). As PE is normally situated on the outer side and PC on the inner side of the membrane, one can imagine that alterations in their ratio would influence the membrane's structure and function (Schaffer et al. 2010). Other potential taurine-mediated effects on lipid-dependent viral replication include the modulation of molecules that impact lipids, such as cholesterol. Indeed, taurine modulates cholesterol metabolism with cholesterol-lowering effects, which may be explained through various mechanisms, including improved LDLR-binding capacity and increased formation of fecal bile acid via upregulating CYP7A1, among others (Chen et al. 2012).

3 Age-Related Mitochondrial Dysfunction in COVID-19 and the Indirect Antioxidant Actions of Taurine

There are multiple age-related contributors to the increased risk of severe disease in COVID-19, including (among others) an increased incidence of comorbidities in older patients, a decline in immune function affecting innate and adaptive

immune responses ("immunosenescence"), and a chronic pro-inflammatory profile ("inflammaging") (van Eijk et al. 2021). As seen in Fig. 2, inflammaging is associated with the deterioration of mitochondrial function, which entails mitochondria that are incompetent to produce sufficient amounts of adenosine triphosphate (ATP) to meet metabolic demands, while producing an excessive amount of reactive oxygen species (ROS), causing oxidative stress to the cell, resulting in chronic inflammation in the elderly. Mitochondrial dysfunction affecting immune cells is associated with immunosenescence, thereby contributing to excessive inflammation (with increased activation of NLRP3, NF-κB, and increased levels of IL-6) and impaired adaptive immunity as seen in COVID-19. Inflammation itself goes along with immune cell infiltration that generates and releases ROS, which normally function in cell signaling pathways important to their immunological functions (Bourgonje et al. 2020b). However, COVID-19 promotes the overproduction of ROS with antioxidant systems being overrun, causing harmful oxidative stress with resulting tissue damage and ongoing inflammation (Ganji and Reddy 2020).

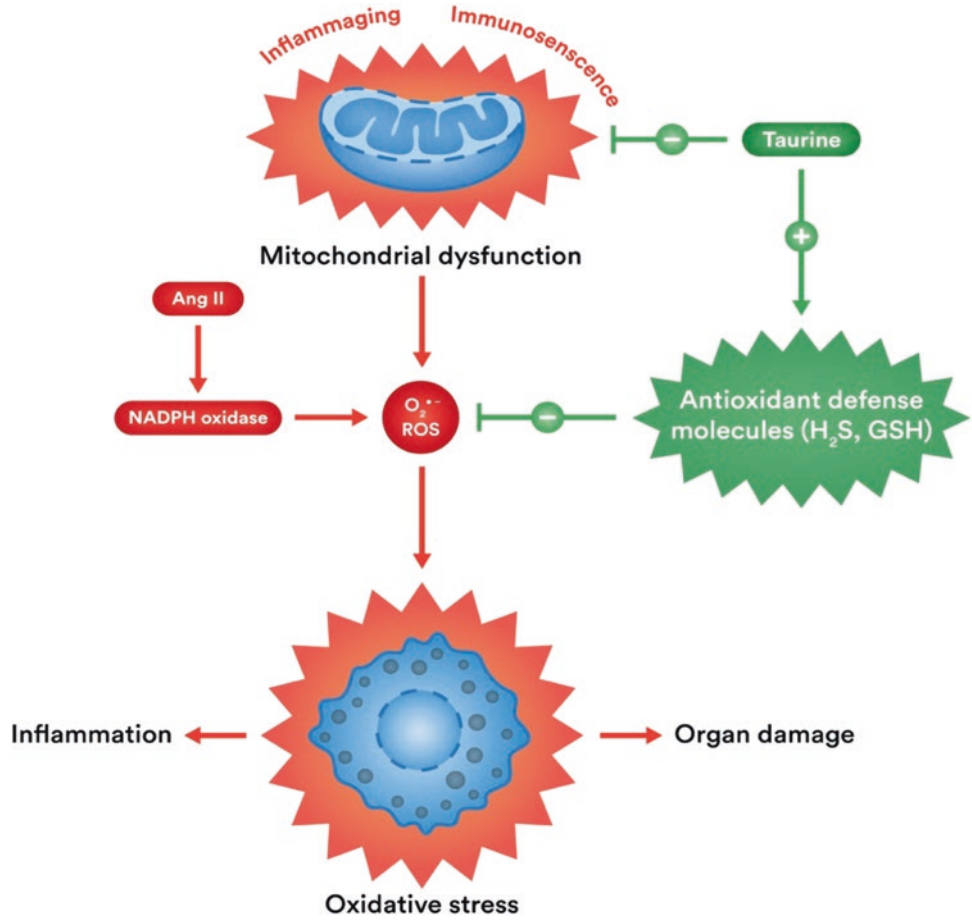

Fig. 2 Interrelationships between age-related mitochondrial dysfunction, oxidative stress, and taurine. Age-related inflammaging and immunosenescence are associated with mitochondrial dysfunction, leading to excessive amounts of reactive oxygen species (ROS). In addition, ROS is promoted by angiotensin II (Ang II) through the activation of NADPH oxidase. ROS in turn cause oxidative stress to the cell, resulting in tissue damage and ongoing inflammation. These effects may be alleviated by taurine because of its indirect antioxidant effects, including its stimulating effect on mitochrondrial functions and by enhancing endogenous hydrogen sulfide (H_2S) and glutathione (GSH) availability

Ang II promotes ROS through the activation of NADPH oxidase, which is counteracted by conversion of Ang II into Ang(1-7) by ACE2 (Gwathmey et al. 2010; Rabelo et al. 2011). Notably, taurine was found to protect against oxidant-induced lung injury and pulmonary fibrosis (Gurujeyalakshmi et al. 2000; Schuller-Levis et al. 1994, 2003). Conversely, taurine concentrations were repeatedly observed to be decreased in oxidative stress-mediated disorders and during aging (Ito et al. 2012; Yoshimura et al. 2021). Although taurine does not appear to have inherent antioxidative capacity, it indirectly increases antioxidation by improving mitochondrial functions (by enhancing electron transport chain activity), thereby attenuating mitochondrial ROS production (Barbiera et al. 2020). Furthermore, taurine displays indirect antioxidant activity through the enhancement of endogenous H_2S and glutathione (GSH) – a tripeptide synthesized from cysteine – availability, both of which are major antioxidants (Bourgonje et al. 2021a). H_2S exerts its antioxidant effects through multiple mechanisms, including the direct scavenging of ROS, increasing levels of other antioxidant defense molecules (such as GSH), and by desulfuration generating sulfane sulfur species (Bourgonje et al. 2021a; Corsello et al. 2018). Finally, taurine is known to neutralize hypochlorous acid (HOCl), which is a major toxic oxidant generated by the myeloperoxidase-halide system within activated leukocytes and is involved in multiple age-related diseases (Casciaro et al. 2017; Chorazy et al. 2002; Goud et al. 2021).

4 COVID-19 Immune Response and the Immune-Related Actions of Taurine

A maladaptive immune response is a hallmark of COVID-19, consisting of a hyperinflammatory innate immune response along with an inadequate adaptive response, eliciting both local and systemic tissue damage (van Eijk et al. 2021). Autopsy data show evidence of an extensive inflammatory response in various organs, including the lungs, kidneys, heart, brain, and liver (Schurink et al. 2020). The type and magnitude of the immune response highly depend on the phase of the disease course. The interstitial infiltrate in the lungs of patients with exudative diffuse alveolar damage (DAD), for example, was found to be CD4+-T-lymphocyte-mediated, whereas in patients with proliferative DAD, this response was primarily CD8+-T-lymphocyte-mediated (Schurink et al. 2020). Furthermore, early COVID-19 is characterized by a more pronounced viral presence and increased neutrophil infiltration, whereas increased macrophage and lymphoplasmacytic infiltration are more often observed when the disease progresses (Nienhold et al. 2020; Rendeiro et al. 2021). The disconnection between viral presence in early disease and inflammatory pathology in late COVID-19 underscores the role of self-perpetuating immunopathology in causing severe disease when the virus usually can no longer be detected.

Similar to other viral infections, the immune response against SARS-CoV-2 starts with the activation of pattern recognition receptors, such as Toll-like receptors (TLRs) that lead to the activation of transcription factors, ultimately resulting in the production of IFN-I, which exerts antiviral actions (Kumar et al., 2021). COVID-19 is characterized by an initial delay in IFN-I response, allowing the virus to replicate and disseminate uncontrollably within the infected host, thereby stimulating a strong activation of the immune response, which promotes hyperinflammatory injury during later stages of COVID-19 (van Eijk et al. 2021). SARS-CoV-2-induced activation of NLRP3 inflammasome was also shown to contribute to this hyperinflammation (Pan et al. 2021). The overactivation of the immune response is characterized by extensive hypercytokinemia – the so-called cytokine storm (Henderson et al. 2020). High serum levels of IL-6, IL-8, and tumor necrosis factor (TNF) at the time of hospitalization were found to be strongly and independently predictive of mortality (Del Valle et al. 2020). Furthermore, the change in ratio of pro-inflammatory IL-6 to anti-inflammatory IL-10 taken 4 days apart, called the Dublin-Boston score, was found to be of prognostic value in COVID-19 (McElvaney et al.

2020). Administration of taurine may be effective in reducing IL-6 and increasing IL-10, either directly or indirectly by affecting other inflammatory modulators, such as NLRP3 (Lak et al. 2015; Liu et al. 2019). Conversely, the SARS-CoV-2 infection especially in patients with moderate to high pro-inflammatory IL-6 levels was associated with decreased taurine levels (Thomas et al. 2020). This decrement could be potentially related to reduced taurine biosynthesis in COVID-19. For example, oxidative stress oxidizes the active form of vitamin B6, pyridoxal-5'-phosphate (PLP), which is involved as cofactor in endogenous taurine synthesis (Mahootchi et al. 2021). Another potential explanation to decreased serum taurine levels includes a shift in taurine distribution with increased body requirements due to its function as conjugator. This hypothesis is supported by a study in which increased levels of taurine were measured in peripheral blood mononuclear cells (PBMCs), where it can neutralize HOCl, thereby favoring anti-oxidation and anti-inflammation (Chorazy et al. 2002; Singh et al. 2021b). Other pro-inflammatory mediators have been shown to be inhibited by taurine, including Ang II, AT_1R, TLR4, IL-1β, NADPH oxidase, and the NLRP3-inflammasome (Han et al. 2016; Liu et al. 2019; Schaffer et al. 2000; Younis et al. 2021). At the same time, taurine can increase anti-inflammatory ACE2 and Ang(1-7), in addition to IL-10 (Lv et al. 2017; Schaffer et al. 2000). Supplementation dosages vary greatly across human studies reporting on the effect of taurine supplementation on inflammatory markers. For instance, studies on obesity (3 gram/day for 8 weeks), type 2 diabetes (1000 mg 3 times/day for 8 weeks), and traumatic brain injury (30 mg/kg/day for 2 weeks) resulted in decreased levels of C-reactive protein (CRP), both TNF-α and CRP, and IL-6 respectively (Maleki et al. 2020b; Rosa et al. 2014; Vahdat et al. 2021).

The aforementioned downregulation of ACE2 with subsequent disrupted orchestration of the Ang II:Ang(1-7) ratio may contribute to the excessive inflammatory response in COVID-19 patients. Ang II binding to AT_1R for instance induces the activation of pro-inflammatory mediators, including TLR4, IL-6, Il-1β, NF-κB, NADPH oxidase, and NLRP3-inflammasome (Fazeli et al. 2012; Wen et al. 2016). Together, these mediators induce the production of superoxide ($O_2^{\cdot-}$), increase vascular permeability, and facilitate leukocyte and thrombocyte adhesion as an excessive response to the infection (Jin et al. 2020; Mittal et al. 2014). The inhibition of Ang II/AT_1R-signaling by taurine may prevent the ACE2-bound virus from entering the cell, limit the pro-inflammatory response, and facilitate anti-inflammation. During a moderate innate immune response, Ang(1-7) activates the MasR, which induces endothelial nitric oxide synthase (eNOS) to produce anti-inflammatory NO (Sampaio et al. 2007). Interestingly, taurine has been found to increase eNOS expression and phosphorylation (Guizoni et al. 2020). Ang II/AT_1R-signaling, however, activates superoxide-producing NADPH oxidase, which causes oxidation of the essential eNOS cofactor tetrahydrobiopterin (BH4), inducing eNOS to produce $O_2^{\cdot-}$ rather than NO – a process called eNOS uncoupling (Bowers et al. 2011; Fazeli et al. 2012). Inhibition of NADPH oxidase by taurine may therefore limit $O_2^{\cdot-}$ production, thereby resulting in an increased availability of BH4 for the activation of eNOS to produce anti-inflammatory NO (Myojo et al. 2014).

Taurine also inhibits TLR4, which has the dual function of initially activating a pro-inflammatory response by way of pro-inflammatory cytokines and an anti-inflammatory response after it is endocytosed into the cell (Kim et al. 2013). Inside the cell, TLR4 activates anti-inflammatory cytokines, such as IL-10, after which TLR4-signaling is ended (Chang et al. 2009; Guven-Maiorov et al. 2015; Kim et al. 2013). In leukocytes, taurine traps and reacts with HOCl to produce taurine chloramine, which inhibits the generation of inflammatory mediators, such as IL-6 and TNF-α (Chorazy et al. 2002). Finally, alteration of the gut microbiota (with its immunomodulatory potential) in response to SARS-CoV-2 infection has been linked to increased levels of inflammatory markers and more severe COVID-19 (Yeoh et al. 2021). Taurine has a modulatory role on the gut

microbiota by potentiating the production of sulfide, an inhibitor of pathogen respiration key to viral invasion into the host, thereby enhancing the resistance against viral infection (Stacy et al. 2021).

5 COVID-19 Vascular Pathology and the Vascular-Related Actions of Taurine

5.1 Prothrombotic State

COVID-19 predisposes individuals to a prothrombotic state, characterized by elevations in D-dimer and fibrinogen levels, both correlates of a poor outcome (Di Micco et al. 2020; He et al. 2021). Consistent with this prothrombotic state, both pulmonary and extrapulmonary microthrombotic and thromboembolic complications are common among severely affected COVID-19 patients, findings which are thought to contribute to disease symptomatology and MOF (Fahmy et al. 2021). For example, microthrombi in the lungs could lead to pulmonary vascular redistribution, thereby contributing to the gas exchange abnormalities observed in COVID-19 (Thillai et al. 2021). The microvascular thrombi have been shown to contain neutrophil extracellular traps (NETs) associated with platelets and fibrin, indicating inflammation as an underlying mechanism for the observed thrombotic complications in COVID-19 – a process also known as "dysregulated immunothrombosis" (Ackermann et al. 2021; Nicolai et al. 2020). Although intravascular microthrombosis is not specific to COVID-19 as it may also occur in sepsis-induced disease states, it was previously shown to occur more frequently in COVID-19-induced respiratory failure when compared to patients with influenza by a ninefold increase (Ackermann et al. 2020). The pathophysiology of the observed thrombosis is complex and assumed to result from an interplay between various underlying mechanisms, including endothelial damage, platelet dysfunction, complement activation associated with the formation of thrombogenic NETs, hypercytokinemia (including IL-6-mediated platelet abnormalities and thrombogenesis), and abnormal blood flow (e.g., due to hyperviscosity or impaired microcirculation in response to hypoxia) (Ackermann et al. 2021; Ahmed et al. 2020; van Eijk et al. 2021).

Taurine may be protective in thrombotic complications of COVID-19 by acting on these different underlying mechanisms. For example, the vasorelaxant functions of taurine (vide infra) may restore hypoxia-induced impaired microcirculation, thereby attenuating thrombosis induced by abnormal blood flow (Ahmed et al. 2020; Nishida and Satoh 2009). Furthermore, the above-described anti-inflammatory effects of taurine will likely attenuate thrombosis associated with the cytokine storm. In COVID-19, cytokine-induced (and possibly virus-induced) endothelial damage, as well as increased Ang II/AT_1R signaling, may stimulate the release of plasminogen activator inhibitor 1 (PAI-1), an inhibitor of fibrinolysis and a risk factor of thrombosis, from primarily endothelial cells lining the blood vessels (Ahmed et al. 2020). Elevated PAI-1 levels have previously been observed among intensive care unit (ICU)-admitted COVID-19 patients, and impaired fibrinolytic activity has further been demonstrated by prolonged clot lysis time in critically ill COVID-19 patients (Nougier et al. 2020; Wright et al. 2020). In ARDS, elevated PAI-1 levels have been shown to be an independent risk factor for poor outcomes and appear to play a role in fibrin deposition leading to fibrosis (Whyte et al. 2020). Taurine may hamper PAI-1 release (e.g., possibly through attenuating cytokine-induced endothelial damage or inhibiting AT_1R), as was previously demonstrated in animal studies, and could therefore restore the fibrinolytic shutdown in severe COVID-19 (Lee et al. 2005; Ruan et al. 2016). Simultaneously, due to its stimulating effect on ACE2, taurine favors the Ang(1-7)/Mas pathway, activation of which leads to the inhibition of platelet adherence and aggregation via the release of NO (Ahmed et al. 2020). Furthermore, ACE2 itself has antithrombotic effects, partly through the activation of tissue plasminogen activator (tPA), a serine protease found on endothelial cells involved in fibrinolysis (Ahmed et al. 2020). Paradoxically, a recent study in 118 hospitalized COVID-19 patients found elevations in not only PAI-1 levels but also tPA levels, findings that are similar to sepsis-induced coagulopathy (Schmitt

et al. 2019; Zuo et al. 2021). These results indicate that fibrinolytic homeostasis in COVID-19 is complicated and provide an explanation for the enhanced bleeding risk among critically ill patients, in addition to the well-known enhanced thrombotic risk (Al-Samkari et al. 2020). An animal study exploring the effect of taurine in combination with delayed tPA on embolic stroke found that this treatment prevented tPA-associated hemorrhage, as well as fibrin/fibrinogen and platelet deposition in (micro)vessels, which could be linked to the inhibition of CD147 by taurine (Jin et al. 2018). Thus, taurine may be protective by regulating fibrinolytic activity, preventing both hemorrhage and thrombosis, through its inhibitory effect on CD147. In addition to improving microvascular patency, the inhibitory effects of taurine on CD147 may potentially limit SARS-CoV-2 uptake and COVID-19-associated hyperinflammation, considering its role in increasing inflammation and fibrosis after a pro-inflammatory insult (Fenizia et al. 2021; Jin et al. 2019; Wang et al. 2020a; Zhu et al. 2014). Although most data come from animal models, the antithrombotic properties of taurine have also been demonstrated in human studies (Santhakumar et al. 2013; Franconi et al. 1995; Ijiri et al. 2013). Findings included a decrease platelet aggregation (Santhakumar et al. 2013; Franconi et al. 1995), a prolongation in prothrombin clotting time (Santhakumar et al. 2013), and an increase in endogenous thrombolytic activity (Ijiri et al. 2013).

5.2 Vasoconstriction

As the endothelial cell layer is a key regulator of vascular homeostasis through its production of vasodilators (e.g., NO, prostaglandins, and endothelium-derived hyperpolarizing factor) and vasoconstrictors, including endothelin-1 (ET-1), endothelial damage in COVID-19 could lead to a prothrombotic and pro-inflammatory state of vasoconstriction (Varga et al. 2020). Release of ET-1 and platelet-activating factor shift the vascular equilibrium toward more vasoconstriction, leading to a reduction in tissue perfusion with resultant ischemic-related tissue damage, thereby further activating inflammation and cytokine release (Eltzschig and Carmeliet 2011; Indranil Biswas 2019). Interestingly, concentrations of ET-1 and ET-1-receptor expression are increased by Ang II, resulting in an increased production of ROS by NADPH oxidase (Lin et al. 2014; Loomis et al. 2005; Moreau et al. 1997). Prevention of conversion of Ang II into Ang(1-7) due to ACE2 deficiency may limit the activation of eNOS via Ang(1-7)/MasR signaling, thereby inhibiting the production of vasodilatory NO. Furthermore, hypoxia is a common feature in severe COVID-19, which itself induces impaired microcirculation in affected organs. For example, hypoxic pulmonary vasoconstriction (i.e., contraction of the vascular smooth muscle of small intrapulmonary arteries in response to hypoxia) likely is a partial contributor to the impaired gas exchange in COVID-19 (Thillai et al. 2021).

Taurine may be beneficial in COVID-19-related vascular dysfunction due to its known modulatory role in homeostatic function of vascular smooth muscle. Through its attenuating effect on Ang II signaling, taurine is assumed to target the vasoconstrictive effects that Ang II exerts via the AT_1R (Schaffer et al. 2000). Furthermore, the increased production of NO as a result of taurine not only exerts anti-inflammatory effects but is also assumed to cause vasorelaxation. NO may interact with H_2S to generate inorganic hydrogen polysulfides (H_2S_n) that activate protein kinase G (PKG)1α, which has vasorelaxant functions (Bourgonje et al. 2021a). NO could also induce relaxation of vascular smooth muscle cells via activation of guanylate cyclase, which is downregulated by increased levels of intracellular Ca2+ (Murad et al. 1987; Serfass et al. 2001). Depending on cellular Ca2+ concentrations, taurine either promotes vasoconstriction to maintain blood pressure or exerts vasodilatory actions during hypoxia, thereby increasing tissue perfusion (Nishida and Satoh 2009). In a study on individuals with prehypertension (i.e., an early stage in the development of hypertension), administration of taurine significantly improved endothelium-dependent and endothelium-independent vasodilation (Sun et al. 2016). Therein, experimental studies on hypertensive rats showed that administration of taurine inhib-

ited transient receptor potential channel 3 (TRPC3) expression in the vasculature, whereas TRPC3 antagonist treatment enhanced H_2S donor-induced vascular relaxation, indicating that taurine exerts vascular relaxation by targeting TRPC3-mediated calcium influx, which is regulated by AT_1R (Sun et al. 2016; Yamaguchi et al. 2018). Next to smooth muscle in the vasculature, taurine has previously been shown to exert a vasorelaxant effect on pulmonary smooth muscle in rats (Ammer et al. 2013). This finding may be especially interesting for long-COVID-19 patients, in which bronchodilators have been suggested as a treatment option to facilitate breathing – even in patients without concomitant obstructive lung disease (Maniscalco et al. 2021).

6 Taurine as Putative Supplementary Therapy for COVID-19

The role of taurine as an immunomodulator has been well-described in the literature. Considering its antiviral, anti-inflammatory, and vascular modulatory effects, as well as its favorable safety profile, taurine could be regarded as a putative beneficial supplement in taurine-deficient patients with COVID-19. To date, no clinical trials of taurine as a treatment for COVID-19 have been conducted. However, other researchers have previously suggested the therapeutic potential of taurine and its derivatives in COVID-19 (Iwegbulem et al. 2021). Potentially suitable derivatives that were mentioned by the authors included taurolidine (TRD) and 1.4.5-oxathiazinane-4,4-dioxide (OTD), both of which contain various administration options (e.g., intravenous, oral, cutaneous) and are well-tolerated. Previously, taurine has been shown to be effective as treatment for diseases such as diabetes and hypertension (Ito et al. 2012; Maleki et al. 2020a; Sun et al. 2016). For example, a clinical trial on 120 prehypertensive individuals reported that taurine supplementation of 1.6 g/day resulted in significant reductions in systolic and diastolic blood pressures (Sun et al. 2016). Furthermore, in a double-blind placebo-controlled study on patients with type 2 diabetes mellitus, taurine supplementation of 3 g/day for 8 weeks improved glycemic control (by reducing fasting blood sugar and insulin levels) and lipid profiles (through decreased total cholesterol and low-density lipoprotein cholesterol levels) (Maleki et al. 2020a). High doses have also been studied, including in a clinical trial in patients with stroke-like episodes of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) in which doses of 9 g/day and 12 g/day were used (Ohsawa et al. 2019). The European Food Safety Agency (EFSA) considers up to 1 gram of taurine per kg of bodyweight per day to be safe (European Food Safety Authority (EFSA), 2009). Moreover, a study on the pharmacokinetics of taurine on healthy volunteers after oral administration of 4 g taurine demonstrated an absorption phase of 1−2.5 h (Ghandforoush-Sattari et al. 2010). In this study, the maximum plasma taurine concentration was 86.1 ± 19.0 mg/L (0.69 ± 0.15 mmol), which returned to normal 6−8 h following digestion. Taurine supplementation may be used in COVID-19 considering its low cost, wide availability, and high safety profile with minimal side effects, all of which contribute to the feasibility of conducting randomized controlled trials to test its clinical effects. Since taurine is hypothesized to reduce SARS-CoV-2 infection, taurine may be recommended in every subject with COVID-19, although the greatest benefit is expected in high-risk groups for severe COVID-19 considering their higher baseline levels of inflammation, oxidative stress, and vascular dysfunction. Based on studies in other disease states, beneficial effects may be expected to occur at dosages of 3 g/day for 8 weeks, although its high safety profile legitimizes its use throughout the entire COVID-19 disease course (Maleki et al. 2020a, b; Rosa et al. 2014). Future studies should be conducted to examine the involvement and applicability of taurine supplementation in COVID-19.

7 Conclusion

The amino sulfonic acid taurine has extensive regulatory versatility and is hypothesized to play a modulatory role in COVID-19. Existing evidence

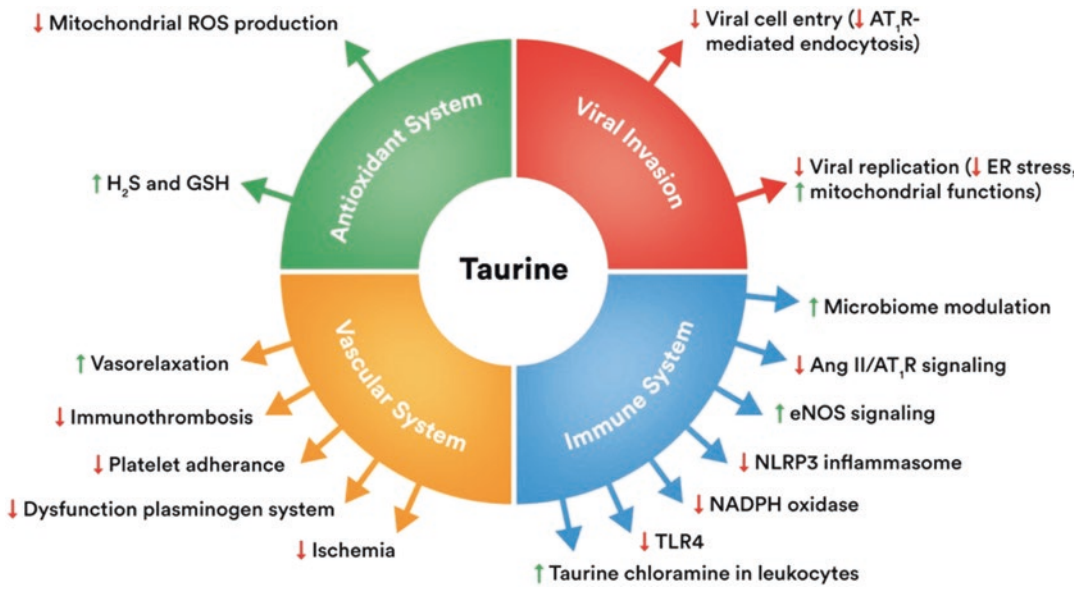

Fig. 3 Overview of stimulatory and inhibitory effects of taurine in coronavirus disease 2019 (COVID-19)

supports antiviral, antioxidant, anti-inflammatory, and vascular modulatory effects of taurine, which could target the multifaceted nature of COVID-19 pathophysiology (Fig. 3). These possible effects are diverse and range from inhibiting viral invasion on account of involvement in (AT_1R-mediated) SARS-CoV-2/ACE2 endocytosis to reducing immunopathology and vascular injury because of its complex involvement in both inflammatory and coagulation pathways. As with other infections, SARS-CoV-2 is associated with decreased levels of taurine, thereby limiting its protective properties normally occurring under physiologic circumstances. Next to its endogenous production, taurine can easily and safely be supplemented to improve body functions. Altogether, taurine should be regarded as a promising supplementary therapeutic option in COVID-19, although future clinical studies are warranted to explore its definite suitability in this disease.

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Examination of Taurine Chloramine and Taurine on LPS-Induced Acute Pulmonary Inflammatory in Mice

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Keywords

Pulmonary inflammation · Taurine chloramine (TauCl) · Cytokine storm · Interleukin

Abbreviations

TauCl	taurine chloramine
LPS	lipopolysaccharide

1 Introduction

The novel coronavirus disease (COVID-19), which is prevalent in the world, develops severe pneumonia, of which 5 % have fatal acute respiratory distress (Cao 2020). It causes acute respiratory distress syndrome (ARDS), which is characterized by symptoms including cough, wheezing, shortness of breath, fever, and chest swelling (Saguil and Fargo 2012). Whereas the immune system is important to defend the body from a microbe, the hyperactivation of the immune response causes systemic inflammation and leads to a life-threatening condition. Local infection can trigger the elevation of circulating cytokine levels and immune-cell hyperactivation, leading to uncontrolled inflammation and multi-organ failure, which is called the cytokine storm syndrome (Fajgenbaum and June 2020). In some cases of COVID-19 patients, it causes fatal hypercytokinemia with multi-organ failure, which is a secondary cause of mortality. Clearly, there is a need to develop a therapeutic drug against ARDS and the cytokine storm syndrome as soon as possible.

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, which can induce inflammatory responses in humans. LPS is commonly used to study pulmonary inflammation and ARDS in mice (Domscheit et al. 2020). It binds to the Toll-like receptor 4 (TLR4) in immune cells and induces cytokine expression through NF-κB activation. Moreover, STAT1 and STAT3 are also involved in the TLR4-regulated pathway and are responsible for inflammation (Greenhill et al. 2011; Metwally et al. 2020).

Taurine (2-aminoethanesulfonic acid) is found in millimolar concentrations in most mammalian cells, including immune cells (Marcinkiewicz and Kontny 2014). Taurine plays an important role in inflammation. Several reports indicate a beneficial effect of taurine treatment against

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inflammatory diseases, such as rheumatoid arthritis and bowel disease in animal experiments (Zhao et al. 2007; Zaki et al. 2011). One potential mechanism of the anti-inflammatory effect of taurine is that it can react with hypochlorous acid generated by neutrophil myeloperoxidase at the site of inflammation (Kim and Cha 2013). This reaction produces taurine chloramine (TauCl), which is less toxic to the surrounding tissues. TauCl prevents the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and nitric oxide in immune cells. Importantly, several studies demonstrated that TauCl blocks LPS-induced production of cytokines. TauCl blocks NF-κB activation by oxidization of its amino acid residue (Kanayama et al. 2002). Moreover, TauCl prevents the phosphorylation of STAT3, which is related to the reduction in IL-6 expression (Kim et al. 2013). Taken together, TauCl is a potential therapeutic agent to prevent acute inflammatory activation.

There is little available information about the in vivo effect of TauCl (Kwaśny-Krochin et al. 2002; Kim et al. 2021), although many reports have demonstrated its immunomodulatory effects during in vitro experiments. In the present study, we investigated the effect of TauCl against LPS-induced acute pulmonary/systemic inflammation in mice.

2 Methods

2.1 TauCl Synthesis

TauCl was synthesized as previously described (Kwaśny-Krochin et al. 2002). Sodium hypochlorite (20 mM) was added dropwise to 24mM taurine dissolved in 50 mM phosphate buffer (pH 7.4) while being vigorously stirred. The synthesis of TauCl was monitored by ultraviolet absorption (220 to 340 nm); Amax for TauCl is 252 nm.

2.2 Animal Experiment

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Fukui Prefectural University. Male C57BL/6J mice (9-week-old, Japan Crea) were used for this study. Mice were fed standard chow (CR-2, Japan Crea, Japan), had access to water ad libitum, and were maintained on a 12-h light/dark cycle.

Pneumonia was induced by intratracheal injection of an LPS solution (1 mg/kg body weight), or an equivalent amount of vehicle (PBS) for the control under anesthesia with a combination of medetomidine hydrochloride (0.3 mg/kg, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam (4.0 mg/kg, Astellas Pharma Inc., Tokyo, Japan), and butorphanol (5.0 mg/kg, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), as previously described (Langen et al. 2012). After injection, Atipamezole (0.3 mg/kg, Nippon Zenyaku Kogyo Co., Ltd.) was injected intraperitoneally to recover from anesthesia. Mice were intraperitoneally pretreated with 250μl TauCl (5mM) or PBS 1 hour before LPS injection. In the taurine-treated group, mice were treated with taurine for 1 week before LPS injection by maintaining them on drinking water containing 0.5% taurine. Two days after LPS injection, mice were euthanized, and tissues were collected. Tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use.

2.3 RNA Isolation and Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from mouse lung by using Sepasol (Nacalai tesque, Kyoto, Japan) according to the manufacturer's protocol. cDNA was generated from total RNA by the reverse transcription with Rever Tra Ace (Toyobo, Japan). Quantitative RT-PCR analyses were performed by using qTOWER^3 (Analytik Jena GmBH, Jena, Germany) with KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Inc., USA). The primers used are as follows; IL-1β; forward, 5′-CCTTG GGCCT CAAAG GAAAG A-3′; reverse; 5′-TTGCT TGGGA TCCAC ACTCT CC-3′, IL-6; forward: 5′-CACTT CACAA GTCGG AGGCT T-3′, Reverse: 5′-GAATT GCCAT TGCAC AACTC TTTTC-3′, IL-17:

Forward: 5′-CCTGG ACTCT CCACC GCAAT-3′, Reverse: 5′-AGCTT TCCCT CCGCA TTGAC-3′, TNF-α; Forward: 5′-CAAAA TTCGA GTGAC AAGCC TGTA-3′, Reverse: 5′-CACCA CTAGT TGGTT GTCTT TGAGA-3′, MCP-1; Forward: 5′-CTGTC ATGCT TCTGG GCCTG-3′, Reverse: 5′-GGCGT TAACT GCATC TGGCT GA-3′. GAPDH was used as an internal control. Data were analyzed using the ΔΔCt method.

2.4 Plasma IL-6 Assay

Plasma IL-6 concentration was measured by using the Mouse IL-6 Quantikine ELISA Kit (R&D Systems, Inc.) according to the manufacturer's protocol.

2.5 Statistical Analysis

The student's t-test or Tukey-Kramer test (for multiple comparisons) were used to determine statistical significance between groups. Differences were considered statistically significant when the calculated P-value was less than 0.05.

3 Results

3.1 Effect of TauCl and Taurine on LPS-Induced Pneumonia in Mice

To evaluate the in vivo effect of TauCl on pulmonary inflammation, we used an LPS-induced acute pneumonia model. Male mice were treated intraperitoneally with PBS or TauCl (10 mg/kg) 1h before the intratracheal injection of LPS (1 mg/kg) or PBS. The body weight was monitored 1 or 2 days later. While body weight was continuously reduced by LPS injection, TauCl treatment did not diminish the effect of LPS (Fig. 1). LPS injection caused to increase the lung weight 2 days after the injection, which is due to immune cell infiltration. TauCl treatment attenuated lung weight gain (Table 1). Moreover, TauCl treatment attenuated the LPS-induced skeletal muscle weight loss.

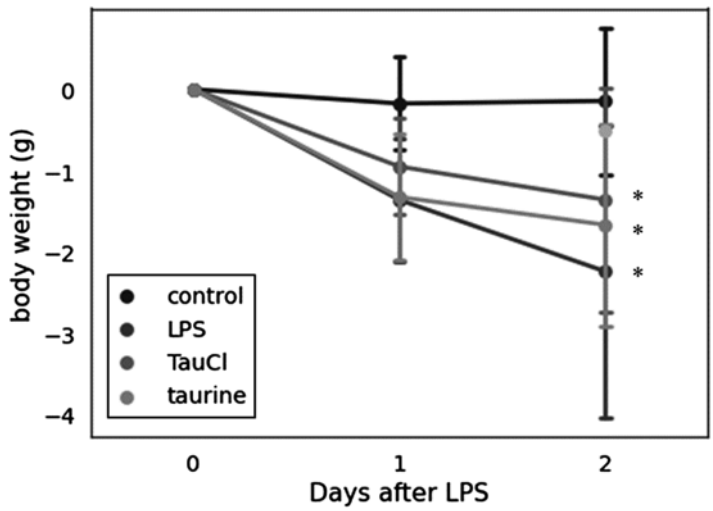

Fig. 1 The changes in body weight after intratracheal LPS injection with TauCl or taurine. Bodyweight was monitored after LPS injection. TauCl was intraperitoneally pretreated 1h prior to LPS injection in the TauCl-treated group (TauCl). Taurine was pretreated by 0.5% taurine-containing drinking water for 1 week prior to LPS injection in the taurine-treated group (taurine). Values shown represent means + SD. n=8 (control), 10 (LPS), 12 (TauCl) and 12 (taurine). *; $p<0.01$ vs control

Table 1 The changes of body weight and tissue weight after LPS injection with TauCl or taurine

	BW (g)	Lung (mg)	Spleen (mg)	TA mus. (mg)	G mus. (mg)
Control	22.9±0.3	133±14	59±2.4	45±8.4	121±1.7
LPS	22.1±1.5	220±50 **	75±7.9	42±13	116±2.5
TauCl	22.6±0.8	159±35 *†	71±7.3	47±8.9††	130±1.6††
Taurine	22.9±0.8	165±19 *†	74±6.3	47±7.6†	125±2.6††

Values shown represent means ± SD. n=5(control), 4-5 (LPS), 6 (TauCl), 5 (taurine). *; p<0.01, **p<0.01, ***;p<0.001 vs control. , †; p<0.05, ††; p<0.01 vs LPS. BW; body weight, G mus.; gastrocnemius muscle, TA mus.; tibial anterior muscle

Furthermore, we evaluated the effect of taurine treatment in the LPS-induced pneumonia model. Mice were treated with taurine from drinking water containing 0.5% taurine from 1 week before LPS injection. Taurine treatment attenuated LPS-induced lung weight gain and muscle weight loss.

3.2 Effect of TauCl and Taurine on the LPS-Induced Cytokine Expression

To determine the effect of TauCl and taurine against cytokine/chemokine expression in acute lung inflammation, we analyzed the mRNA expression of IL-1β, -6, -17, TNF-α**,** and MCP-1 in lung tissue (Fig. 2). While LPS injection caused a marked induction of all those cytokines 2 days after injection, TauCl diminished the expression of IL-6, but not the other genes. Taurine treatment diminished the induction of IL-6 and IL-17.

Next, we measured plasma IL-6 in the LPS-treated mice 2 days after injection (Fig. 3). While it was not detectable in PBS-injected control mice, LPS injection-induced IL-6, leading to concentration varying from 9 to 331 pg/mL. Among the TauCl-treated mice, IL-6 was below the detection limit in some mice (5 of 11), but it was detectable at a comparable level in the LPS-injected group in 6 of the other mice (6 of 11). The average value of the TauCl-treated group was lower than the LPS-injected group, but the difference was not statistically significant. Similarly, plasma IL-6 tends to be lower in the taurine-treated group than LPS-injected mice, but the difference was not statistically significant.

4 Discussion

We tested the effect of intraperitoneal TauCl injection on LPS-induced pulmonary inflammation in mice. TauCl attenuated LPS-induced lung weight gain, suggesting that TauCl reduces the infiltration of immune cells into the lung. Moreover, we found that TauCl diminished the LPS-induced expression of IL-6 mRNA in lung tissue, whereas it did not influence that of the other cytokines/chemokine, TNF-α, IL-1β, IL-17, and MCP-1. Previous studies have demonstrated that TauCl prevents the activation of the DNA-binding activities of NF-κB and STAT3, which are central regulators of cytokine transcription. While LPS-induced transcriptional activation of TNF and IL-1β is directly regulated by NF-κB, the induction of IL-6 is regulated by complex mechanisms. For example, IκB-ζ which is a nuclear member of the IκB family and is induced by LPS is necessary to LPS-induced IL-6 transactivation (Kimura et al. 2018). Moreover, STAT1 phosphorylation is induced by the LPS-TLR4 complex, which is also responsible for the induction of IL-6 expression via induction of ARID5A, a regulator of IL-6 mRNA stability (Metwally et al. 2020). Our findings indicate that TauCl did not diminish NF-κB activity but regulate other specific pathways involved in IL-6 transcription.

In the present study, data for LPS group was very variable; the body weight of some mice (3 of 11) did not change after LPS injection in LPS group. Anastacia during intratracheal injection also affects body weight. One possible cause is that the sensitivity against LPS or the anesthetics is variable.

In the present study, we failed to find a significant effect of TauCl against the elevation of circu-

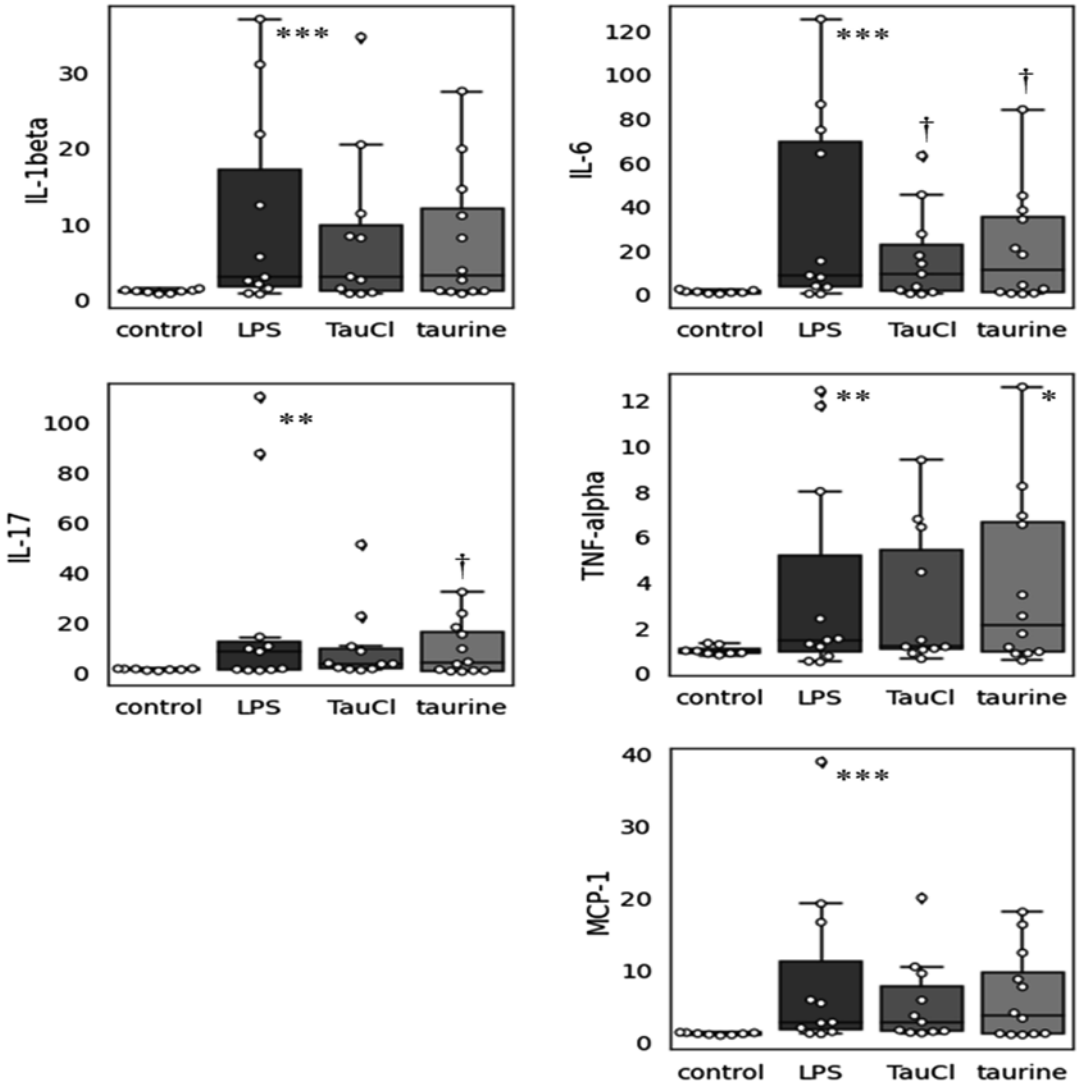

Fig. 2 Effect of TauCl and taurine on the LPS-induced expression of cytokine/chemokine mRNA in the lung. The lungs were isolated from mice 2 days after PBS (control) or LPS (LPS) injection. TauCl was intraperitoneally pretreated 1h prior to LPS injection in the TauCl-treated group (TauCl). Taurine was pretreated by 0.5% taurine-containing drinking water for 1 week prior to the taurine-treated group (taurine). The expression of IL-1β, IL-6, IL-17, TNF-α, and MCP-1 were measured by qRT-PCR. The expression level was normalized by the expression level of GAPDH. Values are shown fold of control. n=8 (control), 10 (LPS), 12 (TauCl) and 12 (taurine). *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs control, †; $p<0.05$ vs LPS

lating IL-6. Plasma IL-6 could not be detected in some mice of the TauCl-treated group, but it was detectable at a particular level in other mice of the LPS group. This observation may be attributed to the variability of the TauCl effect. One problem is the stability of TauCl in the body (Marcinkiewicz and Kontny 2014). TauCl may oxidize various cellular components, such as glycine, methionine, and tryptophan (Kim and Cha 2013). Furthermore, TauCl is eliminated by glutathione (GSH) and ascorbate (Peskin and Winterbourn 2001; Kim and Cha 2013). It is possible that a single injection of TauCl might be insufficient to reach a sustained pharmaceutical effect.

While we did not check whether TauCl affects control mice, we could not find adverse effect,

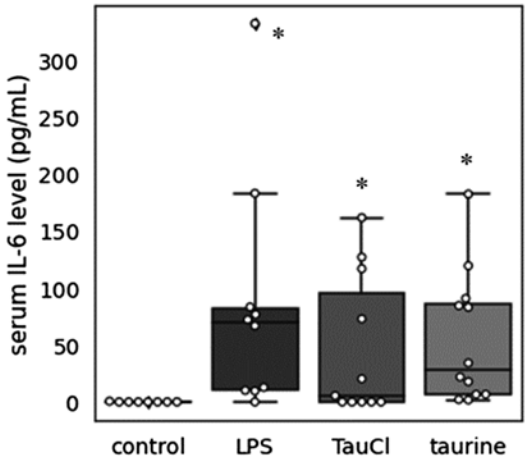

Fig. 3 Effect of TauCl and taurine on serum IL-6 level. Blood was obtained from mice 2 days after PBS (control) or LPS (LPS) injection. TauCl was intraperitoneally pretreated 1 h prior to LPS injection in the TauCl-treated group (TauCl). Taurine was pretreated with 0.5% taurine-containing drinking water for 1 week prior to the taurine-treated group (taurine). Serum IL-6 level was measured by ELISA. Values shown represent means ± SD. n=8 (control), 10 (LPS), 12 (TauCl) and 12 (taurine). *; $p<0.05$

suggesting that TauCl was safety. Concerning the pharmacokinetics of TauCl, there are no information about the pharmacokinetics data in previous reports. Whereas it is important information, measurement of TauCl in blood or animal tissue is difficult due to difficulties in sample preparation and the detection methods. It should be figured out in the future.

In the present study, we demonstrated that taurine administration from drinking water also attenuated LPS-induced lung inflammation. Moreover, the effect of taurine treatment against cytokine transcription is similar to that of the TauCl-treated group; taurine treatment diminished the induction of IL-6 but not that of other cytokines/chemokine. Taurine may react with NaOCl in the inflammation site and produce TauCl.

5 Conclusion

Our data indicate that intraperitoneal TauCl treatment attenuates acute lung inflammation. Further studies are necessary to confirm the clinical utility of this treatment against other pathogen-associated molecular patterns and pathogens themselves.

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Synergism Between Taurine and Dexamethasone in Anti-inflammatory Response in LPS-Activated Macrophages

Chang-Hee Do and Dong-Hee Lee

Keywords

Dexamethasone · Taurine · ER stress · Inflammation · Cytokine · Macrophage

Abbreviations

DEX	Dexamethasone
ELISA	Enzyme-linked immunosorbent assay
ERS	Endoplasmic reticulum stress
HSP	Heat shock protein
LPS	Lipopolysaccharide
TAU	Taurine

1 Introduction

The COVID-19 outbreak, caused by 2019–nCov/SARS-CoV-2, calls for urgent treatment and immediate understanding of the fundamental mechanisms. The pathogenesis is mostly attributed to the profound immune response resulting in the cytokine release syndrome or cytokine storm (Azkur et al. 2020; Bordallo et al. 2020; Ryabkova et al. 2021). Recent studies specify that the deadly pathogenesis is most likely due to endoplasmic reticulum stress (ERS) and the subsequent hypersensitive immune response (Johnson et al. 2019). Infections by SARS-CoV are accompanied by increased levels of reactive oxygen species, and abnormal calcium homeostasis, which are typical of ERS (Sureda et al. 2020). Several therapeutic agents, such as remdesivir, dexamethasone, and hydroxychloroquine derivatives, all of which are apparent ERS modulators, have been tested (Burrage et al. 2020; Banjerjee et al. 2020). Among them, only dexamethasone has been further tested for COVID-19 patients, especially in severe and critical conditions (Johnson and Vinetz 2020; Ahmed and Hassan 2020).

Inflammatory responses encompass a multipart process which is highly conserved throughout animal evolution. Following infection or damage, inflammation begins to restore the tissue as a homeostatic mechanism, and the response may be advantageous to cope with the complications. However, unwarranted or persisting inflammation results in various diseases, and anti-inflammatory approaches are necessary to alleviate the inflammation.

Dexamethasone ($C_{22}H_{29}FO_5$; DEX) is a glucocorticoid and known as a potent clinical drug; however, its mode of action is still complex and

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debatable. It provides protection against respiratory tract disorders during viral infections (Fan et al. 2014; Divani et al. 2020). However, the mechanisms underlying this anti-inflammatory effect remains to be further elucidated, particularly the association between DEX and ERS.

Taurine ($C_2H_7NO_3S$, TAU) is recognized as a potent antioxidant to ameliorate ERS and modulate immune response (Satsu et al. 2019). TAU is a proven anti-inflammatory agent, which counteracts the neutrophil oxidant and hypochlorous acid (HClO) (Posadas et al. 2019; Liu et al. 2017). Following the reaction with HClO, TAU modulates the inflammatory process as taurine chloramine (Kim and Cha 2014; Marcinkiewicz and Kontny 2014). TAU also reduces the generation of superoxide in the mitochondria (Jong et al. 2017).

Macrophages are phagocytes modulating innate immunity as well as cell-mediated immunity. The innate immune response is triggered by toll-like receptors (TLRs) signaling in response to LPS, peptidoglycan, double-stranded RNA, and other microbial products (Zheng et al. 2020). Macrophages play an important role as members of innate immune cells during the course of the inflammatory response. Macrophages show a high sensitivity to a variety of environmental signals and thus appear to be a suitable target for the development of anti-inflammatory drugs because of their essential role in inflammation.

In the present study, using RAW 264.7 murine macrophage cells, we investigated the effects of DEX and TAU on ERS and pro-inflammatory responses in activated macrophage cells. Before activation by LPS, the RAW 264.7 cells were pre-treated with DEX and TAU individually or in combination. The levels of ERS and inflammation were evaluated using Western blot analysis and ELISA, respectively. The expression of ERS markers was analyzed using Western blot against GRP78 and CHOP and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To evaluate the pro-inflammatory response in LPS-stimulated cells, ELISA was used to monitor cytokines interleukin-1 β (IL-1) and IL-6 associated with the pro-inflammatory response.

2 Methods

2.1 Pre-treatment and Activation of Macrophages

The RAW 264.7 cells were typically pre-treated for 3 h with DEX, TAU, or both. The dexamethasone (water soluble, Sigma-Aldrich) concentrations ranged from 0 to 50 nM. The TAU concentrations were 0–500 μM. Following pre-treatment, the macrophages were activated by treating with 1 μg/mL lipopolysaccharide (LPS) solution (Sigma) normally for 6 h. The LPS stock solution (100X) was prepared by dissolving 1 mg in 1 mL PBS at room temperature. The stock solution (1 mg/mL) was divided into 100 μl aliquots and frozen at −20 °C for storage.

2.2 Cell Culture

Murine macrophage cells, RAW 264.7, were cultured in Dulbeco's modified Eagle's media (DMEM, Welgene Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus 5% penicillin-streptomycin (5 μg/mL). All cells were grown at 37 °C in a humidified chamber containing 5% CO_2.

2.3 Western Blot

Protein samples were electrophoresed using 12% SDS-PAGE and transferred onto nitrocellulose membranes, which were then equilibrated in a TBS (TBS-T) buffer containing 5% non-fat milk and 0.1% Tween 20 for 1 h. The blocked membranes were incubated overnight with the relevant primary antibodies: anti-GRP78 (Stressgen), anti-CHOP (Santa Cruz), or anti-β-actin (Santa Cruz), followed by copious washing with TBS-T buffer, and incubation with a secondary antibody.

The membranes were washed four times with TBS-T buffer and visualized using an ECL system (Roche).

2.4 RT-qPCR Analysis of ERS Markers

The transcription of ERS marker protein was analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Treatment with DEX/TAU at different concentrations and LPS activation was followed by extraction of the total macrophage RNA in TRIzol and synthesis of cDNA using a reverse transcription kit purchased from Thermo Fisher, Inc. The qPCR procedure was repeated three times for each sample using a qPCR system (Applied Biosystems). The procedure entailed 40 cycles of 95 °C for 15 s followed by sequential annealing at 60 °C for 1 min. The transcription levels were standardized per β-actin using the ABI 7500 system. The primer sequences (20mer) were as follows (forward and reverse): GRP78 (5′-AGCGACAAGCAACCAAAGAT-3′ and 5′-CCCAGGTCAAACACAAGGAT-3′); CHOP (5′-ACAGAGGTCACACGCACATC-3′ and 5′-CTCCTGCTCCTTCTCCTTCA-3′); β-actin (5′-GTGCTATGTTGCTCTAGACTTCG-3′ and 5′-ATGCCACAGGATTCCATACC-3′).

2.5 ELISA

Following each treatment and LPS-activation, the culture supernatants were collected for analysis. The triplicates of each sample were fixed in 96-well plates (Fisher, Waltham, MA, USA) and analyzed using the murine IL-6 ELISA set (eBioscience). Anti-mouse IL-1β (IL-1) or IL-6 was sequentially added up to 100 μl per well and incubated at 4 °C overnight. The reaction wells were washed three times with PBS-T and supplemented with 1X assay dilution buffer. The diluted samples were incubated for an additional 1 h. Once the reaction was terminated, plates were thoroughly washed three times with PBS-T, followed by additional incubation with streptavidin-HRP diluted in 1X assay buffer. The plates were carefully washed again, and the reaction was ended by adding 50 μl of 2 N H_2SO_4, followed by the addition of TMB peroxidase substrate to the wells. The concentrations of IL-1 and IL-6 were assessed at 450 nm using an ELISA reader (R&D Systems, Inc.).

2.6 Statistical Analyses

Analysis of variance (ANOVA) was used to determine the significance of treatment with DEX, TAU, or DEX/TAU in the two groups, control or treated. The paired sample t-test was also performed to investigate the significance of pairwise differences between these comparisons. Graphs were generated using Excel for Windows. Statistical significance was considered when p is less than 0.05.

3 Results

In the present study, the effects of DEX and TAU on ERS and pro-inflammatory responses in the induced RAW 264.7 cells were analyzed. In addition, potential synergistic effects between the two agents were also analyzed. The macrophages were pre-treated with DEX or TAU, either individually or combined. The cells were induced with LPS (1 μg/mL). Neither DEX nor TAU had any toxic effect on the RAW 264.7 cells within the scope of concentration employed in the experiment (data not shown).

3.1 LPS Treatment-Induced ERS and Pro-inflammatory Responses

When the macrophages were induced by LPS, the corresponding expression of ERS markers (GRP78/CHOP) and pro-inflammatory cytokines (IL-1/IL-6) was apparent in a time-dependent mode. When induced with LPS alone, the macrophages underwent ERS and inflammation according to the expression of ERS and pro-inflammatory

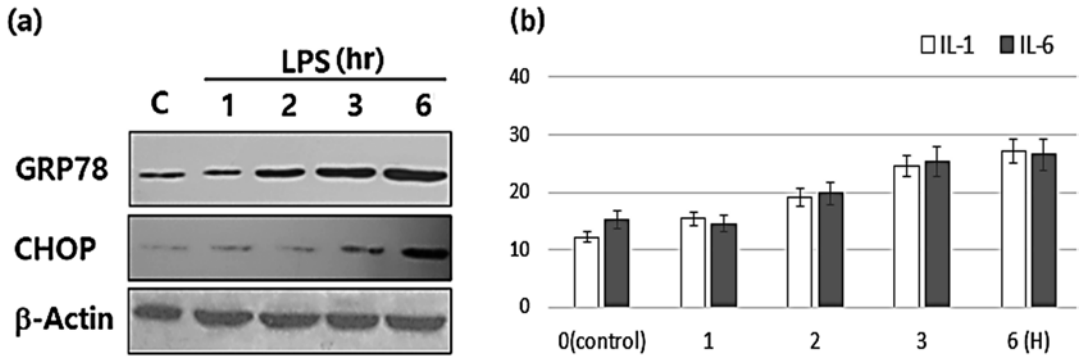

Fig. 1 Effects of LPS on the expression of ERS and inflammatory markers. The RAW 264.7 cells were treated with LPS 1 μg/mL for 1, 2, 3, and 6 h. The cultured cells were lysed for Western blot analysis, and the culture medium was collected for ELISA. (**a**) The expression of ERS markers following induction by LPS. (**b**) The level of inflammation according to IL-1 and IL-6 using ELISA. C: LPS-free treatment control. (GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein). Error bars represent the standard deviation (SD)

markers (Fig. 1). In the absence of pre-treatment with DEX or TAU, ERS was detected based on the ERS marker proteins. Both protein markers showed similar expression patterns. The expression of GRP78 and CHOP was enhanced, which is typical of ERS. The level of GRP78 was increased along with CHOP.

Similar to ERS, the degree of pro-inflammatory responses was also augmented in a time-dependent manner. The cytokine markers, IL-1 and IL-6, increased in a time-dependent mode according to ELISA.

3.2 DEX Attenuates ERS and Pro-inflammatory Responses

When the macrophages were preincubated with DEX, a simultaneous reduction of ERS and pro-inflammatory responses occurred. Before LPS-activation, the cells were pre-treated with DEX for 3 h at 0, 10, 20, or 50 nM concentrations, designated as D0, D10, D20, or D50, respectively.

The expression of ER markers was significantly decreased in the LPS-activated macrophages following pre-treatment with DEX starting from D10 in a dose-dependent manner (Fig. 2). According to the results of Western blot analyses, the ERS markers were greatly decreased. The DEX-treated macrophages also showed a significant reduction in the expression of cytokine markers at D10, D20, and D50, according to the ELISA results ($p < 0.05$).

3.3 Taurine Alleviates ERS and Pro-inflammatory Responses

Pre-incubation with TAU appeared to alleviate both ERS and pro-inflammatory responses in the macrophages. The expression of ERS marker proteins was decreased by treatment with TAU (Fig. 3). The macrophages were pre-treated with TAU at T0, T100, T200, or T500, which denote 0, 100, 200, or 500 μg/mL, respectively. Starting with pre-incubation at T100, the expression of ER markers significantly decreased upon LPS activation of the macrophages. The ERS markers were greatly decreased based on the results of the Western blots. TAU-treated macrophage similarly showed a substantial decrease in the expression of cytokine markers according to ELISA. When activated by LPS, the levels of both IL-1 and IL-6 greatly decreased following pre-treatment with TAU at T100-T500 ($p < 0.05$).

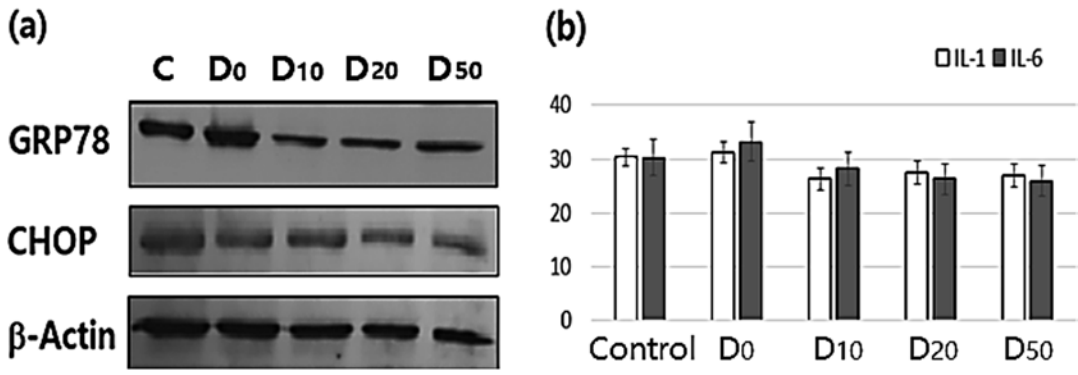

Fig. 2 Reduction of ER stress response and inflammation in RAW 264.7 cells by dexamethasone (DEX). The cells were pre-incubated for 3 hours with four different concentrations of DEX: 0, 10, 20, and 50 (nM). These concentrations were designated as D0, D10, D20, and D50, respectively. The pre-treated groups were further treated with LPS at 1 μg/mL for 6 h. Culture supernatants were collected and analyzed to determine the pro-inflammatory response mediated via IL-1 and IL-6. C: no pre-treatment before LPS-activation. (**a**) The expression of ERS marker proteins in the RAW 264.7 cells following pre-treatment with DEX and induction by LPS. D0 refers to pre-treatment at D0 before LPS activation. (**b**) Pro-inflammatory cytokine expression. Except for D0, D10–50 showed a reduction in ERS and pro-inflammatory response

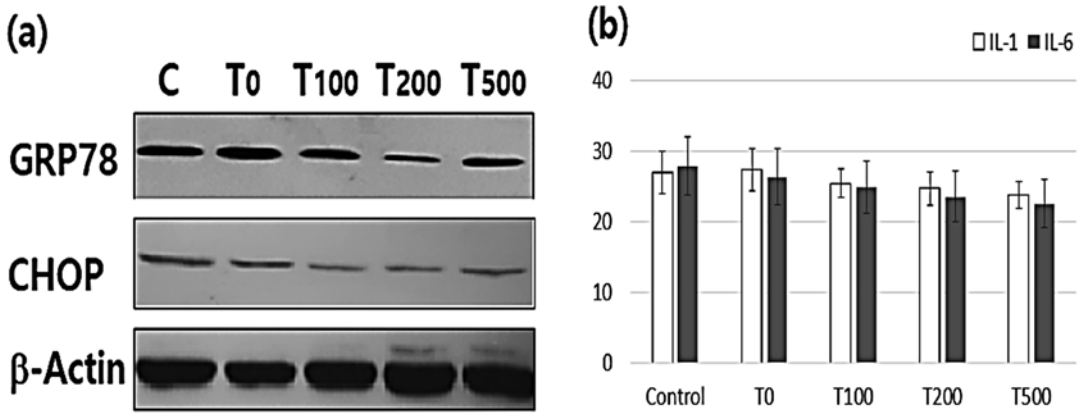

Fig. 3 Effects of taurine (TAU) on ER stress and inflammatory response in RAW 264.7 cells. TAU was added to the culture media at different concentrations (range, 0–500 μg/mL). Effects of TAU on ERS were determined using Western blots. Following pre-treatment with TAU for 6 h, the macrophages were induced with LPS (1 μg/mL) for 6 h. The cell lysates were subjected to SDS-PAGE followed by Western blot with anti-GRP78, anti-CHOP, and anti-β-actin antibodies. (**a**) The expression of ERS marker proteins. Levels of all the above proteins were the highest under treatment with LPS only and lower in the TAU groups. The expression levels decreased as the concentration of TAU increased. (**b**) Pro-inflammatory cytokine expression: The supernatant was collected, and IL-1 and IL-6 levels were detected via ELISA. The pro-inflammatory cytokine levels were consistently reduced in the TAU-treated group

3.4 DEX and TAU Synergistically Ameliorate ERS and Pro-inflammatory Response

Treatment with DEX and TAU synergistically reduced ERS and pro-inflammatory response. When the macrophages were pre-treated with both DEX and TAU, the macrophages showed a significant reduction in ERS (Fig. 4). The macrophages were pre-treated with the two agents via six different combinations in the two groups: [D20/T0, D20/T100, D20/T500] and [T200/D0, T200/D10, T200/D50].

The macrophages were pre-treated either with DEX or TAU (i.e., D20/T0 or T50/D0) and acti-

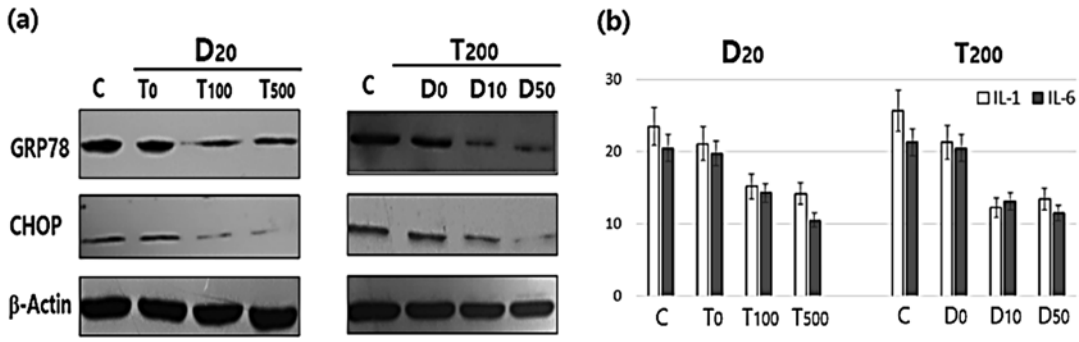

Fig. 4 Effects of dual treatment with dexamethasone (DEX) taurine (TAU). RAW 264.7 cells were jointly pre-treated with DEX and TAU for 1 h and further incubated with LPS for 1 h. The cell lysates and supernatant were collected for Western blot analyses and ELISA, respectively. (**a**) (*Left*) Western blot of lysates derived from LPS-activated cells were pre-treated with DEX/TAU at D20/T0, D20/T100, and D20/T500. The control (C) refers to the samples which were treated only with LPS. (*Right*) Immunoblotting of TAU/DEX-treated cells at T200/D0, T200/D10, and T200/D50 as shown in the left panel. (**b**) IL-1 and IL-6 expressions at the joint concentration as in 4-(a)

vated by LPS at 1 μg/mL. Following pre-treatment with a combination of DEX and TAU, the macrophages showed a greater reduction in ERS at the concentrations of T50/D10, T50/D50, D20/T100, and D20/T500. The combined treatment also resulted in significant reductions in the pro-inflammatory responses. Both IL-1 and IL-6 levels significantly ($p < 0.01$) decreased with the combined pre-treatment of TAU and DEX using the four combinations: [D20/T100, and D20/T500] and [T200/D10, T200/D50].

3.5 Transcriptional Effects of DEX and TAU on ERS and Inflammation

The effects of combined pre-treatment were further evaluated in terms of transcription. RT-qPCR was used to measure the effects of the combined treatment (Fig. 5). Total RNA was isolated via RT-qPCR analysis of GRP78 and CHOP. The levels of transcription decreased significantly in the pre-treated group. The joint treatment group showed a significant reduction in the transcription of GRP78 and CHOP. The size of transcriptional reduction, due to the dual treatment, consistently surpassed the total of single treatments for the two protein indicators both at [T200/D50] and [D20/T100] ($p < 0.01$). This synergistic effect is also evident in the expressions of pro-inflammatory cytokine markers: IL-1 and IL-6, both of which decreased significantly in comparison to the single treatments ($p < 0.01$).

4 Discussion

The present study indicates that LPS treatment induces ERS and inflammation in RAW 264.7 macrophages. The activated macrophages expressed marker proteins (GRP78 and CHOP) and pro-inflammatory cytokines (IL-1 and IL-6). The expression level of ERS and inflammatory markers was decreased by the pre-treatment of macrophages with DEX, TAU or both, indicating that the two agents provide resistance to ERS and decrease pro-inflammatory responses. Especially when the cells were treated with both agents, a synergistic pattern was evident in reduction of pro-inflammatory responses.

DEX is known to increase apoptosis and even block TAU transporter (TAUT) in RAW264.7 when treated at high concentration (Ai et al. 2020, Kim et al. 2003). However, this study provides a unique line of evidence that DEX does not interfere with cell viability and TAUT activ-

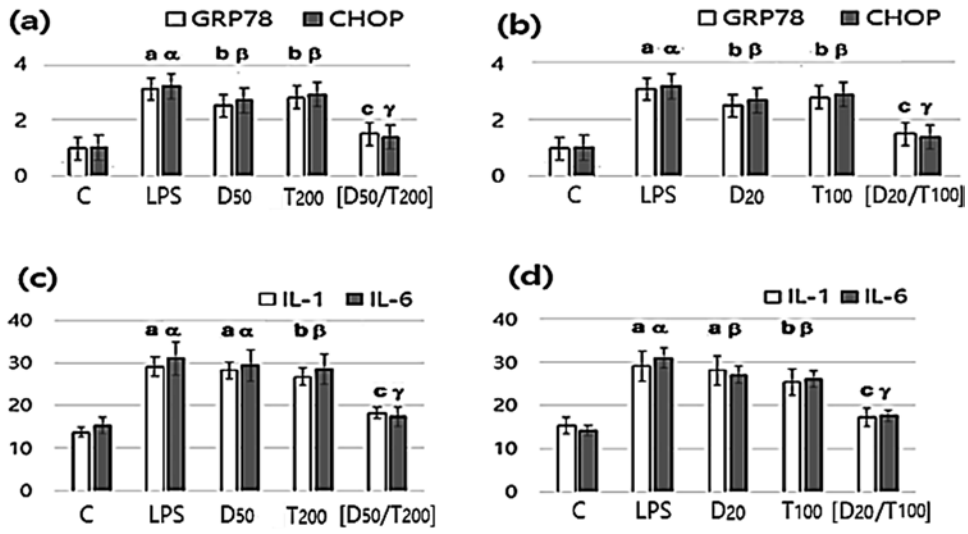

Fig. 5 Effects of combined pre-treatment on the transcription of ERS proteins and inflammation. Using RT-qPCR of total RNA, the transcriptional effects on the ERS marker proteins were analyzed following combined pre-treatment and LPS-activation. Two separate experiments were performed to test for the potential synergism in transcriptions of GRP78 and CHOP between DEX and TAU: (**a**) and (**b**). The reduction levels were categorized into three classes as a-b-c for GRP78 while CHOP as α-β-γ. In terms of cytokine expression, the data is summarized in (**c**) and (**d**). The levels of reduction were interpreted as in 5-(a) and 5-(b): a-b-c to IL-1 and α-β-γ to IL-6, respectively. The data were analyzed according to the paired sample *t-test* (n = 4). Error bars represent S.D

ity in RAW264.7 cells when treated with no more than 50 nM. Rather, DEX decreased the expression of ERS proteins and pro-inflammatory cytokines in the stimulated RAW 264.7 cells. TAU also significantly attenuated the expression of ERS markers without toxic effects on LPS-stimulated RAW 264.7 cells. The expression of ERS-associated proteins and the inflammatory response was also decreased upon treatment with TAU, which alone appears to reduce the inflammatory response. TAU appears to exert anti-inflammatory effects most likely by suppressing ERS or pro-inflammatory mediators, which were induced by LPS treatment of RAW 264.7 cells.

Both DEX and TAU exhibit versatile biological activities on ERS and immunomodulation. Given the anti-inflammatory, antiviral, and ERS modulating properties, the combined treatment may be potentially effective against COVID-19 and its hypersensitive immune response. These results offer a novel insight into the anti-inflammatory effects of DEX and TAU.

GRP78 and other GRPs are vital companions of ER proteins. They belong to the HSP protein family. GRP78 and other GRPs alleviate inflammation, and GRP78 is essential in combating ERS (Ibrahim et al. 2019). As HSPs, they are vital to reduce impairment and irritation during the recovery from cellular stress. HSPs promote protein synthesis in the ER, and their folding and translocation in the cell membrane. The association between ERS and inflammation is not one-sided considering that inflammation can trigger ERS via cytokines.

ERS also prompts the inflammatory response, and the inflammatory mediators may induce ER to discharge calcium ions and retain ROS in the lumen. LPS-elicited similar effects in murine RAW 264.7 cells, indicating that a line of several mechanisms, play a role in the inhibition of LPS-induced gene expression of ERS markers via ER stressors. However, the present study does not undeniably explain whether ERS was a cause or an effect of inflammation, nor does it identify the optimal concentration of DEX and

TAU. Therefore, a series of future investigations are necessary to elucidate the issues of interest.

The COVID-19 pandemic requires rapid elucidation of the underlying pathophysiological mechanisms to develop effective treatments. Infections with SARS-CoV are accompanied by a significant level of ERS when viroporins pass in the host cells. The viral proteins prompt their downstream indicators by triggering ERS mediators such as IRE-1, PERK, and ATF-6. Thus, it is essential to interfere with the ERS pathway to enhance the therapeutic effect of anti-inflammatory drugs. Currently, combination therapies for COVID-19 are used to modulate ERS (Rizk et al. 2020; Shabbir et al. 2020; Perricone et al. 2020). The anti-inflammatory property of both compounds as well as their role in ERS modulation suggests that the combination therapy may have potential efficacy against COVID-19.

DEX has been frequently used in syndromes closely related to COVID-19, such as severe acute respiratory syndrome (SARS), MERS, influenza, and pneumonia (Lansbury et al. 2020). The use of DEX, however, has not been further supported due to insufficiency of data from organized or randomized trials. The positive effect of DEX in patients with COVID-19 or related syndromes depends on the choice of the right dose. High doses may be more harmful than useful at a time when control of viral replication is dominant (Zhou et al. 2020). Under circumstances, the potential synergism between DEX and TAU in lowering inflammatory response might avoid a high dose application of DEX at the stage when inflammatory features are dominant rather than active viral replication.

DEX and TAU decreased the pro-inflammatory response and the expression of ERS-associated proteins. The two agents, individually and jointly, might alleviate the inflammatory response by inhibiting the induction of ERS in the RAW 264.7 macrophages especially when DEX was applied at lower concentrations (<50 nM). However, the present study had shortcomings: It did not clarify whether ERS serves as a cause or an effect of inflammation, nor did it recognize a "best possible" therapeutic concentration of DEX and TAU. Therefore, further investigations employing experimental animals are required to address the urgent questions related to the COVID-19 pandemic.

5 Conclusion

The levels of ERS markers and pro-inflammatory cytokines decreased under single pre-treatment with DEX. TAU also reduced the expression of LPS-induced ERS and pro-inflammatory markers. Under the combined treatment with DEX and TAU, however, the macrophages showed even a greater level of reduction in ERS and pro-inflammatory responses ($p < 0.01$). The RT-qPCR data indicate that the reduction of ERS markers is caused by the inhibition of their own expression at the transcriptional level. The concurrent treatment of macrophages by both agents reduced the level of ERS and pro-inflammatory cytokines in a synergistic fashion. In conclusion, the joint therapy potentially might help the COVID-19 patients overcome so-called cytokine release syndrome.

6 Acknowledgement

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Regulation of CXCR4 Expression by Taurine in Macrophage-Like Cells

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Keywords

CXCR4 · Macrophage · J774.1 · CXCR2 · GABA

Abbreviations

AMPK Adenosine monophosphate kinase
CXCL C-X-C chemokine ligand
CXCR C-X-C chemokine receptor
DSS Dextran sulfate sodium
GABA γ-aminobutyric acid
GAT GABA transporter
IL Interleukin
MIP-2 Macrophage inflammatory protein-2
NAFLD Nonalcoholic fatty liver disease
TAUT Taurine transporter
TLR Toll-like receptor
TXNIP Thioredoxin-interacting protein

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a free sulfur-containing amino acid found at high concentrations in mammalian tissues. Taurine has been found to play important roles in various biological processes, including anti-oxidation, membrane stabilization, detoxification, osmoregulation, and bile acid conjugation (Huxtable 1992; Schaffer et al. 2003). We have previously examined the relationship between taurine and its intestinal absorption via the taurine transporter (TAUT; SLC6A6) and the function of intestinal epithelial cells. Additionally, we have reported that TAUT activity is regulated by various factors such as extracellular taurine concentration (Satsu et al. 1997), hyperosmolarity (Satsu et al. 1999, 2004), inflammatory cytokines including TNF-α (Mochizuki et al. 2002, 2005), and lysophosphatidylcholine, which is one of the inhibitory components in sesame extracts (Ishizuka et al. 2000, 2002).

Although several functional studies on taurine have been reported (Schaffer et al. 2003; Murakami 2017), the detailed regulatory mechanisms underlying taurine function, especially at the molecular and cellular levels, remain unclear. We have previously examined the effect of taurine on the gene expression profile of human intestinal-like Caco-2 cells using DNA microarrays and found that taurine markedly increases the mRNA expression of thioredoxin-interacting protein (TXNIP), which further regulates cell functions such as adenosine monophosphate kinase (AMPK) phosphorylation via TXNIP induction (Gondo et al. 2012; Satsu et al. 2019). Conversely, we found that

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taurine suppressed cell damage of intestinal epithelial cells by activated macrophage-like cells in an in vitro intestinal inflammation model and an in vivo dextran sulfate sodium (DSS)-induced colitis model (Satsu et al. 2006; Zhao et al. 2008). This suggested that taurine has anti-inflammatory effects in the intestine. However, the detail regulatory mechanism by which taurine affects the intestinal immune system and suppresses the intestinal inflammatory response is unknown.

Based on this background, we tried to reveal the relationship between taurine and the intestinal immune system, especially immune cells present beneath the intestinal epithelial cells. In the present study, we analyzed the effect of taurine on the mRNA expression of immune-related genes in macrophage-like cells.

2 Methods

2.1 Cell Culture

J774.1 cells and RAW264.7 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Roswell Park Memorial Institute medium-1640 (RPMI) containing 10% fetal calf serum (FCS), 2% glutamine, and an appropriate amount of penicillin-streptomycin (10,000 U/mL and 10 mg/mL). J774.1 cells and RAW264.7 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2 Real-Time PCR

J774.1 cells were seeded on 24-well plates without or with taurine and used for real-time PCR analysis. Total RNA was extracted from J774.1 cell line using RNA-iso (TAKARA, Shiga, Japan) according to the manufacturer's instructions. Isolated total RNA (0.5 μg) was reverse-transcribed to cDNA using the Primescript RT Reagent Kit (TAKARA), and the first-strand cDNA was amplified using a TB Green Premix Kit (TAKARA). The conditions for PCR were as follows: denaturation at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 72 °C for 15 s. The primers used for real-time PCR are listed in Table 1. The mRNA expression of GAPDH was not affected by taurine at any stage, indicating that GAPDH could be used as a housekeeping gene.

Table 1 Primers used for real-time PCR

Gene	Species		Primer sequence
GAPDH	Mouse	Forward reverse	aacgacccctttcattgac tccacgacatactcagcac
TLR3	Mouse	Forward reverse	tgccaaatactccctttgttgaa cccgttcccaactttgatagatg
TLR4	Mouse	Forward reverse	agaaaatgccaggatgatgc tgtcatcagggactttgctg
TLR5	Mouse	Forward reverse	gcaggatcatggcatgtcaac atctgggtgaggttacagcct
TLR7	Mouse	Forward reverse	cccttaccatcaaccacatacc tacacacattggctttggaccc
TLR8	Mouse	Forward reverse	atgcccctcagtcatggattc ttgacgatggttgcattctgca
TLR9	Mouse	Forward reverse	tctcccaacatggttctccgtcg tgcagtccaggccatga
CXCR2	Mouse	Forward reverse	gctcacaaacagcgtcgtag ccacaggtattttgtcgttgc
MIP-2	Mouse	Forward reverse	cctggttcagaaaatcatcc gctacaggcttgtcactcg
IL-β	Mouse	Forward reverse	tcggacccatatgagctga ccacaggtattttgtcgttgc
IL-10	Mouse	Forward reverse	cagagccacatgctcctaga tgtccagctggtcctttgtt
IL-12	Mouse	Forward reverse	accaaattactcccggacggttc agacagagacgccattccaca
IL-18	Mouse	Forward reverse	caaaccttccaaatcacttcct tccttgaagttgacgcaaga
TNF-α	Mouse	Forward reverse	ctgtagcccacgtcgtagc ttgagatccatgccgttg
CXCR1	Mouse	Forward reverse	ctcccgcacacaaggaac gcagcattcccgtgatattt
CXCR3	Mouse	Forward reverse	cagcctgaactttgacagaacc gcagccccagcaagaaga
CXCR4	Mouse	Forward reverse	gaccgcctttaccccgatagc acccccaaaaggatgaaggagtc

2.3 Western Blot Analysis

J774.1 cells were cultured on 78.5 cm^2 dishes and cultured without or with taurine for 24 h. Then, the membrane fraction was prepared using lysis buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% of an inhibitor cocktail at pH 7.5), and western blotting was performed as previously described (Satsu et al. 2008). The protein assay was performed using a BIO-RAD Protein Assay Solution (BIO-RAD, Hercules, CA, USA). The primary antibody used was rabbit anti-mouse CXCR4 antibody and rabbit anti-mouse β-actin antibody (Abcam, Cambridge, UK). Goat anti-rabbit IgG antibody linked to horseradish peroxidase (Abcam, Cambridge, UK) was used as the secondary antibody. Bound antibodies were analyzed using an ECL chemiluminescent substrate (GE Healthcare, Chicago, IL, USA) and a Lumino Image Analyzer (LAS-4000miniPR; Krefeld, Germany).

2.4 Statistical Analysis

Values are expressed as the mean ± standard error (SE). Data were analyzed using Student's *t*-test or Tukey's test. Statistical significance was set at $P < 0.05$.

3 Results

3.1 Effect of Taurine on Immune-Related mRNA Expression in Macrophage-Like J774.1 Cell Line

To reveal the relationship between taurine and the immune system at the molecular level, the effect of taurine on mRNA expression levels of immune-related molecules was examined using macrophage-like J774.1 cells. J774.1 cells were incubated with 100 mM taurine for 3 h, and then RNA was extracted for real-time PCR analysis. Toll-like receptor (TLR) 3, TLR4, TLR5, TLR7, TLR8, TLR9, CXCR2, TNF-α, interleukin (IL)-1β, IL-10, IL-12, IL-18, and MIP-2 were selected as immune-related molecules. Taurine significantly increased CXCR2 mRNA levels, whereas TLR3, TLR5, TLR9, and IL-18 mRNA levels were significantly decreased by taurine (Fig. 1). The mRNA expression of MIP-2 was not detected in this study. As the change in expression level of CXCR2 was high, we focused on CXCR2 for further study.

3.2 Effect of Taurine on mRNA Expression Level of CXCR Family in Macrophage-Like J774.1 Cell Line

We next focused on CXCR, a chemokine receptor family. Eight CXCR family members have been reported in humans (Cabrero-de Las Heras et al. 2018). Among them, as CXCR1, CXCR2, CXCR3, and CXCR4 are most extensively investigated, we also examined the effect of taurine on mRNA expression of CXCR1, CXCR2, CXCR3, and CXCR4. As shown in Fig. 2, taurine significantly increased the mRNA levels of CXCR2 and CXCR4. In contrast, CXCR3 mRNA expression was decreased following taurine treatment. The mRNA expression of CXCR1 was not detected in this study. These results suggest that taurine regulates the expression levels of the CXCR gene family. As the CXCR4 mRNA level was markedly increased by taurine, we further analyzed the effects of taurine on CXCR4 mRNA.

3.3 Time- and Dose-Dependent Increase in CXCR4 mRNA Levels in Macrophage-Like J774.1 Cell Line

We examined the effect of 0 − 100 mM taurine on mRNA expression of CXCR4. As shown in Fig. 3a, taurine increased the CXCR4 mRNA expression in a dose-dependent manner. Next, we examined the effect of 100 mM taurine on mRNA expression at various time points. Taurine most increased the mRNA expression of CXCR4 after 3 h (Fig. 3b). Therefore, incubation with 100 mM taurine for 3 h was used as the optimal condition for taurine-induced CXCR4 expression for further studies.

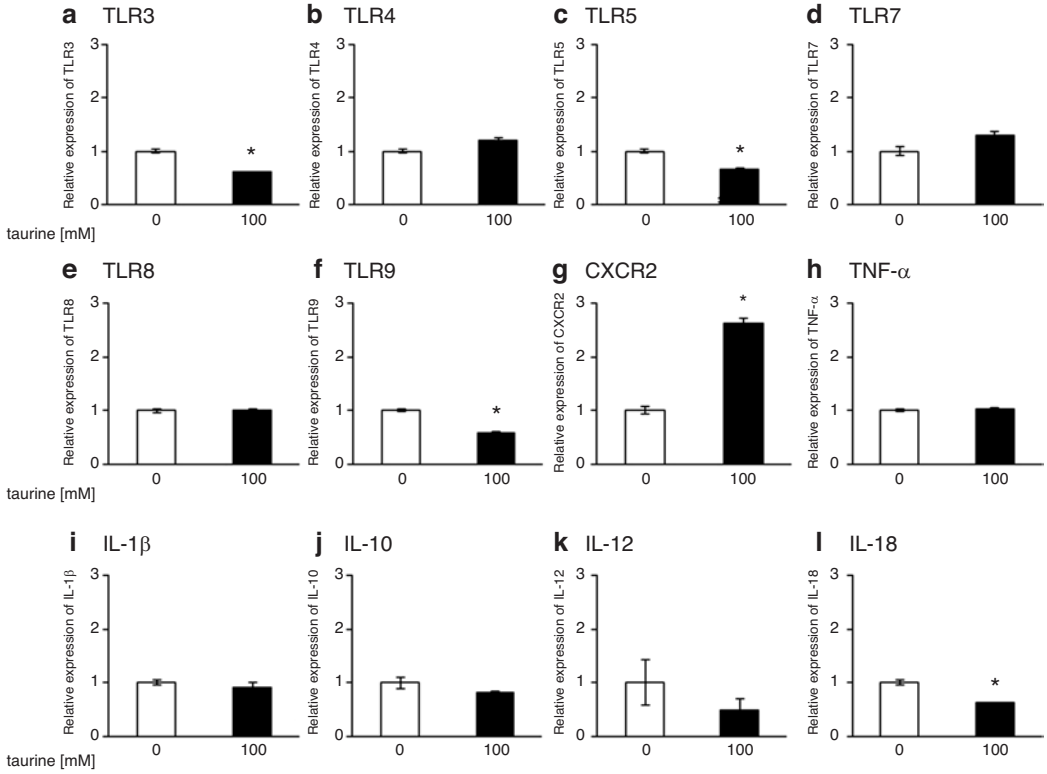

Fig. 1 Effect of taurine on mRNA expression of 13 immune-related genes in J774.1 cells. Each value means ±SE (n = 4), Student's *t*-test *p < 0.05 vs 0 mM

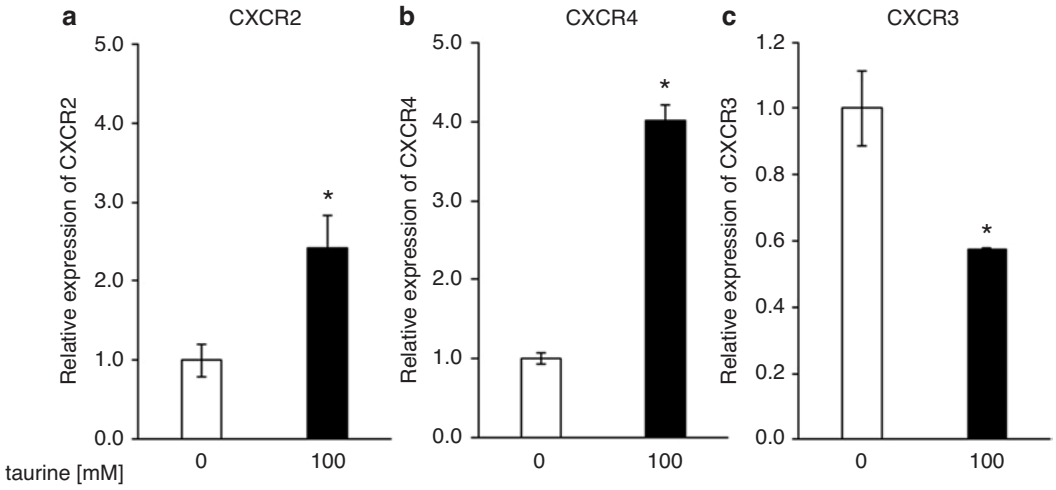

Fig. 2 Effect of taurine on mRNA expression of CXCR family in macrophage-like J774.1 cells. Each value means ±SE (n = 4), Student's *t*-test *p < 0.05 vs 0 mM

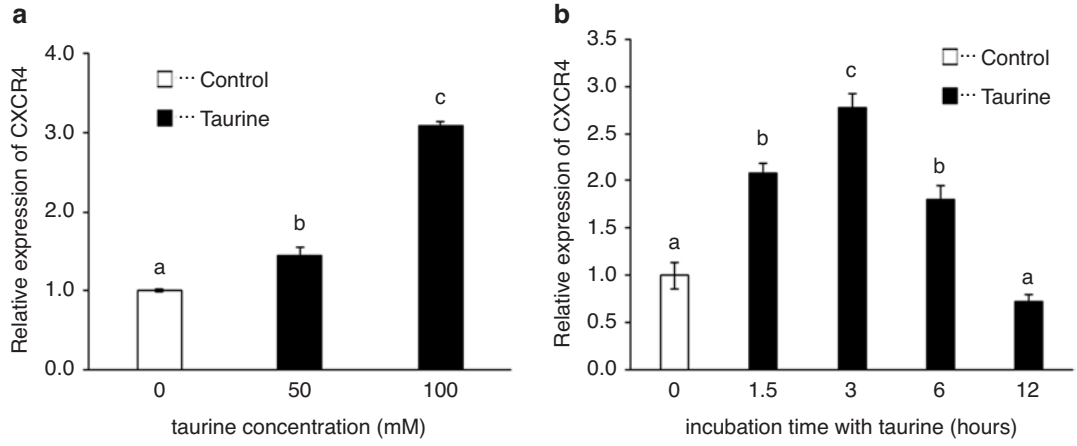

Fig. 3 Effect of taurine on CXCR4 mRNA levels at various concentrations of taurine (0 − 100 mM) for 3 hours (**a**) and various incubation time (0 − 12 h) at 100 mM taurine (**b**). Each value means ± SE (n = 4), a,b,c,d; Tukey's test $p < 0.05$

3.4 Effect of Taurine on Protein Expression Level of CXCR4 in Macrophage-Like J774.1 Cell Line

To confirm whether the protein expression level of CXCR4 was also increased by taurine, western blotting analysis was performed. The protein expression level of CXCR4 was increased by taurine by approximately 1.6-fold (Fig. 4). This indicates that taurine increased CXCR4 expression at both the protein and mRNA.

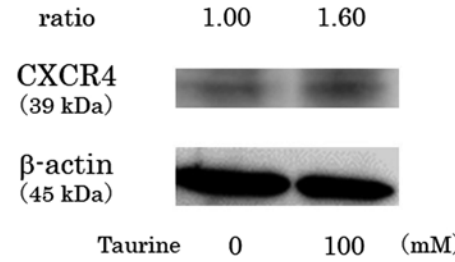

Fig. 4 Effect of taurine on the expression level of CXCR4 protein in J774.1 cells

3.5 Effect of Taurine on mRNA Expression of CXCR4 in Macrophage-Like RAW264.7 Cells

To examine whether taurine-induced increase in CXCR4 mRNA level occurs in other macrophage-like cells, we examined the effect of taurine on CXCR4 mRNA expression in RAW264.7 cells, another mouse-derived macrophage-like cell line. We found that taurine increased CXCR4 mRNA levels in a dose-dependent manner (Fig. 5). This suggests that taurine induces CXCR4 mRNA expression in macrophages.

3.6 Effect of Taurine-Related Compounds on mRNA Expression of CXCR4 in Macrophage-Like J774.1 Cells

To determine whether upregulation of CXCR4 mRNA is specific to taurine treatment or structure, we examined the effect of taurine-related compounds on the mRNA expression of CXCR4 in J774.1 cells. γ-aminobutyric acid (GABA) increased CXCR4 mRNA levels by approximately threefold (Fig. 6). Hypotaurine also significantly increased the mRNA expression of CXCR4, but to a lesser extent than taurine. In contrast, β-alanine had no effect on the mRNA

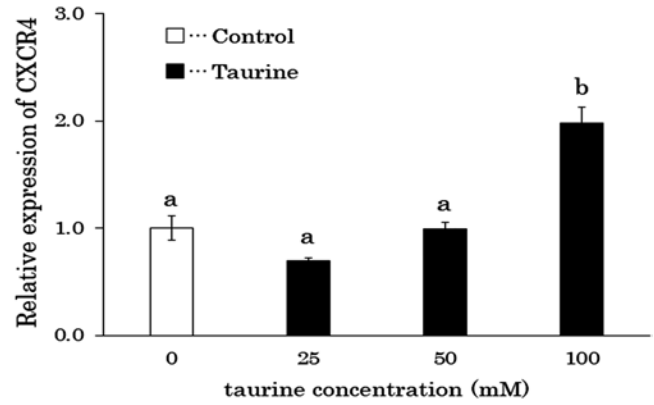

Fig. 5 Effect of various concentrations of taurine on mRNA expression of CXCR4 in RAW264.7 cells. Each value means ± SE (n = 4), a,b,c,d; Tukey's test $p < 0.05$

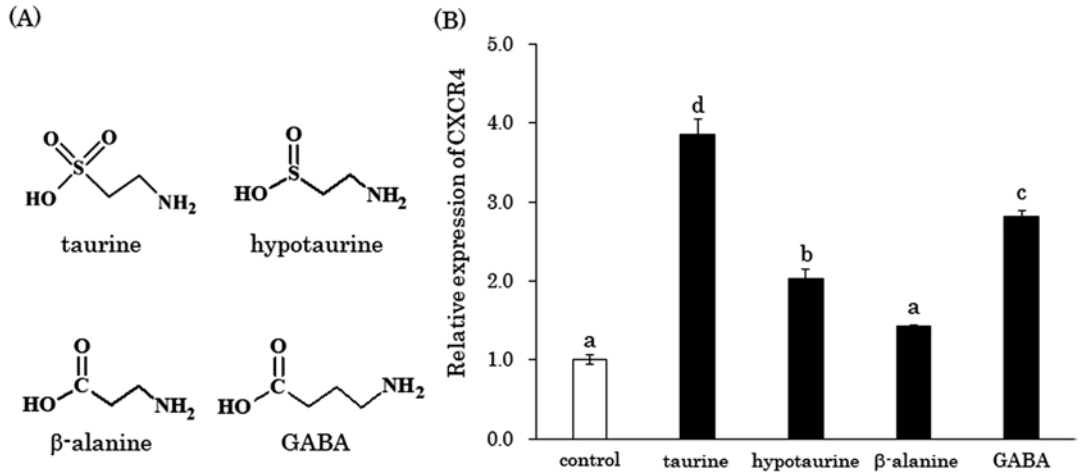

Fig. 6 Chemical structures of taurine and its related compounds (**a**) and effect of taurine-related compounds on CXCR4 mRNA expression (**b**) in J774.1 cells. Each value means ± SE (n = 4), a,b,c,d; Tukey's test $p < 0.05$

levels of CXCR4. These results suggest that GABA and hypotaurine also increase the mRNA level of CXCR4.

4 Discussion

In the present study, we focused on the relationship between taurine and the immune system and examined the direct effect of taurine on gene expression of immune-related genes in macrophage-like J774.1 cells.

We searched for molecules whose mRNA expression levels were regulated by taurine among 13 immune-related molecules. The results showed that taurine increased the mRNA expression of CXCR2 and decreased the mRNA expression of four genes, and the expression of eight genes remained unchanged. We then focused on the CXCR family and found that CXCR4 mRNA level was most increased by taurine, and further detailed analysis was performed.

CXCR is a rhodopsin-like seven-transmembrane trimeric G-protein-coupled receptor, whose major ligand is CXC chemokine (Susek et al. 2018). CXC chemokine is a low molecular weight protein of 6 − 14 kDa (Guo et al. 2019), and binding of CXCR to CXC chemokine results in processes such as neutrophil migration (Cavalera and Frangogiannis 2014) and angiogenesis (Addison et al. 2000).

It has been reported that CXCR1, whose mRNA expression was not detected in J774.1 cells, is not expressed in mouse macrophages

under normal conditions, but its expression is induced when they are infected with *Staphylococcus aureus* (Bishayi et al. 2015), suggesting that CXCR1 was not expressed under the steady-state experimental conditions in this study. CXCR2 is mainly expressed on neutrophils, which together with CXCR1 function as receptors for IL-8 and MIP-2, and inflammatory cytokines secreted from inflammatory sites induce CXCR2-expressing cells at inflammatory sites (Liu et al. 2016). Macrophages are also antigen-presenting cells that feed on and digest foreign substances, such as degenerative substances and invading bacteria or virus, and present captured antigens. Therefore, taurine is thought to increase CXCR2 mRNA expression in macrophages, enabling faster induction of macrophages to the site of inflammation for prompt self-defense. In contrast, CXCR3, whose mRNA expression was suppressed by taurine (Fig. 2c), has been reported to be an important receptor in several diseases that are expected to be improved by taurine. For example, it has been reported that taurine reduces triglycerides in nonalcoholic fatty liver disease (NAFLD) (Murakami et al. 2018) and that CXCR3 knockout mice have reduced NAFLD symptoms (Du et al. 2017), suggesting that taurine may improve NAFLD through suppression of CXCR3 expression.

In addition, we focused on CXCR4, whose mRNA expression was most enhanced by taurine, and further analyzed the enhancement of CXCR4 expression by taurine using J774.1 cells. CXCR4 binds to its ligand CXCL12 (also known as stromal-cell-derived factor-1α, SDF-1α) and exerts a variety of functions, including angiogenesis and the establishment of hematopoietic stem cells and their progenitors in bone marrow (Janssens et al. 2018; Teixido et al. 2018). Among them, taurine has been reported to be involved in the homing of *hematopoietic stem cells* from the liver to the bone marrow during fetal life (Doring et al. 2014) and in fetal survival, as indicated by the perinatal death of knockout mice (Hughes and Nibbs 2018). This may be related to the fact that taurine is an important substance for fetal development (Tochitani 2019). However, the mechanism by which taurine affects the fetus remains unclear. In addition, one of the tissues in which the ligand CXCL12 is secreted is the subventricular zone. The subventricular zone contributes to the production of neurons during embryonic development and plays an important role in brain formation (Mirzadeh et al. 2008). Thus, both CXCR4 and CXCL12 may be important for fetal survival. Since fetal-derived cells were not used in this study, future studies using fetal-derived cells, such as primary cultures from pregnant mice, will be necessary to accurately analyze the relationship between taurine and CXCR4 in the fetus.

Hypotaurine, β-alanine, and GABA, which were used in this study, are all common substrates of TAUT (Han et al. 2006), and even though they are all thought to be taken up into cells via TAUT, their degree of enhancement of CXCR4 mRNA expression is different. Furthermore, GABA increased the CXCR4 mRNA expression (Fig. 6), while the mRNA expression level of TXNIP was increased by taurine but not GABA (Gondo et al. 2012). Therefore, this strongly suggests that the regulatory mechanisms of taurine-induced upregulation of CXCR4 and TXNIP are different. In the case of CXCR4, as both GABA and taurine increased CXCR4 mRNA levels, it is likely that the receptors or signaling molecules with affinity for taurine as well as GABA are involved in this regulation. One candidate is the GABA receptor. GABA is an inhibitory neurotransmitter that binds to $GABA_A$ receptors (Taber et al. 1986; del Olmo et al. 2000; Hussy et al. 1997), and the GABA transporter (GAT). $GABA_A$ receptors are transmembrane ion channel receptors that have been reported to have a high affinity for taurine (Albrecht and Schousboe 2005). There are four subtypes of GATs, one of which is GAT-2. GAT-2 is also known to take up taurine (Paul et al. 2014). Therefore, we plan to study the involvement of GABA receptors in this phenomenon using an antagonist of the GABA receptor or via knockdown of GABA receptors using RNA interference. Further, we will analyze the intracellular regulatory mechanism of taurine-induced upregulation of CXCR4 in a future study. We will also perform animal studies through oral administration of taurine to mice to determine whether the increase in CXCR4 by taurine also occurs in vivo.

5 Conclusion

The direct effect of taurine on the expression levels of immune-related genes such as interleukin, chemokine, its receptor, and TLR was examined using the macrophage-like J774.1 cell line. We revealed that the CXCR family was regulated by taurine and that taurine upregulated CXCR4 mRNA expression in a dose-dependent manner. Furthermore, taurine was found to increase the protein levels of CXCR4 as well as mRNA levels. Among taurine-related compounds, we found that GABA and hypotaurine also induced CXCR4 mRNA expression, but taurine induced the mRNA expression of CXCR4 most. These findings are expected to provide a better understanding of the physiological properties of taurine and lead to a new function of taurine in the immune system.

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Taurine Chloramine Inhibits Leukocyte Migration by Suppressing Actin Polymerization and Extracellular Signal-Regulated Kinase

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Keywords

Taurine chloramine · Inflammation · Neutrophils · Macrophages · Migration · Actin polymerization

Abbreviations

BSA	bovine serum albumin
BTM	brewer thioglycollate medium
DAPI	4′,6′-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
fMLP	N-formyl-methionyl-leucyl-phenylalanine
HBSS	Hanks' balanced salt solution
IL	interleukin
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MPO	myeloperoxidase
NIH	National Institute of Health
NO	nitric oxide
PBS	phosphate-buffered saline
Tau-Cl	taurine chloramine
TNF	tumor necrosis factor

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is one of the most abundant free amino acids in the animal kingdom and is ubiquitously distributed in mammalian tissues, with the highest levels being reported in the heart, brain, and leukocytes (Huxtable 1992). Taurine is involved in various physiological functions, including osmoregulation, membrane stabilization, calcium mobilization, neurotransmission, reproduction, inflammation, and detoxification (Huxtable 1992; Schuller-Levis and Park 2003). In leukocytes, taurine has been reported to react with hypochlorous acid ($HOCl/OCl^-$), which is released by the myeloperoxidase (MPO) system, to form taurine

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chloramine (Tau-Cl) (Weiss et al. 1982). Tau-Cl suppresses the production of inflammatory mediators, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, superoxide anion (O_2^-), and nitric oxide (NO) and upregulates the expression of antioxidant enzymes, such as heme oxygenase, glutamate-cysteine ligase, and catalase, thereby protecting cells from inflammatory cytotoxicity (Kim and Cha 2014; Marcinkiewicz and Kontny 2014).

Inflammatory cells, such as neutrophils and macrophages, play critical roles in immune surveillance and defense, as well as in the progression and resolution of inflammation. During infection, neutrophils, which are the most prevalent leukocytes in the blood, rapidly migrate to infection sites, engulf invading microorganisms, and release oxidants and enzymes that kill and degrade the microorganisms (Segal 2005; Amulic et al. 2012; Kolaczkowska and Kubes 2013). Using MPO, neutrophils produce microbicidal HOCl as a first line of defense against infections (Nauseef 2014). Moreover, neutrophils contain large amounts of taurine, which accounts for 30–75% of the cells' total free amino acids (10–70 mM in humans) (Fukuda et al. 1982) and which upon activation reacts with HOCl to produce Tau-Cl, thereby ameliorating inflammation through the elimination of highly toxic HOCl. Meanwhile, macrophages infiltrate infection and inflammation sites, either as tissue-resident macrophages or as monocytes that migrate from the blood. Upon activation by invading microorganisms and dying cells, macrophages produce pro-inflammatory factors such as IL-1β, IL-6, TNF-α, prostaglandin E_2, and NO and recruit other immune cells to inflamed tissues (Duque and Descoteaux 2014). In addition, macrophages modulate adaptive immune responses by presenting foreign antigens.

Neutrophil and macrophage functions are dependent on timely recruitment from the blood stream, followed by directed migration to the site of inflammation. The cells migrate to infection sites by following concentration gradients of chemoattractants that are produced by microorganisms, the complement system, and other acute inflammatory cells. Efficient migration occurs in sequential steps (i.e., tethering, rolling, crawling, adhesion, and transmigration), which all require cytoskeleton reorganization. Migrating cells extend a leading edge by assembling a branched network of actin filaments that produces physical force as the polymers grow beneath the plasma membrane (Pollard and Borisy 2003). Filamentous actin (F-actin) is a double-helical polymer of globular subunits that are arranged head-to-tail and play an important role in regulating membrane plasticity and cell motility. The extravasation of neutrophils and macrophages from the blood to target tissues involves the tethering and rolling of cells on the blood vessel wall, firm adhesion, and crossing through the endothelial barrier (Vicente-Manzanares and Sánchez-Madrid 2004). Extracellular signal-regulated kinase (ERK) signaling is a critical regulator of cell motility. The ERK pathway inhibitors PD98059 and U0126 and a dominant negative ERK mutant have all been reported to block cell migration (Huang et al. 2004). ERK influences cell motility by regulating genes involved in cell migration or through the direct phosphorylation of myosin light chain kinase, calpain, paxillin, or focal adhesion kinase (Hunger-Glaser et al. 2003; Tanimura and Takeda 2017). Moreover, ERK influences cell migration by regulating expression and activation of integrin (Lai et al. 2001; Takami et al. 2002).

In the present study, we investigated the effect of Tau-Cl on the migration of inflammatory cells. Tau-Cl inhibited leukocyte migration to the peritoneal cavity in vivo and in a transwell system and inhibited actin polymerization, adhesion, and ERK activation in RAW 264.7 macrophages. These findings demonstrate the inhibitory effect of Tau-Cl on ERK-dependent F-actin polymerization and migration in inflammatory cells.

2 Methods

2.1 Tau-Cl Synthesis

Tau-Cl was freshly synthesized on the day of use by mixing equimolar amounts of sodium hypochlorite solution (NaOCl; Sigma-Aldrich,

St. Louis, MO, USA) and taurine (Sigma-Aldrich). The formation and concentration of Tau-Cl were monitored by measuring the UV absorption at 200–400 nm (Kim and Kim 2005).

2.2 Preparation of Peritoneal Exudate Cells from Mice

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed under pathogen-free conditions at the animal facility of Inha University (Incheon, Korea). Animal study was conducted according to the guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH, Bethesda, MD, USA) and the ARRIVE guidelines and was approved by the Institutional Animal Care and Use Committee of Inha University (INHA200107-680-1).

Sterile peritonitis was induced using thioglycollate as described previously (Kim and Dinauer 2001). Briefly, mice were received intraperitoneal injections of 3% Brewer thioglycollate medium (BTM) (Sigma-Aldrich) at 12 h before administration of saline or 2–20 mM Tau-Cl. Then, at 6 h after saline or Tau-Cl treatment, the mice were sacrificed, and peritoneal cavity was lavaged twice with 10 mL of phosphate-buffered saline (PBS). Total peritoneal exudate cell numbers were counted, and after cytospin were performed using Shandon-Cytospin 3 (Astmoor, England), the cells were stained with Diff-Quik (Dade Behring, Malvern, PA, USA). Differential cell numbers of the peritoneal exudates were determined by analyzing 300 cells on each slide.

2.3 Preparation of Bone Marrow Neutrophils

Bone marrow neutrophils were isolated from the femurs and tibias as described previously (Kim and Dinauer 2001). Bone marrow cells were suspended in 3 mL of 45% Percoll (GE Healthcare, Uppsala, Sweden) and layered on top of a Percoll gradient (81, 62, 55, and 50%). After centrifugation at 1600 × g for 30 min at 10 °C, the cells between the 81% and 62% layers were collected, washed with HBSS-BG (Hanks' balanced salt solution with 0.1% bovine serum albumin (BSA) and 1% glucose), and suspended in HBSS-BG. The cells were layered over Histopaque 1119 (Sigma-Aldrich) and centrifuged at 1600 × g for 30 min at 10 °C to remove contaminating red blood cells. The cells between the Histopaque and HBSS-BG layers were harvested and washed with HBSS-BG. The final cell preparation was kept in ice cold PBS until processed further.

2.4 RAW 264.7 Cell Culture

Murine macrophage RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA), which was supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin (HyClone), and 100 μg/mL streptomycin (HyClone) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.5 Transwell Migration Assay

Bone marrow neutrophils (1×10^5) were placed in the upper chamber of a transwell (3.0–5.0 μm pore size; Corning Costar, Corning, NY, USA), along with 0–1.0 mM Tau-Cl, and 10 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich) in DMEM was added to the lower chamber. The transwell system was incubated for 10–60 min at 37 °C. To measure macrophage migration, the lower surface of transwell membrane was coated with 0.1% poly-L-lysine (Sigma-Aldrich) for 2 h at room temperature. RAW 264.7 cells (1×10^5) in DMEM were placed in the upper chamber of a transwell (8.0 μm pore size; Corning Costar), along with 0-1.0 mM Tau-Cl, and 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich) was added to the lower chamber. The transwell system was incubated for 16 h at 37 °C, and the non-migrated cells were removed from the upper membrane using a cotton swab. Migratory neutrophils and

macrophages on the underside of the membrane were stained using Diff-Quik and counted under a microscope (Olympus, Tokyo, Japan).

2.6 Fluorescence Staining of F-Actin

Changes in actin organization of the RAW 264.7 cells were determined. Cells were seeded onto sterile coverslips in 24-well plates and treated with 0–1.0 mM Tau-Cl in the presence of 1 μg/mL LPS at 37 °C for 30 min. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min, blocked using 1% BSA in PBS, permeabilized with 0.1% Triton X-100/PBS for 15 min, washed twice with PBS, and stained with 100 nM Alexa Flour 555-phalloidin (Invitrogen, Carlsbad, CA, USA) and 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at 25 °C for 30 min in the dark. Coverslips were washed twice with PBS before they were mounted to the slides using Dako fluorescent mounting medium (Agilent, Santa Clara, CA, USA), and the slides were examined using a fluorescence microscope (Olympus) or an LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany). The fluorescence intensity of F-actin was quantified by ImageJ software (NIH).

2.7 Western Blotting

RAW 264.7 cells were treated with 0-1.0 mM Tau-Cl for 20 min and stimulated with 1 μg/mL LPS for 15 min at 37 °C. Cell lysates were prepared as described previously (Kim and Kim 2005). The 20–30 μg of total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the resolved proteins were transferred onto polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were then probed with antibodies against ERK and phospho-ERK (Cell Signaling, Danvers, MA, USA) with horseradish peroxidase-linked anti-rabbit IgG secondary antibody, and the signals were developed using the ECL method (Amersham, Arlington Heights, IL, USA).

2.8 Statistical Analysis

Two-sample comparisons were performed using the two-tailed Student's t-test with Microsoft Excel (Redmond, WA, USA). The results are presented as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Tau-Cl Inhibited Leukocyte Migration into Peritoneum

Neutrophils are the most abundant leukocytes in the bloodstream and migrate rapidly to infection sites. To determine whether Tau-Cl affects neutrophil migration, mice were subjected to sterile peritonitis induction with 3% BTM, followed by injection with 2–20 mM Tau-Cl (Fig. 1a). Intraperitoneal thioglycollate injection induced significant leukocyte migration into the peritoneal cavity (control $1.7 \pm 0.2 \times 10^6$ cells vs. BTM $14.5 \pm 3.1 \times 10^6$ cells), and Tau-Cl inhibited the migration in a dose-dependent manner, with 10 mM Tau-Cl inhibiting 63% of migration ($5.4 \pm 1.7 \times 10^6$ cells) when compared to that in BTM mice (Fig. 1b–c).

The proportions of monocytes and neutrophils among the thioglycollate-induced peritoneal cells were 13% and 60%, respectively, and the proportions were not affected by Tau-Cl (Fig. 2a–b). The monocyte proportion seemed reduced in BTM mice (control 38% vs. BTM 13%); however, the converted cell numbers were 0.7×10^6 in control mice and 2.0×10^6 in BTM mice, which suggests that enough number of monocytes reside in the peritoneal cavity and monocytes rarely migrate to the peritoneum by thioglycollate at 16 h after injection. Although migration of monocytes did not occur sufficiently at 16 h, Tau-Cl inhibited monocyte migration by 65% at a concentration of 10 mM to control levels (Fig. 2c).

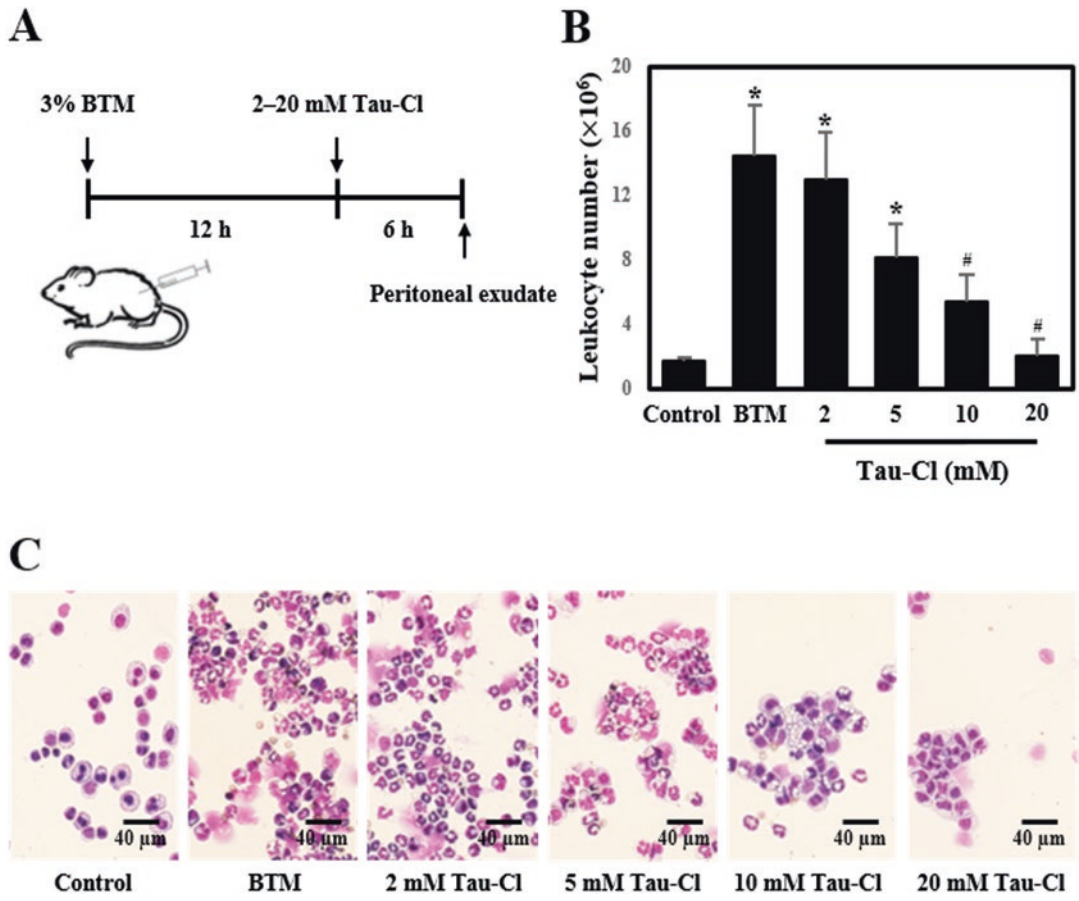

Fig. 1 Tau-Cl inhibited leukocyte migration in the thioglycollate-induced murine sterile peritonitis model. (**a**). Mice were administered intraperitoneally with 1 mL of 3% Brewer thioglycollate medium (BTM) for 12 h followed by saline or 2–20 mM Tau-Cl for 6 h, and peritoneal exudates were collected. (**b**). The number of total leukocytes in peritoneal exudates was counted. (**c**). Representative peritoneal exudate cells after Diff-Quik staining (scale bar, 40 μm). Data are expressed as the mean ± SEM of five independent experiments. *$p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with BTM

However, thioglycollate significantly increased neutrophil migration at 16 h (control $0.2 \pm 0.02 \times 10^6$ cells vs. BTM $8.8 \pm 1.9 \times 10^6$ cells), and 10 mM Tau-Cl inhibited neutrophil migration by 54% ($4.1 \pm 1.3 \times 10^6$ cells) (Fig. 2d).

3.2 Tau-Cl Inhibited Leukocyte Migration In Vitro

The findings of the in vivo study were then examined using a transwell migration system. Bone marrow neutrophils migrated to DMEM in the absence of a stimulus, and migration of neutrophils was increased when using fMLP (at 30 min, control 264 ± 8 cells vs. fMLP 572 ± 61 cells). fMLP is a neutrophil chemotactic factor that attracts leukocytes by binding to the formyl peptide receptor. Furthermore, Tau-Cl (0.2, 0.5, 0.7, and 1.0 mM) inhibited fMLP-induced neutrophil migration by 20, 37, 55, and 60%, respectively (Fig. 3a, c). LPS induces the reorganization of actin cytoskeleton and accelerates macrophage migration (Kleveta et al. 2012). LPS promoted the migration of RAW 264.7 cells (control 121 ± 15 cells vs. LPS 528 ± 116 cells), and

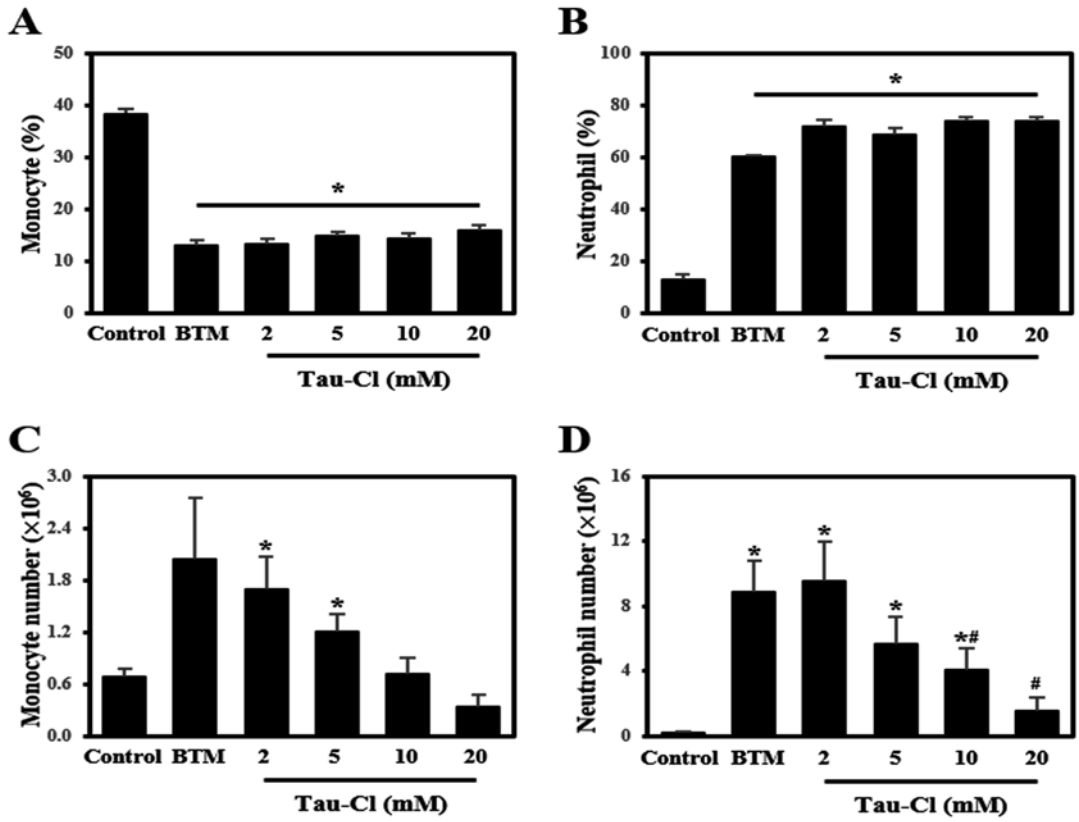

Fig. 2 Tau-Cl inhibited thioglycollate-induced neutrophil migration. Mice were administered BTM for 12 h followed by saline or 2–20 mM Tau-Cl for 6 h, and peritoneal exudates were collected. (**a–b**). The proportions of monocytes and neutrophils in peritoneal exudates were determined by Diff-Quik staining. The numbers of monocytes (**c**) and neutrophils (**d**) in peritoneal exudates were counted. Data are expressed as the mean ± SEM of five independent experiments. *$p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with BTM

Tau-Cl significantly inhibited the migration in a concentration-dependent manner (0.5 mM Tau-Cl 182 ± 8, 0.7 mM Tau-Cl 95 ± 23, and 1.0 mM Tau-Cl 57 ± 26 cells) (Fig. 3b, d).

3.3 Tau-Cl Inhibited Actin Polymerization and Adhesion of RAW 264.7 Cells

The actin cytoskeleton plays important roles in cell shape definition, migration, and invasion. To evaluate the effect of Tau-Cl on actin polymerization, the F-actin contents of LPS- and Tau-Cl-treated RAW 264.7 cells were measured using phalloidin staining. LPS treatment increased the number of cells with greater F-actin staining as well as the fluorescence intensity of stained cells (Fig. 4). Tau-Cl significantly reduced LPS-induced F-actin staining (LPS 2.8 ± 0.4 vs. 0.5 mM Tau-Cl 1.5 ± 0.3). Tau-Cl also decreased the number of adherent cells on the coverslips (LPS 278 ± 13 cells vs. 0.5 mM Tau-Cl 96 ± 5 cells) (Fig. 4d). These results suggest that Tau-Cl inhibits leukocyte migration by reducing actin polymerization and adhesion.

3.4 Pharmacological Actions of Taurine on Metabolic Flux and ATP Biosynthesis

Activation of the ERK pathway is required for actin polymerization and leukocyte migration

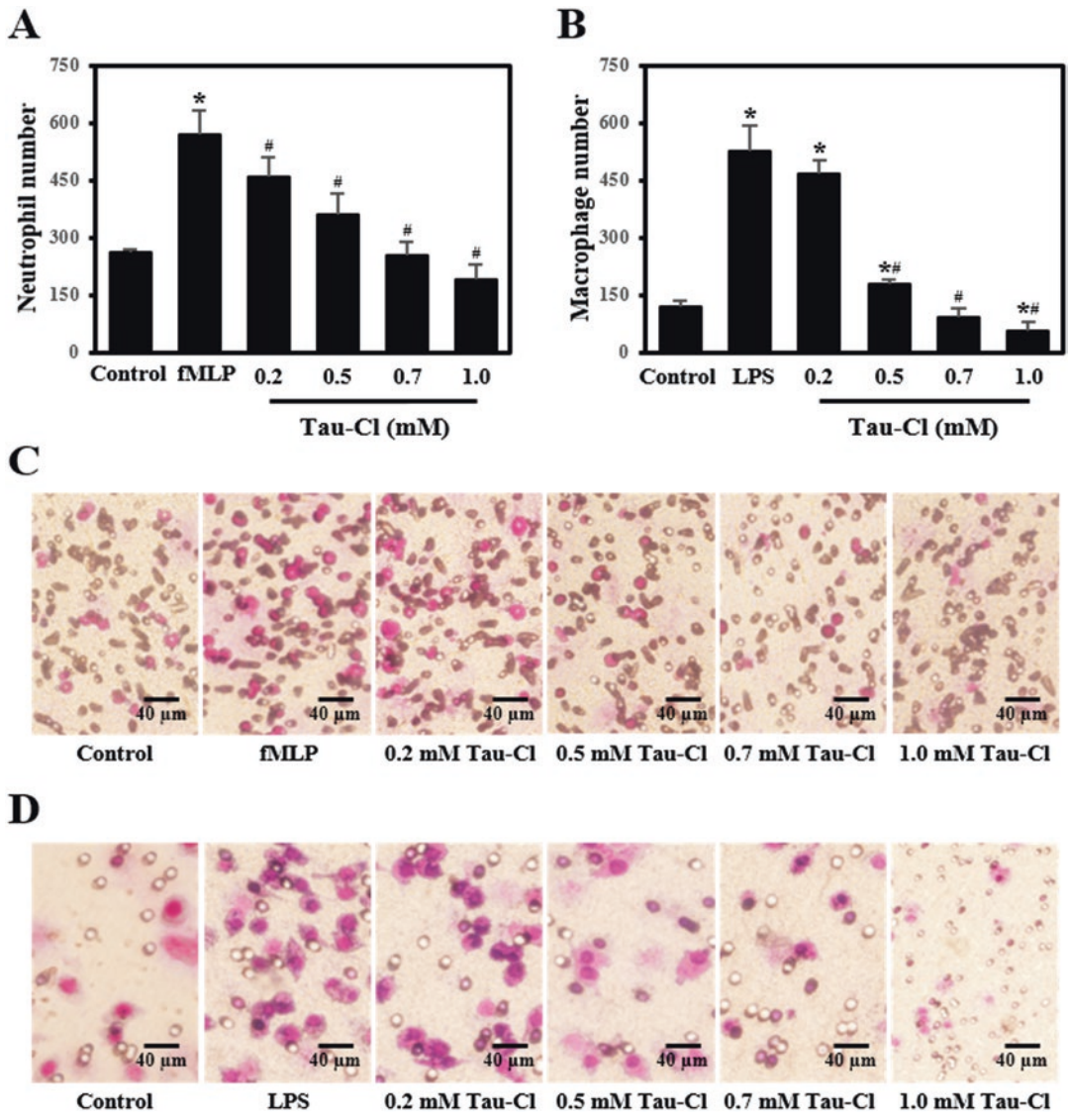

Fig. 3 Tau-Cl inhibited the migrations of LPS-stimulated macrophages and fMLP-induced neutrophils in vitro. (**a**). Bone marrow neutrophils were incubated with Tau-Cl for 30 min in a transwell system. (**b**). RAW 264.7 cells were incubated with Tau-Cl for 16 h. Cells on the lower surface of the membrane were stained with Diff-Quik and counted. Representative images of bone marrow neutrophils (**c**) and RAW 264.7 cells (**d**) on the lower surface of membranes (scale bar, 40 μm). The experiment was performed in triplicate, and results are from three independent experiments. Data are expressed as mean ± SEM, and $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with fMLP or LPS

(Tanimura and Takeda 2017). To evaluate whether Tau-Cl regulates ERK activation, RAW 264.7 cells were treated with Tau-Cl and then stimulated with LPS. Tau-Cl significantly inhibited LPS-stimulated ERK phosphorylation, by 53 and 76% at 0.5 and 1.0 mM concentrations, respectively (Fig. 5), which suggests that Tau-Cl regulates leukocyte migration by inhibiting actin polymerization and ERK activation.

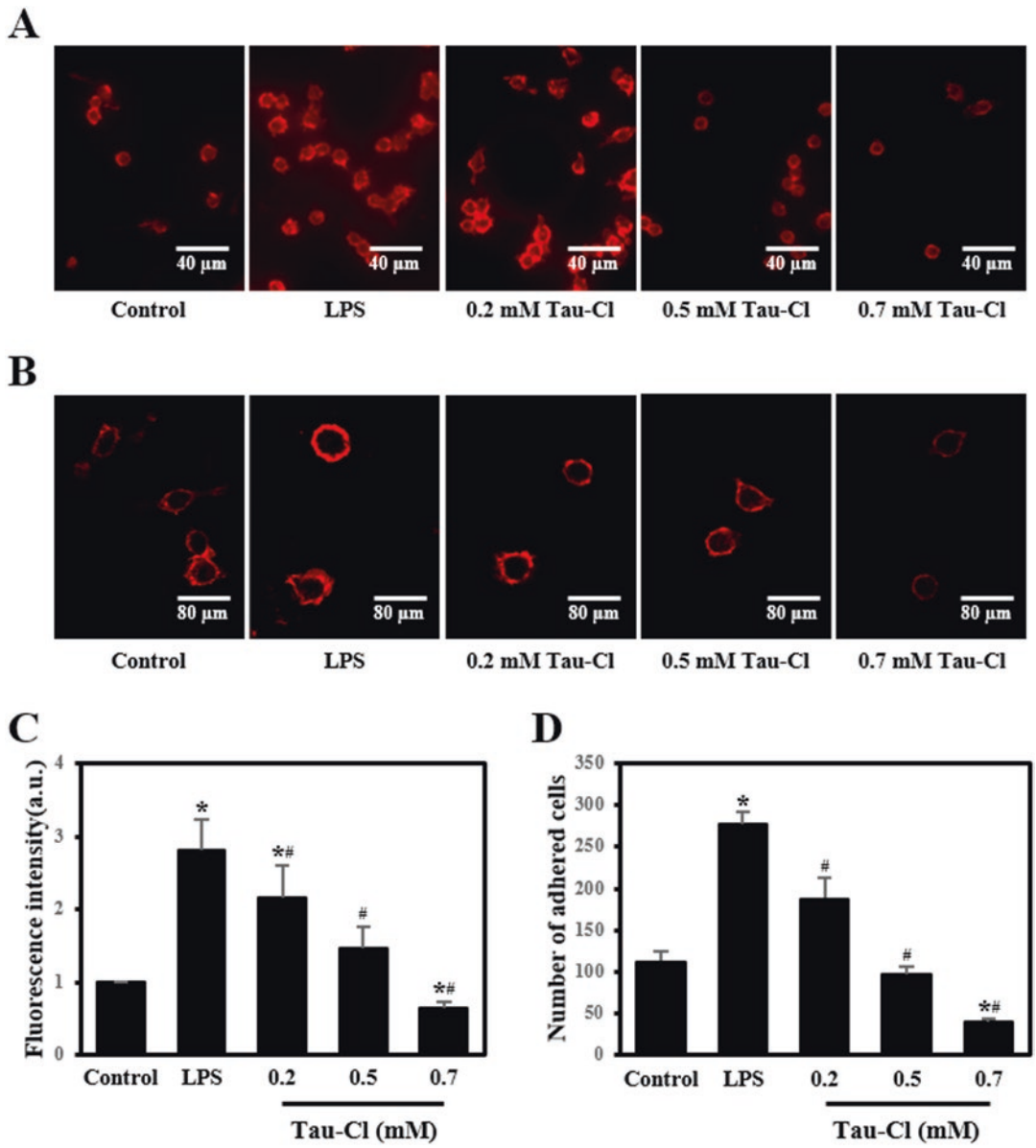

Fig. 4 Tau-Cl inhibited LPS-induced actin polymerization in RAW 264.7 cells. RAW 264.7 cells were treated with Tau-Cl for 30 min in the presence of 1 μg/mL LPS and F-actin content labeled with Alexa Fluor 555-phalloidin was analyzed. (**a**). Representative fluorescence images of F-actin (scale bar, 40 μm). (**b**). Confocal images of F-actin (scale bar, 80 μm). (**c**). Fluorescence intensity of F-actin was quantified using ImageJ and represent as arbitrary unit (a.u.). (**d**). The numbers of adhered cells on coverslips were counted. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with LPS

4 Discussion

During infection, neutrophils and macrophages infiltrate infected tissues to engulf and destroy microorganisms and apoptotic cells. The neutrophils and macrophages that accumulate at infection sites then release reactive oxygen and nitrogen species, granular enzymes, and cytokines (Kolaczkowska and Kubes 2013), which, together, increase chemoattraction and inflam-

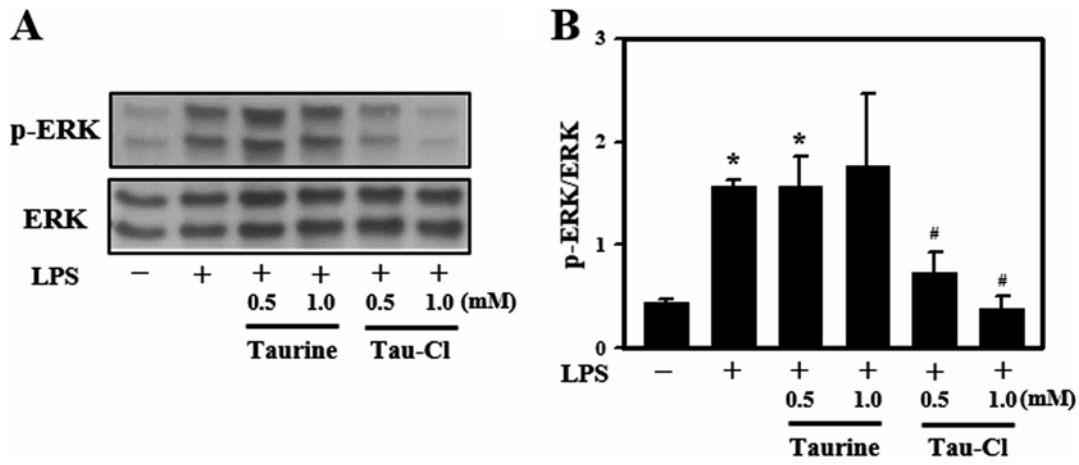

Fig. 5 Tau-Cl inhibited LPS-induced ERK phosphorylation in RAW 264.7 cells. (**a**). RAW 264.7 cells were treated with 0.2–1.0 mM Tau-Cl in the presence of 1 μg/mL LPS for 30 min. Phosphorylation of ERK was measured by Western blotting. (**b**). Blots were quantified by densitometry, as represented by arbitrary density units. Data are expressed as the mean ± SEM of three independent experiments. *$p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with LPS

mation and activate adjacent immune cells, thereby inducing additional immune cell infiltration and chronic inflammation. Neutrophils contain a large amount of primary granule MPO, an enzyme that catalyzes the formation of HOCl, which subsequently reacts with taurine to generate Tau-Cl. Therefore, the production of Tau-Cl by the MPO system results in the elimination of the powerful oxidant, HOCl. Moreover, Tau-Cl also has anti-inflammatory and antioxidant properties that protect itself and surrounding cells (Kim and Cha 2014).

Neutrophils and macrophages migrate to target sites by responding to chemoattractants through polarization and directional sensing. We previously reported that injection with a Tau-Cl sodium solution (Gottardi and Nagl 2002) reduced the number of total leukocytes and neutrophils whereas increased the number of monocytes in the peritoneal exudates of zymosan A-treated mice (Kim et al. 2015), thereby suggesting that Tau-Cl potentiated the efferocytosis of macrophages engulfing apoptotic neutrophils. However, the effect of Tau-Cl on leukocyte migration itself was not completely elucidated. In the present study, soluble thioglycollate was used, instead of zymosan A, to prevent zymosan-induced signaling. Zymosan binds dectin-1 or Toll-like receptor 2 and activates various signaling pathways (Brown et al. 2002; Sato et al. 2003). Phagocytosis of zymosan particles activates signaling pathways and induces actin polymerization, and phagocytosed neutrophils undergo apoptosis. Therefore, these processes (direct receptor binding, phagocytosis, and efferocytosis) activate leukocytes, thereby stimulating the secretion of inflammatory mediators, including TNF-α, IL-8, hydrogen peroxide, and arachidonic acid (Nobel et al. 1993; Sato et al. 2003). Thus, the use of soluble thioglycollate medium should considerably reduce particle-induced cell activation. In the present study, Tau-Cl reduced the number of peritoneal monocytes, as well as total leukocyte and neutrophil numbers, and also inhibited macrophage migration in a transwell, which suggests that Tau-Cl inhibits the migration of leukocytes from the blood.

Cell motility is regulated by multiple processes that are spatiotemporally controlled by a variety of intracellular signaling pathways, including mitogen-activated protein kinases (MAPKs) signaling. MAPKs influence focal adhesion dynamics, microtubule reorganization,

and actin polymerization, which together regulate cell spreading, lamellipodium extension, and tail retraction during cell migration. Indeed, p38 MAPK has been reported to activate neutrophil migration (Zu et al. 1998; Cara et al. 2001). However, contradictory results have been reported among researchers regarding the role of ERK on leukocyte migration. ERK has been reported to positively regulate leukocyte migration (Boehme et al. 1999; Chen et al. 2013). For example, inhibition of ERK signaling abolished neutrophil migration induced by *Candida albicans*, whereas inhibition of p38 MAPK and JNK failed to impair neutrophil migration (Wozniok et al. 2008). However, Liu et al. (2012) reported that ERK inhibited neutrophil migration, whereas p38 MAPK facilitated it. The present study demonstrates that Tau-Cl inhibits LPS-stimulated ERK activation, actin polymerization, adhesion, and migration in RAW 264.7 macrophages.

5 Conclusion

The present study demonstrates that Tau-Cl inhibits actin polymerization and leukocyte migration by inhibiting ERK activation. In this study, Tau-Cl reduced both the thioglycollate-induced migration of leukocytes in vivo and the chemoattractant-induced migration of leukocytes in a transwell system. Tau-Cl also inhibited actin polymerization, adhesion, and ERK phosphorylation. These results suggest that neutrophils that infiltrate inflamed tissues produce Tau-Cl and that Tau-Cl alleviates chronic inflammation by regulating the excessive and persistent infiltration of inflammatory cells.

Acknowledgments This work was supported by INHA University Research Grant.

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Taurine Alleviates LPS-Induced Acute Lung Injury by Suppressing TLR-4/NF-κB Pathway

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Keywords

Taurine · ALI · LPS · Prevention

Abbreviations

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
LPS	lipopolysaccharide

1 Introduction

ALI often leads to hypoxic respiratory failure and clinical acute respiratory distress syndrome (ARDS), which leads to serious harm to the body (Danielson et al. 2010). Studies have shown that ALI can be caused by a variety of factors, such as sepsis, pneumonia, trauma, acute pancreatitis, gastric aspiration, drowning, and other diseases and complex stress conditions (Mokra and Kosutova 2015). Since the pathogenesis of ALI is not clear, it is only classified by pathological features, which are the formation of pulmonary edema and pulmonary inflammation. During the initial phases of pathogenesis, the lung is strongly stimulated, which induces the lung tissue to secrete a large number of chemokines, attract inflammatory cells to migrate and gather within lung tissue, together with activating the inflammatory pathway and promoting the release of a large number of inflammatory factors, resulting in inflammation. Concomitantly, the Qi blood barrier is destroyed, the pulmonary vascular endothelial cells are damaged, the pulmonary vascular permeability is enhanced, the coagulation activation function of the body is destroyed, and thrombus is formed in the lung, resulting in the occurrence of pulmonary edema (Lou et al. 2018; Shah et al. 2014).

Taurine is a conditionally restricted amino acid in free form in the body. It has a variety of physiological functions such as membrane stability, regulation of calcium homeostasis, anti-apoptosis, anti-arrhythmia, and other roles. It has been proven that taurine can effectively reduce oxidative stress-induced injury, alleviate acute/chronic inflammation, and maintain cell homeostasis in a variety of disease models (Jakaria et al. 2019; Szymanski and Winiarska 2008). Studies have shown that the level of taurine in cells is 10−50 times higher than that outside cells.

Dongdong Zhao, Xiaozhou Zhang and Ying Feng contributed equally with all other contributors.

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Following lipopolysaccharide (LPS) injection, tissue cells are damaged, leading to a large amount of taurine being released from cells into the blood, resulting in the increase of taurine blood levels. Consequently, taurine is considered as a potential marker of acute lung injury (Hofford et al. 1996; Chesney 1985; Huxtable 1992). In this study, LPS-directed ALI model was used for monitoring possible taurine influence on inflammatory cell infiltration, inflammatory factors, and Toll-like receptor 4 (TLR-4) and NF-κ B (TLR-4/NF-κ B) signaling pathway in lung tissue. The main objective of this study was to elucidate the mechanistic roles of taurine directed at ALI, together with providing a theoretical basis for the application of taurine as a nutritional and immune additive to regulate inflammatory reactions and achieve the purpose of disease prevention and resistance.

2 Methods

2.1 Reagent

LPS was purchased from Sigma, Britain. Anti-CD68 and MCP-1 antibodies were purchased from Abcam, Britain. MyD88, NF-κ B p65, and NF-κ B p-p65 antibodies were purchased from Cell Signaling Technology, USA.

2.2 Fauna

Sixty SPF grade male SD rats (age, 6−7; weight, 140−160 g) were purchased from China Liaoning Changsheng Biotechnology Co., Ltd.

2.3 ALI Modeling and Experiment Grouping/Protocols

The bodyweight of rats was measured and LPS (25 mg/kg) was injected into the trachea of rats to establish ALI model induced by LPS. SD rats were segregated at random into six arms, with 10 rats/group. The blank control-group (C Group), taurine control-group (T group), ALI model-group (LPS group), and taurine prevention-group (LPST1 LPST LPST3 group). Among them, C group was fed diet and drinking water; T group was fed diet and drinking 2% taurine; LPS group was fed with diet and drinking water, while LPST1 group was fed with diet and drinking 1% taurine; LPST group was fed with diet and drinking 2% taurine; and LPST3 group was fed with diet and drinking 3% taurine. On day 14 and 28, LPS group and LPST1, LPST, and LPST3 group were subjected to injection of LPS (25 mg/kg) from *E. coli* to establish ALI model, and C-/T--groups were injected with an equivalent dose of standard saline. Blood, tissue, and BALF were collected 6 hours post-intraperitoneal injection of LPS. All animal experiments were approved by the Animal Ethics Committee of Shenyang Agricultural University, and the study was carried out in compliance with the ARRIVE guidelines.

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2.4 HE

Multiple 5 μm sections were dissected/fixed on glass slides. The histopathological analysis and images were taken by a fluorescence biomicroscope [Leica, Cologne, Germany].

2.5 Inflammatory Cytokines/Taurine Serum/Lung Homogenate Level Analysis

Rats were anesthetized, followed by treatment with intraperitoneal injection of equivalent volumes of normal saline or LPS for 3 h. The levels of TNF-α, IL-1 β, IL-4, IL-6, IL-10, and IL-18 in serum and lung homogenate were detected by ELISA.

2.6 Measurement of Lung W/D Ratio

Lung tissue wet weight (W) was measured. The lung was dried in a 70 °C oven overnight and

weighed twice until the weight difference was less than 1%. The dry weight (D) of the lung was measured, followed by lung W/D ratio calculation.

2.7 Bronchoalveolar Lavage and Anticoagulant Test

Firstly, the trachea from exposed rats was dissected, and 5 mL sterile PBS was infused into the lung tissue through the bronchial tube. The needle plug was withdrawn to recover the perfusion fluid, with this technique repeated three times. The average recovery of perfusate was over 75%. BALF was centrifuged at 4 °C and 15000 rpm for 15 min. The supernatant was placed at 80 °C for cytokine and protein content analysis. Cell particles were resuspended in PBS to count the number of various inflammatory cells and calculate the proportion of various inflammatory cells. 1 mL of peripheral blood was collected and treated with EDTA as anticoagulant. The number of inflammatory cells in peripheral blood was detected by an EDX automatic hematology analyzer [Beijing, China].

2.8 Immunohistochemical Staining of TLR-4/NF-κ B Pathway-Related Proteins Within Lung Tissue

The middle lobe of the left lung tissue was perfused with 4% formaldehyde through the bronchus, followed by placing at room temperature for 48 h. The tissue was paraffin-embedded and dissected into 5 μm-wide segments. The expression levels of TLR-4, NF-κ B p65, MCP-1, and CD68 in the middle lobe of left lung tissue were detected by immunohistochemistry. Under a light microscope, the brownish-yellow particles were positive expression products, with each segment randomly observed at five different fields.

2.9 Expression of Key Proteins of TLR-4/NF-κ B Pathway in Lung Tissue

Expression levels for TLR4, MyD88, NF-κ B p65, NF-κ B p-p65, MCP-1, and CD68 within lung tissue were detected through Western blotting. The lower-lung tissue of the left lung was collected, and the total protein of lung tissue was extracted by BCA protein concentration assay kit of China Beyotime Biotechnology. The total protein was detected by 12 alkyl sulfate polyacrylamide gel electrophoresis. Consequently, the protein separated by electrophoresis was placed onto a PVDF membrane, sealed in 5% skim milk within room-temperature blocking buffer for 1 h and ultimately incubated overnight at 4 °C with TLR4-, MyD88-, NF-κ B p65-, NF-κ B p-p65-, MCP-1-, and CD68-specific antibodies (diluted 1:1000−3000). Consequently, the membrane was cleaned; the secondary antibodies (with dilution of 1:3000) were added and incubated for 60 min at room temperature. Gray values of TLR4, MyD88, NF-κ B p65, NF-κ B p-p65, MCP-1, and CD68 were analyzed by target optical analysis with the Jena UVP Chem studio 515 software system in Germany. The ratio of gray values of TLR4, MyD88, NF-κ B p65, MCP-1, and CD68 were compared with that of β-actin.

2.10 Statistical Analysis

All data were analyzed by SPSS v.23.0 [IBM, Amon, New York, USA], n = 6 in each group. The results were expressed as mean ± standard deviation (SD). Variations between the experimental groups were analyzed by one-way ANOVA and then compared by t-test. $P < 0.05$ was considered to be statistically significant.

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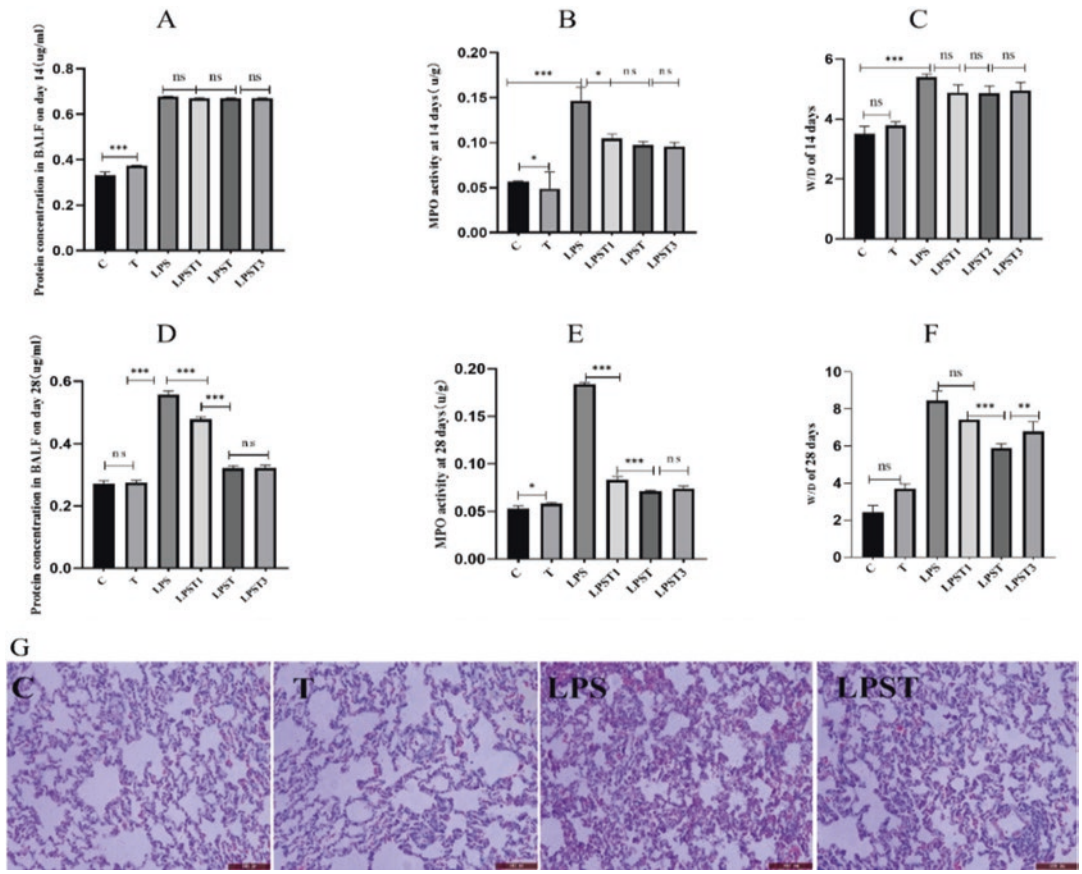

Fig. 1 After 14 days of taurine feeding, LPS (25 mg/kg) was injected into the trachea of rats. Six hours later, the protein concentration in BALF was detected (**a**). Lung tissue was collected to detect MPO activity (**b**). The W/D was also analyzed (**c**). After 28 days of taurine feeding, LPS (25 mg/kg) was injected into the trachea of rats. Six hours later, the protein concentration in BALF was detected (**d**). Lung tissue was collected to detect MPO activity (**e**). The W/D was also detected (**f**). The morphological changes of lung tissue were also studied (**g**) (400 ×)

3 Results

3.1 Taurine Can Reduce Acute Lung Injury in Rats

On day 14, the total protein concentration, MPO activity, and W/D ratio in group LPS were significantly increased, but taurine had no significant effect (Fig. 1a, b, c). After 28 days of taurine prevention, taurine in LPST group could significantly reduce the total protein concentration, MPO activity, and W/D ratio of BALF (Fig. 1d, e, f). The HE staining results for lung tissues demonstrated that lung injury induced by LPS was characterized by smaller alveoli, congestion of alveoli, and infiltration of inflammatory cells. Taurine pretreatment significantly alleviated the pathological changes of lung tissue. Taurine pretreatment had a moderate prophylactic effect on LPS-induced ALI in rats (Fig. 1g). These results suggest that LPS (25 mg/kg) can successfully establish an ALI model and 2% taurine pretreatment can prevent LPS-induced ALI.

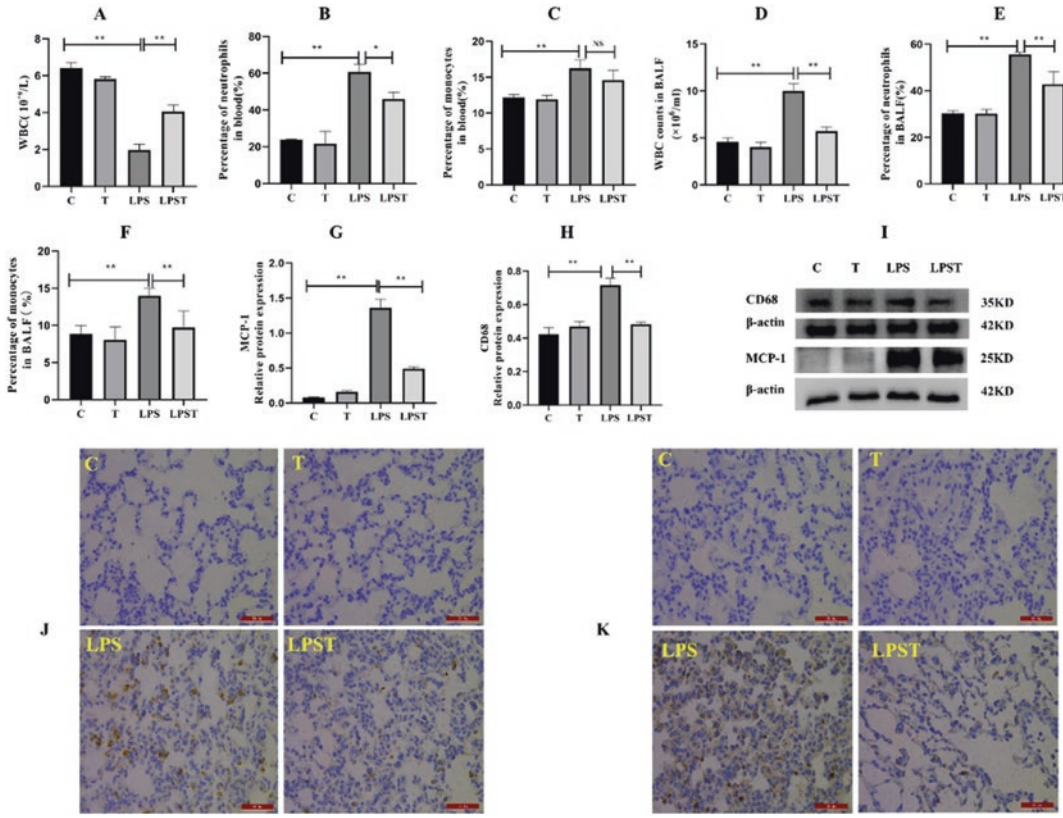

Fig. 2 After 28 days of taurine feeding, LPS (25 mg/kg) was injected into the trachea of rats. Six hours later followed by peripheral blood collection from all rats in order to determine relevant/total white blood cell population densities (**a–c**). BALF samples were collected from all rats in order to determine relevant/total white blood cell population densities (**d–f**). Expression levels of MCP-1 and CD68 protein were analyzed in lung tissues (**g–i**). Protein expression levels of CD68 (400×) (**j**) and MCP-1 (400×) (**k**) were analyzed in lung tissue samples

3.2 Taurine Can Inhibit the Infiltration of Inflammatory Cells Within ALI Lung Tissue of Rats

The total number of WBC in LPS group was markedly reduced compared to group C, ($P < 0.01$), while the total number of WBC in LPST group was found to be markedly elevated compared to the LPS group ($P < 0.01$) (Fig. 2a). Neutrophil population within the LPS group was significantly more elevated than for the C group ($P < 0.01$), with the neutrophil population within the LPST group being significantly reduced, compared to the LPST group ($P < 0.01$) (Fig. 2b). Ratios of monocyte/macrophage were highly differing (though not statistically significant) in all groups (LPS > C; LPST < LPS) (Fig. 2c). Leukocyte populations within BALF demonstrated statistically significant variations among all study groups (LPS > C; LPST < LPS) ($P < 0.01$) (Fig. 2d). Similarly, neutrophil populations within BALF demonstrated statistically significant variations among all study groups (LPS > C; LPST < LPS) ($P < 0.01$) (Fig. 2e). The BALF monocyte/macrophage ratios also demonstrated statistically significant variations among all study groups (LPS > C; LPST < LPS) ($P < 0.01$) (Fig. 2f). Similarly, protein expression levels (CD68 and MCP-1) differed among all groups (LPS > C; LPST < LPS; C = T) ($P < 0.01$) (Fig. 2g, h, i, g, k). These results indicate that taurine can inhibit the accumulation of inflammatory cells in lung tissue by regulating the ratio of

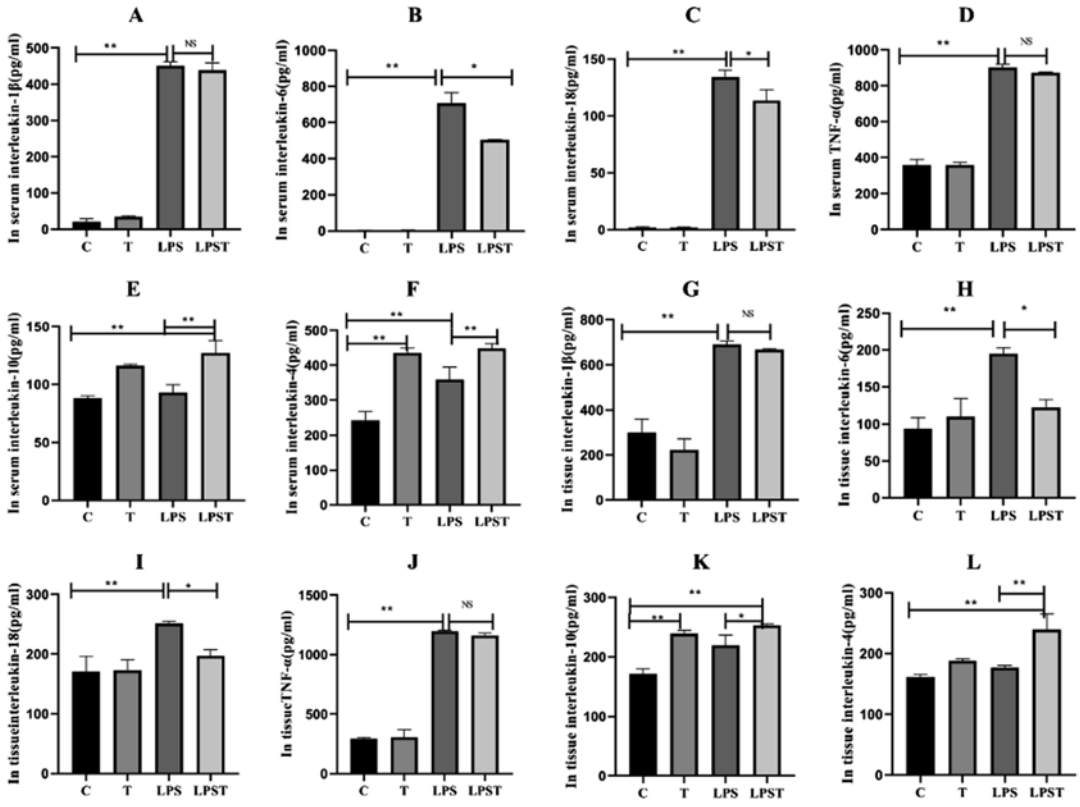

Fig. 3 After 28 days of taurine feeding, LPS (25 mg/kg) was injected into the trachea of rats. Six hours later, the levels of IL-1 β, IL-6, IL-18, TNF-α, IL-10, and IL-4 were analyzed in the serum (**a–f**) and lung tissue (**g–l**)

leukocytes in peripheral blood and lung tissue, in order to inhibit the occurrence of acute lung injury and protect lung tissue.

3.3 Taurine Reduces Pro-inflammation Factors and Increases Inflammation-Inhibiting Factors Within Serum and Lung Tissue of ALI Rats

ELISA results demonstrated that the serum levels of TNF-α, IL-1 β, IL-6, and IL-18 varied markedly across all study groups (LPS > C; LPST < LPS) ($P < 0.01$) (Fig. 3a, b, c, d). lLung tissue levels for such cytokines also demonstrated differences (LPST < LPS) ($P < 0.01$) (Fig. 3g, h, i, j). Serum levels (Fig. 3e, f) and lung tissue levels (Fig. 3k, l) of IL-10 and IL-14 demonstrated differences between the LPST and LPS groups (LPST > LPS) ($P < 0.01$). Taurine can alleviate the occurrence of pneumonia by inhibiting pro-inflammatory cytokines and activating anti-inflammatory cytokine factors.

3.4 Taurine Inhibits ALI in Rats by Inhibiting the TLR4-NF κ B Signaling Pathway

Western blotting analyses demonstrated variations among most groups regarding the protein expression levels of TLR4, MyD88, NF-κ B p65, and NF-κ B p-p65 (LPS > C; LPST < LPS; C = T) ($P<0.01$) (Fig. 4a, b, c, d). Interestingly, TLR4 and NF-κ B p65 exhibited unique expression profiles (LPS > C; LPST < LPS; C =T) ($P < 0.01$) (Fig. 4e, f).

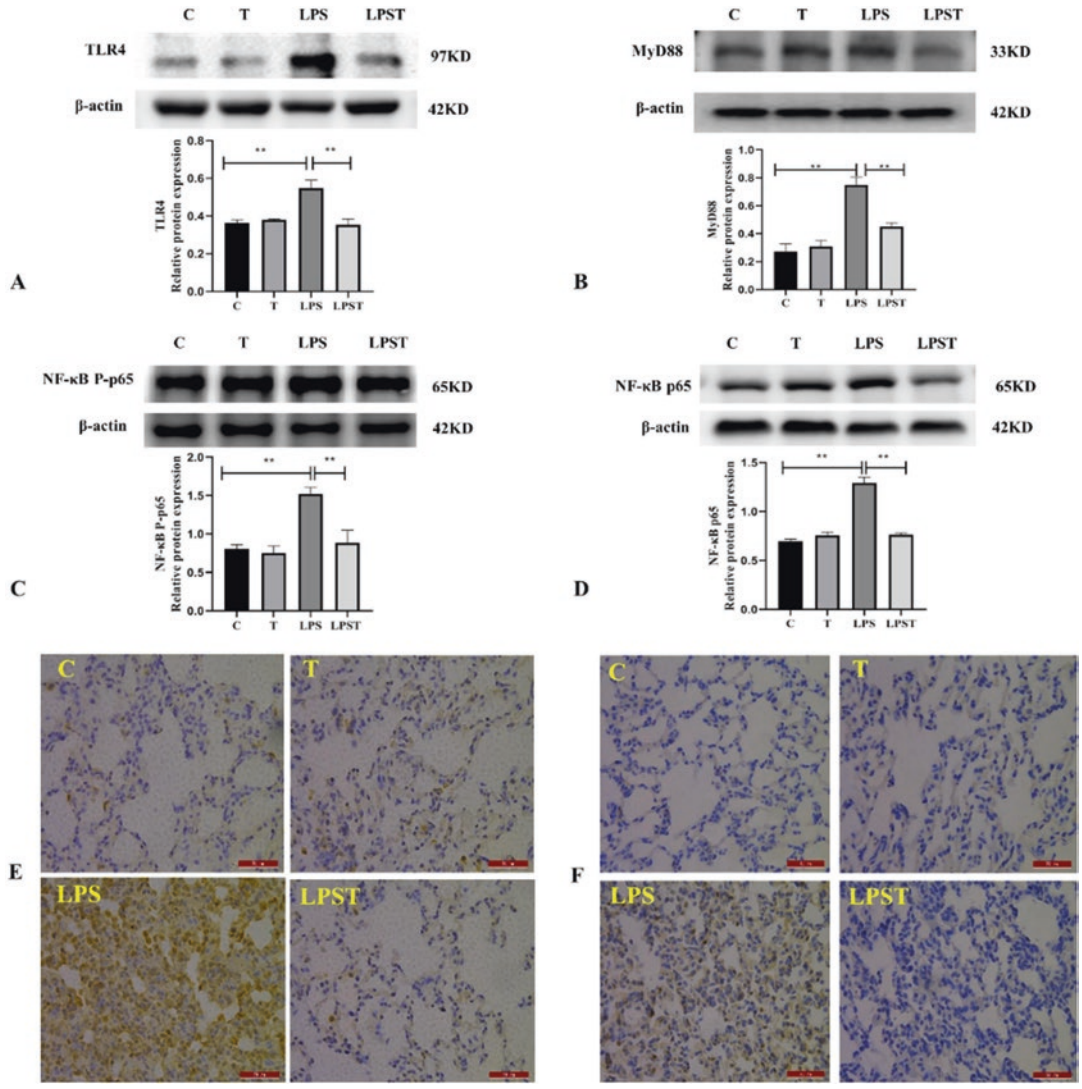

Fig. 4 After 28 days of taurine feeding, LPS (25 mg/kg) was injected into the trachea of rats. Six hours later, rat lung tissues were sampled, followed by Western blotting procedures for determining protein expression levels of TLR4 (**a**), MyD88 (**b**), NF - κ B p-p65 (**c**), NF- κ B p65 (**d**). TLR4 protein (**e**) and NF-κ B p65 protein (**f**) lung tissue expression levels were determined through immunohistochemistry (400 ×)

4 Discussion

The lung is an important part of the respiratory system. Due to the close relationship between the lung and the external environment, it is the main place for the invasion of pathogenic microorganisms from the environment. Many factors can lead to lung inflammation, resulting in lung injury.

The main manifestations of ALI were dysfunction of the epithelial barrier and decrease in alveolar fluid clearance, which eventually led to pulmonary edema. The results showed that within 3 h post-intraperitoneal injection of LPS, the wet to dry ratio of lung tissue and protein levels in BALF were significantly increased. LPS can lead to diffuse inflammatory cell infiltration, interstitial widening, alveolar cavity narrowing, and neutrophil infiltration within lung tissue, which

can lead to inflammatory cascade of pro-inflammation cytokines (Niu et al. 2019). This demonstrates that LPS can effectively simulate the natural occurrence and development process of ALI which provides a good-model to study the protective effect of taurine on ALI. Peripheral blood leukocyte count (WBC) is an important marker of acute infection, which can play an early warning role. One hour post-LPS injection, the number of white blood cells decreased significantly (Li et al. 2016a, b). The results demonstrated that 3 h after LPS injection, the total number of leukocytes within peripheral blood of the LPS group decreased significantly (in comparison with other study groups), while in BALF, the population level was significantly higher than that of other groups, with the proportion of various types of inflammatory cells increased in varying degrees.

Studies have confirmed that when ALI occurs, excessive chemokines are produced in the lung to induce the directional migration of related inflammatory cells, and a large number of inflammatory mediators are released to trigger inflammatory reaction. When inflammation occurs, white blood cells in the blood migrate to the tissue where the inflammation is located (De Boever et al. 2009). Results in the early stage of inflammation, the number of white blood cells in the blood decreased, while the number of white blood cells within localized inflamed tissues increased (Li et al. 2016a, b). The excessive activation and release of MPO cause neutrophils to migrate to the lesions, and neutrophils migrate to the tissues through paracellular or transcellular pathways and enter the interstitial and bronchoalveolar spaces, causing lung inflammation (Aratani 2018; Hsieh et al. 2018). Neutrophils are not only messengers but also effectors. They participate in the pathogenesis of ALI and are key to induce ALI (Wu et al. 2020; Blazquez-Prieto et al. 2018). Studies have confirmed that macrophage over-activation also plays a pivotal part in the occurrence and pathology of lung injury (Lu et al. 2018).

There are a certain number of resident macrophages in healthy lung tissue, which have high plasticity to immune response. MCP-1 is a key chemokine involved in regulating migratory/infiltration properties for both macrophages and monocytes. Under its action, monocytes in blood migrate and gather within the inflammatory and injury sites, thus controlling the number of macrophages in the inflammatory and injury sites (Deshmane et al. 2009; Ajuebor et al. 1998; Ribeiro et al. 2015). When MCP-1 is overproduced due to external stimulation, a large number of monocytes in peripheral blood are transferred to the alveolar cavity and differentiate into macrophages with M1 phenotype in the alveolar cavity, which can activate the immune response and trigger inflammatory reaction. The expression of CD68 on the surface of macrophages is a characteristic marker of macrophage activation, which is used to evaluate the degree of macrophage activation in lung tissue (Fei et al. 2019).

Acute inflammation is the most important innate immune mechanism in animals. Inflammatory response includes activation of many kinds of cells and directional migration from blood to the invasion site. When the body is stimulated by infection, the immune mechanism is activated rapidly, producing a variety of inflammatory mediators, which is conducive to inflammatory response and wound repair, consequently involving a spectrum of pro-inflammation cytokine players, such as TNF-α, IL-1 β, IL-6, and IL-18 (Shie et al. 2015; Jia et al. 2019; Grommes and Soehnlein 2011). However, when the body's defense function is too strong, it will cause excessive production of inflammatory mediators, leading to cascading and widespread damage of tissues and cells. ALI is related to the excessive release of a variety of pro-inflammatory cytokines, while the anti-inflammatory cytokines IL-4 and IL-10 have the ability to mitigate effects of a variety of inflammatory cells, inhibit inflammatory responses, and reduce the release of pro-inflammatory cytokines IL-1 β and IL-6, ultimately reducing LPS-induced acute lung injury (Chen et al. 2018). Therefore, when the body has a serious inflammatory reaction, balancing the levels of pro-inflammatory factors and anti-inflammatory factors can prevent inflammatory damage and protect the body.

Taurine can reduce the expression of CD68, MCP-1, and MPO activity in lung tissue; it can reduce the expression of chemokines in the lesion site, combined with the number and proportion of inflammatory cells in bronchoalveolar lavage fluid and peripheral blood. This indicates that neutrophils and monocytes are affected by their chemokines and migrate from blood to lung when ALI occurs. The increased permeability of visceral capillaries leads to alveolar infiltration, which increases the number of neutrophils and macrophages in BALF and leads to the release of pro-inflammatory factors and the production of anti-inflammatory factors. However, the number of neutrophils and macrophages in BALF decreases after the preventive addition of taurine, which inhibits the excessive release of pro-inflammatory factors while also promoting anti-inflammatory factor production. This suggests that taurine may reduce lung injury and inhibit the development of inflammation by inhibiting the infiltration of inflammatory cells and regulating the production of inflammatory factors.

TLR-4/NF-κ B pathway plays an essential part within LPS-induced ALI. As a natural ligand of TLR4, LPS can activate TLR-4/NF-κ B pathway and induce the release of pro-inflammatory factors (Li et al. 2014; Ju et al. 2018). LPS binds with the TLR4 exon to form a complex, realizing extracellular "pattern recognition," completing transmembrane transport, and entering the cell membrane (Jerala 2007). After receiving the cascade signal, MyD88 is recruited to IL-1 receptor after TLRs. At this time, IRAK is phosphorylated, and IRAK-1 is activated. TNF receptor-related factor 6 (TRAF6), which triggers the signaling cascade, is recruited and bound, resulting in the activation of two different signaling pathways. JNK and NF-κ B are activated eventually (Lomaga et al. 1999; Hamesch et al. 2015; Takeda and Akira 2004). NF-κ B is an important cascade signal of ALI (Jain and Darveau 2000; Kraus et al. 2012). The inflammatory reaction can lead to the activation of NF-κ B, in which a large number of inflammatory cytokines are produced, such as TNF-α, IL-1 β, IFN-γ, and chemokines (Cavaillon 2018).

5 Conclusion

Our results suggest that ALI, induced by LPS, can increase the expression of TLR4, MyD88, NF-κ B p65, and NF-κ B p-p65 in lung tissue, which is consistent with previous studies. Concomitantly, the expression of corresponding proteins in the LPST group was significantly lower than that in LPS group, indicating that taurine can inhibit the occurrence of inflammatory factor storm by inhibiting the activation of TLR-4/NF-κ B pathway and regulating the release of inflammatory factors.

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Intervention Effect of Taurine on LPS-Induced Intestinal Mechanical Barrier Injury in Piglets

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Keywords

Taurine · LPS · Piglet · Intestinal mucosal structure · Mechanical barrier

Abbreviations

LPS	lipopolysaccharide
DAO	diamine oxidase
ET	endotoxic serum
DLA	D-lactic acid

1 Introduction

The intestinal tract is a location in which most toxins accumulate. When physiological function and growth state are normal, the structure of the tight junctions of the intestinal epithelial cells is relatively stable, and the integrity of the animal's intestinal barrier remains intact. Under those circumstances, it is challenging for toxins to penetrate the intestinal mucosal barrier and enter the bloodstream. However, when the intestinal barrier is damaged, toxins pass through the intestinal wall and circulate throughout the bloodstream to other locations, thereby aggravating the animal's inflammatory response and causing toxemia. LPS is an endotoxin that is expressed on the cell wall surface of Gram-negative bacteria and causes an inflammatory response (Jeon et al. 2009). LPS stimulation can induce effector cells to produce a large number of bioactive molecules and accelerate the release of multiple factors, such as pro-inflammatory and anti-inflammatory cytokines. This acute and sudden stress leads to activation of the body's immune system, accelerates the phosphorylation of myosin light chains, breaks acidified myosin light chain, reduces the tightness of intestinal tight junction structures, and ruptures the intestinal mucosal barrier. Typical symptoms in animals exposed to include fever, lethargy, and anorexia, which lead to growth hindrance and reduced body development (Potoka et al. 2002; Vaquero et al. 2001). Therefore, during actual production, the animal rearing industry employs LPS to establish an acute stress model to assess damage inflicted upon the intestinal barrier (Sukhotnik et al. 2004); common markers of the damage in the serum are DAO (diamine oxidase, DAO), ET (endotoxic serum, ET), DLA (D-lactic acid, DLA), and endotoxin. Tight junctions are the connecting structures between adjacent epithelial

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cells located at the top of the cell junction, which close the intercellular space and prevent toxic and harmful substances within the intestinal cavity from entering the submucosa through the epithelial intercellular space. Concomitantly, it serves the additional function of selectively transporting nutrients. Tight junction structures are composed of a variety of tight junction proteins, including ZOs, occludin, and claudins. The immune and digestive systems of piglets weighing < 30 Kg are easily affected by various pathogenic factors. The intestinal barrier structure of piglets ends up being damaged, allowing normal bacteria living in a healthy state to become pathogenic. The tight junctions that constitute intestinal epithelial cells are the most important structural links ensuring regular digestion and absorption of food, as well as resistance to invasion of toxins into the intestinal mucosa.

Taurine, 2-aminoethanesulfonic acid, which was first discovered in bovine bile, is the most abundant sulfur-containing amino acid in mammals. It largely exists in mammals in a free state. Its physiological concentration is approximately 10 mmol/L, which is necessary for normal physiological activity in mammals. Taurine exerts a variety of physiological actions, such as maintaining homeostasis of internal environments, regulating calcium stability, anti-inflammation, anti-oxidation, enhancing immunity, regulating lipid metabolism, and other functions. Taurine is classified as an amino acid, but unlike other neuroactive amino acids, its acidic moiety is a sulfonic acid rather than a carboxylic acid (Vanitha et al. 2018). Taurine has been widely used in animal production research due to multiple advantages, such as safety, efficiency, stable physical and chemical properties, and diversified functions. In this study, taurine was added to the diet of piglets, which were subsequently treated with LPS in order to establish the acute stress model. The expression of tight junction protein within intestinal mucosal cells and the shifts in intestinal permeability were examined. The effect of taurine on intestinal mechanical barriers was also assessed, in order to lay a foundation for its use in livestock and poultry production.

2 Methods

2.1 Animal Model

Twenty-four piglets with similar body weight and good health and mean age of 28 (± 3) days were randomly divided into 4 groups: control group (C), taurine group (T), LPS group (M), and taurine + LPS group (MT). Each group had six biological replicates, the pre-feeding period was 7 days, and the formal trial period was 28 days. Group C and M were fed a basal diet, while groups T and MT were fed the basal diet supplemented with 0.3% taurine. On the last day of the study, piglets in group M and group MT were intraperitoneally injected with 100 μg/kg body weight of LPS, while groups C and T were injected with the same dose of sterilized 0.9% saline. One hour postinjection, a D-xylose solution (0.1 g/kg body weight) was reapplied into the piglet stomachs. Blood was collected from the anterior vena cava 3 h later, in order to detect intestinal mucosa permeability. After blood collection, the individual piglet was sacrificed, and the duodenum, jejunum, and ileum were collected. The accumulated chyme within the intestinal tissue was washed with 0.9% normal saline and trimmed on filter paper, with the remaining tissue fixed in 4% paraformaldehyde solution to prepare paraffin sections. The morphology of the intestinal villi and the ratio of (villus length, VL) VL to (crypt depth, CD) CD were observed, and all remaining tissue samples were packed in frozen tubes and stored at -80 °C. Such procedures were used for analyzing gene expression of proteins involved in tight junction structural development within varying segments of the intestinal tract.

3 Results

3.1 Effect of Dietary Taurine on Intestinal Morphology of LPS-Stimulated Piglets

As shown in Fig. 1, the morphology of small intestinal villi in the M and MT groups was damaged to varying degrees, and the apical struc-

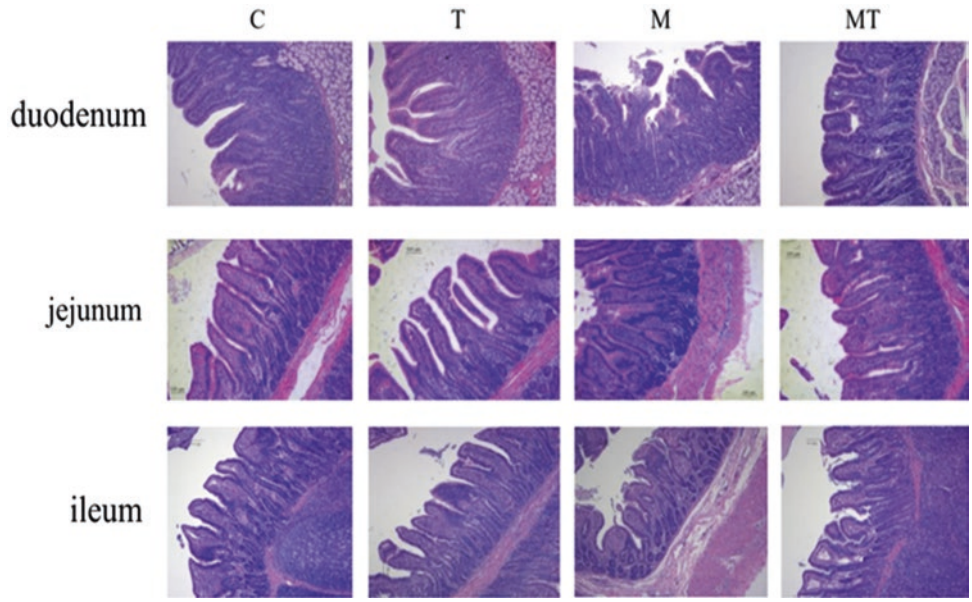

Fig. 1 Duodenum, jejunum, and ileum were collected for HE staining. The change of organizational structure was observed

ture of the intestinal villi was incomplete, especially within the duodenum. The intestinal wall of group C was clear, the structure of the mucosal epithelium was intact, and intestinal villi were neatly arranged and consistent. The villi of group T were longer and neatly arranged, though group T villi width was significantly lower than for that of group M, and the latter group's villi were found to be wider and deeper than for those of CD. Intestinal villi structure was damaged in group MT though was significantly improved in comparison to those of group M. Regarding ileal tissue, the CD of group T demonstrated obvious shallowness, while the VL of group M was reduced though villi width were increased and contained more profound CD. Compared with those of group M, the length and width of intestinal villi, together with CD outline in group MT, were clearer.

As shown in Fig. 2, compared with group T, the VL of duodenum in group M decreased significantly, the CD increased significantly, and the VL/CD ratio decreased significantly. The VL of duodenum in MT group was significantly higher than that in M group. There was no significant difference in the other indexes in duodenum among the other groups; there was no significant difference of CD in jejunum between group T and group C. There was a significant difference of VL in the jejunum between group M and group C. There were also significant differences in the other indexes in jejunum among the other groups. However, there was no significant difference in VL of the ileum between group M and group C. There was no significant difference of CD in ileum between group T and group C, between group M and group C, and between group MT and group M. A significant difference in VL/CD of the ileum was observed between group M and group C. There were significant differences in the other indexes in ileum among the other groups.

3.2 Effect of Taurine on Intestinal Permeability of LPS-Stimulated Piglets

As illustrated in Fig. 3, there was no significant difference in the contents of diamine oxidase (DAO), endotoxin (ET), and D-lactic acid (D-LA)

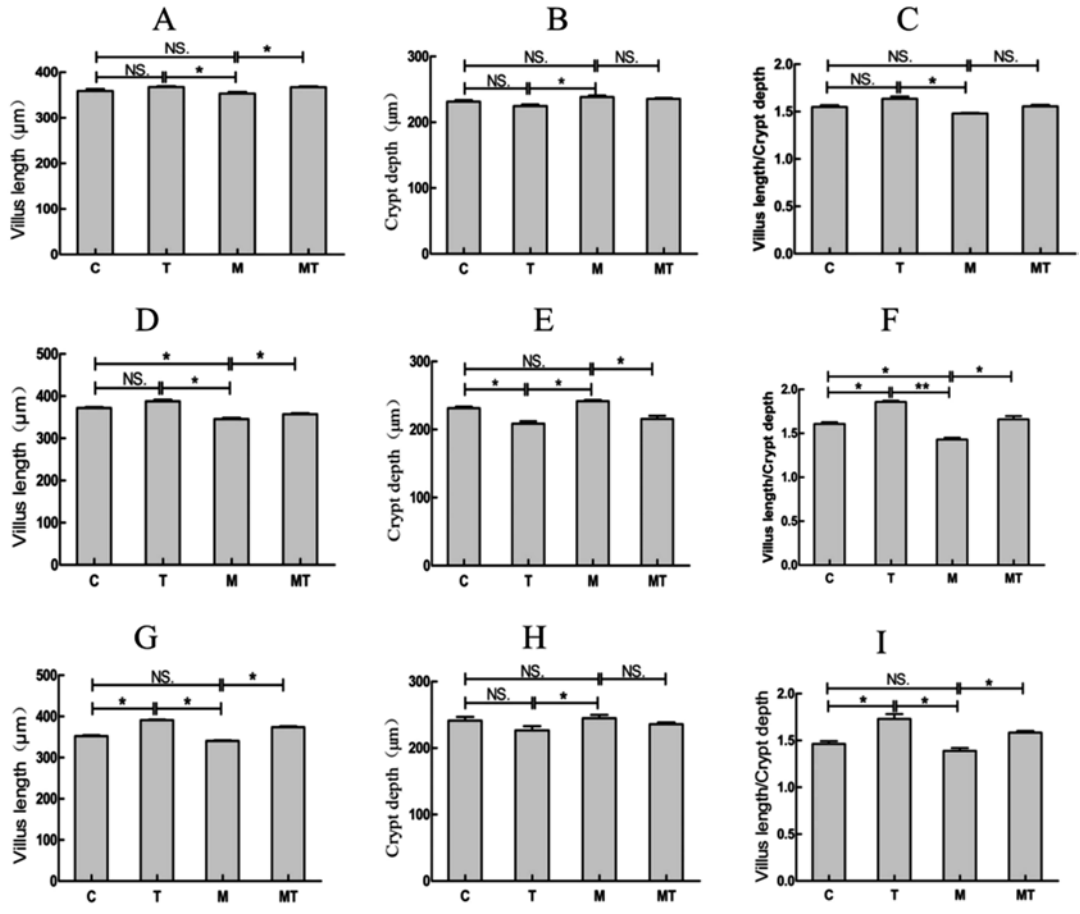

Fig. 2 Villus length (**a**, **d**, **g**), crypt depth (**b**, **e**, **h**), and villus concealment ratio (**c**, **f**, **i**) of duodenum (**a**, **b**, **c**), jejunum (**d**, **e**, **f**), and ileum (**g**, **h**, **i**). In the figure, an asterisk (*) reveals a significant difference between groups ($P < 0.05$), and two asterisks (**) reveal an extremely significant difference between groups ($P < 0.01$). There was no significant difference between the two groups ($P > 0.05$) when the comparison is indicated by NS

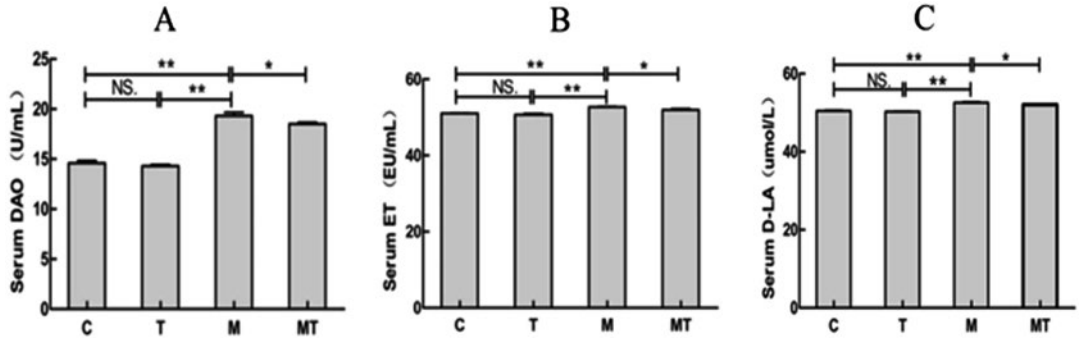

Fig. 3 Contents of diamine oxidase (**a**), endotoxin (**b**), and D-lactic acid (**c**) in serum. In the figure, an asterisk (*) reveals a significant difference between groups ($P < 0.05$), and two asterisks (**) reveal an extremely significant difference between groups ($P < 0.01$). There was no significant difference between the two groups ($P > 0.05$) when the comparison is indicated by NS

between groups T and C, though the contents of DAO, ET, and D-LA in group M were significantly higher than those for group C, and the contents of DAO, ET, and D-LA in group M were significantly higher than for those in group T. In comparison to the MT group, the contents of DAO, ET, and D-LA in the serum of the MT group were lower than for those for the M group. The results demonstrated that taurine could inhibit the increase of serum DAO, ET, and D-LA, induced by LPS.

3.3 Effect of Taurine on Tight Junction Protein Expression in LPS-Stimulated Piglets

As shown in Fig. 4, there was no significant difference in the expression of ZO1 and claudin-1, occludin mRNA in the duodenum, jejunum, and ileum between group T and group C, though there was a significant downregulation of ZO-1 and claudin-1, occludin in the duodenum, jejunum, and ileum between group M and group C

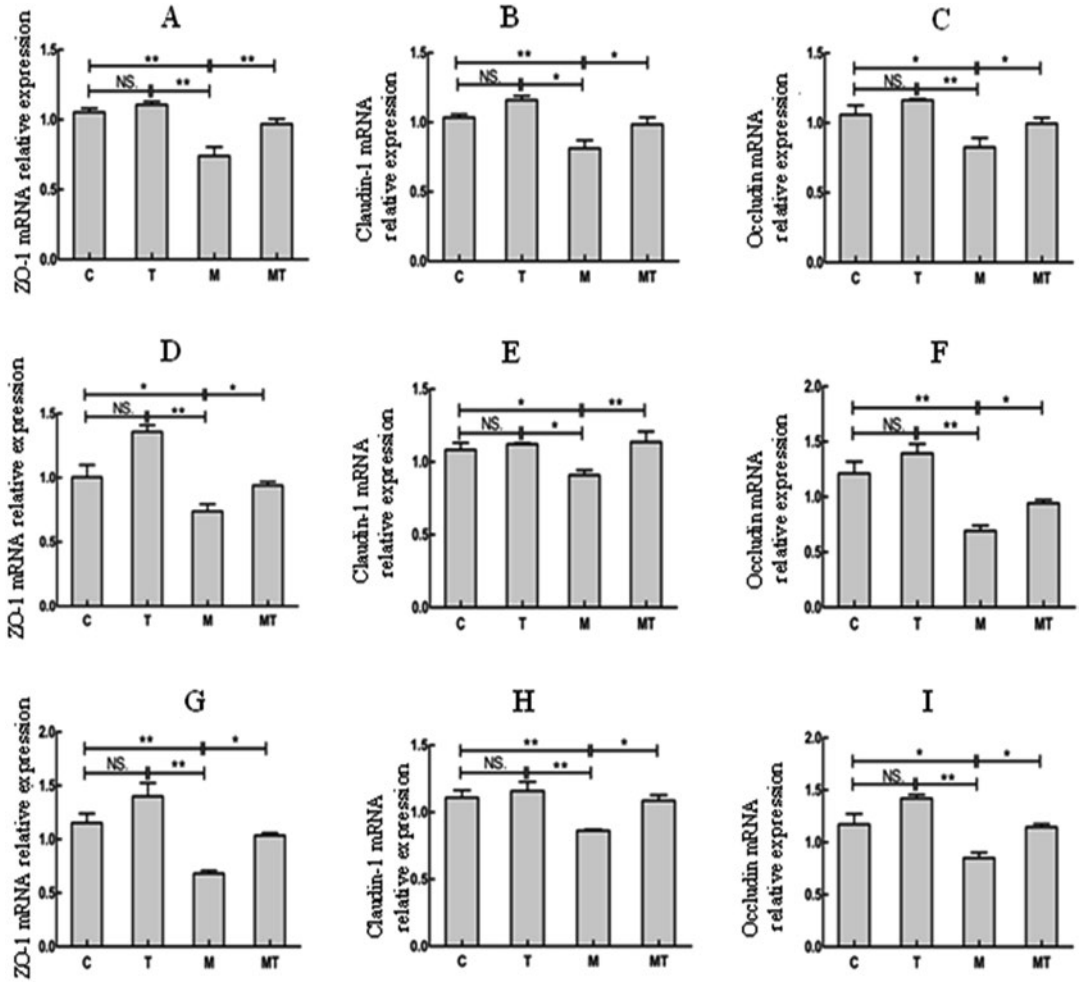

Fig. 4 Relative mRNA levels of ZO-1 (**a**, **d**, **g**), claudin-1 (**b**, **e**, **h**) and occludin (**c**, **f**, **i**) in the duodenum (**a**, **b**, **c**), jejunum (**d**, **e**, **f**), and ileum (**g**, **h**, **i**). In the figure, an asterisk (*) reveals a significant difference between groups ($P < 0.05$), and two asterisks (**) reveal an extremely significant difference between groups ($P < 0.01$). There was no significant difference between the two groups ($P > 0.05$) when the comparison is indicated by NS

due to the decrease in mRNA content. In comparison to the T group, the content of mRNA for ZO-1, claudin-1, occludin mRNA in the duodenum, jejunum, and ileum in the M group decreased significantly, while the expression of ZO-1 and claudin-1, occludin mRNA in the duodenum, jejunum, and ileum in MT group increased significantly, in comparison to the M group. The results show that taurine increases ZO-1, claudin-1, occludin mRNA content in LPS-stimulated duodenum, jejunum, and ileum.

4 Discussion

Presently, one particular method that is effective in causing intestinal mucosal barrier injury in piglets is intraperitoneal or intravenous administration of LPS, which is part of the cell membrane of Gram-negative bacteria. It can induce acute bacterial infection symptoms in piglets, such as anorexia, somnolence, and fever. In addition, LPS leads to intestinal villus edema, mucosal cell necrosis and exfoliation, destruction of tight junction protein structure, increased intestinal mucosal permeability, bacterial endotoxin translocation, and eventually a systemic inflammatory response and multiple organ dysfunctions. Typically, the intestinal villi demonstrate a banded structure (Thulesen et al. 2001) with varying widths and heights. The integrity of the morphology and structure of intestinal mucosa directly reflect the intestinal health of the animal. Crypt depth is a tubular gland formed by the epithelium of the small intestine, which extends to the lamina propria at the bottom of the villi, with the adjacent villi forming a gap (Taras et al. 2006) that comes into contact with food. The depth of the crypt reflects the proliferation rate of intestinal mucosal epithelial cells, and any decrease in crypt depth indicates that the ability of cell proliferation and differentiation of the crypt is accelerated, and the intestinal villus cells which fall off due to normal metabolism can be replaced in time. The cashmere hidden ratio can comprehensively reflect small intestine health status, with higher ratios reflecting a more efficient digestion and absorption capacity in piglets, which is consequently beneficial to the growth and development of the animal (Taras et al. 2006). Adding 0.3% taurine to the diet significantly increased the VL of ileum, decreased the CD of jejunum, and increased VL/CD of jejunum and ileum, which indicated that taurine could improve the structure of intestinal mucosa and enhance the digestion and absorption capacity of piglets. Compared with the normal control group, the VL of jejunum and VL/CD of jejunum decreased in LPS-spiked group, which indicated that LPS could damage the structure of intestinal mucosa. Compared with LPS-spiked group, the VL of jejunum and ileum in taurine prevention group was significantly increased, the CD of jejunum and ileum was significantly decreased, and VL/CD of jejunum and ileum was increased. That is to say, taurine alters VL, CD, and VL/CD of both the control and the LPS-spiked animal in some intestinal segments.

DAO, which is an enzyme involved in normal intestinal metabolism, is mainly distributed in human or mammalian intestinal mucosal villus cells (Obrosova and Stevens 1999). During disease/external stimulation, the intestinal mucosal barrier function is destroyed, and the Toll-like receptor and nod-like receptor pathways of intestinal mucosal epithelial cells are activated by harmful substances (Matsuda et al. 2004). Following stressful events, intestinal epithelial villus cells continue to secrete DAO, which consequently dissociate in the intercellular space, allowing a degree of infiltration into the bloodstream through capillary walls. Serum DAO levels act as a biomarker for injury/recovery of the intestinal mucosal barrier (Ruan et al. 2004). Post-LPS injection, DAO serum levels significantly increase, indicating that damage to the intestinal barrier results in increased permeability of intestinal mucosa, resulting in DAO entry into the bloodstream. This study found that taurine moderately inhibits excessive DAO secretion, presumably by preventing the increase in intestinal permeability and protecting the integrity of the intestinal barrier.

Bacterial ET, which is mainly distributed on the cell wall of Gram-negative bacteria, can stimulate innate immune responses (Walker et al.

2011). It has been established that the ET levels of animals depend on the physiological state of the animal itself. The intestinal barrier containing proper morphological structure can resist the invasion of pathogenic microorganisms and bacterial toxins. If the body is subjected to acute stress, the intestinal mucosa can be damaged, causing the morphological structure of the inner wall of the intestinal epithelial vessels to be altered. Bacteria, antigens, and other harmful substances enter the damaged capillaries and reach various body compartments via the bloodstream, leading to bacterial translocation. The results revealed that intraperitoneal injection of LPS significantly increased ET serum levels in piglets, while taurine protected the integrity of the intestinal barrier, inhibiting the increase in intestinal permeability and ET.

Lactic acid (D-LA) is produced by the metabolism of a variety of bacteria in the intestinal tract. Since this substance can be produced and metabolized only in the intestinal tract, it is typically employed as an important biomarker for intestinal mucosal permeability. When an animal is in a state of hypoxia, malnutrition, stress, or disease, bacteria within the intestinal cavity massively proliferate. Moreover, bacteria having elevated metabolic rates excrete a larger amount of D-LA, and this excess D-LA is absorbed into the bloodstream, along with other small molecular compounds in the intestine. In this study, LPS stimulation led to an increase in serum D-LA levels, but taurine treatment prevents the LPS-mediated increase in serum D-LA content, presumably by partially blocking the increase in intestinal permeability and improving intestinal barrier integrity. The intestinal mechanical barrier is the most basic barrier among all intestinal epithelial mucosal barriers. The tight junctions between intestinal epithelial cells are evenly arranged, forming a natural and closed intestinal mechanical barrier (Blair et al. 1991). The types of intestinal epithelial cells mainly include endocrine cells, absorptive cells, undifferentiated stem cells, goblet cells, and phagocytes. These cells are involved in food digestion and absorption, together with maintenance of intestinal barrier function (Baumgart and Dignass 2002). Healthy and intact intestinal mucosa effectively resists the invasion of pathogenic microorganisms, toxins, and antigens into the intestinal tract (Chahine and Feng 1998). Under the influence of stress factors, intestinal mucosal barrier function is typically reduced, allowing harmful microorganisms to pass through intestinal regions with impaired integrity into the blood, which circulate to distal body organs, causing bacterial translocation or sepsis. Therefore, preventing the injury to the intestinal epithelial barrier and strengthening the repair of damaged intestinal mucosa are crucial to maintaining mechanical barrier integrity of the intestinal tract. This mechanical barrier is the least complex of all intestinal epithelial mucosal barriers. The tight junction proteins, which are attached to the cell membrane of the intestinal mucosa and serve as biomarkers for intestinal barrier function, are ZO-1, claudin, and occludin. Dysregulation of occludin directly affects cell membrane permeability (Wachtel et al. 1999). The tight junction attachment protein ZO consists of mainly ZO-1, ZO-2, and ZO-3. During stimulation of intercellular signaling, ZO-1 binds with both the actin cytoskeleton and occludin, to form an intestinal mucosal mechanical barrier. Different subtypes of claudin protein also play varying roles in maintaining the tight junction barrier. Claudin-1, which is located in the intestines, contains two extracellular rings of identical structure, which allow the extracellular loop between two adjacent cells to form the shape of a tight junction, and atresia, which intensifies the gap restrictions between intestinal epithelial cells. Due to early weaning stress, the expression of the intestinal tight junction protein decreases, leading to destruction of the intestinal mechanical barrier structure and an increase in intestinal permeability (Hu et al. 2013). In this study, LPS administration significantly decreased the mRNA levels of ZO-1, occludin, and claudin-1 within each intestinal segment in piglets. However, taurine significantly upregulated the expression of tight junction proteins ZO-1, occludin, and claudin-1 within each intestinal segment. Through that mechanism, taurine helps preserve the mechanical barrier of the intestinal mucosa.

5 Conclusion

LPS stimulation leads to intestinal mucosal damage, increased intestinal permeability, altered tight junction structure of intestinal epithelial cells, and destruction of the intestinal mechanical barrier. Conversely, the prophylactic treatment by addition of 0.3% taurine into feed blocks the downregulation of ZO-1, claudin-1, and occludin by LPS-spiked intestinal epithelial cells, improves the intestinal mucosal structure of piglets, reduces intestinal mucosal permeability, and enhances the intestinal mucosal mechanical barrier.

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Part II

Therapeutic Potential of Taurine and Its Derivatives

Current Opinion on the Therapeutic Capacity of Taurine-Containing Halogen Derivatives in Infectious and Inflammatory Diseases

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Keywords

Taurine derivatives · Inflammation · Infection diseases · Taurine chloramine · N-chlorotaurine · Taurine bromamine · N-bromotaurine · COVID-19

Abbreviations

ARDS	acute respiratory distress syndrome
ASM	artificial sputum medium
CFU	colony-forming unit
EKC	epidemic keratoconjunctivitis
HOBr	hypobromous acid
HOCl	hypochlorous acid
MIC	minimum inhibitory concentration
MPO	myeloperoxidase
NBT	N-bromotaurine
NCT	N-chlorotaurine
ROS	reactive oxygen species
RSV	respiratory syncytial virus
Tau	taurine
TauBr	taurine bromamine
TauCl	taurine chloramine

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1 Introduction

1.1 Generation of Endogenous Taurine Derivatives (Taurine Haloamines)

Taurine endogenous derivatives, taurine chloramine (TauCl) and taurine bromamine (TauBr), are generated at a site of inflammation in stimulated neutrophils and eosinophils, respectively. TauCl (N-chlorotaurine, NCT) is the effect of the reaction of hypochlorous acid (HOCl) with taurine, the most abundant free amino acid in the cytosol of neutrophils (Zgliczynski et al. 1968; Weiss et al. 1982).

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Taurine $HOCl / OCl^{-}$ NCl Tau NCT
H_2O

On the other hand, TauBr (N-bromotaurine, NBT) is generated primarily in the cytosol of activated eosinophils containing high levels of very toxic hypobromous acid (HOBr) (Thomas et al. 1995).

Taurine $HOCl / OCl^{-}$ NCl Tau NCT
H_2O

1.2 A Role of Taurine Derivatives at a Site of Inflammation

Both taurine haloamines, NCT and NBT, are less toxic than hypohalous acids (HOCl, HOBr). Their generation protects cells from death caused by free extracellular HOCl and HOBr, the major products of the MPO-halide system. Importantly, NCT and NBT show antimicrobial activities at noncytotoxic concentrations (Nagl et al. 1998a, b, c; Marcinkiewicz et al. 1995; Walczewska et al. 2017). Therefore, endogenous NCT and NBT are important components of innate immunity and the nonspecific host defense system (Marcinkiewicz et al. 1995; Nagl and Gottardi 1996; Jang et al. 2009) Moreover, NCT can regulate the course of acute inflammation (Park et al. 1997; Marcinkiewicz et al. 1995) and has a great impact on the fate of neutrophils (Kim and Cha 2014).

1.2.1 Effects of NCT on Efferocytosis and Formation of Neutrophil Extracellular Trap (NET)

The primary role of neutrophils at a site of acute inflammation is to phagocytize and to kill pathogens (Klebanoff 2005). Neutrophils, after elimination of invaders, commit apoptosis and no longer generate ROS and proinflammatory mediators. Apoptotic neutrophils express scavenger receptors and are eliminated by macrophages. Such phagocytic engulfment of apoptotic neutrophils is called efferocytosis and is necessary for the effective resolution of acute inflammation. If the dying neutrophils are not completely removed, chronic inflammation may develop. Kim et al. (2015) have demonstrated that NCT increases the efferocytic activity of macrophages through upregulation of heme oxygenase-1 (HO-1), one of the stress-response proteins (Olszanecki et al. 2008). This mechanism confirms the diverse anti-inflammatory properties of NCT.

Neutrophils, in addition to phagocytosis and extracellular degranulation of proteases and ROS, use formation of NETs (neutrophil extracellular traps) to immobilize and kill pathogens (Papayannopoulos and Zychlinsky 2009). NETs contain decondensed nuclear chromatin, MPO, and granule-derived anti-microbial peptides. It has been shown that free MPO-driven extracellular HOCl is necessary for NETs release and its conversion to NCT diminished NETs activity (Palmer et al. 2012). NETs can play a dual role in inflammation, a beneficial one to kill microbes or a detrimental one responsible for tissue injury. Thus, their neutralization by taurine may provide a novel model of cytoprotection in some chronic infections.

1.3 A Hypothetical Role of NCT in Inhibition of the Cytokine Storm

Well-known anti-inflammatory properties of taurine derivatives, such as inhibition of a wide range of proinflammatory mediators, are of great value for the inhibition of the cytokine storm, the state of hyperinflammation responsible for a severe outcome of various viral infectious diseases (COVID-19, Ebola). NCT at non-cytotoxic concentrations inhibits the production of IL-6 from stimulated neutrophils and macrophages (Marcinkiewicz et al. 1995).

Namely, the fatal outcome of COVID-19 is associated with the cytokine storm, pulmonary hyperinflammation, systemic hypercoagulation, and ARDS (acute respiratory distress syndrome) (Tang et al. 2020). IL-6 is considered to be the key cytokine in the pathogenesis of the cytokine

storm. Moreover, in patients with ARDS, the massive IL-6 production predicts poor survival. Furthermore, IL-6 is the most frequently reported cytokine to be increased in severe COVID-19, and IL-6 elevated levels have been associated with a higher rate of mortality (Gubernatorova et al. 2020; Han et al. 2020). In line with these observations, various anti-IL-6 therapies have been proposed (Tabll et al. 2021). Our previous studies have shown that NCT strongly inhibits production of IL-6 by inflammatory cells (Marcinkiewicz et al. 1995). Therefore, we propose that inhalation of NCT in the early stages of COVID-19 may have an important role into protecting patients from the severe forms of the disease. We suggest that NCT may play a dual therapeutic role; it will act as an anti-coronavirus and an anti-IL-6 agent. However, further studies are necessary to confirm this idea.

In this review, we present the recent investigations showing the therapeutic capacity of taurine derivatives (NCT, NBT) in selected infectious and inflammatory diseases, including COVID-19.

2 Taurine Derivatives and Infectious Diseases

2.1 Taurine Derivatives and Bacterial Infections

Today, the major clinical problem of bacterial infections is prevalence of antibiotic resistance. Consequently, an inability of full eradication of bacteria at the early stages of infections results in a conversion of the planktonic form of bacteria into the more dangerous biofilm form (Marcinkiewicz et al. 2013a). Herein, we summarize the data concerning bactericidal properties of N-chlorotaurine (NCT/TauCl) and N-bromotaurine (NBT/TauBr) against various bacterial strains. Importantly, the bactericidal properties of NCT and NBT have been demonstrated at non-cytotoxic concentrations, well tolerated by mucous membranes and skin. The bactericidal activity of NCT was shown in vitro against both Gram (-) bacteria (*Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) (Nagl et al. 2000a, b) and Gram (+) bacterial strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*) (Nagl et al. 2000a, b). NCT also has a microbicidal effect against *Mycobacterium terrae*, especially in the presence of ammonia, which is due to the formation of the stronger bactericidal monochloramine (NH_2Cl) by the transhalogenation reaction (Nagl and Gottardi 1998). On the other hand, strong bactericidal activity of NBT was shown against the following bacteria strains: Gram (-) bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Porphyromonas gingivalis*) and Gram (+) bacteria (*Streptococcus mutans*, *Staphylococcus epidermidis*, *Propionibacterium acnes*) (Marcinkiewicz et al. 2005, 2006a, b, 2008, 2009, 2013a, b; Pasich et al. 2013).

Importantly, NCT as a sodium salt has been used in clinical studies as an effective and well-tolerated antiseptic and anti-inflammatory agent for topical treatment of infections at different body sites (e.g., the skin and mucous membranes, the eye, the ear, and the urinary bladder) (Gottardi and Nagl 2010). In contrast to NCT, the therapeutic applicability of NBT is limited due to its relatively poor stability. To overcome this disadvantage, a synthesis of the stable N-bromotaurine compounds, N-monobromo-2,2-dimethyltaurine (Br-612) and N-dibromo-2,2-dimethyltaurine (Br-422), has been performed. It has been shown that both Br-422 and Br-612 retained anti-inflammatory properties of NBT. Both Br-422 and Br-612 compounds exerted stronger bactericidal activity than NBT against all the tested bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*). On the other hand, Br-422 exerted higher activity than Br-612 in most in vitro tests (Walczewska et al. 2017).

Antibiotic resistance of a wide range of bacteria strains is a very important problem of the global health. Resistant microbes are more difficult to treat, requiring higher doses of antibiotics or alternative medications. For example, NCT

demonstrates bactericidal activity against multidrug-resistant bacteria strains. Namely, NCT used at 1% concentration reduced the number of CFU of strains of methicillin-resistant *Staphylococcus aureus*, linezolid-resistant *Staphylococcus epidermidis*, vancomycin-resistant, and linezolid- and vancomycin-resistant *Enterococcus faecium*, 3MRGN and 4MRGN *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* (Anich et al. 2021). The activity of NCT, in contrast to other active halogen compounds (NBT), is not decreased but even enhanced in the presence of organic fluids (e.g., body fluids, purulent exudation, serum) (Gottardi and Nagl 2010; Gottardi et al. 2014; Gruber et al. 2017).

Therefore, the anti-inflammatory properties of NCT and NBT, combined with their bactericidal activity, without causing drug resistance, confirm that both taurine haloamines have great therapeutic potential in the treatment of infectious diseases such as otitis externa, urinary tract infections, and acne vulgaris (Gottardi and Nagl 2010). Clinical trials confirmed that NCT applied topically to the external ear canal has high against otitis externa caused by *P. aeruginosa* and *S. aureus*. Moreover, clinical trials have shown a beneficial effect of NCT, used as an antiseptic, in postoperative prophylaxis after plastic surgery of the ear tympanic membrane (Neher et al. 2004, 2007). It has also been shown that NCT can be used in topical treatment of urinary tract disorders caused by *P. aeruginosa*. When used in daily lavages of the urinary bladder, it was well tolerated and did not cause any side effects (Nagl et al. 1998a, b, c). In a double-blind pilot study, the efficacy and safety of NBT cream against *P. acnes* were evaluated by Marcinkiewicz et al. (2009). Clindamycin gel was used as a control. Forty patients with mild to moderate inflammatory facial *acne vulgaris* were randomly treated with either TauBr or clindamycin. After 6 weeks, both treatments showed comparable results. More than 90% of patients improved clinically with a similar reduction in the number of acne lesions (~65%), and with no side effects. Results from this clinical pilot study definitely show that NBT could be considered a new therapeutic option in inflammatory acne. Due to the microbicidal activity of NCT and NBT against bacteria that cause tartar formation and caries, both substances can be used as ingredients in mouth rinses or as an ingredient in toothpaste (Lorenz et al. 2008; Mainnemare et al. 2004; Pasich et al. 2013).

For years, the antibacterial properties of NCT and NBT were tested against the planktonic form of bacteria. In the last decade, their anti-biofilm properties have been also determined (Grimus et al. 2021; Marcinkiewicz et al. 2013b). It is of great importance as the majority of chronic infections are characterized by the biofilm formation. Biofilm is a structured, long-lived form of bacteria organized community. The microbes in biofilms are hidden in a self-produced polymeric matrix. The matrix contains polysaccharides, proteins, and DNA originating from the microbes; biofilm may be composed of one or more species. Biofilm-growing bacteria cause chronic infections because they show high resistance to antibiotics and to phagocytosis and other mechanisms of the innate and adaptive immune system (Marcinkiewicz et al. 2013a,b). Biofilm-related infections are characterized by persistent inflammation and tissue damage (chronic rhinosinusitis, chronic wounds, periodontal diseases), and they are difficult to treat (Grimus et al. 2021; Marcinkiewicz et al. 2013a,b). Biofilm structures are significantly more resistant to antibacterial substances than the planktonic forms of bacteria. The effective therapeutic concentrations of some antibiotics to bacteria in biofilm may be even up to 100–1000-fold higher than that to planktonic bacteria (Høiby et al. 2011).

Anti-biofilm activity of taurine derivatives has also been tested. It has been shown that viability of young biofilms of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* is largely reduced by a concentration of 1% (55 mM) NCT, after incubation times of 15 min to 1 h (Ammann et al. 2014; Coraca-Huber et al. 2014). The activity of NCT against bacteria in longer-lasting biofilms formed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella variicola* was also tested. The results revealed that older biofilms retained

constant susceptibility to NCT up to several months despite their age (Grimus et al. 2021). It may explain the clinical efficacy of NCT in infected non-healing chronic wounds and purulently coated ulcerations (Grimus et al. 2021). Because bacteria hidden in a biofilm matrix are much more resistant than planktonic bacteria, the killing incubation time of NCT is much longer. Namely, it has been shown that destruction of the biofilm matrix and loss of bacteria viability in the presence of NCT at a pH 7 and at 37 °C takes 0.5–1 h, while killing of planktonic bacteria requires less than 20 min. (Gottardi and Nagl 2005; Grimus et al. 2021).

In clinical studies, NCT has shown significant therapeutic effects in some infections where biofilms play an important role: purulent leg ulcers (Nagl et al. 2003), external otitis (Neher et al. 2004), and dental plaque (Lorenz et al. 2008). Moreover, it has been shown that both haloamines, NCT and NBT, are effective in the local treatment of skin and mucosa infections, including biofilm-related infections (Nagl et al. 2000a, b; Marcinkiewicz et al. 2008; Marcinkiewicz 2009).

Both taurine haloamines, NCT and NBT, can inhibit the formation of *P. aeruginosa* biofilm in vitro at its early stages of development. However, they cannot destroy mature biofilm and kill sessile bacteria at low, subtherapeutic concentrations (Marcinkiewicz et al. 2009, 2013b). Finally, these preliminary data suggest that taurine derivatives are good candidates as local therapy against various bacterial infections including biofilm-related bacterial infections at the early stage of biofilm formation (Marcinkiewicz et al. 2013a, b).

2.2 Taurine Derivatives and Fungal Infections

Fungal infections are one of the most common diseases affecting people all over the world. As estimated by the Global Action Fund for Fungal Infections (GAFFI), "over 300 million people are afflicted with a serious fungal infection and 25 million are at high risk of dying or losing their sight" (GAFFI 2021). Fungal infections affect different groups of patients, both healthy and people with various underlying diseases, and the course of the disease largely depends on the immune status of the host. The spectrum of infections is very diverse, from superficial, mucocutaneous, subcutaneous to life-threatening deep-seated mycoses including infections of the central nervous system and disseminated mycoses with accompanying fungemia (Brown et al. 2012). In particular, mycoses caused by drug-resistant strains are currently of most concern (Whaley et al. 2017; Vahedi Shahandashti and Lass-Flörl 2019; Ramirez-Garcia et al. 2018; Riat et al. 2018; Taghipour et al. 2020; Forsberg et al. 2019; Zhao et al. 2021), especially that the number of antimycotic medications available is very limited (Campoy and Adrio 2017). The problem of drug resistance of fungi is associated with the natural (innate) resistance of some species to certain antimycotics and also with prolonged use of antifungal preparations in treatment of infections, prophylaxis of mycoses, and as plant protection agents (acquired resistance) (Revie et al. 2018; Berger et al. 2017). The need to search for new substances with antifungal properties, a broad spectrum of action and low toxicity for the patient, is indisputable, and even more so nowadays since the current progress in medicine allows severely ill patients to survive, which often results in immunity disorders substantially increasing the risk of development of mycoses.

Taurine derivatives with anti-inflammatory effects and potential antimicrobial activity represent a new alternative to commonly used antimycotics. The research conducted so far has focused mainly on N-chlorotaurine (NCT) and clearly shows the high potential of this compound as an antifungal agent. The antimycotic action of NCT has been demonstrated for some of the most common human fungal pathogens, including drug-resistant species. Nagl et al. studied the antimycotic effect of 1% NCT against various *Candida* species: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. dubliniensis* (Nagl et al. 2002). This compound showed fungicidal activity against these yeasts and also influenced

their metabolism through an impact on the production of secreted aspartyl proteinases (sap). Sap are important virulence factors and play a significant role in the pathogenesis of candidiasis, particularly in digesting host molecules for nutrient acquisition, protection against host immune system, facilitating adhesion, and host tissue invasion (Naglik et al. 2003; Rodríguez-Cerdeira et al. 2020). Sap production was clearly impaired after incubation of fungi in the presence of NCT at concentrations that even did not inhibit fungal growth.

Reeves et al. (2006) tested the antifungal action of NCT against *Aspergillus* species: *A. fumigatus*, *A. flavus*, *A. niger*, and its influence on the production and stability of gliotoxin, which is an important determinant of pathogenicity of *Aspergillus*, modulates host immune response and induces apoptosis in different cell types (Kwon-Chung and Sugui 2009; Scharf et al. 2012). NCT MIC_{90} ranged from 16.0 ± 0.2 to 37.8 ± 1.4 μM for *A. fumigatus* strains and 28.2 ± 4.6 μM and 65.2 ± 4.5 μM for *A. flavus* and *A. niger*, respectively. The concentration of 75 μM significantly reduced *Aspergillus* survival and 150 μM resulted in total killing of all tested strains. Furthermore, exposure of fungi to NCT for a period of 1 h led to a significant decrease in gliotoxin.

Lackner et al. (2015) confirmed the fungicidal activity of NCT against multiresistant fungi from the genera *Scedosporium* and *Lomentospora*. In in vitro studies, scientists showed that 1.0% NCT killed both conidia and hyphae of *Scedosporium apiospermum*, *Scedosporium boydii*, and *Lomentospora prolificans* and caused a reduction in fungal colony-forming units by 1- to 4-$\log_{10}$ and 4- to >6-$\log_{10}$ after 4 h and 24 h incubation, respectively. Moreover, NCT-pretreated *Scedosporium* and *Lomentospora* conidia exhibited delayed germination and a reduced rate of germination, even after 10 min incubation. The antimycotic activity of NCT against *Scedosporium* was confirmed in an in vivo model of *Galleria mellonella* larvae. Preincubation of *Scedosporium* conidia with 1.0% NCT for 30 min before larvae inoculation resulted in a loss of virulence of fungi, which became manifest in a reduced rate of larvae mortality of 20–50% in comparison to 90–100% by nontreated fungal cells after 8–12 days after infection (Lackner et al. 2015).

It has also been proven that the presence of organic material increases the antifungal activity of NCT. This is unusual for antiseptics, which generally in body fluids or exudates partially lose their effect. The mechanism of enhancing the antifungal activity of NCT in an environment containing organic material is associated with formation of stronger microbicidal N-chloro derivatives by transferring the chlorine atom from NCT to ammonium and amino acids (Nagl et al. 2001; Gruber et al. 2017). Particularly, formation of the more lipophilic monochloramine (NH_2Cl) is important in this regard, which penetrates pathogens better than NCT. Nagl et al. compared the influence of NCT on *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Candida parapsilosis*, *Fusarium moniliforme*, and *Penicillium commune* in both aqueous solution and human nasal mucus (Nagl et al. 2001). The team confirmed the broad-spectrum antimycotic activity of NCT and found significantly more rapid killing of fungi in nasal mucus than in a buffer solution. This was confirmed in in vitro experiments when the combination of NCT and NH_4Cl was used in antifungal susceptibility testing. The enhanced antifungal effect and the reduction in the incubation time required to kill fungi were noted (Nagl and Gottardi 1996; Nagl et al. 2001; Reeves et al. 2006; Lackner et al. 2015). Similar results indicating better antifungal activity in the presence of organic compounds were obtained by Gruber et al. who studied NCT against *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans*, *Exophiala dermatitidis*, *Geotrichum* sp., *Lomentospora prolificans*, *Scedosporium apiospermum*, *Scedosporium aurantiacum*, *Scedosporium boydii*, *Scedosporium minutisporum* in artificial sputum medium (ASM), mimicking the composition of mucus of cystic fibrosis patients (Gruber et al. 2017). The compound showed antifungal activity against all tested strains, and the effect in ASM was stronger than in phosphate buffer solution. 1% NCT caused the reduction of fungal spores below 10^2 CFU/mL

within 15 min, except *Scedosporium minutisporum* for which it was 30 min. Lower concentrations of NCT (0.5% and 0.3%) had a similar effect but after a longer incubation period. The most susceptible species was *C. albicans* which was killed by 0.3% NCT within 60 min at a detection limit of 10^2 CFU/mL. The clinical efficacy of NCT against fungal infection was confirmed by its use in a combination with dexamethasone in a patient with therapy-resistant otitis externa with additional tympanic membrane perforation from whom *Aspergillus fumigatus* was cultured (Lumassegger et al. 2010).

The antifungal properties of bromine taurine derivatives – N-bromotaurine (TauBr) and more stable N-monobromo-2,2-dimethyltaurine (Br-612) and N-dibromo-2,2-dimethyltaurine (Br-422) – are still poorly investigated. Walczewska et al. (2017) evaluated bromine taurine compounds against a single *Candida albicans* strain. Among the tested compounds, TauBr showed the weakest fungicidal activity, exceeding non-cytotoxic concentrations of up to 300 μM, while Br-422 was the strongest antimycotic. Minimal fungicidal concentrations for TauBr, Br-612, and Br-422 ranged from 2500 to 10000 μM, 7 to 30 μM, and 1.5 to 7 μM, respectively, depending on the starting fungal inoculum (10^5 CFU/mL or 10^8 CFU/mL) (Walczewska et al. 2017). The strongest antimycotic effect of Br-422 could be explained by the presence of two oxidizing bromine atoms compared to the single one of both other compounds.

2.3 Taurine Derivatives and Viral Infections (COVID-19)

2.3.1 Mechanism of Action Against Viruses

Taurine active halogen derivatives possess a non-specific oxidizing mechanism of action so that they are microbicidal against all kinds of microorganisms, including viruses (Eitzinger et al. 2012; Gottardi and Nagl 2002; Gottardi and Nagl 2010; Peskin et al. 2005; Peskin et al. 2009; Thomas et al. 1986; Fernandez et al. 2013; Tatsumi and Fliss 1994). This has been known for decades for other chloramines, for instance, dichloroisocyanurate and chloramine T (McDonnell and Russell 1999). Insight into the virucidal mechanism of a taurine derivative was provided by the work of Yoon et al. (2011), who investigated the action of *N,N*-dichloro-2,2-dimethyltaurine (NVC-422) against adenovirus type 5, which is causative for epidemic keratoconjunctivitis. They found oxidative inactivation of key proteins of the virus, for instance, hexon and fiber proteins and oxidation of sulfur-containing amino acids such as methionine as an important molecular mechanism. Loss of viral structural integrity was seen in electron microscopy, namely, dissociation of proteins from the capsid, appearance of circular spots and cavities on the surface, deformation of the capsid structure, aggregation of adjacent viruses, and loss of fiber shafts (Yoon et al. 2011). This is in agreement with the inactivation of bacterial toxins by *N*-chlorotaurine (NCT, TauCl) whereby oxidation of cysteines and methionines and chlorination of tyrosines, phenylalanines, and histidines was found (Eitzinger et al. 2012). Similar results with NCT-treated proteins from the new coronavirus from our research group have just been submitted for publication. A major conclusion from the mechanism of action with many sites of attack is the absence of resistance of viruses against active halogen compounds, which is naturally valid for the taurine derivatives, too.

2.3.2 In Vitro Activity Against Viruses

Considerations and initial studies on the application of NCT in the eye (Koyama et al. 1996; Nagl et al. 1998a, b, c) led to investigations on its activity against adenoviruses and herpes simplex viruses (HSV) 1 and 2, which play a major role in keratoconjunctivitis and keratitis. Pharmacological concentrations of NCT, generally about 1% (55 mM), reduce the number of infectious particles of these viruses within a few minutes, and 0.1% still has virucidal activity (Nagl et al. 1998a, b, c). The infection spread of HSV-1 and vaccinia virus in human explanted corneas can be prevented, and the release of virus is reduced by 2 – 3 $\log_{10}$ (Huemer et al. 2010). A panel of adenovirus serotypes 1, 2, 3, 4, 5, 7a, 8,

and 19, playing a role in epidemic keratoconjunctivitis (EKC), was tested subsequently with similar results, i.e., concentration- and incubation time-dependent inactivation (Romanowski et al. 2006). Another study confirmed the inactivation of adenovirus type 5 in drinking water (Garcia et al. 2019). In the same study, a strong virucidal effect was found for bromamine T, the bromine analog of chloramine T (Garcia et al. 2019). The broad-spectrum virucidal activity of taurine active halogen derivatives was demonstrated with the abovementioned *N,N*-dichloro-2,2-dimethyltaurine against adenovirus types 5, 8, 19, and 37, HSV-1, coxsackievirus A24, and enterovirus 70, which are all causative for human keratoconjunctivitis (Jekle et al. 2013). The activity was maintained in the presence of synthetic tears (Jekle et al. 2013).

A highly interesting approach came from the group of H. Fliss in 2008. They published on the strong activity of 2−5 mM NCT against high titers of human immunodeficiency virus (HIV) 1 (Dudani et al. 2008). Moreover, they used NCT-inactivated HIV in host cell lysate as a vaccine in a murine AIDS model. Actually, both preventive and therapeutic efficacy of the vaccine could be shown. Several advantages were proposed compared to conventional vaccine manufacture, a rapid and easy preparation without harsh inactivation or purification steps, no problems with surviving or mutated viruses, and the possibility of a preparation of autologous or custom-made vaccines for individual patients (Dudani et al. 2008).

The recent and continuous pandemic with COVID-19 prompted us to test the activity of NCT against SARS-CoV-2 and other respiratory viruses, namely, influenza virus H3N2 and H1N1pdm and respiratory syncytial virus (RSV). The preprint has been published (Lackner et al. 2020) the updated manuscript submitted for publication. As expected, 1% NCT and also 0.1% NCT had time-dependent virucidal effects against all of these viruses, with high concentrations acting within 1 to a few minutes. It was also shown with viruses – similar to bacteria, fungi, and protozoa (Arnitz et al. 2009; Fürnkranz et al. 2011; Fürnkranz et al. 2008; Lackner et al. 2015; Nagl et al. 2018) – the activity of NCT is enhanced in the presence of proteinaceous material (Lackner et al. 2020). This is explained by formation of (NH_2Cl) in equilibrium from NCT plus ammonium chloride. Monochloramine penetrates the microorganisms more rapidly than NCT because of its higher lipophilicity (Arnitz et al. 2009; Nagl et al. 2018; Grisham et al. 1984).

2.3.3 In Vivo Activity Against Viruses

It is a logical consequence to think of a therapeutic use of antiseptics as well tolerated as NCT against viral infections, too. As mentioned, one of the first approaches was the application in ophthalmology. After having shown the tolerability of NCT in phase 1 and phase 2a (Nagl et al. 1998a, b, c, 2000a, b), efficacy was seen in a controlled, randomized, double-blind phase 2b study in epidemic keratoconjunctivitis (EKC) (Teuchner et al. 2005). Subjective symptoms were improved more rapidly in the NCT group compared to that of the gentamicin group in both the whole study population and in a subgroup with particularly severe infections by adenovirus type 8, with objective parameters only in the latter subgroup in the study. The frequency of subepithelial infiltrates was similar in both groups (Teuchner et al. 2005). The influence of early treatment in this infection and the addition of ammonium chloride to NCT with production of a better penetrating monochloramine remain to be investigated in humans. With iodine, which is known to penetrate tissue well, a good therapeutic effect evidenced by EKC was found in one study (Kovalyuk et al. 2017). In the adenovirus 5 New Zealand White (ad5/NZW) EKC rabbit model, therapeutic efficacy of NCT plus monochloramine ammonium chloride was superior to that of plain NCT, and corneal penetration of active chlorine was better with the combination (Romanowski et al. 2006).

The activity of chlorine and bromine taurine derivatives may be useful in the treatment of skin infections caused by herpes viruses. In a multiple sclerosis patient with a herpes zoster skin infection in the thoracic area unresponsive to systemic valaciclovir and topical lidocaine, topical application by spray of 0.8% NCT rapidly caused a

marked decrease in pain within 1 day and a marked regression of papules after 2–4 days (Kyriakopoulos et al. 2016). On day 5, 1.0% *N*-bromotaurine (TauBr) was added to potentiate the anti-inflammatory activity of NCT and to induce reepithelialization, which was achieved on day 7. Treatment with NCT once in the morning and TauBr once in the evening was continued to prevent a relapse for 1 month. During an observation period of 8 months in the following, no more signs of herpes occurred (Kyriakopoulos et al. 2016). In a few other named patient uses, clear hints of therapeutic effects could be seen without adverse events except for short and transient local itching and slight burning. Performance of controlled clinical trials is indicated to obtain reliable results for further development.

A highly interesting and important field is the topical treatment of frequent viral upper airway infections with antiseptics and antiviral substances in general. Under the COVID-19 pandemic, this concept obtained topicality and has been propagated (Carrouel et al. 2021; Cegolon et al. 2020; Kramer and Eggers 2020; Mitchell et al. 2020; Kofler et al. 2020; Kofler and Nagl 2020). Although the concept is not new, there is a lack of controlled confirmatory studies. Investigations with iodine and, for instance, carageenan nose spray, are encouraging (Kramer and Eggers 2020; Eccles et al. 2015; J.M. F and Group C-C 2021; Koenighofer et al. 2014). Our own experiences from case applications with NCT nose spray, throat spray, and gargling solution are encouraging, too. Tolerability is very good, confirmed by a pilot study (Neher et al. 2005), and the observations strongly indicate attenuation of the course of common cold infections, particularly after rapid application in the first stages of infection. This is in agreement with influenza antivirals zanamivir and oseltamivir, which should be used early, too (Hayden et al. 1997). The intention is to rapidly reduce the viral load in early stages to prevent a severe course and to found to shorten the course of infection. In an exemplary case, a 53-year-old male suffering from a COVID-19 infection with elevated body temperature (37.9 °C) and strong rhinopharyngitis, a 1% NCT solution was applied as a nose spray three times daily, as well as gargling the solution three times daily and inhaling for 10 min (to prevent infection of the lower airways). The symptoms markedly improved within 1 day and vanished on the third day, resulting in the termination of therapy at the end of the third day. Interestingly, the viral load measured from nose and throat swabs by RT-qPCR was low under therapy on the first day (cycle threshold (ct)-value 30-32) and increased again 2 days after termination of the therapy to a ct-value of 25 and 27 another 2 days later. Since there were no more symptoms, no further therapy was performed, and the ct-value came to 36.5 on day 13, and no viral RNA could be detected a week later. No more symptoms occurred in the following time. In another case (male, 52-year-old) with COVID-19 rhinopharyngitis, the initial ct-value without therapy was 14. After the start of nose spray and gargling solution 3 times daily, the ct-value came to 21 1 day later, and to 33 3 days later. Both courses of ct-values indicate a rapid decrease of viral load by therapy compared to that without therapy (Jang et al. 2021). The advantage of NCT is that it can be used for inhalation, too, because of its high tolerability and absence of systemic distribution and therefore absence of systemic adverse effects (Arnitz et al. 2018). Respective clinical studies are needed to clearly determine the efficacy of NCT in respiratory tract infections.

2.3.4 Perspectives

There are several possibilities for the use of taurine active halogen derivatives in viral infections, which should be investigated in randomized, controlled, double-blind clinical studies. Skin infections are near at hand, such as herpes simplex and herpes zoster. A broad field is upper respiratory tract infections caused by many viruses that may be treated by nose and throat sprays, as well as gargling solutions. The usability of NCT for inhalations also opens the field of bronchopulmonary infections, for instance, caused by SARS-CoV-2, influenza virus, and RSV.

According to the initial experience and logical considerations for topical therapy, the dosing should be started as early as possible in the course

of the infection, with the intention to rapidly decrease the viral load and attenuate the severity of clinical symptoms and of the postinfection syndromes. Besides the antiviral activity of taurine active halogen derivatives, their anti-inflammatory properties (downregulation of proinflammatory cytokines and chemokines, upregulation of hemoxygenase-1, and others (Kim and Cha 2014; Marcinkiewicz 2010; Marcinkiewicz and Kontny 2014; Walczewska et al. 2017)) are of high interest for viral infections. These anti-inflammatory effects have actually been shown in vivo to have clear curative potential (Kim et al. 2021a, b; Kwasny-Krochin et al. 2002; Neher et al. 2007) and are thought to be beneficial, for instance, in herpes and COVID-19 infections.

General advantages of local therapy with taurine active halogen derivatives are efficacy against all kinds of pathogens including viruses without resistance development, absence of systemic toxicity or systemic adverse effects (valid at least for NCT), and a very low potential for allergic reactions as endogenous amino acid derivatives. More specific for NCT is enhancement of the antimicrobial, including antiviral, activity in the presence of an organic load.

In summation, taurine active halogen derivatives are highly interesting compounds for topical therapy of infections of different body sites including viral infections of the skin and the whole respiratory tract. Their main advantages are broad-spectrum activity against pathogens and high tolerability.

3 Taurine Derivatives and Skin Inflammatory Diseases

3.1 The Clinical Use of Taurine Active Halogen Derivatives: From Inflammatory Disorders and Infections to Cancer

The taurine active halogen derivatives, namely, N-chlorotaurine (NCT) and N- bromotaurine (NBT), produced by myeloperoxidase and eosinophil peroxidase of neutrophils and monocytes (Thomas et al. 1995; Henderson et al. 2001), are powerful essential constituents of innate immunity (Henderson et al. 2001). Numerous investigations disclosed their anti-inflammatory (Marcinkiewicz et al. 2005) and anti-infective activity (Gottardi and Nagl 2010).

Although both NCT and NBT are abundant molecules located at the sites of inflammation (Thomas et al. 1995), it took two decades to provide deep insight into the pathways of their activity (Olszanecki et al. 2008). A main mechanism of anti-inflammatory activity of NCT is inhibition of the production of prostaglandin E_2 (PGE2), among other proinflammatory mediators. Additionally, it was found that NCT inhibits the production of nitric oxide, interleukin 6, and tumor necrosis factor α (Marcinkiewicz et al. 1995), and later it was shown that it downregulates the activity of NF-kappa B (NF-kappa B) and I kappa B (Barua et al. 2001). Later studies investigated and revealed further molecular interactions exerted by haloamines. Olszanecki et al. (2008) discovered that both haloamines upregulate heme oxygenase activity that leads to a decrease in cyclooxygenase activity halting the production of PGE_2. In this study, however, the decrease in PGE_2 was not linked to the reduction in cyclo-oxygenase activity but was attributed to the differential cellular response to haloamines. Taurine is known to possess anti-inflammatory properties due to its extreme antioxidant activity (Marcinkiewicz and Kontny 2014). Significantly, taurine also exerts potent anticancer activity by induction of mitochondrial apoptosis and regulation of autophagy through the mitogen-activated protein kinase (MARK) pathway (Baliou et al. 2021a, b). The basic anti-inflammatory properties of NCT and NBT are well described in detailed original studies (De Carvalho et al. 2016; Walczewska et al. 2017) and reviews (Marcinkiewicz and Kontny 2014; Baliou et al. 2020; Kyriakopoulos et al. 2017; Marcinkiewicz 2010).

Notably, the anti-inflammatory and bactericidal effect of NBT, as shown by laboratory investigations (Marcinkiewicz et al. 2005; Marcinkiewicz and Kontny 2014, Marcinkiewicz 2010), has also been clinically demonstrated by

its successful application against *acne vulgaris* (Marcinkiewicz et al. 2008). In this study, topical application of 3.5 mM of NBT showed a strong therapeutic effect against acne skin lesions (papules and pustules) comparable to that of topically used antibiotic clindamycin (1%). Thereon, a series of clinical case studies clearly indicated beneficial effects of both NCT and NBT. In an immune-compromised patient due to treatment against multiple sclerosis, the combination of topical NCT and NBT proved life-saving, as his herpes zoster virus reinfection was refractory to systemic valacyclovir treatment (Kyriakopoulos et al. 2016). The remarkable effect of NCT was noted during the initial stage of treatment that led to rapid removal of herpetic pain and in lesions. The equally remarkable effect of NBT was on rapid wound healing of the patient's lesions. The exceptional observation of this haloamine combination treatment was that it lasted for a short period of 7 days compared to the long period of a patient that had received systemically valacyclovir with no therapeutic benefit. The importance of this therapy with the haloamine combination relates to a curative outcome that allowed the patient to continue the immunosuppressive treatment for multiple sclerosis. Equally exceptional was the activity of NCT to rapidly result in the immediate alleviation of herpetic pain in this patient.

A thorough laboratory analysis of NBT anticancer activity on glucocorticoid resistant skin cancer cells revealed a notable antiproliferative effect on these cells by restoration of cell cycle inhibition (cell cycle arrest at both G1 and G2 stages) (Logotheti et al. 2016). In a follow-up study, it was discovered that taurine and NBT exert antiproliferative effects on psoriasis skin cells as well. Based on these findings, topical treatment of a localized psoriasis and a general psoriasis case that were resistant to glucocorticoid agents but biological factor systemic use was successful with NBT (Kyriakopoulos 2020).

Further, the activity of both topical NCT and NBT was found to be remarkable for healing chronic wounds, especially in elderly patients, and truly preventing clinical worsening conditions like amputation of lower limbs and skin graft removal. In a case of an elderly patient with stage 5 renal disease and chronic diabetes mellitus 2, the otherwise unavoidable amputation of the lower extremity due to a chronic wound infected with a multiresistant *Staphylococcus aureus* strain could be avoided by the curative effect of the combination of NCT and NBT within hours rather than days (Kyriakopoulos et al. 2019). Similarly, in a unique case of a chronic polymicrobial bacterial infection of the scalp of an elderly patient, refractory over many years of systemic and local antibiotic and glucocorticoid treatments, the sole solution of skin grafting was overcome by the successful topical use of the combination of NCT, NBT, and bromamine T within days (Kyriakopoulos et al. 2020). Bromamine T used in this study is a synthetic bromine compound with an antimicrobial (Walczewska et al. 2017), anti-inflammatory (Baliou et al. 2021a), and anti-cancer activity similar to NBT (Baliou et al. 2021a, b; Logotheti et al. 2016), but of higher stability (Walczewska et al. 2017).

Overall, the taurine derivatives NCT and NBT may become valuable compounds to overcome the clinical failures of topical antibiotic treatment and glucocorticoid resistance in treating skin disorders and deserve further clinical trial investigations. In most terms, both agents offer significant efficacy with mild transient or even absent adverse effects. Therefore, their clinical use is more than promising in view of the increasing global resistance of microbes against antibiotics (Baliou et al. 2021a) and because glucocorticosteroid usage is becoming increasingly restricted due to its long-term side effects (Oray et al. 2016). Moreover, the antiproliferative effects of NBT and bromamine T, especially in combination with established anti-malignancy compounds, such as cisplatin, might become valuable in treating cancer in the near future (Baliou et al. 2021a, b; Kyriakopoulos et al. 2016; Logotheti et al. 2016; Kyriakopoulos 2020).

4 Conclusions

A number of laboratory investigations and clinical studies have shown that both NCT and NBT can be used as antimicrobial and anti-inflammatory agents. They exhibit a broad spectrum of antimicrobial activity against bacteria, yeast, and viruses, including SARS-CoV-2. The major advantages of taurine derivatives are their stable antimicrobial activity and great tolerability in clinical settings. Importantly, neither bacteria nor fungi acquire resistance to their antimicrobial properties. On the other hand, the major limitation of the clinical use of NCT and NBT is their instability in the presence of body fluids. Therefore, patients cannot be treated with those substances by systemic administration (e.g., intravenously). However, local treatment by topical application, irrigations, and inhalations of taurine derivatives has been well documented. Special attention should be paid to NCT inhalation as a new therapeutic option for treatment of COVID-19 patients.

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Taurine and N-Bromotaurine in Topical Treatment of Psoriasis

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Keywords

N-bromotaurine · Taurine · Olive oil · Topical psoriasis treatment · Sulfur metabolism

Abbreviations

GR	Glucocorticoid receptor
MTX	Methotrexate
PWESI	Psoriasis weighted extend severity index
SPOO	Stable produced olive oil
TauNH-Br	N-bromotaurine

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1 Introduction

Successful psoriasis treatment remains an unsolved problem in clinical practice. Long-term effectiveness of biological factor systemic treatment is questioned over established therapies in relation to serious side effects (Patel et al. 2009; Peleva et al. 2018). The high percentages of discontinuation even in the first week of systemic treatment with biological agents (about 85%) raise questions to what extent patients can be compliant (Galván-Banqueri et al. 2013). For corticosteroids, systemic treatment is not recommended for psoriasis (Mrowietz and Domm 2013). Systemic methotrexate (MTX) use as a steroid sparing agent involves considerable side effects in particular to the bone marrow (Djerassi et al. 1967), the liver (Conway and Carey 2017), and the lung (especially in association with antineoplastic drugs) (Willson 1978), among others. MTX modifies glucocorticoid receptor (GR) expression to produce GR resistance (Stevens et al. 2004; van der Heijden et al. 2007). Nevertheless, GR sensitivity is important to obtain an optimum clinical response to therapy (Barnes and Adcock 2009; Inaba and Pui 2010). Keratinocytes selectively GR ablated are predisposed to cancer development (Latorre et al. 2013; Liang et al. 2017). Literature therefore concludes to the need for further clinical investigation efforts to identify different pathways and

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agents to improve treatment safety and efficiency of psoriasis and further associated pathologies.

At a site of inflammation, taurine reacts with HOCl and HOBr to produce N-chlorotaurine (TauNH-Cl) and N-bromotaurine (TauNH-Br), respectively (Marcinkiewicz and Kontny 2014; Thomas et al. 1995). Both taurine haloamines exert anti-inflammatory effects by downregulating prostaglandin E_2 without altering the expression of COX-2 enzyme while inducing heme oxygenase-1 activity (Olszanecki et al. 2008). TauNH-Cl therapeutic efficacy against infections and inflammations together with its remarkable tolerability has been documented in a series of clinical studies (Gottardi and Nagl 2010; Nagl et al. 2003; Neher et al. 2004; Teuchner et al. 2005; Arnitz et al. 2018). On the other hand, TauNH-Br can bypass GR resistance in cancer cells while exerting significant antiproliferative activity against numerous cancer cell lines including skin cancer cells and immortalized keratinocytes (Logotheti et al. 2016). Furthermore, TauNH-Br has been indicated as a valuable therapeutic agent in a clinical study for *acne* vulgaris (Marcinkiewicz et al. 2008). A combination of TauNH-Cl and TauNH-Br regimen was used successfully to cure a herpes zoster virus infection case in an unresponsive to valacyclovir in a multiple sclerosis comorbidity patient. In this case report, the addition of TauNH-Br was significant to the healing and rapid reepithelialization of HZV lesions (Kyriakopoulos et al. 2016). Moreover, the use of TauNH-Br was found essential in combination with TauNH-Cl to close a chronic wound co-infected with multiresistant *Staphylococcus aureus* in an elderly patient with stage 5 renal disease (Kyriakopoulos et al. 2019), and also, the use of TauNH-Br in combination with TauNH-Cl and Bromamine T (a TauNH-Br analogue) has successfully treated and closed efficiently a unique chronic multi-bacterial scalp infection of an elderly patient (Kyriakopoulos et al. 2020).

Acne vulgaris is regarded as an inborn defect of the innate immune system and an autoinflammatory disorder of the skin (Brydges and Kastner 2006; Gurung and Kanneganti 2016). Psoriasis is also regarded as an autoimmune and auto-inflammatory disorder (Liang et al. 2017) with abnormal differentiation and hyperproliferation of keratinocytes as a central pathogenesis factor (Lowes et al. 2014). Importantly, psoriatic skin during therapy with systemic biologicals that target IL12 is predisposed to loss of tumor control (Tugues et al. 2015) and development of skin cancer (Langley et al. 2013). Our investigations for TauNH-Br so far suggest a mechanism involving anti-autoimmunity activities (Lipnik and Levy 1959; Marcinkiewicz and Kontny 2014), antiproliferative properties against glucocorticoid-resistant skin cancer cells (Logotheti et al. 2016), anti-infectious activity (Kyriakopoulos et al. 2016, 2017, 2019, 2020), and potent therapeutic efficacy against the skin auto-inflammatory disorder *acne vulgaris* (Marcinkiewicz et al. 2008).

These findings led us to investigate a possible therapeutic value of TauNH-Br in psoriasis cases unresponsive to corticoids and biologicals. In this report, we present two therapy-refractory cases of pustular-plaque psoriasis, one localized and one generalized, that were efficiently treated locally with 1% TauNH-Br. TauNH-Br use was optimized using a patented combination treatment protocol (Kyriakopoulos 2014, 2017, 2020). With this innovative treatment approach, psoriasis symptoms withdrew in a very short time circumventing side effects to the advantage of both patients and physicians.

2 Methods

2.1 N-Bromotaurine and Taurine Solutions and Emulsions with Stable Produced Olive Oil

2.1.1 1% (49 mM) N-Bromotaurine Solution

For the preparation of 1% N-bromotaurine (TauNH-Br) solution according to Olszanecki et al. (2008), at first a NaOBr solution was needed. Equimolar amounts of NaOCl solution (Sigma-Aldrich, Gremany) and NaBr, pH 10, (Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) reacted to give 49 mM NaOBr solution. Equal volumes of 49 mM NaOBr were added dropwise to

490 mM taurine (Panreac-Applichem, USA) solution in PBS to obtain an approximate 49 mM TauNH-Br solution that equals to 1%.

The TauNH-Br concentrations were estimated by UV absorption spectra according to Olszanecki et al. (2008). Stock solutions were kept at 4 °C for a maximum of 3 days prior to dilution to 1% in pyrogen-free sterile water and immediate use.

2.1.2 40 mM NaOBR Solution

Separate NaOBr solutions were synthesized as described in production of 1% N-bromotaurine. These were kept at 20–28 °C at dark bottles to avoid light exposure prior to use.

2.1.3 1% (49 mM) N-Bromotaurine Plus Taurine (400 mM) Plus 50% Stable Produced Olive Oil (SPOO)

For the preparation of 1% TauNH-Br solution plus taurine (approximately 5%) plus 50% SPOO, first 98 mM NaOBr solution was added to 1900 mM of taurine solution according to the patented methodology of Kyriakopoulos (2017, 2020). The SPOO (Nasco AD Biotechnology Laboratory, Greece) was made according to the patented methodology of Kyriakopoulos (2014). Emulsion of TauNHBr, taurine with SPOO, was prepared by vigorous mixing of 1:1 parts of (a) aqueous solution of 2% TauNH-Br and surplus of taurine (approximately 10%) and (b) SPOO, at the same day of use. Patients were advised to adequately shake the emulsion prior to each application. For dosing schemes and duration of application, see the results section. Patients gave written informed consent. All authors declare that the study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Special approval from a committee was not applicable.

3 Results

3.1 The Localized Psoriasis Case

In order to test TauNH-Br as an agent for psoriasis treatment, a localized case was selected initially. This patient was a 53-year-old male suffering from pustular-plaque psoriasis diagnosed by biopsy and clinical examination. He had a long history of unsuccessful topical corticosteroid use. The patient was unmanageable by systemic methotrexate 20–30 mg/week, too, as he repeatedly relapsed after consecutive treatment approaches. Also a biological factor administration, etanercept 25 mg twice a week for 6 weeks, resulted only in a small decrease of Psoriasis Weighted Severity Index (PWESI) (Wittkowski et al. 2011) and in incompliance of the patient for continuation of therapy. Due to ineffectiveness of corticosteroid treatments and intolerability of biological factor administration, the patient was considered to receive 1% TauNH-Br local treatment by direct aerosol spraying onto the lesions of his left arm. He gave written informed consent and was managed according to the Declaration of Helsinki. A 10% TauNH-Br solution was prepared according to Olszanecki et al. (2008) and diluted to 1% in sterile and pyrogen-free water. Lesions were mostly located on both arms (elbows and lower arm) with each arm having almost an identical clinical presentation. The patient had a free period of 3 weeks without any other treatment previously received. The PWESI of the patient prior to TauNH-Br treatment was scored 4 with a Weighted Total Extent Score (WTEC) of 2. WTEC equals to the sum of regional scores/4 for each of the ten separate body domains investigated prior to treatment. The Mean Global Severity Score for this patient was rated 2, and the simplified PWESI score was rated 16. The simplified PWESI score equals to the total extend score multiplied by the mean global severity score (Wittkowski et al. 2011).

3.1.1 Left Arm: Treatment with 1% TauNH-Br Solution

The patient was advised to apply three puffs onto the affected regions of the left arm (elbow and lower arm) two times a day at 12 h apart. Each puff released approximately 130 μL of 1% TauNH-Br solution. The patient showed remarkable improvement after the first 2 weeks of application (Fig. 1a, b). Between the second and third week (as noticed at the 21st day of application), however, a slower withdrawal of symptoms was recorded (Fig. 1c). The patient had regular itch-

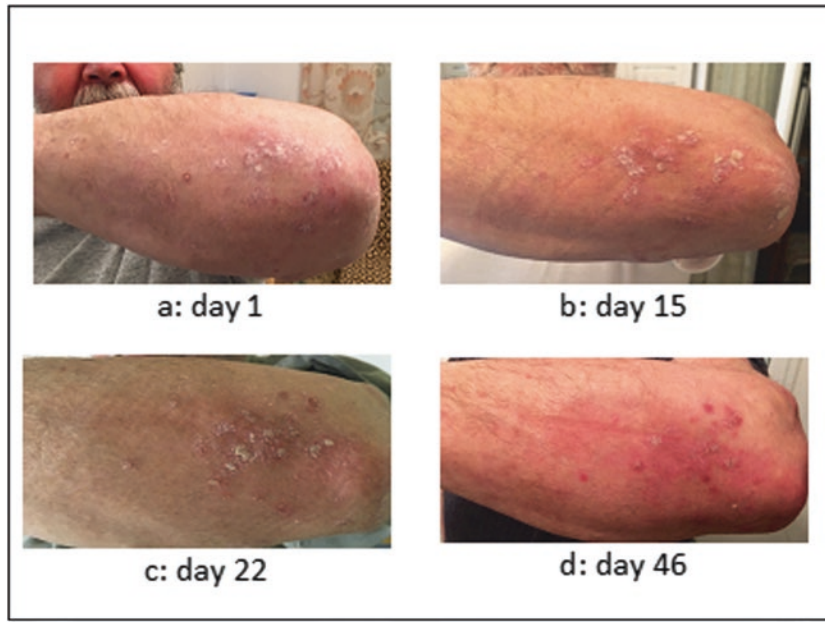

Fig. 1 46-day period of the left arm of the localized psoriasis patient lesions upon treatment with 1% N-bromotaurine solution. (**a**) Typical plaque-pustular presentation of psoriasis extending from elbow to lower arm prior to 1% TauNH-Br application, (**b**) after 2 weeks of 1% TauNH-Br topical application, a significant withdrawal mostly of pustular lesions was noticed, plaque lesions remained, but in a more localized fashion, patient suffered from itching and bleeding episodes during lesion regression, (**c**) a slow withdrawal of lesions was noticed as compared to the first 2 weeks. Patient complained for itching and bleeding episodes. Stable Produced Olive Oil (SPOO) topical application was introduced after the third week to alleviate discomfort (**d**), lesions continued to withdraw, and patient managed itching and bleeding episodes with SPOO application. After 6.5 weeks of 1% TauNH-Br topical application, almost all lesions including plaques had regressed. Patient consented to publication of images

ing episodes that were coupled with some bleeding occurrences at the lesions. Application of Stable Produced Olive Oil (SPOO), a natural antioxidant olive oil derived from molecular filtration of Extra Virgin Olive Oil (Kyriakopoulos 2014), after the third week of TauNH-Br treatment attenuated these adverse effects. When SPOO was smeared on dry skin surface, there was an immediate relief of itching that lasted for more than 4 h, skin softened and redrew from bleeding in minutes. Therefore, the therapy could be continued until the sixth to seventh week when nearly all lesions on the right arm regressed (Fig. 1d). SPOO was applied by simply smearing onto the lesions 5 min after each aerosol application of 1% TauNH-Br. Lesions should have been dried up from TauNH-Br solution to smear the oil. Between the third and fourth week, intolerable itching events stimulated the patient to use the oil for more than two times per day and between intervals of TauNH-Br application to mitigate these symptoms.

3.1.2 Right Arm: Treatment with 1% TauNH-Br, Surplus of Taurine Emulsion with SPOO

To overcome side effects encountered with the left arm and to make the TauNH-Br treatment more efficient for the right arm, an innovative formulation of TauNH-Br emulsion was applied. Briefly, a 2% TauNH-Br solution was prepared with a five times surplus of taurine in sterile and pyrogen-free water. This aqueous solution was mixed 1:1 with SPOO to make an emulsion and to reach the 1% TauNH-Br concentration previously tested on the left arm (Kyriakopoulos 2017, 2020). Separately, a 40 μM NaOBr solution in sterile and pyrogen-free water was prepared

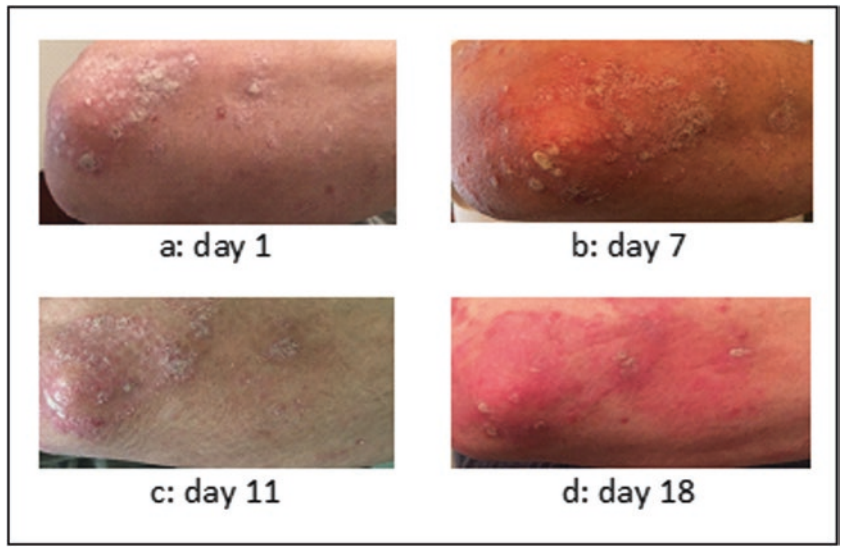

Fig. 2 18-day period of the right arm of localized psoriasis patient lesions upon treatment with 1% TauNH-Br, surplus of taurine emulsion with SPOO. (**a**) Right arm presentation with pustular-plaque psoriasis lesions prior to treatment, lesions were of equal extent and severity to the left arm, located mostly on elbows (**b**) pustular and plaque lesions regressed without any complications of itching and bleeding, NaOBr (40 μM) solution was introduced to the treatment protocol, (**c**) an accelerated and fine dissolving of psoriasis lesions was recorded. The patient did not complain for any side effects as lesions regressed, (**d**) an almost complete regression of lesions was recorded in a remarkably shorter time period needed for the left arm without any itching and bleeding episodes. Patient consented to publication of images

(Kyriakopoulos 2017, 2020). This solution was added to the treatment protocol for the right arm after 1 week of application of 1% TauNH-Br and surplus of taurine emulsion with SPOO.

For the first week of treatment, the patient sprayed three puffs (130 μl each, same volume as on the left arm) of 1% TauNH-Br and surplus of taurine emulsion with SPOO twice a day within 12-h intervals (Fig. 2a). From day 8 onwards, the patient also sprayed three puffs (130 μl each) of 40μM NaOBr solution after each TauNHBr/taurine/SPOO application as soon as the skin had completely dried up from the emulsion (Fig. 2b). This protocol was followed by the patient for the rest of the treatment period.

In less than 3 weeks of treatment with 1%TauNH-Br, surplus of taurine (approximately 5% taurine) emulsion with SPOO, and NaOBr solution (18 days), the psoriasis lesions almost completely regressed from the right elbow with minimized side effects encountered with TauNH-Br treatment of the left arm, as presented in Fig. 2c, d, leaving a slight erythema with edges on the previous psoriatic plaque area. Patient treatment of the right arm lasted for 18 days. Monitoring, however, continued for 3 months, and, remarkably, both arms remained free of psoriatic lesions.

3.2 The Generalized Psoriasis Case

Following the successful local treatment with TauNH-Br/taurine/SPOO emulsion with subsequent co-application of a mild NaOBr (40 μM) solution after 1 week on the right arm of the localized psoriasis case, a generalized psoriasis patient was selected to be treated with the same protocol. The patient was a 23-year-old female with numerous pustular lesions that disseminated to more than 50% of the whole body surface area some of which exceeded 1 × 1 cm. She had a mixed presentation of pustules and typical silvery plaques of extended area located on the upper and lower limbs. Also, psoriatic plaques covered most of the whole area of the right hand (Fig. 3a,

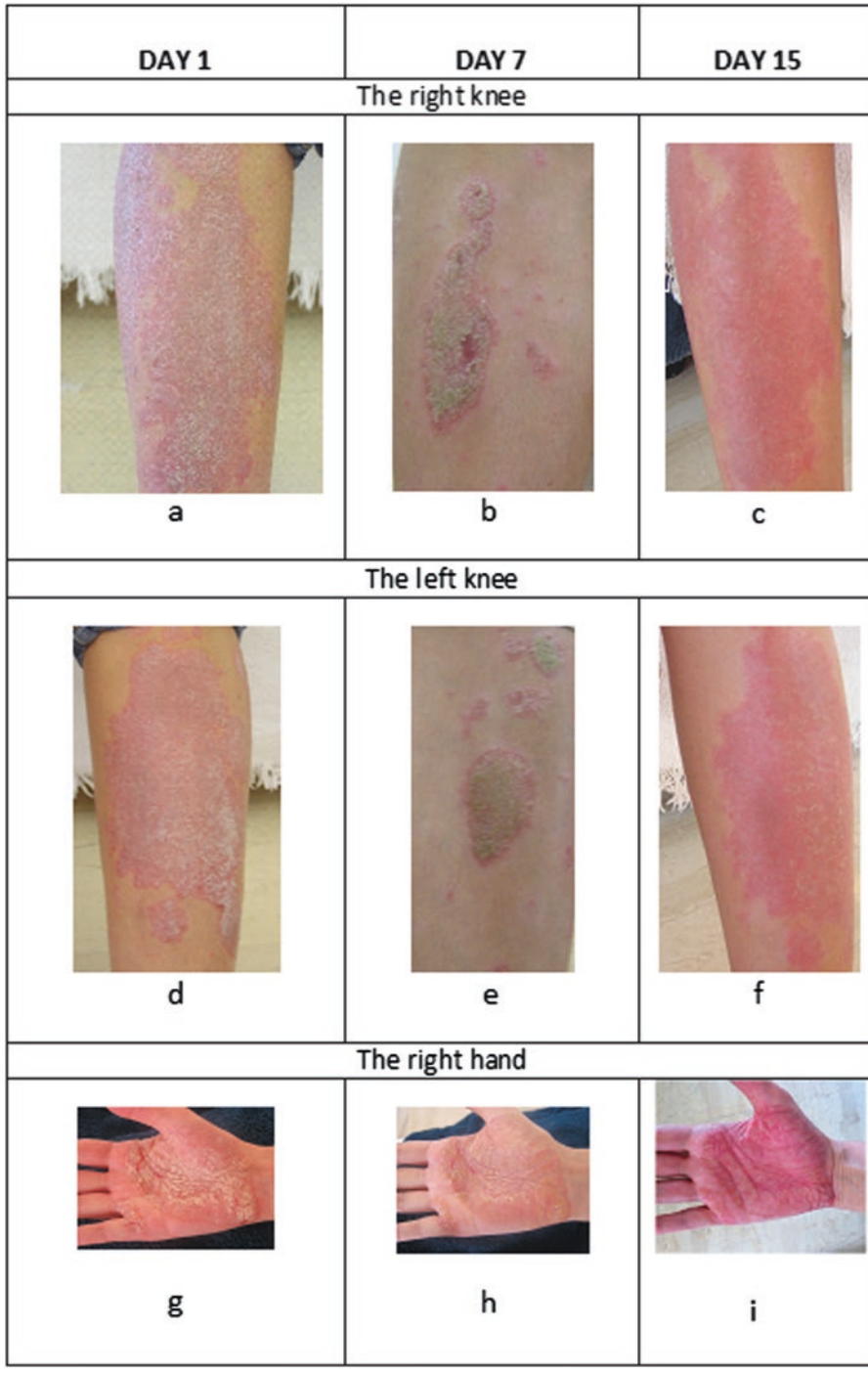

Fig. 3 15-day period of lesions of the knees and right hand of the general psoriasis patient upon treatment with 1% TauNH-Br, surplus of taurine emulsion with SPOO and subsequent application of 40 μM NaOBr solution. (**a**) The posterior surface of the right knee was covered almost in full with typical silvery unified psoriasis plaque prior to treatment; plaque was desquamating and erythematic; lesion has not been resolved by more than 2 years by any previous treatment; (**b**) significant withdrawal of lesions leaving white traces and ulcerative lesions, NaOBr co-administration commenced; (**c**) plaque and pustules have almost completely regressed leaving a slight erythema and

d, g). Severe lesions were also located on the neck, the abdomen, and the lower trunk (Fig. 4a, d, g).

The patient had received in the past systemic therapy and on regular intervals methotrexate 25–40 mg/week and biological factors, etanercept 50 mg twice per week for 6 weeks on a later stage and twice subcutaneous administrations of 90 mg ustekinumab, every 12 weeks. Local application of dexamethasone creams was performed, too. These treatments failed to improve her overall condition, and she relapsed.

The PWESI of the patient prior to local treatment with TauNH-Br and after 3 weeks of any other treatments previously received was scored 19 with a Weighted Total Extent Score of 6.50. The mean global severity score for this patient was rated 3, and the simplified PWESI score was rated 78. She gave written informed consent and was managed according to the Declaration of Helsinki. The patient was treated on most lesions with 1% TauNH-Br and 5% taurine emulsion with SPOO as it was used on the right arm of the local psoriasis patient. NaOBr solution was supplementary administered after the first week of treatment. Overall, treatment lasted for 2 weeks.

After the first week, all treated psoriasis lesions showed regression (Figs. 3b, e, h and 4b, e) with no complaints from the patient. The PWESI score of the patient after 1 week of treatment was reduced to 10.75 from 19 and the weighted total extend score to 4.75 from 6.5. However, transformation to ulcerative plaques occurred on both lower legs (Fig. 3b, e). After 2 weeks, further regression of the lesions was remarkable (Figs. 3c, f, i and 4c, f, h). Notably, the psoriatic plaques almost vanished in both arms and legs where, moreover, the ulceration had disappeared. The most evident result was noticed for the extinguished plaque of the right hand (Fig. 3i). Satisfaction was also best for the genital lesion regression where pictures were refused to be taken. The patient could not reach lesions at the back, which remained almost unaltered. The PWESI score after 2 weeks of treatment was reduced to 2.75, the weighted total extent score to 2, the mean global severity score to 1, and the simplified PWESI to 10. The therapeutic effect remained for more than 3 months, during which the patient was continuously monitored.

4 Discussion

The use of TauNH-Br as a local therapeutic agent against psoriatic lesions demonstrated a significant effect on withdrawal of pustules and plaques in both the localized and generalized psoriasis patient in this study. Due to a decrease of the therapeutic effect and occurrence of itching and bleeding adverse effects after 2 weeks, it was considered reasonable to provide a physical ointment to alleviate side effects. Indeed addition of SPOO allowed extending the TauNH-Br treatment for 48 days, for almost complete regression (Fig. 1a–d) in the localized psoriasis patient. Encouraged by this result, the formulation containing TauNHBr, taurine, and SPOO was used for comparison in a part of the lesions in the general psoriasis patient from the beginning and showed a significantly more rapid therapeutic effect with an almost complete regression within 14 days. Additional application of 40 μM NaOBr solution that commenced after the first week possibly contributed to the overall clinical improvement of this patient since more rapid healing was a consequence.

Fig. 3 (continued) condition completely tolerable by patient; (**d**) silvery plaque on left knee was thicker; bleeding episodes were regular especially at the edges of psoriasis lesion contact with the remaining of normal skin; (**e**) withdrawal of lesions leaving white traces and ulcerative lesions, co-application of 40 μM NaOBr solution commenced; (**f**) plaque and pustules have almost completely regressed leaving a slight erythema; (**g**) right hand was probably the most severe from psoriatic lesions, an extensive psoriatic plaque with prominent scaling, that remained resistant and as shown for more than 3 years, from all previous systemic treatments; (**h**) withdrawal of psoriasis plaque with minimal side effects, skin surface turned to smooth and skin gained elasticity for everyday activities for patient; (**i**) an almost complete withdrawal of plaque lesion was remarkable showing an evident therapeutic result. Patient consented to publication of images

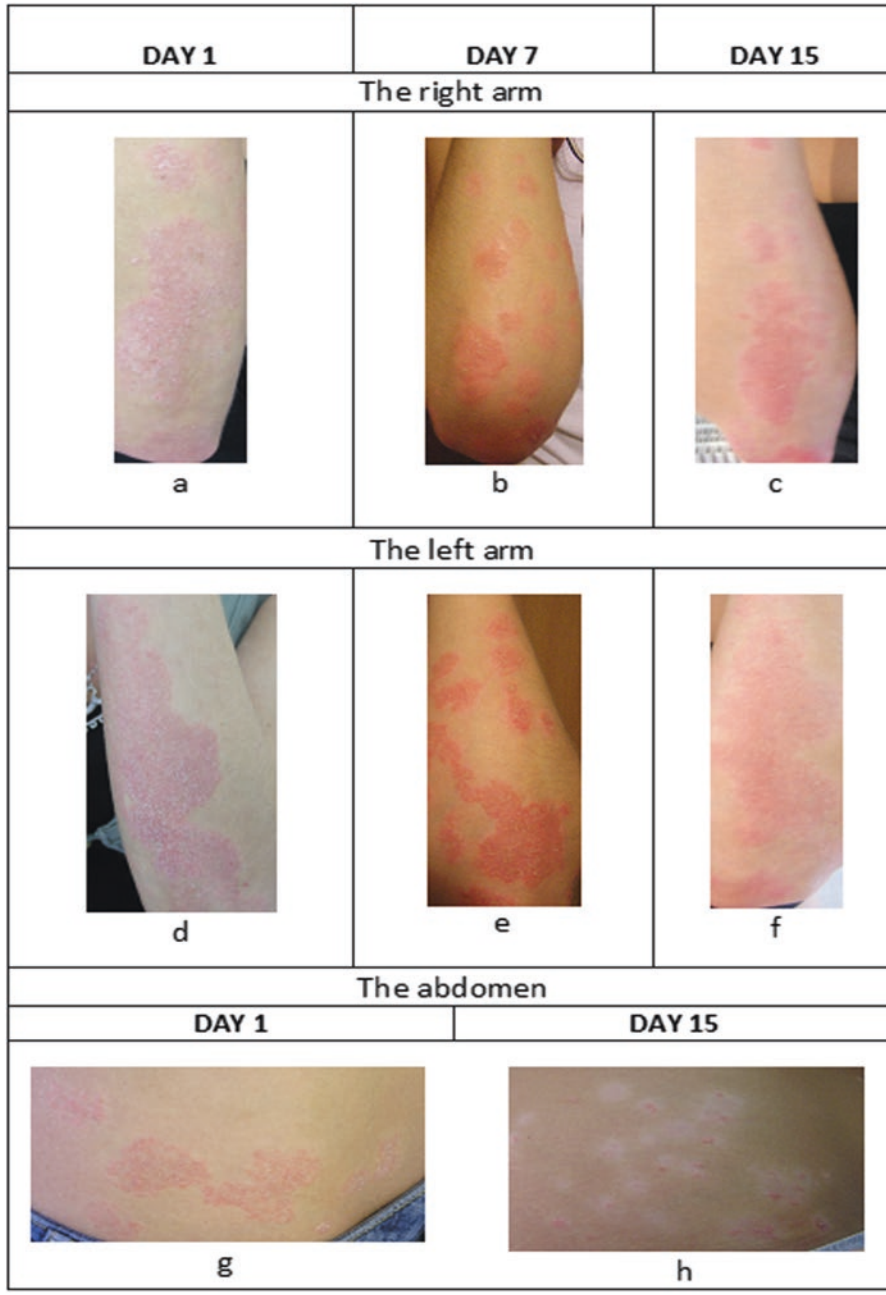

Fig. 4 15-day period of the arms and abdomen of general psoriasis patient lesions upon treatment with 1% TauNH-Br, surplus of taurine emulsion with SPOO, and subsequent application of 40 μM NaOBr solution. (**a**) Similar to the knees, the right lower arm was evidently covered with silvery plaque prior to treatment; (**b**) withdrawal of plaque that turned to erythematic; (**c**) significant withdrawal of plaque leaving a slight erythema after co-administration of 40μM NaOBr solution; (**d**) the left lower arm had an intense erythematic presentation with periodic rash episodes that turned to bleeding; (**e**) significant lesion withdrawal leaving some erythema spots, co-administration of NaOBr; (**f**) significant withdrawal of plaque leaving a slight erythema; (**g**) abdominal sides showed erythematic plaques that were considerably irritating for patient prior to treatment; (**h**) abdominal sides were cleared from erythematic plaque lesions leaving white spots on previous psoriasis areas. Patient consented to publication of images

The evident therapeutic effect of TauNH-Br observed in these psoriasis cases is considered to be attributed to both its anti-inflammatory (Kontny et al. 2007; Kyriakopoulos et al. 2016, 2017; Marcinkiewicz et al. 2008; Marcinkiewicz and Kontny 2014) and antiproliferative properties (Logotheti et al. 2016; Kyriakopoulos 2020). Moreover, TauNH-Br downregulates tumor necrosis factor alpha, prostaglandin E2, interleukin 12, nitric oxide, and reactive oxygen species, while heme oxygenase-1 is upregulated (Olszanecki et al. 2008). Together with its inhibition of cell-cycle progression, which was found in skin cancer mouse carcinogenesis model (Logotheti et al. 2016), TauHNBr obviously also blocks the enhanced immune reaction in psoriasis as results showed. In addition, the potent broad-spectrum microbicidal activity of TauNH-Br (Gottardi et al. 2014; Walczewska et al. 2017) as an antiseptic may have contributed significantly as the psoriasis pathogenesis is linked to the microbial colonization and chronic infection (Barnes and Adcock 2009; Balci et al. 2009; Marcus et al. 2011). As TauNH-Br – remarkably at a higher concentration than for the bactericidal effect (Gottardi et al. 2014; Walczewska et al. 2017) – has a direct cytotoxic activity to produce mutagenic nucleobases, it may be also lethal for rapidly dividing skin cells in psoriasis (Henderson et al. 2001; Krokan et al. 2002). Nevertheless, specific studies will be needed to evaluate the contribution of each of these properties of TauHNBr to the therapeutic effect in psoriasis.

Taurine is a sulfur containing amino acid. Epidermal retention of sulfur containing compounds has been substantiated to be involved to the pathogenesis of psoriasis (Lipnik and Levy 1959) by means of sulfur accumulation of methionine in psoriatic over normal or over psoriatic skin cleared from lesions. In this early experimentation, it was strikingly shown that psoriatic skin lesions retain the sulfur containing methionine for a considerable time over the normal areas of skin in the same individuals. It has been then hypothesized by Roe 1962, that "Effective therapy might require a stimulus to the catabolism of such substances on the skin and the induction of their excretion from the body." The accumulated methionine in psoriasis skin is subject to the increased presence of hydrogen peroxide (H_2O_2) as the catalase activity decrease with increased severity of the disease (Kadam et al. 2010). Methionine when reacting with H_2O_2 generates methionine sulfoxides. These are associated with age-related diseases, neurodegeneration, and shorter life span (Moskovitz 2005) and may have a worsening effect on psoriasis lesions. TauNH-Br may reduce these sulfoxides on psoriatic skin by activating heme oxygenase-1 (HO-1) (Kyriakopoulos et al. 2017; Olszanecki et al. 2008; Walczewska et al. 2017). Since the HO-1 draws electrons from P450 oxido-reductases and triggers formation of NADPH (Sugishima et al. 2014), this may be important for the therapeutic effect of TauNH-Br on psoriasis. In psoriasis skin, NADPH is essential to reduce the toxic methionine sulfoxide proteins to nontoxic methionine proteins and hence to be able to revert to normal the methionine and other sulfur amino acid metabolism in psoriatic skin (Kyriakopoulos et al. 2017; Moskovitz 2005; Sakagami et al. 2001; Sugishima et al. 2014). Therefore, in Fig. 5, a mechanism of the therapeutic effect seen by TauNH-Br on alleviation of psoriasis lesions due to reversal of methionine sulfoxide accumulation is hypothesized and presented accordingly.

Due to the combined application of several agents in our patients, it is presently impossible to reliably estimate the contribution of the single components of treatment to the therapeutic effect. This has to be investigated in future studies. Nevertheless, the additional components of the combination therapy consisted of (a) TauHN-Br, (b) taurine, (c) stable produced olive oil, and (d) separate use of NaOBr, and these were used due to their following properties. Apart from the aforementioned activity of TauNH-Br, the addition of taurine was founded in its ability to scavenge free oxygen (O_2^-) radicals (Hanna et al. 2004), which may have enhanced the anti-inflammatory effect. Notably, the TauNH-Br antiproliferative activity was significantly enhanced by the presence of taurine and subsequent addition of NaOBr during cancer cell culturing with no evident cytotoxicity

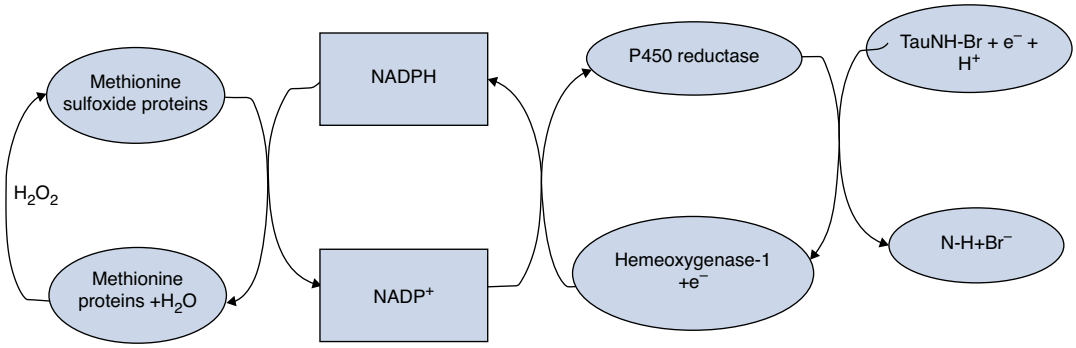

Fig. 5 Methionine metabolism is being restored by N-bromotaurine. The liberation of bromide anions from the reduction of oxidative TauNH-Br may provide the necessary stimulus by electron transport from P450 reductase to induce heme oxygenase-1. NADPH produced may convert methionine sulfoxides to methionine. (Moskovitz 2005; Sakagami et al. 2001; Sugishima et al. 2014)

to normal cells (Kyriakopoulos 2020). NaOBr, when applied after the other components in the psoriasis patient lesions, may have elevated the level of active bromine (Henderson et al. 2001) over a longer period between dosing, which may be important, particularly in severe cases of intense scaling.

The adverse reactions of TauNH-Br on psoriatic tissue (itching and bleeding) recorded during therapy may be due to its oxidative mechanism of action and high reactivity with proteinaceous material (De Carvalho Bertozo et al. 2016; Gottardi et al. 2014; Henderson et al. 2001). To treat these irritating effects, the novel formulation of olive oil was used (Kyriakopoulos 2014). The Extra Virgin Olive Oil (EVOO) is a unique mixture of lipophilic and lipophobic antioxidants (Bendini et al. 2007). Due to self auto-oxidation and instability of antioxidant molecule concentrations in time, however, its use for pharmaceutical applications is bounded (Krichene et al. 2015; Psomiadou and Tsimidou 2002). By the innovative molecular filtration of EVOO, the final oil (SPOO) retains the concentration of antioxidants rendering it ideal for use in medicinal preparations (Kyriakopoulos 2014). Complementary, the application of SPOO with TauNH-Br was recorded to have restored the cracking of the skin that caused bleeding of psoriasis lesions and to have withdrawn itching during intense episodes that occurred instantaneously even at night sleeping. As the TauNH-Br has only a low potential to react with linoleic acid (De Carvalho Bertozo et al. 2016), it is supposed that it will not be consumed by SPOO. Therefore, it is considered that the synergy of oxidative and antioxidative activity of the components should have led to a good therapeutic effect and alleviation of side effects at the same time.

5 Conclusion

An aberrant balance between reactive oxygen species, nitric oxide, and HO-1 activity exists in psoriasis. Indicatively, this may result in abnormal proliferation of epidermal cells and conservation of the chronic inflammatory state in the skin (Wojas-Pelc and Marcinkiewicz 2007). The clinical response to the combination of TauHN-Br, taurine, SPOO, and NaOBr indicates restoration of the imbalance of oxidants and antioxidants in psoriatic skin, and this requires further laboratory and clinical investigation. Particularly remarkable is the fact that there was no relapse of psoriatic lesions in both patients for a monitoring period of at least 3 months. Since the remnant oxidative effect after topical application of active halogen compounds at maximum lasts only for a few hours (Gottardi and Karl 1990), a sustained change in the disordered immune state of psoriasis may be speculated. Overall, the short period to psoriatic lesion regression and the remarkable durability of the therapeutic effect after termina-

tion of treatment in this study indicate a clinically meaningful improvement of disease by the combination of agents used. This was tested in patients with a disease nonresponsive to other therapies including systemic MTX and biologicals. The localized treatment presented is of considerable lower toxicity compared to systemic therapies, which appears to be a great advantage. Systematic studies to further elucidate the mechanisms of action and the therapeutic effect of bromotaurine, taurine, and olive oil in psoriasis are strongly indicated.

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Conflict of Interest The authors state no conflict of interest.

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Taurine Deficiency in Tissues Aggravates Radiation-Induced Gastrointestinal Syndrome

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Keywords

TauTKO mice · Radiation damage · Crypt stem cells · PCNA cells · Gastrointestinal syndrome

Abbreviations

PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
TauT	Taurine transporter

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1 Introduction

Exposure to ionizing radiation generates reactive oxygen species (ROS) and free radicals, which, in turn, can cause oxidative stress in the irradiated 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). Free radicals can cause double-stranded breaks (DSBs) and single-stranded breaks (SSBs) in DNA, promote apoptosis and modulate inflammatory processes (Duan et al. 2017; Smith et al. 2017). Radiation-induced injuries can easily induce DSBs and SSBs in organs such as the intestines, bone marrow, and skin. Radioprotective agents provide protection by removing free radicals and have been studied for a long time for their ability to reduce cell damage caused by free radicals in normal tissues (Weiss and Landauer 2009; Poggi et al. 2001).

Research on the radioprotective effect of taurine has been carried out since the 1960s (Sugahara et al. 1969), and the physiological roles of taurine include antioxidant activity and protection against ROS and free radical formation (Johnson et al. 2012). Thus, taurine appears to be an attractive candidate for use as a radioprotector and radiation mitigator. We previously reported that taurine can promote recovery from

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radiation-induced injuries (Yamashita et al. 2017). However, at present, the effect of taurine on radiation-induced injuries remains poorly understood.

Taurine is taken up by cells via the taurine transporter (TauT) (Kwon and Handler 1995). In TauT KO mice that lack the TauT gene, taurine levels are markedly decreased in several tissues. Compared to wild-type mice, taurine levels in TauT KO mice are decreased by 96–98%, in skeletal and cardiac muscle, and by 70–90% in the brain, kidney, and liver (Ito et al. 2010; Heller-Stilb et al. 2002). Taurine depletion is likely harmful because it blocks the recovery of physiological functions that depend on cellular growth, the immune system, and intestinal mucosa function.

Exposure to ionizing radiation causes injury to organs containing rapidly proliferating cells, resulting in acute radiation syndromes, such as hematopoietic syndrome and gastrointestinal syndrome (Suman et al. 2012). Gastrointestinal syndrome leads to death within 10–12 days after ionizing radiation exposure (Rosen et al. 2015). A loss of villus epithelial cells or crypt stem cells has been suggested as the potential cause of gastrointestinal syndrome (Qiu et al. 2008). Taurine has been reported to be essential for optimal proliferation, development, and maturation of brain cells. Furthermore, taurine increases neural stem/progenitor cell proliferation in the developing brain (Shivaraj et al. 2012). Thus, taurine might be involved in the proliferation of stem cells. Tissue depletion of taurine might be associated with the impairment of the proliferating ability of intestinal stem cells after X-irradiation. Therefore, it is of interest to investigate whether knockout of the taurine transporter in mice aggravates radiation-induced gastrointestinal syndrome. In this study, we evaluated the role of taurine on survival, the crypt-villus structure in the small intestine, and the proliferation of proliferating cell nuclear antigen (PCNA)+ cells in the small intestine using a taurine-deficient mouse model generated by knocking out the taurine transporter (Ito et al. 2008).

2 Methods

2.1 Animals

TauTKO (TauT−/−) mice and their wild-type (TauT+/+) littermates were obtained by breeding heterozygous (Tau+/−) males and females. Female, 6-week-old TauTKO mice and WT littermates were handled according to the Guidelines for the Regulation of Animals, from the Animal Ethics Committee of Suzuka University of Medical Science (Suzuka, Mie-ken, Japan). The animals were maintained under controlled conditions at 22 ± 3 °C with 65 ± 5% relative humidity and a 12 h light/dark cycle (light from 08:00 to 20:00).

2.2 X-Irradiation of Mice

Mice were placed in well-ventilated boxes (five mice in each box) and irradiated with 5 Gy of whole-body irradiation at a dose rate of 0.331 Gy/min at 200 kV and 9 mA (Phillips MG226, Tokyo, Japan). The beam was filtered through a 0.2 mm copper and 1 mm aluminum board. After irradiation, the mice were returned to their cages and maintained on food and water ad libitum.

2.3 Survival Studies

Two groups of 10 mice each were used in the survival experiments. Mice were exposed to whole-body X-irradiation (5 Gy/mouse) and then monitored continuously for a period of 30 days for survival and apparent behavioral deficits.

2.4 Immunohistochemical Studies

TauTKO (TauT−/−) mice and wild-type (TauT+/+) mice exposed to 5 Gy of radiation were killed on day 3 ($n = 3$ per group) for immunochemical analysis of the small intestine. After sacrifice, the small intestines were removed, fixed with 3.7% paraformaldehyde overnight,

and then embedded in paraffin. Then, 7 μm thick paraffin sections were stained with hematoxylin and eosin (H&E). Rabbit taurine-specific antibodies were prepared as described previously (Ma et al. 1994). A PCNA antibody (bs-2007R) was obtained from Bioss Inc. (Woburn, MA, USA). PCNA antibody immunoreactivity in intestinal sections from the mice was observed using the peroxidase anti-peroxidase (PAP) method. Briefly, paraffin sections (6 μm thick) were incubated with rabbit anti-PCNA polyclonal antibody (2 μg/ml) overnight at room temperature. Then, the sections were incubated with a goat antibody against rabbit IgG (1:200) for 2 h, followed by peroxidase anti-peroxidase complex (1:200) for 2 h. The sections were incubated for 10 min at RT with 3, 3′-diaminobenzidine tetrahydrochloride as a chromogen that had been freshly prepared as a 20 mg solution in 100 ml of PBS containing 0.01% H_2O_2. Images of the developed tissue sections were captured using an optical microscope (Olympus, Tokyo, Japan).

2.5 Statistical Analysis

Means were compared using the t-test for two-group comparisons. Survival was assessed by the Kaplan Meier method. Data are expressed as the means ± SEM. For all tests, significance was set at $P < 0.05$.

3 Results

3.1 Tissue Depletion of Taurine Decreased the Survival Rate of Mice After X-Irradiation

Exposure to high-dose radiation affects the gastrointestinal system. To investigate whether tissue depletion of taurine aggravates radiation-induced gastrointestinal syndrome, the 30-day survival of TauT+/+ and TauT−/− mice following 5 Gy of whole-body X-ray irradiation was analyzed (Fig. 1). The survival rate of mice in the TauT−/− group was significantly lower than that of the TauT+/+ group. Moreover, the bodyweight of mice in the TauT−/− group was reduced rapidly. These results suggested that the intestinal epithelium of TauT−/− mice was more sensitive to radiation, causing lethal gastrointestinal syndrome.

3.2 Tissue Depletion of Taurine Aggravates Radiation-Induced Gastrointestinal Syndrome

Radiation-induced gastrointestinal syndrome is primarily caused by the death of epithelial stem cells in the crypts of the small intestine (Ghosh et al. 2012). In this study, the blood feces of mice in the TauT−/− group was observed. Therefore, H&E staining was performed to assess the damage in the small intestine after X-irradiation (Fig. 2a). The villi in the small intestines of both TauT+/+ and TauT−/− mice 3 days after irradiation were shorter than those in normal nonirradiated mice. Moreover, the villi in the small intestine of TauT−/− mice were significantly shorter than those of TauT+/+ mice. Mice in the TauT−/− group exhibited significant, severe villous epithelial atrophy and a loss of normal crypt architecture (Fig. 2b).

3.3 Tissue Depletion of Taurine Decrease the Number of Proliferating Cells in Crypts After X-Irradiation

Intestinal stem cells are indispensable for intestinal regeneration following radiation exposure. Staining for PCNA was performed to assess the proliferation ability of intestinal stem cells after X-irradiation (Fig. 3a). The results showed that the numbers of PCNA+ cells in TauT+/+ mice and TauT−/− mice were lower than the numbers in normal nonirradiated mice. Moreover, the numbers of PCNA+ cells in the TauT−/− group were significantly lower than those in the TauT+/+ group. These data show that the loss of TauT and taurine in TauT−/− mouse resulted in reducing the number of epithelial proliferating cells in the villi.

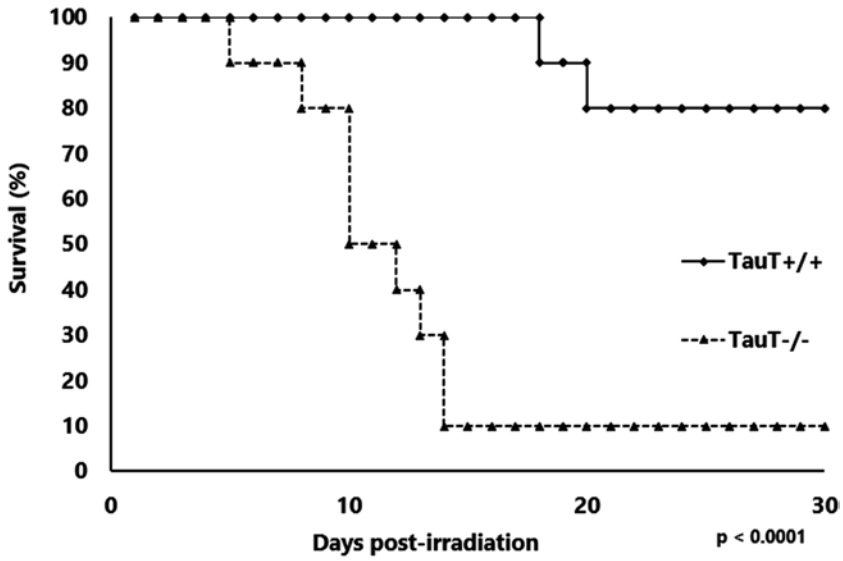

Fig. 1 Effect of TauT knockout on the survival rates of mice after exposure to whole-body X-ray irradiation (5 Gy/mouse). Kaplan Meier survival curve of TauT+/+ and TauT−/− mice after X-ray irradiation. Female mice (n = 10 mice per group) were treated with 5 Gy of whole-body X-ray irradiation and monitored continuously for 30 days to determine the survival rates. Data are expressed as the percentage of surviving mice. The survival rate of the TauT−/− group was significantly lower than that of the TauT+/+ group

4 Discussion

The mechanisms underlying radiation-induced cell damage are complex and varied and mainly include ROS, DNA damage, inflammation, and oxidative stress. ROS destroy large molecules in cells, such as DNA, proteins, and lipids, leading to cell necrosis and apoptosis (Chen et al. 2012). Radiation exposure can injure hematopoietic and gastrointestinal systems, depending on the dose of radiation received (Suman et al. 2012). The morphological changes that occur in the intestinal mucosa after high-dose radiation exposure have been well documented (Driák et al. 2008; Labéjof et al. 2002). However, the molecular events that regulate the radiosensitivity of the intestinal epithelial cells and radiation-induced gastrointestinal syndrome are not fully understood (Li et al. 2015).

Taurine (2-aminoethanesulfonic acid) is a major intracellular amino acid with several important functions, including antioxidant and anti-inflammatory activities (Oliveira et al. 2010; Ma et al. 2010; Kato et al. 2015). Taurine is taken up by cells via taurine transporter. Therefore, loss of the taurine transporter can aggravate radiation-induced gastrointestinal syndrome. We previously reported the critical role of taurine by showing that increasing the expression of taurine transporter had a mitigating effect on radiation exposure (Yamashita et al. 2019). Here, we explored the effect of taurine on radiation-induced intestinal injury to determine whether tissue depletion of taurine aggravates radiation-induced gastrointestinal syndrome. To this end, we established mouse models of radiation-induced gastrointestinal syndrome in TauT+/+ and TauT−/− mice by exposure to whole-body X-irradiation. The survival rate of the TauT−/− mice was significantly lower than that of the TauT+/+ mice. These results suggested that the intestinal epithelium in TauT−/− mice was more sensitive to radiation, causing lethal gastrointestinal syndrome. This result is most likely due to incomplete recovery from intestinal injury and the death of epithelial stem cells in the crypts. Therefore, we examined the crypt-villus structure of the small intestine and the proliferation of PCNA+ cells in the small intestine.

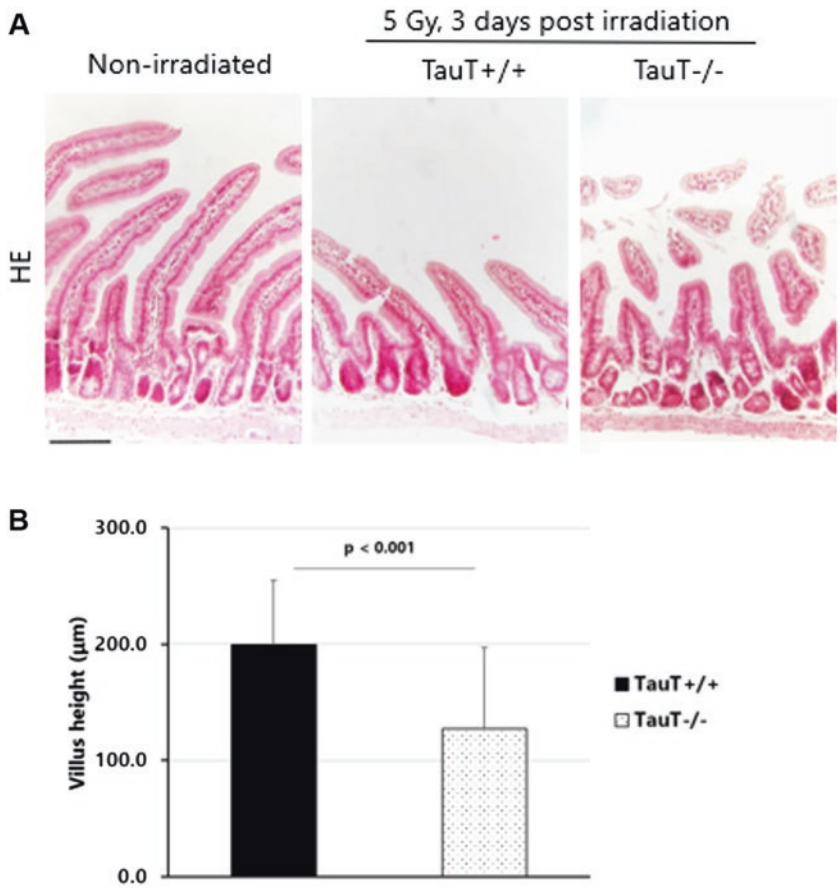

Fig. 2 Histological analysis of intestinal injury in mice after 5 Gy of whole-body X-ray irradiation. (**a**) Representative intestinal sections (top panel) stained with H&E (200×) at 3 days postirradiation. Scale bar = 100 μm. The villi in the small intestine of mice in the TauT+/+ and TauT−/− groups were shorter than those in the normal, nonirradiated small intestine of control mice. (**b**) Compared to Tau+/+ mice, TauT−/− mice showed significant villous epithelial atrophy ($p < 0.001$). Data are shown as means ± SE

Radiation exposure inflicted severe damage to the villi in the small intestine. This evidence proved that failure to absorb nutrients can affect the metabolic function after X-irradiation. Depletion of taurine transporter in the small intestine, and the resulting taurine depletion, reduced the number of proliferating cells in the crypts. Taurine deficiency can aggravate the damage in intestinal epithelium in TauT−/− mouse, and taurine deficiency can also enhance the irradiation damage in intestinal epithelium. The severe pathological damage was observed in TauT−/− mouse intestine after X-irradiation, while PCNA+ cells were reduced, which proves taurine can also affect regeneration after irradiation damage. Exposure to ionizing radiation induces apoptotic cells, which are associated with a loss of villus epithelial cells and crypt stem cells. Yang (2017) reported that taurine reduced the percentage of apoptotic spermatocyte-derived GC-2 cells after exposure to ionizing radiation. Taurine also significantly suppressed UVB-induced apoptosis in lens epithelial cells (Dayang and Dongbo 2017). Thus, the protective effect of taurine against organ damage may stem from its ability to suppress oxidative stress and apoptotic responses (Nagai et al. 2016).

Taurine prevents arsenic-induced oxidative stress and apoptotic damage by inhibiting JNK signaling pathways (Ghosh et al. 2009; Das

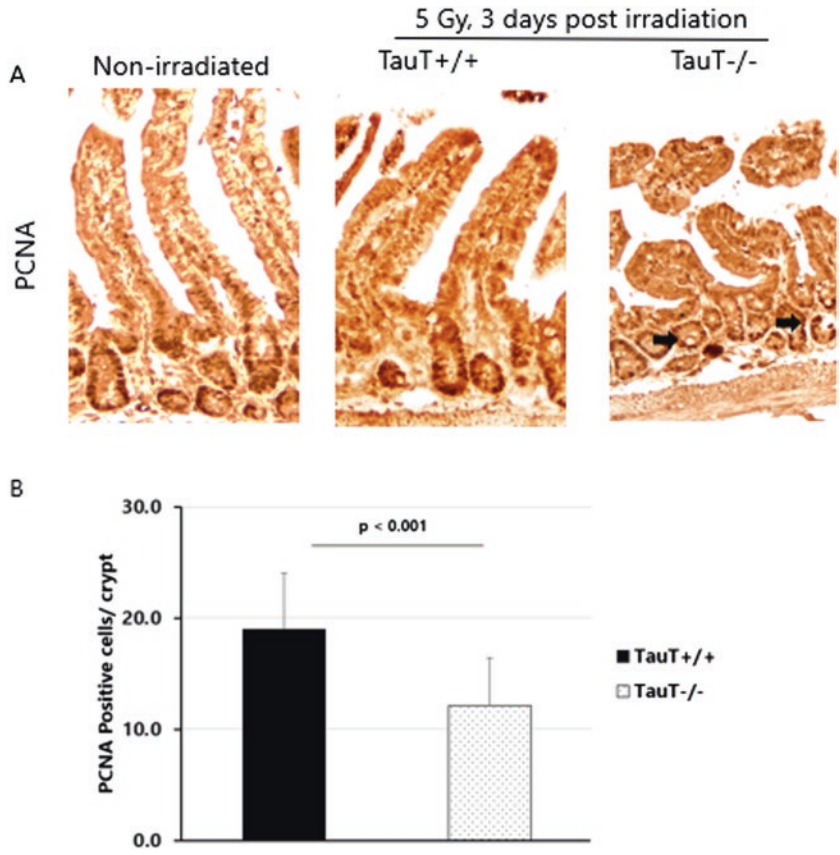

Fig. 3 Histological analysis of the proliferation ability of intestinal stem cells in mice after 5 Gy of whole-body X-ray irradiation. (**a**) Representative immunohistochemical images of PCNA stained sections of the small intestine (200×). (**b**) At 3 days postirradiation, the numbers of PCNA+ cells in TauT−/− mice were significantly lower than those in TauT+/+ mice ($p < 0.001$). Data are shown as means ± SE

et al. 2010). Jun N-terminal kinase (JNK) is one of the three main members of the mitogen-activated protein kinase (MAPK) superfamily (Gururajan et al. 2005). JNK is activated in response to certain stresses, such as γ radiation, UV-C, and arsenic, and stress-induced activation of JNK leads to cell death through activation of the mitochondrial apoptotic pathway (Chen et al. 1996).

It was reported that taurine deficiency reduces life span by promoting mitochondrial-dependent and ER stress-mediated apoptosis (Jong et al. 2017). Taurine depletion causes cardiomyocyte atrophy, mitochondrial and myofiber damage, and cardiac dysfunction (Ito et al. 2008). Schaffer et al. (2009) reported that taurine may inhibit the production of ROS by regulating mitochondrial function. Thus, taurine might contribute to the recovery from radiation-induced gastrointestinal syndrome by regulating mitochondria, and JNK signaling pathways.

The results observed in the TauT−/− mice suggest that taurine plays a role in protecting against radiation-induced gastrointestinal syndrome. Taurine modulates the kinetics of crypt cell proliferation, reduces radiation-induced DNA damage, and promotes crypt regeneration.

Our data demonstrate that taurine transporter and taurine are important factors in the radiation response of normal tissue.

5 Conclusion

This study showed that tissue depletion of taurine aggravates radiation-induced gastrointestinal syndrome in mice. The results observed in the radiation-exposed TauT−/− mice indicate that taurine and taurine transporter are important factors in the radiation response of normal tissue. Taurine modulates crypt cell proliferation kinetics, reduces radiation-induced villus structural damage, and promotes crypt regeneration. Our data demonstrate that taurine is a key regulator of crypt stem cells that has important roles in intestinal cell proliferation and survival.

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Taurine and Its Anticancer Functions: In Vivo and In Vitro Study

Ning Ma, Feng He, Jun Kawanokuchi, Guifeng Wang, and Takenori Yamashita

Keywords

Taurine · Anticancer · Azomethane · Ki-67 · PTEN · PUMA

Abbreviations

Akt	Phosphorylated protein kinase B
AOM	Azomethane
CDDP	Cisplatin
COX-2	Cyclooxygenase
CTX	Cyclophosphamide
DOX	Doxorubicin
FAS	Fatty acid synthase
IFO	Ifosfamide
MST1	Human mammalian sterile 20-like kinases 1
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
PUMA	p53-upregulated modulator of apoptosis
QOL	Quality of life
TauT	Taurine transporter
YAP	Caspase-3 and downregulated yes-associated protein

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1 Introduction

Since ancient times, natural products have been used in traditional medicine to prevent and treat various diseases, including cancer. Taurine (2-aminoethanesulfonic acid) is a natural amino acid that is found widely in all mammalian tissues. It is crucial for proper function of the central nervous system (Kilb and Fukuda 2017), retinal neurons (Gaucher et al. 2012; Schaffer et al. 2010), and cardiac and skeletal muscle (Ito et al. 2010; Khalil et al. 2017). Several studies have demonstrated that taurine has anti-inflammatory (Marcinkiewicz and Kontny 2012), antioxidant (Shimada et al. 2015), and hypoglycemic (Pandya et al. 2017) effects. Recently, taurine not only mitigates the side effects of

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chemotherapy but also possesses antitumor properties and has been shown to inhibit proliferation and induce apoptosis in certain cancers by differentially regulating proapoptotic and antiapoptotic proteins (Zhang et al. 2014a, c). The antitumor study of taurine is still in its infancy, and the mechanism of its antitumor effect is not fully understood. In this mini review, we summarize the main effects of taurine that have shown suppressing actions in the initiation and progression of cancers. The underlying molecular mechanism was also elucidated confirming that taurine can provide to provide evidence of the potential clinical application to taurine in tumor therapy.

Its biological functions are to maintain osmotic pressure balance, stabilize cell membrane, lower blood sugar, prevent tissue ischemia-reperfusion injury, improve immunity, regulate lipid secretion, protect myocardial cells, and antioxidation. With the in-depth study of different biological functions of taurine, we found that many systemic diseases are associated with taurine (Redmond et al. 1998; Ito et al. 2011; El Idrissi et al. 2009). It is undeniable that the research on the antitumor effect of taurine is still in the primary stage. In this review, the research progress of taurine's antitumor effect is briefly summarized.

2 Taurine as a Metabolomic Marker of Malignant Tumorigenesis

Metabolomics is a complete set of metabolites that provide potential information by measuring and quantifying the end products of cell metabolism. The disorder of any cell process will appear with the change of metabolite level. Therefore, the differential expression of metabolites between normal people and cancer patients is of great significance for the early diagnosis of diseases and the discovery of metabolomic biomarkers. Therefore, the identification of differentially expressed metabolites between noncancer and cancer patients is of great significance for the early diagnosis and treatment of diseases as well as metabolomic biomarker discovery.

Agouza's study found that the serum taurine level of breast cancer patients was significantly lower than that of high-risk breast cancer patients and patients with benign breast lesions. In addition, the study also confirmed that the determination of serum taurine level in high-risk patients has important value in the early diagnosis of breast cancer (El Agouza et al. 2011). A study of 50 patients with irregular uterine bleeding found that the serum taurine level in patients with endometrial cancer was significantly lower than that in healthy patients, suggesting that the detection of serum taurine level in patients with irregular uterine bleeding may be helpful for the early diagnosis of endometrial cancer (Refai et al. 2019). Therefore, the identification of differentially expressed metabolites between noncancer and cancer patients is of great significance for the early diagnosis and treatment of diseases as well as metabolomic biomarker discovery.

3 Antitumor Effect of Taurine

3.1 Taurine Mitigates Side Effects on Cancer Chemotherapy

The efficacy of the existing antitumor drugs is significant. However, the adverse reactions cannot be ignored. For example, ifosfamide (IFO) and cyclophosphamide (CTX) are common clinical antitumor drugs with severe side effects. IFO and CTX can cause myelosuppression and cisplatin (CDDP)-induced gastrointestinal toxicity.

Therefore, it is urgently needed to find ancillary drugs which have synergism and attenuation effects on IFO and CTX. Taurine maintains the balance of the internal environment by reducing the excretion of electrolytes, albumin, and glucose, and increasing the clearance of creatinine, which significantly improves the nephrotoxicity caused by IFO. In addition, the use of taurine not only maintains the antitumor activity of IFO but also alleviates weight loss due to treatment with IFO and could result in lower mortality in mice study (Badary 1998). Zhao et al. also reported that co-treatment with CTX and taurine in S180-bearing mice could synergistically exert antitumor

effects and attenuation of the side effects of CTX by increasing the activity of immune-related cells (Zhao et al. 2009). Some research groups found that after using taurine and CTX in S180 tumor-bearing mice, taurine enhanced the antitumor effect of CTX and improved the adverse reactions of CTX by inhibiting bone marrow proliferation and immune system, suggesting that taurine had synergistic and attenuating effects on CTX (Zhang et al. 1997). Kim et al. study revealed that co-treatment of cisplatin with taurine significantly increased cervical cancer cells apoptosis significantly better than taurine or cisplatin alone. This effect may be related to the activation of p53 and caspases (Kim and Kim 2013).

In our previous study, we investigated the effects of taurine on cisplatin (CDDP)-induced acute nephrotoxicity (Tsunekawa et al. 2017) by immunohistochemical methods. A single intraperitoneal injection of CDDP (15 ~ 25 mg/kg) deteriorated the kidney functions as reflected by histopathological changes. These changes were observed in all CDDP groups. In the CDDP group, oxidative stress was evident in the cisplatin group by observing an increase in 8-OHdG expression, an indicator of oxidative DNA damage. CDDP also resulted in an increase of CD68 expression in the renal tissues of CDDP groups. Taurine transporter (TauT) was downregulated, and p53 was upregulated in renal tissues as indicated by immunohistochemical analysis. Administration with taurine prior to a CDDP injection was able to protect against deterioration of kidney function, abrogate the decline of antioxidant, and suppress the DNA damage. Moreover, taurine inhibited p53 activation and improved the pathological changes induced by CDDP. This study demonstrates the protective effects of taurine in attenuating the expression of pro-inflammatory mediators and improving antioxidant capacity in the kidney of CDDP-injected rats. Thus, taurine could be a beneficial dietary supplement to attenuate CDDP-induced nephrotoxicity in vivo (Tsunekawa et al. 2017).

Severe inflammation can be induced by azomethane (AOM) and sodium sulfate (DSS), which leads to colonic epithelial cancer. Thereby they can be used in an inflammation-related carcinogenic mouse model for colon carcinogenesis study. Our study also indicated that taurine significantly inhibited AOM + DSS-induced tumor formation, the increase of cleaved caspase-9 level, and the decrease of Ki-67 level, a marker of cell proliferation, in mouse colon tissues (Wang et al. 2020). The intensive Ki-67 immunoreactivities were observed in a large proportion of colon cancer cells in the AOM-DSS model mice. Ki-67 was expressed in the nuclei of cancer cells and showed relatively weak Ki-67 immunoreactivities in taurine administration mice (Fig. 1). Cyclooxygenase (COX-2), an inflammation marker, was observed in the cytoplasm of epithelial cell. The immunoreactivities of COX-2 in control mice showed weak staining. COX-2 expression in the AOM-DSS model mice colon was much higher than that in the AOM-DSS taurine administration mice (Fig. 2). It is concluded that taurine not only reduces the side effects caused by antitumor drugs but also inhibits tumor inducers.

3.2 Taurine Inhibits Tumor Growth by Improving Antioxidant Capacity

Doxorubicin (DOX) is used to treat many types of cancer, includes breast cancer, bladder cancer, Kaposi's sarcoma, lymphoma, and acute lymphocytic leukemia (Johnson-Arbor and Dubey 2021). However, the clinical application of doxorubicin is compromised by its serious side effects, including cardiotoxicity and nephrotoxicity (Songbo et al. 2019). Sadzuka et al. (2009) reported that taurine did not affect the DOX influx into M5076 ovarian sarcoma cells and inhibited DOX efflux significantly, which maintained the DOX level in tumor cells. Taurine decreased tumor weight by 40% compared with the DOX alone group and significantly increased its antitumor effect. Taurine did not increase DOX concentration in normal tissue; it is suggested that it increased the antitumor effect without enhancing DOX-induced adverse effects. DOX efflux was inhibited by beta-alanine as a taurine transporter inhibitor. Therefore, enhancement of the DOX

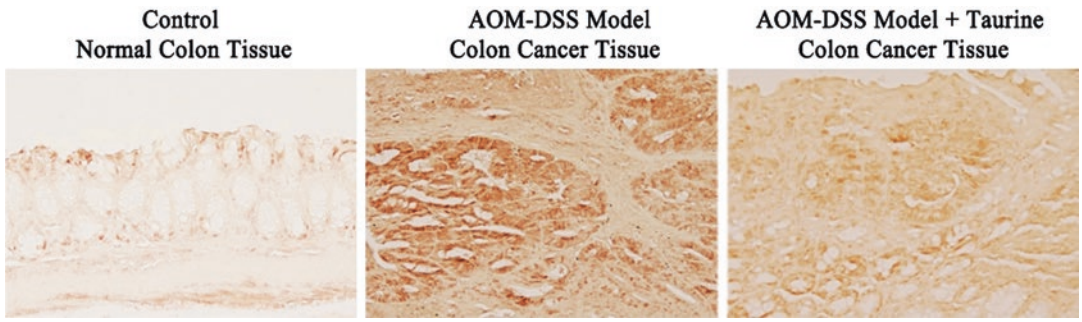


Fig. 1 Immunohistochemistry of Ki-67 in the AOM-DSS-induced colon cancer. The expression of Ki-67 was assessed by avidin-biotin kits with peroxidase-based detection (brown). Original magnifications 100x

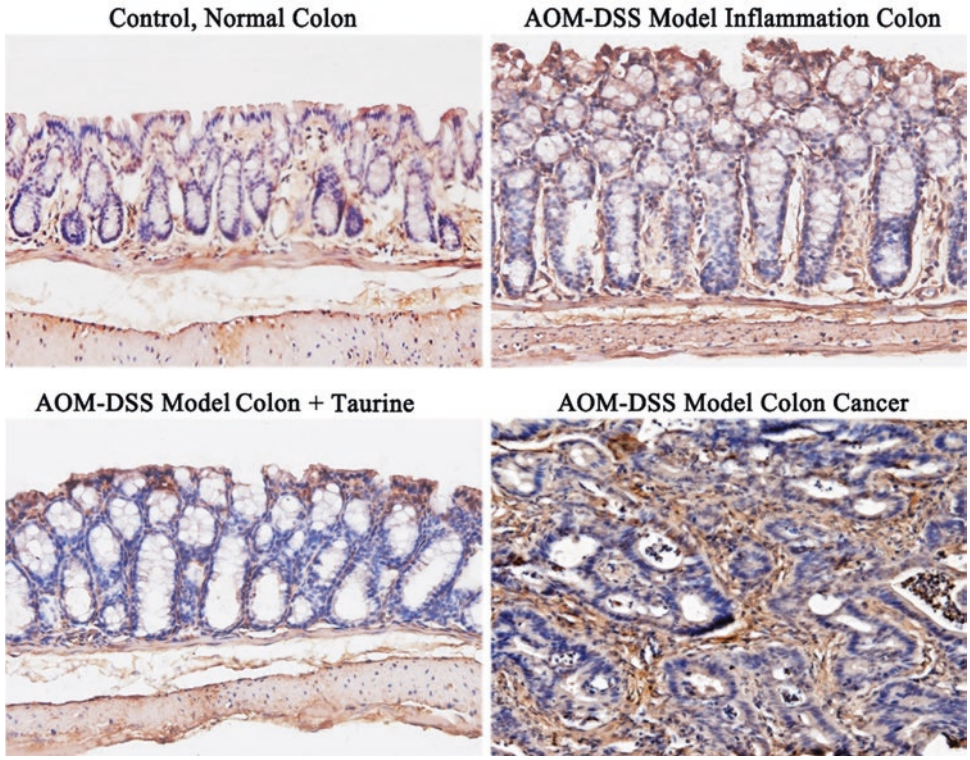


Fig. 2 Immunohistochemistry of COX-2 in the AOM-DSS-induced colon cancer. The expression of COX-2 was assessed by avidin-biotin kits with peroxidase-based detection (brown). Nuclei were counterstain with hematoxylin. Original magnifications 100x

level by taurine was suggested to act via taurine transport. Taurine can enhance the therapeutic index of cancer patients and improve quality of life (QOL) of a modulator (Sadzuka et al. 2009). Interestingly, DOX co-treatment with taurine can induce apoptosis, attenuate doxorubicin-induced cytotoxic side effects, and reduce ROS production in melanoma cells (Kim et al. 2017).

3.3 Taurine Exerts Antitumor Effect by Regulating Immunity

Immune surveillance is the main means against cancer, which enables immune cells to recognize and eliminate tumor cells (Vesely et al. 2011). Maher's study found that taurine can inhibit T-cell apoptosis by reducing the expression of fatty acid synthase (FAS) and may reverse the lymphopenia induced by interleukin-2 treatment, so taurine has the potential to enhance the effect of immunotherapy (Maher et al. 2005). $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$T cells were involved in antigen-specific tumor clearance. Ibrahim's team found that taurine combined with chemotherapy drugs can increase the percentage of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells in the spleen of mice to achieve better immune surveillance of tumor cells (Ibrahim et al. 2018).

3.4 Taurine Induces Apoptosis in Tumor Cells

Apoptosis is a form of programmed cell death that has been described as a key strategy for the elimination of neoplastic cells. Apoptosis is a highly ordered protective mechanism by which unwanted or damaged cells are eliminated before malignancy manifests (Hassan et al. 2014). It is essential for normal development, turnover, and replacement of cells in the living system. Cell apoptosis is characterized by typical morphological and biochemical hallmarks including chromatin condensation, membrane blebbing, cell shrinkage, nuclear DNA fragmentation, and the formation of apoptotic bodies. The cells then divide into membrane-enclosed fragments to form apoptotic bodies, which are then rapidly recognized and engulfed by adjacent cells or macrophages (Ghavami et al. 2014). Apoptosis could be induced by two major pathways, the mitochondrion-mediated pathway (intrinsic pathway) and the death receptor-medicated pathway (extrinsic pathway).

In our recent research, we performed cell viability and colony formation assays on taurine-treated nasopharyngeal carcinoma cell lines. The apoptotic cells were quantified by flow cytometry and expression levels of apoptosis-related proteins were evaluated by western blot. The results showed that taurine markedly inhibited cell proliferation in nasopharyngeal cells, but only slightly in an immortalized normal nasopharyngeal cell line. Taurine suppressed colony formation and induced apoptosis of nasopharyngeal carcinoma cell lines in a dose-dependent manner. Furthermore, taurine increased the active form of caspase-9/3 in a dose-dependent manner. Taurine downregulated the antiapoptotic protein Bcl-xL and upregulated the proapoptotic protein Bax and GRP78, a major endoplasmic reticulum chaperone. These results suggest the involvement of mitochondrial and ER stress signaling in apoptosis. In addition, taurine increased the levels of phosphatase and tensin homolog deleted on chromosome 10 and p53 and reduced phosphorylated protein kinase B (Akt) (He et al. 2018). In conclusion, taurine may inhibit cell proliferation and induce apoptosis in nasopharyngeal carcinoma through PTEN activation with concomitant Akt inactivation (He et al. 2019).

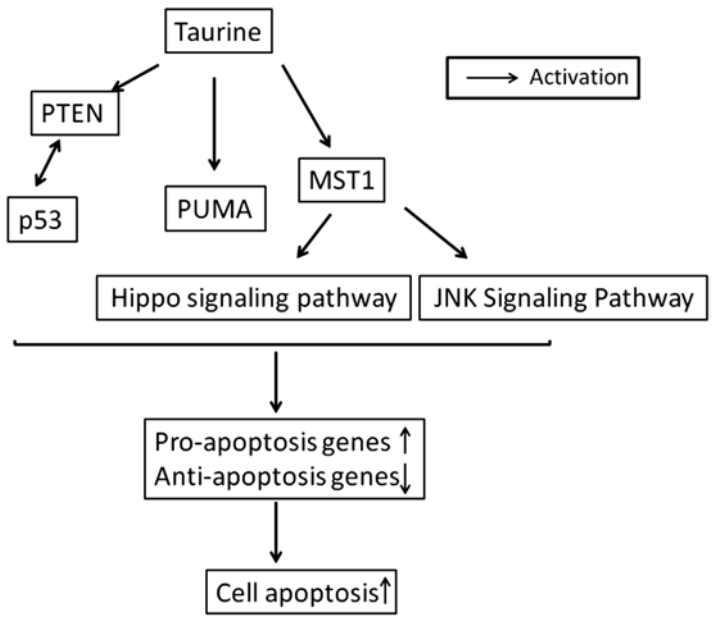
Many recent studies demonstrated that taurine can inhibit cell growth and induce cell apoptosis in nasopharyngeal carcinoma cells (He et al. 2018), human lung cancer cells (Tu et al. 2018), colon cancer cells (Zhang et al. 2014b), breast cancer cells (Zhang et al. 2014a, b), and cervical cancer cells (Li et al. 2019). Caspases, a ubiquitous family of cysteine proteases, play key roles both as upstream initiators and downstream effectors in apoptosis. This cascade leads to proteolytic cleavage of a variety of cytoplasmic and nuclear proteins, thereby favoring the prevalence of proapoptotic activities on antiapoptotic activities (Cossu et al. 2019). Taurine abrogated the expression of antiapoptotic Bcl-2 and/or Bcl-XL proteins and enhanced the levels of proapoptotic BAX proteins followed by caspase-3/9 activation (Vanitha et al. 2018; Tu et al. 2018).

Tumor suppressor genes phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and p53 are well known and play a vital role in tumorigenesis (Nakanishi et al. 2014). Some studies revealed that high concentration of taurine induces the expression of PTEN and p53

(He et al. 2018; Vanitha et al. 2018). They assumed that PTEN and/or p53 is the main pathway for taurine-induced tumor cell apoptosis. P53-upregulated modulator of apoptosis (PUMA) plays an important role in the process of apoptosis induction in a variety of human tumor cells in both p53-dependent and p53-independent manners (Puzio-Kuter 2011). Tu and Zhang et al. studies (Tu et al. 2018; Zhang et al. 2014b) found that taurine PUMA serves an important role in taurine-induced apoptosis pathway. Human mammalian sterile 20-like kinases 1 (MST1) is one of the major members of the Hippo signaling pathway and JNK signaling pathway, which could regulate cell proliferation and apoptosis (Taha et al. 2018; Zhang et al. 2018). MST1 not only enhanced taurine-induced apoptosis but also upregulated the expression of p73, p53, PUMA, and caspase-3 and downregulated yes-associated protein (YAP) in cervical carcinoma cells. Therefore, taurine exhibits antitumor effects through the MST1-mediated Hippo signaling pathway. Liu et al. research also found that taurine could induce the apodosis after treat by taurine in colorectal cancer cells, as well as MST1 and phosphorylated-JNK could be activated. Taurine could reverse the decrease of tumor cell apoptosis caused by knocking out MST1 (Li et al. 2019). Finally, they concluded that the MST1-JNK signaling pathway plays an important role in taurine-induced colorectal cancer cell apoptosis. In addition to the induction of tumor cell apoptosis, taurine also inhibits the metastasis of prostate cancer cells by regulating the expression of PSA, MMP-9, TIMP-1, and TIMP-2 under dihydrotestosterone (DHT)-stimulated conditions (Tang et al. 2015).

4 Conclusion

In conclusion, taurine can be used as a marker for the early diagnosis of some tumors, which is of great significance for tumor prevention. In addition, taurine shows antitumor effect by improving antioxidant capacity, enhancing immunity, and inducing apoptosis of tumor cells. At the same time, taurine combined with chemotherapy drugs can improve the efficacy of chemotherapy drugs and reduce their adverse reactions. However, the mechanism of taurine's antitumor effect is still in its infancy, and there are few studies on the signaling pathway involved in taurine-induced tumor cell apoptosis. With the deepening of the



research, the antitumor effect of taurine will present a huge potential as a promising therapeutic strategy for cancer in the future.

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Taurine Supplementation Inhibits the Expression of Atrogin-1 and MURF-1, Protein Degradation Marker Genes, in Skeletal Muscle of C26-Induced Cachexia Mouse Model

Hari Madhuri Doss, Jung Yeon Kim, and Kyoung Soo Kim

Keywords

Sarcopenia · Skeletal muscle · Atrogin-1 · MuRF-1

Abbreviations

mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING finger 1
TauT	Taurine transporter

1 Introduction

Sarcopenia is defined as age-associated progressive depletion of muscle mass with consequential loss of function and disability. The causes are multifactorial and involve many risk factors, including a decline in physical function, inflammation, nutritional deficiencies, hormonal imbalances, and other diseases (Ali and Garcia 2014). Changes in body composition with a decline in important functional and metabolic activity are primarily recognized with increasing age (Lo et al. 2020). An existing disproportionate rise in aging population and current circumstances have only contributed to a more sedentary lifestyle and diminished health status among the elderly (Kirwan et al. 2020).

However, there are no effective treatments for sarcopenia other than exercise and nutritional supplementation (Lo et al. 2020). In fact, overexertion might negatively impact physical health. Nutritional therapy with amino acids, such as leucine, is currently employed as an important intervention for the treatment of sarcopenia. Specific amino acids have been shown to influence protein turnover under physiological conditions (Ko et al. 2020). However, the hunt for better treatment options is still ongoing for better management of age-related muscle loss. Physiologically, muscle mass is maintained by a balance between protein synthesis and breakdown. The best-defined muscle metabolic pathway that favors protein anabolism involves the activation of serine/threonine kinase Akt, which further amplifies mammalian target of rapamycin (mTOR) that leads to increased muscle protein

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synthesis. The axis of the phosphatidylinositol 3-kinase (PI3K)-Akt kinase-mTOR kinase determines the balance between protein synthesis and breakdown pathways (Martinez-Arnau et al. 2020; Egerman and Glass 2014; Sandri et al. 2013). Two genes, both muscle-specific ubiquitin-ligases, are involved in muscle protein degradation. The expression of those genes is increased in the elderly and triggers the onset of sarcopenia (Gumucio and Mendias 2013). However, sarcopenia pathophysiology involves an imbalance in these processes in favor of protein degradation rather than protein synthesis. In this signaling pathway, the translocation of FoxO into the nucleus is initiated, which activates the expression of atrogin-1 (MAFbx) and muscle RING finger 1 (MuRF1) (Martinez-Arnau et al. 2020; Egerman and Glass 2014; Sandri et al. 2013). These genes are muscle-specific ubiquitin ligases known to be involved in muscle protein degradation. The increased expression of these genes in the elderly has been reported to trigger the onset of sarcopenia (Gumucio and Mendias 2013). Studies in cancer patients have also shown a relatively high prevalence of gradual muscle wasting associated with a condition called cancer cachexia. These patients show progressive loss of skeletal muscle induced by tumorigenesis and tumor growth.

Taurine is a sulfur-containing semi-essential amino acid abundantly present in many mammalian tissues, including skeletal muscle, in free form. This amino acid is known to exert numerous cellular processes, including membrane stabilization and calcium ion-dependent excitation-contraction mechanisms (Spriet and Whitfield 2015). It also possesses anti-inflammatory and antioxidant effects, which attenuate the chronic low-grade inflammatory state associated with aging and serve as a precursor to the consequential sarcopenic phenotype. Evidence supporting a link between changes in cellular taurine levels in skeletal muscle and different pathological conditions, such as disuse-induced muscle atrophy, likely contribute to senescence-mediated muscle loss (De Luca et al. 2015). Such findings only reinforce the interest of researchers and clinicians toward its external dietary supplementation as a potential treatment module. Although further studies are necessary to fill the gaps in translational research, the benefit of taurine appears promising due to its influence on multiple cellular functions while exhibiting relatively low toxicity.

Studies have demonstrated that taurine attenuates catabolic processes involved in the onset of sarcopenia, which is associated with the inhibition in myogenic differentiation and increase in the expression of atrogin-1 and MuRF1 in skeletal muscle (Barbiera et al. 2020). Research studies have shown that taurine stimulates the differentiation of myogenic cells downregulated by inflammation and also modulates dysregulated autophagic pathways and apoptosis (Zhou et al. 2021). However, the studies highlighting the beneficial effects of taurine on sarcopenia have mostly been tested in vitro.

Colon-26 carcinoma tumor-bearing mice are a well-established system to investigate the molecular causes of skeletal muscle wasting induced by tumor growth (Bonetto et al. 2016). In this study, the effect of taurine as a potential prophylactic treatment module of sarcopenia was tested using C26 tumor-bearing mice. We hypothesized that muscle wasting caused by tumor expansion and growth exhibit similar molecular processes as those underlying age-induced muscle wasting. We tested if the expression of two genes, namely, atrogin-1 and MuRF-1, which are primarily responsible for protein degradation, might be increased in C26 tumor-bearing mice but attenuated by taurine supplementation.

2 Methods

2.1 Animal Model

Male Balb/c mice, 8 weeks of age, were divided into three experimental groups (n = 5), namely, normal group, control group (mice injected with C26 carcinoma cell suspension), and taurine

group (mice injected with C26 carcinoma cell suspension treated with taurine). The mice were maintained on a 12-h light-dark cycle. Animals were allowed to acclimatize to their environment for 3 days before the start of the experiment. Mice were subcutaneously injected between the scapulae with 200 µl suspension (1×10^6 cells per mouse) of C26 carcinoma cells in PBS.

The treatment group of mice were orally administered 200 µl of taurine solution (200 mg/kg body weight) every day for 2 weeks, while the control group received 200 µl of saline solution instead of the taurine solution. The mice were euthanized by cervical dislocation. Femur muscle tissue was excised, gonadal fat tissue, spleen, and tumor tissues were also collected. The tissue samples were weighed simultaneously.

2.2 Histopathological Characterization

Muscle tissues excised from experimental mice were initially fixed for 24 h in 10% formalin solution. The samples were then dehydrated through graded ethanol series, cleared in xylene followed by paraffin embedding for cross-sectioning. Sample sections of 8 mm thickness were processed for hematoxylin and eosin staining using standard procedures. The stained sections were then analyzed using a confocal laser scanning microscope (Olympus BX53, Olympus Corporation, Tokyo, Japan).

2.3 Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 100 mg of gastrocnemius muscle tissue using 1 ml TRIzol (Thermo Fisher Scientific Korea, Seoul) according to the manufacturer's instructions. 1 µg RNA was converted into cDNA using a cDNA synthesis kit (Thermo Fisher Scientific Korea, Seoul). Real-time PCR was performed using the following primer pairs for atrogin-1-forward 5′-CAA CAT TAA CAT GTG GGT GTA T-3′, atrogin-1-reverse 5′-GTC ACT CAG CCT CTG CAT G-3′, MuRF1-forward 5′-GAG AAC CTG GAG AAG CAG CT-3′, MuRF1-reverse 5′-CCG CGG TTG GTC CAG TAG-3′, 18S-forward 5′-GTA ACC CGT TGA ACC CCA TT-3′, 18S-reverse 5′-CCA TCC AAT CGG TAG TAG CG-3′, and SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.4 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. Prism software v.5 (Graphpad Software, San Diego, CA, USA) was used for statistical analysis and graphing. Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 Effect of Taurine Supplementation on Organ Mass in C26-Induced Cachexia Mouse Model

At the end of the experimental period, tissue samples were excised and weighed. A considerable increase in spleen size and tumor growth was observed in C26-induced cachexia mice, while muscle mass and gonadal fat tissue mass were diminished. The effect of taurine supplementation over a period of 2 weeks showed a modest recovery from the loss of gonadal fat mass but had no effect on muscle mass loss. Similarly, taurine mediated a reduction in spleen tissue, but tumor mass was not significantly affected as shown in Fig. 1.

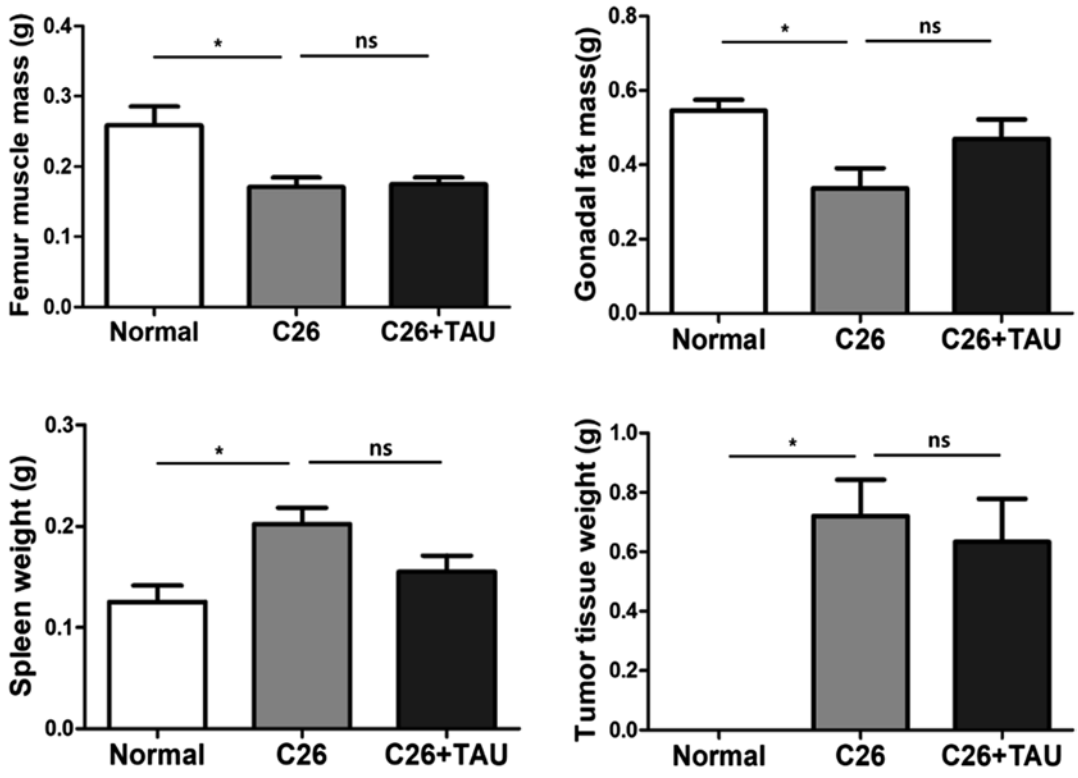

Fig. 1 Taurine supplementation recovers loss of organ mass in C26-induced cachexia mouse model. C26 carcinoma cells (1×10^6 cells) were injected into the scapulae of Balb/c mice. Taurine was orally (200 µl) administered for 2 weeks daily at a dose of 200 mg/kg of body weight. Taurine-induced weight maintenance was comparable between spleen mass and tumor mass for the C26-induced group, although femoral muscle mass and gonadal fat tissue did not show significant changes. Values shown represent means ± S.E.M. of five samples: $^*p < 0.05$, ns no significance, C26 C26 carcinoma cells, TAU taurine

3.2 Effect of Taurine Supplementation on the Histological Structure of Muscle Tissue in C26-Induced Cachexia Mice

Histopathological examination of the gastrocnemius muscle tissue sections showed regular morphological muscle structure in normal group animals. When the muscle tissues of C26-induced cachexia mice were examined, it showed evidence of muscle atrophy associated with malformation in muscle cells. Taurine supplementation partially restored muscle tissue integrity in C26 experimental mice as seen in Fig. 2a but did not completely restore morphological structure to normal.

3.3 Effect of Taurine Supplementation on Transcriptional Expression of Atrogin-1 and MuRF-1 in Muscle Tissues of C26-Induced Cachexia Mice

Gene expression analysis of muscle-specific ubiquitin-ligases, atrogin-1 and MuRF-1, known to be involved in muscle protein degradation showed increased expression in C26-induced mice when compared to normal mice (Fig. 2b). This indicated the onset of muscle hypertrophy in tumor-bearing experimental animals. Taurine supplementation in experimental mice showed a downregulated expression of atrogin-1. However, the expression of MuRF-1 was only modestly

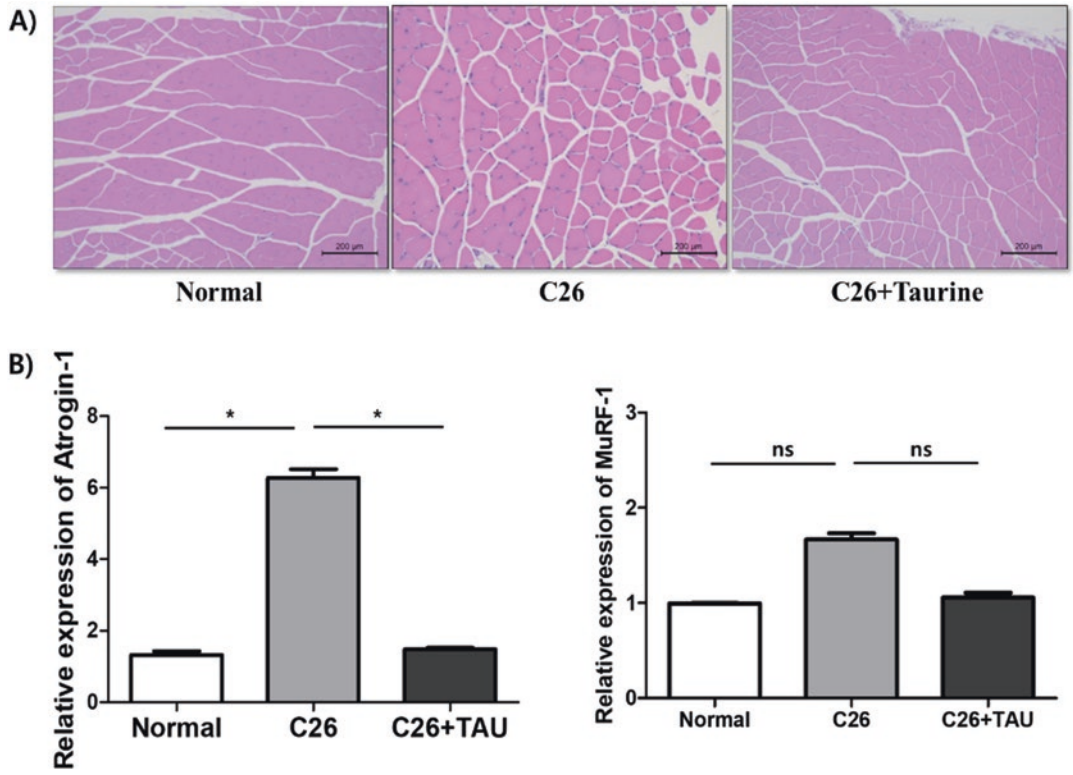

Fig. 2 Taurine supplementation downregulates the expression of genes involved in muscle atrophy. C26-induced cachexia resulted in atrophic muscle and significantly increased the expression of atrogin-1 and MuRF-1. In contrast, only a slight downregulation in the MuRF-1 gene expression was observed upon taurine supplementation compared to a significant decrease in atrogin-1 expression. Values shown represent the means ± S.E.M. of five samples. *$p < 0.05$, ns not significant, C26 C26 carcinoma cells, TAU taurine. Decreased the transcriptional expression of atrogin-1

downregulated in taurine supplemented experimental animals.

4 Discussion

Sarcopenia is increasingly being recognized as a geriatric syndrome. It is characterized by loss of muscle mass and strength. Reports show a loss of about 1% of muscle per year in individuals starting from the age of 30 with an overall loss of 30% muscle mass by the age of 80 (Lo et al. 2020; Fielding et al. 2011). Reductions in muscle function are associated with an increased risk of impairment and disability in the elderly (Lexell et al. 1988). Sarcopenia greatly affects the quality of life due to higher rates of falls and incidence of hospitalization, leading to a higher rate of mortality in the elderly population (Beaudart et al. 2017). Over the years, the exponential rise in the demographic of the aging population has made sarcopenia a cause of concern for public health. Standard treatments include regular exercise and nutritional strategies to improve muscle health. Other sarcopenia medications, such as ACE inhibitors, myostatin inhibitors, testosterone, and growth hormone, are known to have serious side effects (Lo et al. 2020). So far, no intervention in the field has shown superior results than exercise training. However, the incidence of overexertion and a lack of motivation have been major barriers for the aging population.

There is a substantial amount of research on nutritional therapies which include the use of

antioxidants and specific amino acids for improving muscle buildup (De Luca et al. 2015; Ko et al. 2020; Schaffer et al. 2010). In particular, studies have shown that intake of a protein-rich diet containing a high proportion of the amino acid leucine can rescue protein synthesis in the elderly, restoring skeletal muscle protein metabolic balance (Ko et al. 2020). However, the results cannot be considered universally conclusive at this stage. Although in the present scenario there are only a limited number of options to counter the effects of age-related as well as disease-induced declines in muscle mass. Nonetheless, there are still a number of less explored therapies that show promising potential. It is therefore impertinent to explore better treatment options for sarcopenia that can greatly improve the quality of life of the elderly. Taurine (2-aminoethane-sulfonic acid) is the most abundant free sulfur-containing amino acid in mammalian tissues that is not utilized for protein synthesis. Intracellular concentrations of taurine range between 5 and 20 μmol/g in excitable tissues such as skeletal muscle, brain, and heart (De Luca et al. 2015; Schaffer et al. 2010). It is known that skeletal muscle tissue is able to concentrate the largest reserve of taurine in the body, via TauT (taurine transporter) activity. Extensive studies have identified several essential biological functions of taurine which include calcium homeostasis, membrane stabilization, osmoregulation, antioxidant, and anti-inflammatory actions (Olson and Martinho 2006). Transgenic mice lacking the TauT gene have shown a variety of abnormalities in tissues, such as kidney, heart, skeletal muscle, and nociceptive system, which are similar to pathophysiological conditions induced by altered taurine tissue content or by administration of taurine transporter inhibitors.

In line with the key role of taurine in maintaining steady physiological functions, preclinical studies have also shown beneficial effects of taurine supplementation. However, the therapeutic use of taurine is still very limited. Taurine is commonly known for its claimed anti-fatigue and energizing effects and is a major constituent of commercial energy drinks. The relatively low toxicity of taurine in this context with respect to other active ingredients makes it a preferred choice among researchers to explore further (Schaffer et al. 2014).

Studies have demonstrated improved electrical and contractile function of skeletal muscle fibers in aged experimental rats upon treatment with taurine (Pierno et al. 1998). Age-induced muscle loss is associated with underlying low-grade inflammation. This remains a major cause for the onset of sarcopenia among the elderly. Recent research demonstrated the potential anti-inflammatory and antioxidant activity of taurine in relieving low-grade inflammation and prevention of sarcopenia (Sanada et al. 2018). Recent reports supporting the therapeutic potential of taurine showed that myogenic rat cells administered with high levels of taurine downregulated the expression of inflammatory factors and stimulated cell differentiation. Taurine administration also exhibited modulatory effects on autophagy and cell apoptosis (Seidel et al. 2019). Furthermore, taurine treatment protected myoblasts from impaired cell proliferation and myotube differentiation in cisplatin-induced ROS exposure (Zhou et al. 2021). These reports suggest that taurine might play an important role in restoring muscle function and further reinforce its potential in treating disorders associated with muscle loss.

In our study, we examined the effect of taurine on muscle loss caused by cancer-induced cachexia. Our findings showed a modest restoration of muscle morphology that was identified by tissue histopathological analysis. The results showed a modest recovery as compared to muscle tissue breakdown in C26-induced cachexia mice. However, taurine administration did not show any effects on muscle mass loss. This might be attributed to the short-term treatment regimen in our study that needs to be addressed in our future research. Also, the cachexia model in our study was selected to demonstrate accelerated muscle atrophy, but the muscle atrophy was so extensive that taurine at the administered dose could not reverse muscle atrophy to a large extent. It is possible that a different muscle atrophy rodent model might better demonstrate the beneficial effects of taurine supplementation.

Interestingly however, we found that transcriptional expression of genes, responsible for muscle degradation, namely, atrogin-1 and MuRF-1, were downregulated by taurine supplementation. This indicated that taurine indeed possesses the potential in preventing and delaying the onset of age-induced sarcopenia.

5 Conclusion

In conclusion, our findings demonstrate preliminary effects of taurine in sarcopenia muscle loss. Although taurine treatment did not show recovery in the loss of muscle mass in C26-induced cachexia model at the molecular level, transcriptional downregulation of muscle degradative genes, atrogin-1 and MuRF-1, was observed. This suggests the definitive potential of taurine in attenuating muscle loss. Research is required to further confirm these effects in other sarcopenia models. Above all, taurine being a safe amino acid certainly has the potential to be developed as a therapeutic agent against sarcopenia. It can also be developed as a combinatorial treatment with other compounds.

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Bioavailability of Tauropine After Oral Ingestion in Mouse

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Keywords

Tauropine · Mass spectrometry · Bioavailability

1 Introduction

Sea animals and seaweed produce various taurine derivatives, such as methyltaurine and tauropine) (Impellizzeri et al. 1975; Sato et al. 1985). There is some evidence that taurine derivatives demonstrate health benefits in mammals. For example, homotaurine inhibits amyloid aggregation and is effective against Alzheimer's disease (Caltagirone et al. 2012). Moreover, N-methyltaurine attenuates steroid-induced muscle atrophy in mice (Nguyen et al. 2020).

Tauropine (D-rhodoic acid) is a kind of opine, which are anaerobic metabolites in abalone and algae (Sato et al. 1985, 1987). Tauropine is produced from reductive condensation of taurine and pyruvate, as catalyzed by tauropine dehydrogenase (TADH, Fig. 1) (Gäde 1986). We are interested in the health effects of tauropine in mammalians. However, it has not been elucidated whether orally ingested tauropine from seafood can be absorbed by the intestines and transported into the blood. In the present study, we tested the bioavailability of tauropine in mice.

2 Methods

2.1 Synthesis of Tauropine

Taurine was synthesized as previously reported (Haque et al. 2000). Taurine (12 g) was reacted with L-bromopropionic acid (25 g) in 350 mL of 1M NaOH solution for 7 days. The reaction mixture was applied to a Dowex 1-X2 column (OH-form, Fuji film, Tokyo, Japan). The column was washed by water and then by 0.1M HCl. Then tauropine was eluted with 0.2M HCl. The eluant was concentrated by rotary evaporator, and the residue was recrystallized by adding ethanol and then lyophilized. The yield was confirmed by electrospray ionization mass spectrometry (ESI-MS) (Esquire 4000, Bruker) (Fig. 2).

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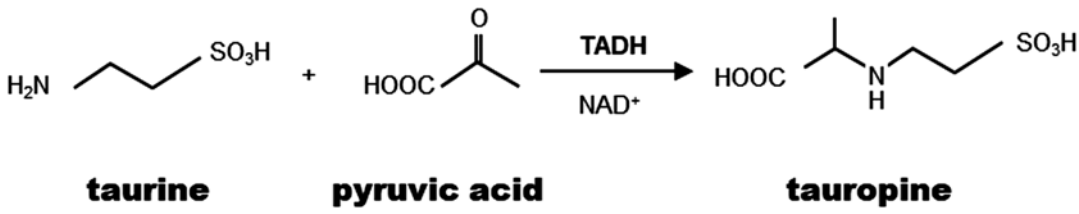

Fig. 1 Production of tauropine from taurine and pyruvic acid in abalone

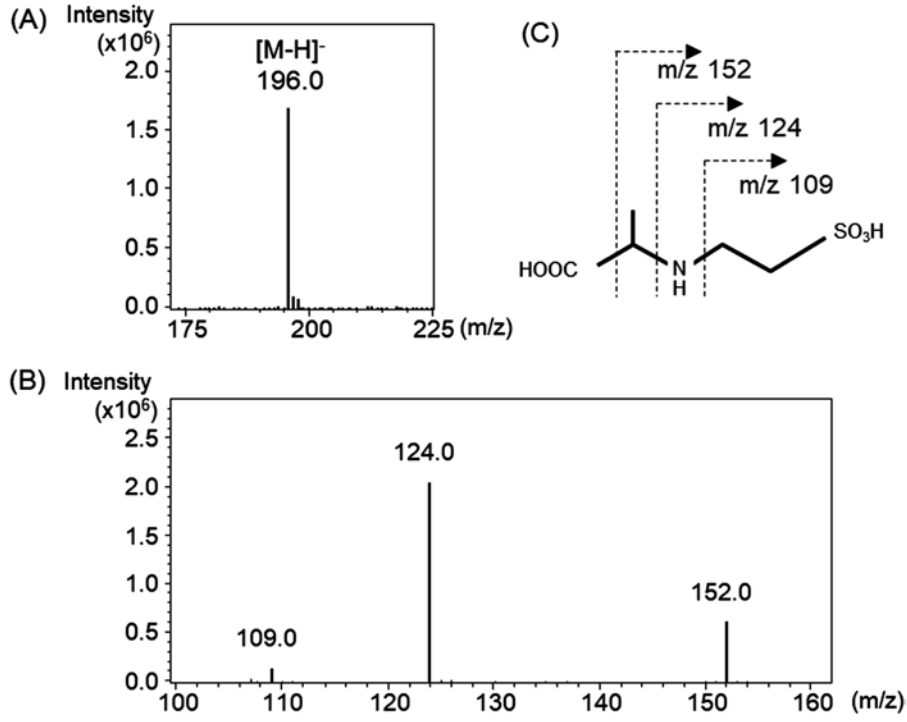

Fig. 2 Detection of tauropine. (**a**) MS spectrum of synthesized tauropine (negative ion mode). (**b**) MS/MS spectrum of daughter ion of tauropine ([M-H] = 196.0). (**c**) Fragmentation of tauropine expected from daughter ions

2.2 HPLC Determination of Tauropine by Phenyl Isothiocyanate Derivatization

Tauropine was derivatized with phenyl isothiocyanate (PITC) as previously reported (Sato et al. 1988). Tauropine solution was mixed with equal amounts of ethanol-water-triethanolamine (2:2:1) and dried by a concentrating centrifuge. The dried sample was dissolved by ethanol-water triethanolamine-PITC (7:1:1:1) and incubated for 20 min at room temperature. The sample was dried again and kept at 4 °C until measurement.

The PITC derivatives were analyzed by high-performance liquid chromatography (HPLC)-ESI-MS (maXis plus, Bruker) equipped with a reversed-phase column (Shim-pack XE-ODS 3 μm, 75 × 2.6 mm; Shimazu, Kyoto, Japan) at 40 °C at a flow rate of 0.3 mL/min and with a linear gradient of acetonitrile in 20 mM acetic acid run over 20 min (5–40% (v/v) acetonitrile for 20 min).

2.3 Animal Experiment

The experimental procedures were approved by the Institutional Animal Care and Use Committee of Fukui Prefectural University. Male ICR mice (8-week-old) was purchased from Japan Crea Inc. (Japan). Mice had access to water ad libitum and maintained on a 12-h light/dark cycle. Tauropine solution in saline was administered intraperitoneally (i.p.) or orally (p.o.) to mice at 50 mg/kg body weight. The mice were anesthetized to collect blood sample and then killed by cervical dislocation. Tissues were isolated and were kept at −80 °C until use.

2.4 Blood Sample Preparation for HPLC Analysis

Samples were deproteinated by using 5% sulfosalycylic acid (SSA, Nacalai). Serum samples were mixed with equal volume of 5% SSA. Tissues were homogenized in 10 volumes of 5% SSA using a polytron homogenizer. Samples were centrifuged, and the supernatant was filtered with a 0.45 microm filter and then neutralized with NaHCO3. Next, the primary amine compounds were removed after being derivatized with o-phthalaldehyde (OPA, 5 mM) in boric acid buffer (pH 10.4) containing 2-mercaptoethanol (29 microM); the OPA-derived amines were removed by a Monospin C18 Column (GL Science, Tokyo, Japan). The flow-through eluant was then derivatized with PITC as described above.

2.5 HPLC Determination of Tauropine-PITC in Blood Samples

The PITC-derivatized blood samples were analyzed using an HPLC-photodiode array detector (Shimazu) equipped with a reversed-phase column (COSMOSIL 5C18-MS-II Packed Column 5 μm, 250 × 4.6 mm; Nacalai tesque, Kyoto, Japan) at 40 °C at a flow rate of 1 mL/min and with a linear gradient of acetonitrile in 10 mM phosphate buffer (pH 7.3) run over 45 min (3–97% (v/v) acetonitrile for 45 min). PITC-derivatized tauropine was detected by absorption at 254 nm.

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3 Results

3.1 Detection of Tauropine by HPLC Method

Since tauropine contains a secondary amine, tauropine can be derivatized by PITC. First, we analyzed whether PITC-tauropine can be separated using HPLC-MS (Fig. 3a, b). We confirmed the peak of PITC-tauropine, and the molecular weight of the compound included in this peak was 314. Therefore, we expected that the reaction of PITC with tauropine may have yielded a dehydration product as shown in Fig. 3c.

Meanwhile, we tested whether FMOC-Cl, another amine-derivatizing agent that is readily detectable by a fluorometric detector, could be used to measure tauropine. However, it did not produce FMOC-tauropine (data not shown). Therefore, we decided to use PITC to measure tauropine.

Next, we tried to separate the peak of tauropine from that of the other amino acids found in the blood. Since the blood contains many primary and secondary amines, as well as amino acids, which all can react with PITC, many HPLC peaks could overlap with the peak of PITC-tauropine. Therefore, we removed the primary amines from the blood samples by using OPA, which reacts with primary amines but not secondary amines. We then reacted the samples with PITC. This protocol allowed successful separation of the PITC-tauropine peak from that of the other compounds in the blood sample (Fig. 4).

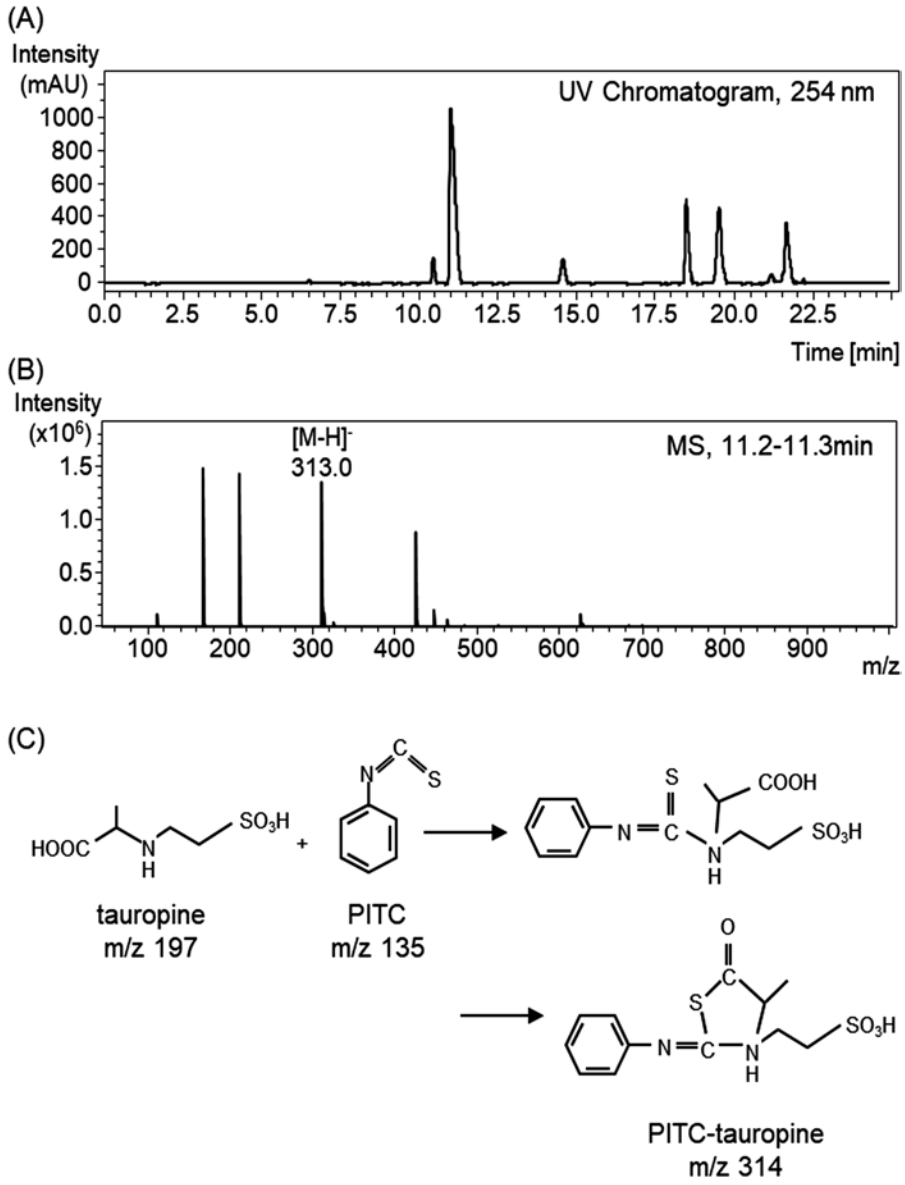

Fig. 3 Detection of PITC-labeled tauropine by HPLC-MS. (**a**) HPLC chart of PITC-labeled tauropine. (**b**) MS spectrum of PITC-labeled tauropine. (**c**) The reaction between tauropine and PITC, which is expected from the m/z of the product

3.2 Pharmacokinetics Study of Tauropine After Oral Injection in Mouse

Tauropine (500 mg/kg body weight) was orally or intraperitoneally injected into mice, and then blood was collected after 1 h. We then confirmed by HPLC whether tauropine was present in the blood (Fig. 4). Although PITC-tauropine was found in the blood of mice intraperitoneally injected with tauropine, it was not detected in the blood of mice administered tauropine orally. Moreover, we maintained mice for 5 days on drinking water containing tauropine (0.5%), and

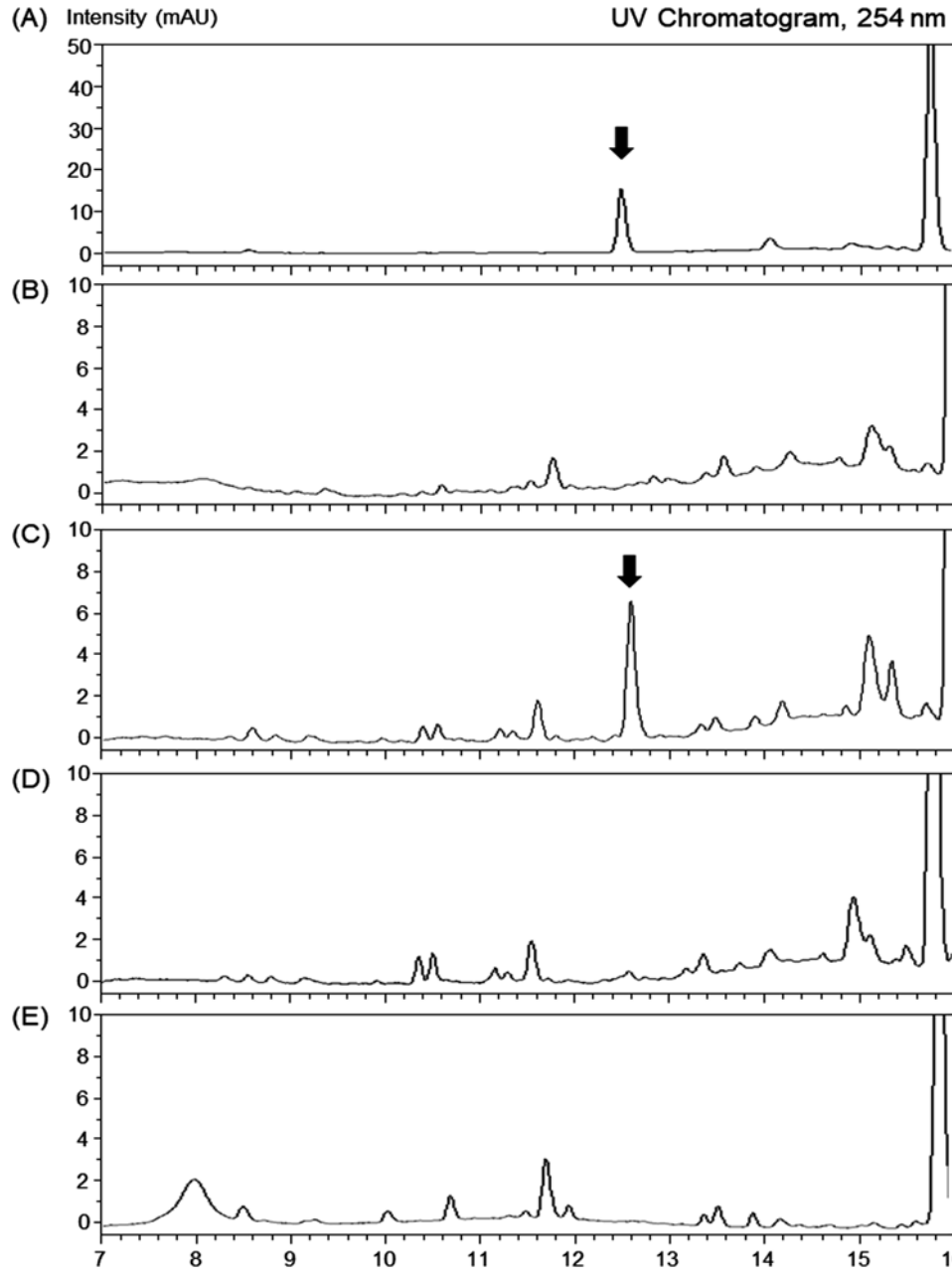

Fig. 4 HPLC charts of PITC-labeled tauropine and blood metabolites. (**a**) Synthesized tauropine. (**b**) Blood from untreated mice. (**c**) Blood from tauropine-treated (i.p.) mice. (**d**) Blood from tauropine-treated (p.o.) mice. (**e**) Blood from mice drinking 0.5% tauropine-containing water for 5 days. Arrows indicate the peaks for PITC-labeled tauropine

then blood tauropine was measured. However, tauropine was not detected in these mice (Fig. 4e). These results indicate that orally administered tauropine cannot be detected in the blood.

There are several possible reasons why orally administered tauropine cannot be detected in the body. (i) It may be degraded in the gastrointestinal tract. The pH of the GI tract is very acidic.

Moreover, there are a lot of enzymes and microbiota that may cause tauropine destruction. Tauropine is stable in acidic condition, since it was eluted by 0.1M HCl during purification. Moreover, since it has no ester-bond, general esterases cannot alter it. Tauropine is formed from pyruvate and taurine in the presence of the enzyme, tauropine dehydrogenase. The catalytic enzymes for tauropine would exist in the gut or gut microbiota. (ii) It may not be absorbed by the intestines and then transferred to the blood. Taurine is hydrophilic; it should be transported by specific transporter to be absorbed by the intestines. We expected that it would be transported by the taurine transporter, but that proved not to be the case.

4 Conclusion

Orally ingested tauropine from certain types of seafood is barely present in the blood. Therefore, the potential health benefit or toxicity of tauropine in the body would not be detected. Interestingly, another opine compound, strombine, contributes to the taste of dried scallops (Starkenmann et al. 2009). There is the possibility that tauropine contributes to the taste of the abalone and other tauropine-containing seafood. If so, information of the pharmacokinetics obtained from this study would be useful in applying tauropine to umami seasoning.

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Part III

Taurine-Mediated Protection Against Temperature Stress in Farm Animals

Taurine Prevents Liver Injury by Reducing Oxidative Stress and Cytochrome C-Mediated Apoptosis in Broilers Under Low Temperature

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Keywords

Taurine · Low temperature · Antioxidative ability · Cytochrome c · Apoptosis

Abbreviations

Apaf-1	Apoptotic protease-activating factor-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
GSH-PX	Glutathione peroxidase
Hsp 27	Heat shock protein 27
Hsp 70	Heat shock protein 70
Hsp 90	Heat shock protein 90
MDA	Malondialdehyde
T-AOC	Total antioxidant capacity

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1 Introduction

Cold temperature is one of the main restraining factors for the poultry industry in cold areas (Morris et al. 2017), which leads to a decrease in growth and increased morbidity of some diseases, such as the ascites syndromes (AS) (Julian et al. 1989; Daneshyar et al. 2009; Wideman et al. 2013). AS is a common metabolic disorder of modern, fast-growing strains of broilers. It is an important cause of mortality in the chicken industry, which leads to an enormous economic loss to the broiler breeding industry (De Smit et al. 2005).

The reasons that cold temperature leads to ascites mainly lie in the mismatch between oxygen demand and the restrictive pulmonary vascular capacity of the modern broilers (Wideman et al. 2001, 2013), as well as the fact that cold temperatures can aggravate cellular hypoxia (Wideman et al. 2013). As hypoxemia develops, the production of reactive oxygen species (ROS) increases and mitochondrial dysfunction is exacerbated (Hamanaka and Chandel 2009). Previous

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studies have shown that oxidative stress is involved in the pathophysiological progression leading to ascites (Maxwell et al. 1986; Wang et al. 2012; Yang et al. 2014).

Taurine, a free sulfur-containing amino acid, is widely distributed in the body. As a conditionally essential amino acid, many studies have shown that taurine has antioxidant activity and inhibits apoptosis (Schaffer et al. 2009; Yang et al. 2015; Jamshidzadeh et al. 2017; Wu et al. 2018; Li et al. 2020). In our previous study, we found that taurine increases the antioxidant capacity of heart and inhibits cardiomyocyte apoptosis in broilers under low temperature (Li et al. 2020). The liver is the most important and active metabolic organ. It is not clear if taurine is capable of protecting the liver under low temperature.

In this study, the effects of taurine on the antioxidant capacity, the mRNA levels of proapoptotic and antiapoptotic factors related to cytochrome c-mediated apoptosis pathway, were examined.

2 Methods

2.1 Animal Treatment

240 arbor acres (AA) chickens (*Gallus*) were randomly divided into three groups on day of 1: a control group (C), a model group (M), and a taurine group (T). The broilers in the control group were raised in normal environment. The broilers in the model group were housed at a low ambient temperature (10 °C ~ 12 °C) from 21 to 42 days, the temperature was decreased 1–2 °C from day of 15 to day of 21. The broilers in the taurine group were given 1% taurine dissolved in the drinking water from 12 days and were raised under the same conditions as the model group. At 28 and 42 days, ten hepatic tissues of each group were randomly collected, snap frozen in liquid N_2 and stored at −20 °C or −80 °C.

2.2 Detection of Antioxidant Capacity of Liver in Broilers

About 10% tissue homogenate was prepared and total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-PX), catalase (CAT), and malondialdehyde (MDA) were assayed with commercial kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the protocols.

2.3 Total RNA of Liver Extraction and Quantitative Real-Time PCR

The operation sequences were the same as described by Li et al. (2020). The sequences of the primers are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression which was normalized to the expression of GAPDH (F:GATGGGTGTCAACCATGAGAAA R: CAATGCCAAAGTTGTCATGGA).

2.4 Statistical Analysis

All data from the assay above were analyzed by ANOVA in SPSS 16.0 and expressed as means ± SEM. A *P*-value of <0.05 was considered significant.

3 Results

3.1 The Effect of Taurine on the Antioxidant Capacity of Liver in Broilers Under Low Temperature

At 28 and 42 days, the level of T-AOC in the low temperature group was significantly lower than that of the control group ($P < 0.01$) (Fig. 1a), but the activity of GSH-PX was significantly

Table 1 The primers of genes

Gene	Primer	Tm
Apaf-1	R:5′ -TCTTTAGTTGGTGCGCTTTTAC-3′	55.5 °C
	F:5′ -ATGGAGAGGTCTGTGTAGTAGT-3′	
Bcl-2	R:5′ -GGGATGCCTTTGTGGAACTATA-3′	53.1 °C
	F:5′ -CTTTTGCATATTTGTTTGGGGC-3′	
Bax	R:5′ -TTTTTGCTACAGGGTTTCATCC-3′	55.4 °C
	F:5′ -CCAGTTCATCGCCAATTCG-3′	
Caspase-3	R:5′ -TGCCTCATTTTTCTCCCAAAAG-3′	53.2 °C
	F:5′ -CAGTTCGAAATCCTTGCTTCAC-3′	
Caspase-8	R:5′ -ATCGGATCAATCGAATAG -3	44.9 °C
	F:5′ -CTGAACGGAGACACCC -3	
Caspase-9	R:5′ -TGCCTCATTTTTCTCCCAAAAG-3′	56.9 °C
	F:5′ -CAGTTCGAAATCCTTGCTTCAC-3′	
Hsp27	R:5′ -ACACGAGGAGAAACAGGATGAG-3′	56.5 °C
	F:5′ -ACTGGATGGCTGGCTTGG-3′	
Hsp90	R:5′ -TCCTGTCCTGGCTTTAGTTT-3′	53.2 °C
	F:5′ -AGGTGGCATCTCCTCGGT-3′	
Hsp70	R:5′ -CGGGCAAGTTTGACCTAA-3′	52.3 °C
	F:5′ - TTGGCTCCCACCCTATCTCT-3′	

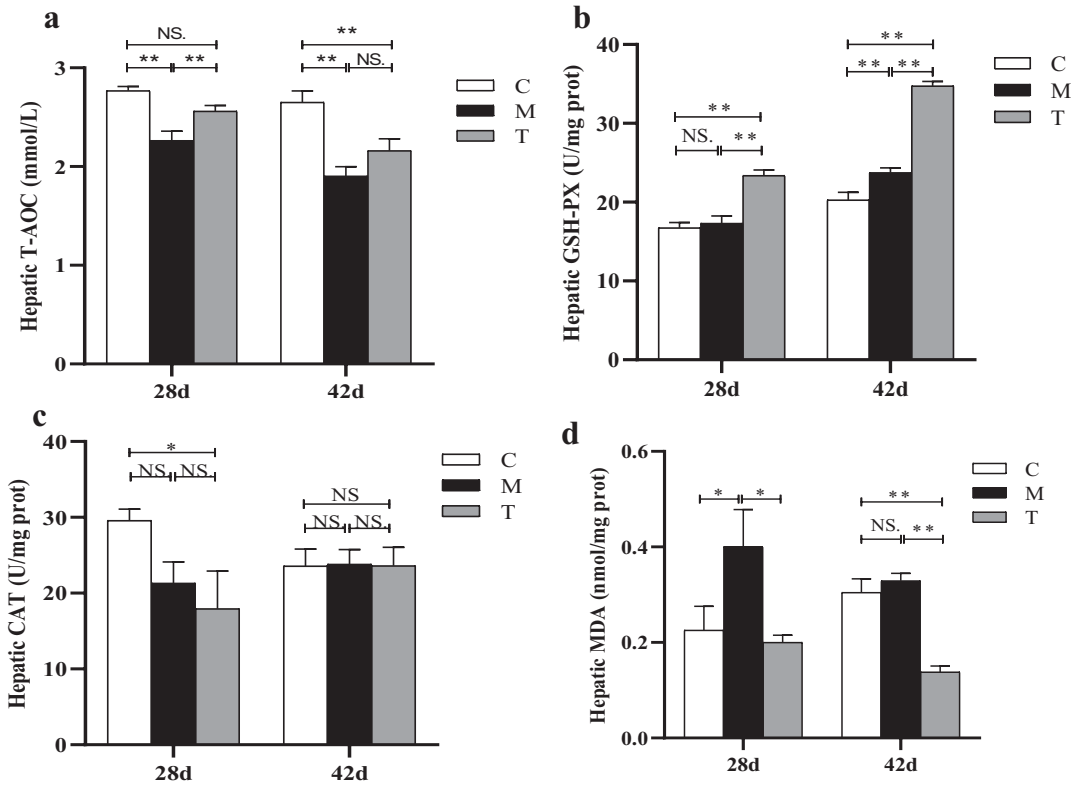

Fig. 1 Effect of taurine on antioxidant capacity of liver in broilers under lower temperature ($n = 8$). (**a**) T-AOC. (**b**) GSH-PX. (**c**) CAT. (**d**) MDA. Values shown represent means ± SEM. One asterisk denotes a significant difference between two groups ($P < 0.05$), double asterisks denote a significant difference between two groups ($P < 0.01$), NS denotes no significant difference between two groups ($P > 0.05$)

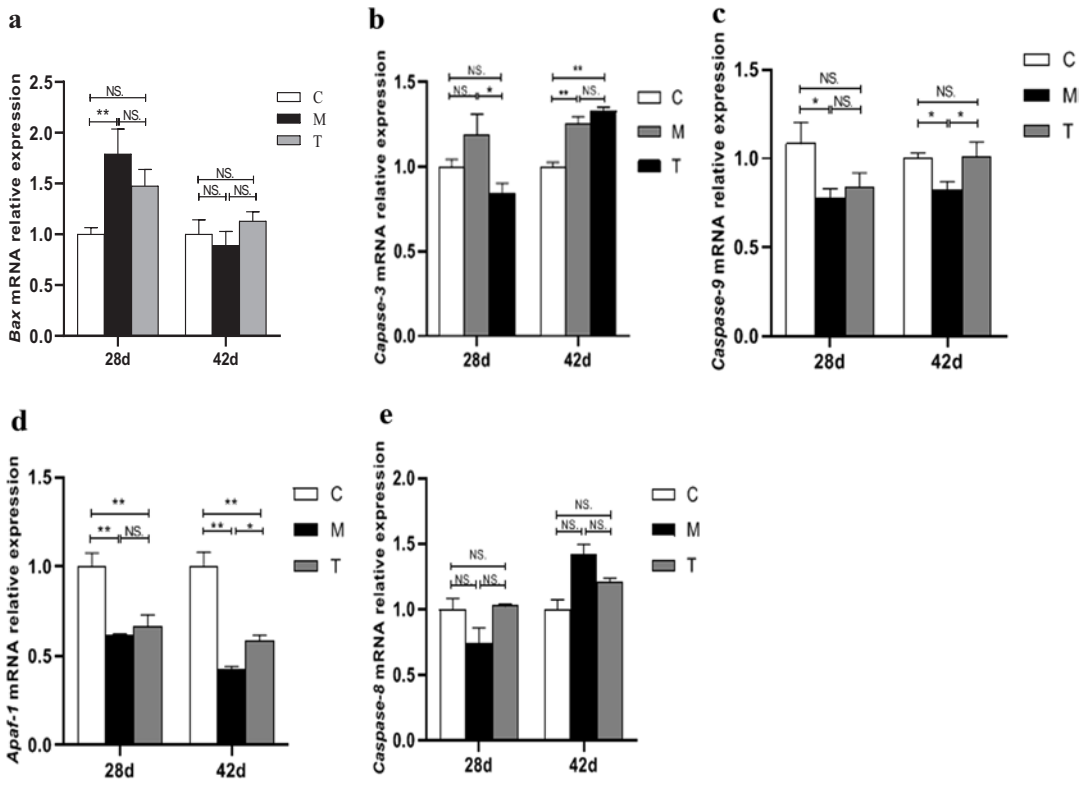

Fig. 2 Effect of taurine on the mRNA expression levels of proapoptotic proteins of liver in broilers under lower temperature ($n = 5$). (**a**) Bax. (**b**) Caspase-3. (**c**) Caspase-9. (**d**) Apaf-1. (**e**) Caspase-8. Values shown represent means ± SEM. The one asterisk denotes a significant difference between two groups ($P < 0.05$), double asterisks denote a significant difference between two groups ($P < 0.01$), NS denotes no significant difference between two groups ($P > 0.05$)

increased at 42 days, and MDA concentration was significantly increased at day of 28, respectively, compared with control group ($P < 0.01$, $P < 0.05$) (Fig. 1b, d). In contrast with the low temperature group, taurine extremely increased the level of T-AOC at 28 d ($P < 0.01$) (Fig. 1a) and the activities of GSH-PX at day 28 and 42 ($P < 0.01$) (Fig. 1b); taurine also decreased the level of MDA at 28 and 42 days ($P < 0.05$, $P < 0.01$) (Fig. 1d).

3.2 The Effect of Taurine on mRNA Expression Levels of Hepatic Proapoptotic Factors Related with Cytochrome c-Mediated Apoptosis Pathway in Broilers

In contrast with the control group, the expression level of Bax mRNA in broilers under low temperature was significantly elevated at 28 days ($P < 0.01$) (Fig. 2a); the mRNA level of caspase-3

was increased significantly at 42 days ($P < 0.01$) (Fig. 2b); but the mRNA expression levels of caspase-9 and Apaf-1 were obviously decreased at 28 and 42 days ($P < 0.05$) (Fig. 2c, d). Compared with the low temperature group, the broilers in taurine group showed a significant decrease of the level of caspase-3 mRNA at 28 days ($P < 0.05$) (Fig. 2b); taurine also significantly promoted the mRNA expression levels of caspase-9 and Apaf-1 at 42 days ($P < 0.05$) (Fig. 2c, d). There are no significant changes on the mRNA level of caspase-8 between different groups.

3.3 The Effect of Taurine on the mRNA Expression Levels of Hepatic Antiapoptotic Proteins Related with Cytochrome c-Mediated Apoptosis Pathway in Broilers

In contrast with the control group, the broilers in the low temperature group showed a significant decline in the mRNA level of Hsp 90 at 28 days ($P < 0.01$) (Fig. 3c) and a significant decline of the mRNA level of Bcl-2 ($P < 0.01$)

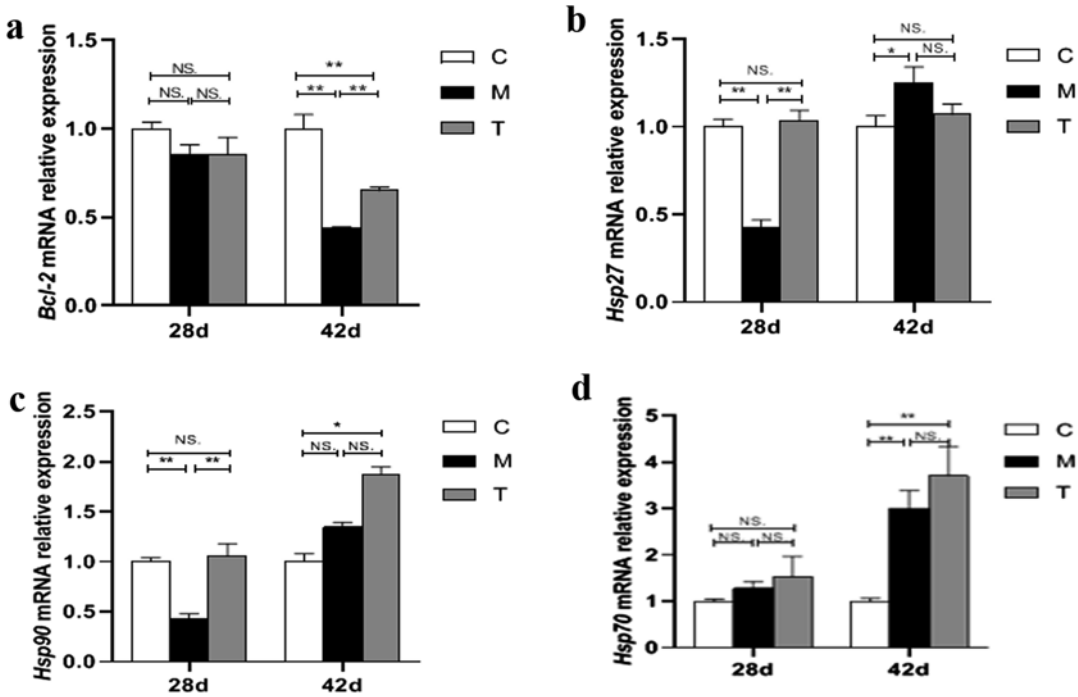

Fig. 3 Effect of taurine on the mRNA expression levels of antiapoptotic proteins of liver in broilers under lower temperature ($n = 5$). (**a**) Bcl-2. (**b**) Hsp 27. (**c**) Hsp 90. (**d**) Hsp70. Values shown represent means ± SEM. One asterisk denotes a significant difference between the two groups ($P < 0.05$), double asterisks denote a significant difference between the two groups ($P < 0.01$), NS denotes no significant difference between the two groups ($P > 0.05$)

(Fig. 3a) at 42 days. As seen in Fig. 3b, the mRNA expression level of Hsp 27 was extremely decreased at 28 days ($P < 0.01$), but it was significantly elevated at 42 days ($P < 0.05$) under the low temperature condition. At 28 days, taurine mediated an upregulation of the mRNA levels of Hsp 27 ($P < 0.01$) (Fig. 3b) and Hsp 90 ($P < 0.01$) (Fig. 3c) compared with the low temperature group. Taurine also significantly increased the mRNA expression level of Bcl-2 at day 42 ($P < 0.01$) (Fig. 3a). Compared with the control group, taurine obviously elevated the mRNA expression levels of Hsp 90 ($P < 0.05$) (Fig. 3c) and Hsp 70 ($P < 0.01$) (Fig. 3d) at 42 days.

4 Discussion

Oxygen-derived free radicals play an important role in the genesis of tissue damage. As the most important and active organ, the liver is easily attacked by reactive oxygen species (ROS). In the present study, the activity of T-AOC at 28 and 42 days was significantly decreased by chronic cold stress, while the levels of GSH-PX at 42 days and MDA at 28 days were significantly increased. The changes of T-AOC and MDA showed that oxidative stress was elevated in the liver of broiler under chronic cold stress. The results are consistent with the reports by Wang et al. (2012) and Yang et al. (2014). The activity of GSH-PX in broilers under low temperature showed an extreme increase at 42 days, which is inconsistent with the results reported by Geng et al. (2004). This difference deserves further study. Taurine significantly increased the level of T-AOC at 28 days and the ability of GSH-PX at 28 and 42 days and decreased the MDA level at 28 and 42 days; these results indicated taurine acts as an antioxidant to alleviate hepatic damage due to lipid peroxidation, which was confirmed in some reports (Schaffer et al. 2009; Yang et al. 2015; Jamshidzadeh et al. 2017; Wu et al. 2018; Li et al. 2020).

Many studies have shown that hepatic apoptosis exists along with hepatic injury (Yalcinkaya et al. 2009; Liu et al. 2016; Wu et al. 2018), whereas taurine protects the liver by inhibiting mitochondria-dependent apoptosis (Das et al. 2010; Lakshmi Devi and Anuradha 2010). The cytochrome c-mediated apoptotic pathway plays an important role in mitochondria-dependent apoptosis. The cytochrome c-mediated apoptotic pathway involves the release of cytochrome c from the mitochondria into the cytosol, followed by the assembly of the apoptosome complex of cytochrome c—Apaf-1—procaspase-9 (Cain et al. 1999; Hu et al. 1999). Procaspase-9 is autoactivated by being recruited to this complex and cleaved to activated caspase-9, which then activates procaspase-3 (Cain et al. 1999; Hu et al. 1999). Bcl-2 and Bax are the key regulators in mediating the release of mitochondrial cytochrome c into the cytosol (Kluck et al. 1997, Karch et al. 2013; Kalkavan and Green 2018). Heat shock proteins (Hsps) are a family of highly conserved stress proteins that play important roles during the stress response (Lindquist et al. 1988; Zhao et al. 2017). Many studies have shown that Hsp70, Hsp90, and Hsp27 play as antiapoptotic proteins to downregulate cytochrome c-mediated apoptosis in vivo or in vitro (Beere et al. 2000; Stankiewicz et al. 2005; Hoter et al. 2018; Bruey et al. 2000). In this study, taurine extremely increased the mRNA expression levels of Hsp27 and Hsp90 and significantly reduced the mRNA abundance of caspase-3 at an early stage of chronic cold stress. At the late stage of cold stress, taurine significantly elevated the mRNA level of Bcl-2 and obviously promoted the mRNA expression levels of Hsp70 and Hsp90.

5 Conclusion

The results indicated that taurine could enhance the antioxidant ability and alleviate cytochrome c-mediated apoptosis through upregulating the mRNA levels of Bcl-2, Hsp 27, and Hsp 90 in broiler hepatic tissues under chronic cold stress.

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Effects of Taurine on Serum Indexes of Broilers with Chronic Heat Stress

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Keywords

Broiler · Chronic heat stress · Serum indexes · Taurine

Abbreviations

ALT	Alanine transaminase
AST	Aspartate transaminase
CK	Creatine kinase
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
LDH	Lactic dehydrogenase
T3	Triiodothyronine
T4	Thyroxine
TP	Total protein

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1 Introduction

In the intensive breeding environment, heat stress is a common occurrence in summers with high temperature and humidity (Rajkumar et al. 2011). There are no sweat glands and feathers on the surface of broilers. When the animal body is under heat stress, liver function decreases, which seriously affects the health and productive performance of the animal and causes serious economic losses (He et al. 2017; Zhong et al. 2018). Many studies have shown that heat stress can affect physiological and biochemical indices (Mashaly et al. 2004; Sun et al. 2015; Liu 2011). The content of uric acid in serum reflects the balance of amino acids and protein metabolism in animals (Malmlof et al. 1989; Huang et al. 2006). Lyu et al. (2020) found that the content of thyroid hormone in the blood of heat-stressed broilers decreased significantly, while the content of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased significantly (Lyu et al. 2020). Thyroxine can promote the metabolism of material and energy in the body, mainly to promote the oxidation and breakdown of energy substances in the body, namely, sugar, protein, and fat, so as to increase oxygen consumption and release energy. Huang et al. (2006) found that the levels of T3 and T4 in the blood of roosters decreased significantly under acute heat stress.

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Taurine is a β-sulfur-containing amino acid which has a wide range of physiological effects and can participate in the metabolism of nutrients. It also has good antioxidant, immune regulatory, and nerve nutritional actions (Hayes et al. 1975). Regarding hormone regulation, He (1998) reported that taurine can antagonize the changes in myocardial tissue T3 and T4 levels in a rat stress model induced by isoproterenol and protect the myocardium from damage. Zhang et al. (2011) found that taurine can increase the content of T3 in rat serum. Gao et al. (2007) also found that taurine could increase the content of T3 and T4 in serum of Yellow River carp. It has been reported that the activities of ALT and AST increased under heat stress (Hosseini-Vashan et al. 2016; Xiaoli et al. 2017). Taurine not only shows anti-stress effects on experimental animals but also has good anti-stress effects on poultry.

The aim of this study was to investigate the effects of taurine on tissue damage, protein metabolism, and basal metabolism of broilers after chronic heat stress by detecting serum physiological and biochemical indices, so as to provide a theoretical basis for the application of taurine in acute heat stress of broilers.

2 Methods

2.1 Experimental Design

AA broilers were purchased from Shenyang Yunken Livestock Co., Ltd. 240 1-day-old AA broilers were routinely bred and were randomly divided into 5 groups at 7-day-old, namely, a normal temperature control group (NT), two heat stress control groups (HS), and three taurine groups low concentration heat stress group (0.5 g/L taurine +HS added to drinking water), four groups taurine medium concentration heat stress group (2 g/L taurine +HS added to drinking water), and high taurine group 5 concentration heat stress group (add 8 g/L taurine + HS to drinking water). All test chickens were free to drink water and feed freely throughout the day. The taurine-supplemented group started drinking taurine water at 8 days of age. The temperature of the control group was controlled so that it gradually decreased from 35 to 30 °C in the first 7 days, and then by 2–24 °C every week, and then maintained at 22–26 °C. The temperature of each group of heat stress was the same as that of the normal temperature group in the first 14 days. The temperature of the 15-day-old group began to rise to 34 °C for 2 h, and the temperature was maintained at 34 ± 2 °C. All groups in the test were maintained with normal ventilation.

2.2 Experimental Material

Chicken T3 and T4 kits were purchased from Shanghai Guchen Biotechnology Co., Ltd.; HSP70 and HSP60 kits were purchased from German IBL aliquots; protein quantification kit, uric acid detection kit triglyceride, CK, LDH, AST, ALT kits were purchased from Nanjing Jiancheng Bioengineering Research Institute.

2.3 Sample Collection

From the age of 15 days, the experiment was carried out formally. 12 hours before the start of each experiment, the broilers were not allowed to drink water. In the experiment, two broilers were randomly selected for each repetition at 6h, 12h, 7d, 14d, 21d, and 28d after heat stress.

2.4 Testing Indicators and Method

2.4.1 Determination of Protein Metabolism-Related Indexes in Serum

The content of total protein (TP) was determined by BCA method, and the content of uric acid was detected by ELISA method.

2.4.2 Determination of the Related Indexes of Basic Metabolism in Serum

Enzyme-linked immunoassay was used to detect the content of serum triiodothyronine (T3) and thyroid hormone (T4).

2.4.3 Determination of Tissue Injury-Related Indexes in Serum

Serum creatine kinase (CK), lactate deoxygenase (LDH), alanine aminotransferase (ALT/GPT), and aspartate aminotransferase (AST/GOT) activities were measured.

2.5 Statistical Analysis

SPSS16.0 statistical software was used for one-way analysis of variance. Multiple comparisons were performed using the DUNCAN method (the difference was significant at $P < 0.05$). The experimental data were expressed as mean ± standard error (SE).

3 Results

3.1 Effects of Taurine on Serum Protein Metabolism in Broilers with Chronic Heat Stress

Figure 1 shows the changes in serum total protein content of broilers. Compared with the NT group, the HS group was significantly lower than the NT group at each time period ($P < 0.05$). Compared with the HS group, the heat stress 7d taurine-supplemented groups, the heat stress 14d and 28d groups 4 and 5 were significantly higher than the HS group ($P < 0.05$). The taurine supplementation group also showed a downward trend compared to the NT group, but the decrease was smaller than that of the HS group. Among the taurine-supplemented groups, the heat stress at 6h was significantly higher in group 4 than in group 3 ($P < 0.05$). There was no significant difference between the other groups ($P > 0.05$).

Figure 2 shows the change in serum uric acid content in broilers. Compared with the NT group, the serum uric acid content increased significantly after 6 hours of heat stress ($P < 0.05$). Compared with the HS group, the heat stress 6h and 12h taurine-supplemented groups 7d, 28d, the fourth and fifth groups were significantly lower than the HS group ($P < 0.05$). Compared with the NT group, the taurine-supplemented groups were significantly higher in the heat stress group 12h, 7d, and 28d than the NT group ($P < 0.05$). Between the taurine-supplemented groups, the heat stress group 7d and 28d were significantly lower than group 3 ($P < 0.05$). The difference between other groups was not significant ($P > 0.05$).

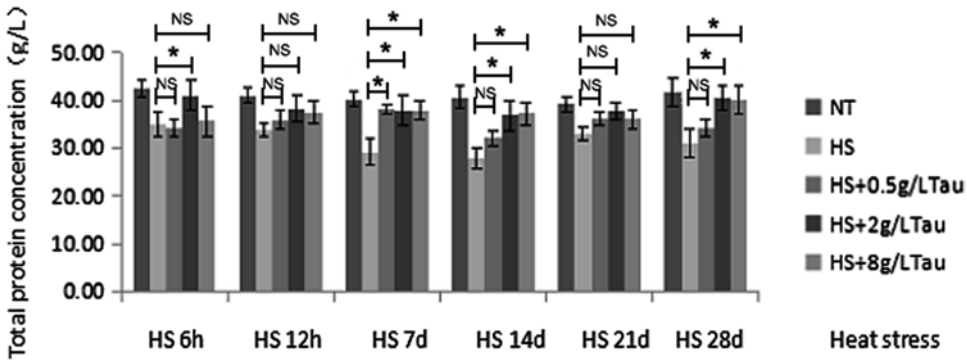

Fig. 1 Effect of taurine on serum total protein of broilers with chronic heat stress

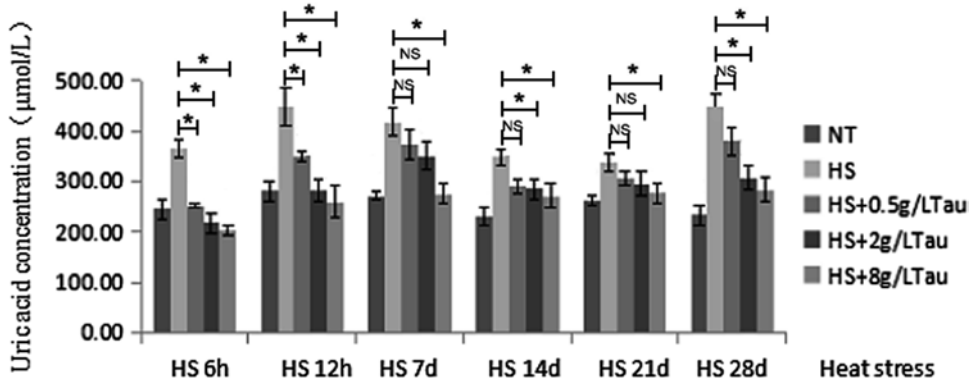

Fig. 2 Effect of taurine on serum uric acid in broilers with chronic heat stress

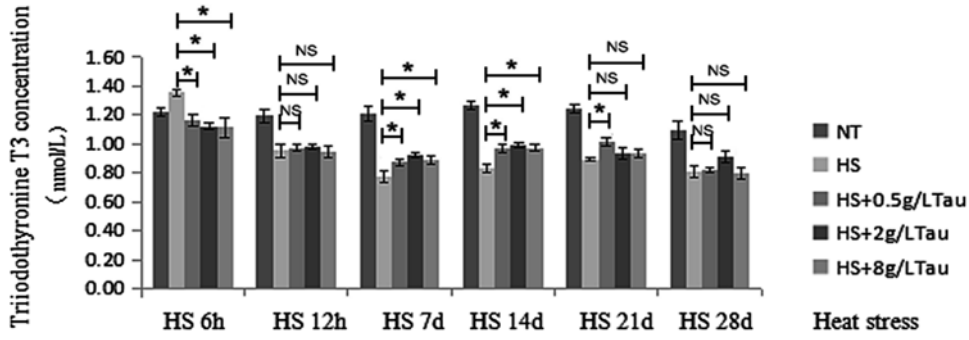

Fig. 3 Effect of taurine on serum T3 in broilers with chronic heat stress

3.2 Effects of Taurine on Serum Basic Metabolic Indices in Broilers with Chronic Heat Stress

Figure 3 shows the changes in broiler serum T3. Compared with NT, the HS group showed a process of first increasing briefly and then decreasing continuously. Compared with the HS group, the T3 concentration of each group of heat-stressed 6-hour taurine treatment group was significantly decreased, and the heat-stressed groups of 7d and 14d taurine and the third group of 21d taurine were significantly higher than the HS group ($P < 0.05$). The heat stress 12h, 7d, and 14d, 28d taurine-supplemented groups were significantly higher than the NT group ($P < 0.05$). There was no significant difference between the taurine-supplemented group and the other groups ($P > 0.05$).

Figure 4 shows the effect of broiler serum thyroid hormone T4. Compared with the NT group, the T4 concentration of the HS group showed a change that increased first and then decreased. Compared with the HS group, the difference between the taurine-treated group and the HS group was not significant ($P > 0.05$). Compared with the NT group, the taurine-supplemented group was significantly lower in the heat stress group 14d at 14d and the heat stress group 28d 3d and 4d ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

3.3 Effects of Taurine on Serum Tissue Injury-Related Indexes in Broilers with Chronic Heat Stress

Figure 5 shows the change in serum creatine kinase (CK) content of broilers. Compared with the NT group, the serum CK activity of the HS group increased significantly at 6h, 12h, 7d, 14d, 21d, and 28d ($P < 0.05$). The heat stress 7d, 14d

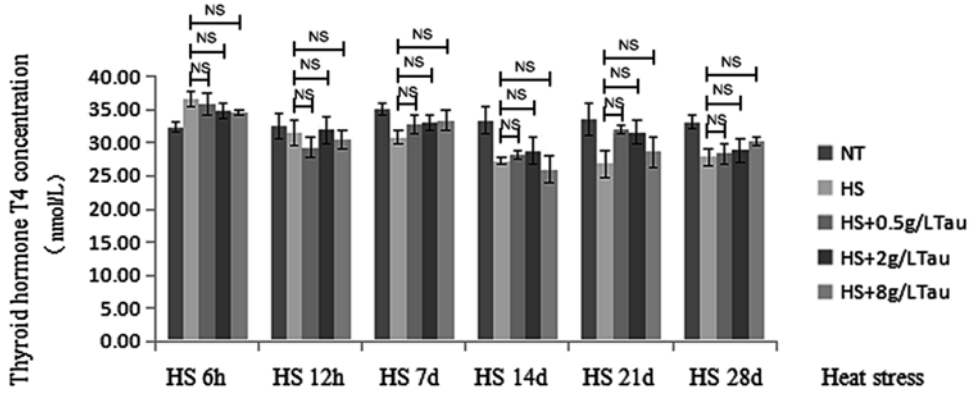

Fig. 4 Effect of taurine on serum T4 in broilers with chronic heat stress

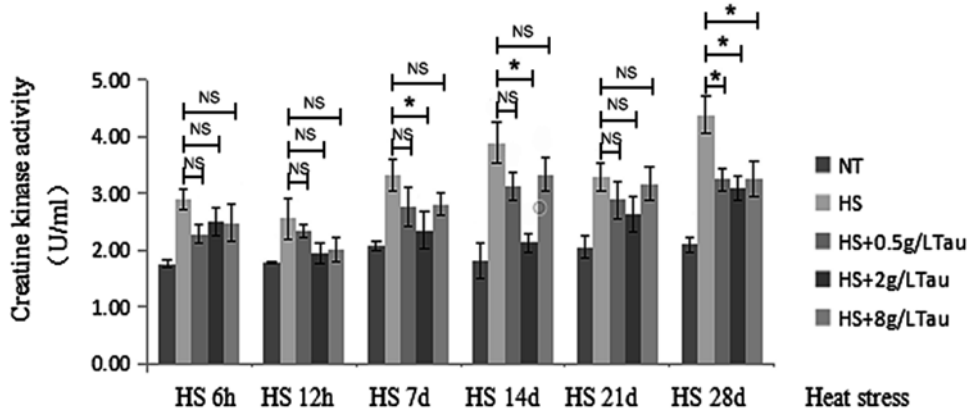

Fig. 5 Effect of taurine on CK activity in serum of broilers with chronic heat stress

group 4, and heat stress 28d taurine-supplemented groups were significantly lower than the HS group ($P < 0.05$). The heat stress 14d, 21d group 5, and heat stress 28d taurine-supplemented groups were significantly higher than those in the NT group ($P < 0.05$). The difference between the remaining groups was not significant ($P > 0.05$).

Figure 6 shows the change in serum lactate dehydrogenase (LDH) content of broilers. Compared with the NT group, the HS group increased significantly after 6 hours of heat stress ($P < 0.05$). Heat stress 6h, 7d taurine groups 3 and 4 added, heat stress 14d, 21d taurine groups 4, 5, and heat stress 28d taurine-supplemented groups were significantly lower than the HS group ($P < 0.05$). Compared with the NT group, taurine-supplemented groups also increased significantly ($P < 0.05$). The difference between taurine-added groups and the other groups was not significant ($P > 0.05$).

It can be seen from Fig. 7 that the change in serum aspartate aminotransferase (AST) content of broiler chickens, compared with the NT group, the HS group serum AST, has a significantly increased trend. Compared with the HS group, heat stress 12h groups 4, 5 groups, heat stress 7d, 14d taurine-supplemented groups, heat stress 21d group 5, and heat stress 28d groups 4 and 5 were significantly reduced ($P < 0.05$). The third group of heat stress at 12h, 7d, and 21d was significantly higher than that of NT group ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

It can be seen from Fig. 8 that the changes in serum alanine aminotransferase content (ALT) of broilers are significantly higher than those in the

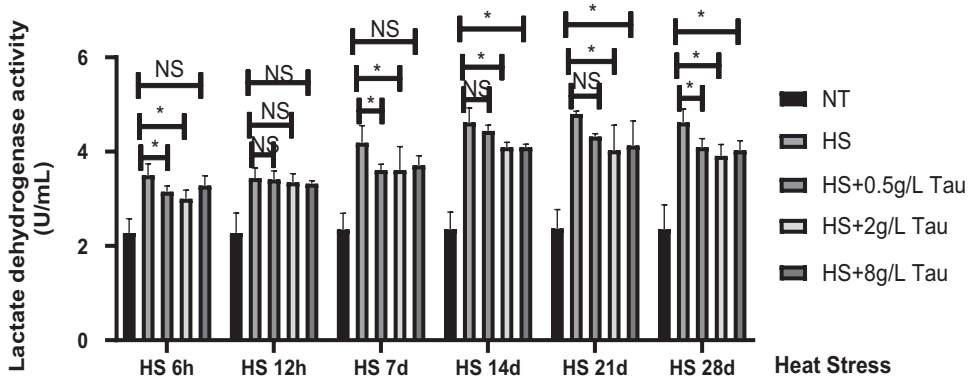

Fig. 6 Effect of taurine on LDH activity in serum of broilers with chronic heat stress

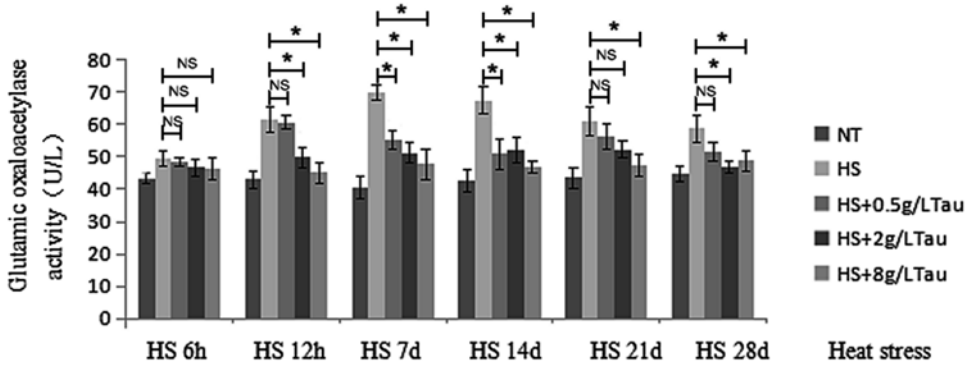

Fig. 7 Effect of taurine on AST activity in serum of broilers with chronic heat stress

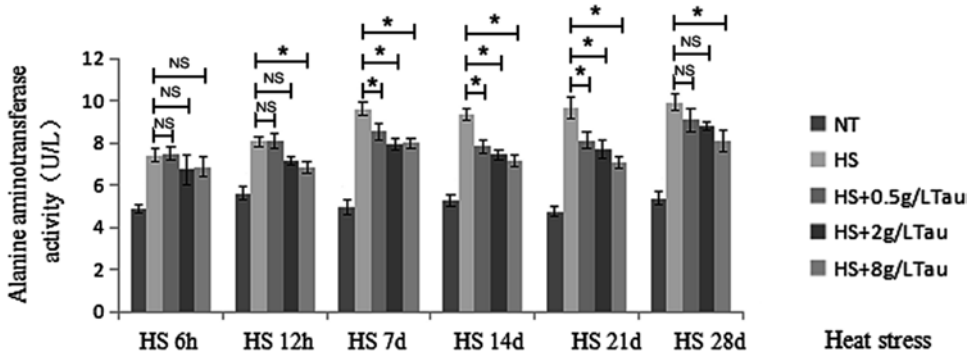

Fig. 8 Effect of taurine on ALT activity in serum of broilers with chronic heat stress

NT group after 6 hours of heat stress ($P < 0.05$). Compared with the HS group, the heat stress 12h, 28d group 5, heat stress 7d, 14d, and 21d taurine supplemented groups were significantly lower than the HS group ($P < 0.05$). Compared with NT group, taurine-supplemented groups were significantly increased ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

4 Discussion

4.1 Effects of Taurine on Serum Protein Metabolism in Broilers with Chronic Heat Stress

Figure 1 shows the changes in serum total protein content of broilers. Compared with the NT group, the HS group was significantly lower than the NT group as serum total protein changes during heat stress. Most studies believe that the serum protein content decrease after heat stress. Liu (2014) reported that serum total protein decreased significantly during heat stress. The heat stress control group had significant changes in serum total protein levels after 7 days of heat stress. This is consistent with the decrease in serum total protein levels reported by Liu (2003) after 5 days of heat stress treatment. In this test, the taurine-supplemented group, the heat-stressed 7d taurine-supplemented groups, 14d, 28d 2 g/L, and 8 g/L added groups were significantly higher than the heat-stressed control group, and there was no significant difference from the normal temperature control group. Therefore, it can be inferred that taurine increases serum albumin during heat stress. Ostrowski reported that acute heat stress causes an increase in plasma uric acid (Ostrowski-Meissner 1981). Studies suggest that uric acid can be considered as a parameter indicator of severe heat stress in broilers (Azad et al. 2010).

4.2 Effects of Taurine on Serum Basic Metabolic Indexes in Broilers with Chronic Heat Stress

Thyroid hormone is an important hormone for animals to regulate heat production and maintain body heat balance. Tao (2003) found that after heat stress, T3 and T4 concentrations in broilers decreased, reducing heat production, T3 decreased rapidly, and T4 decreased slowly. It was recommended to set serum T3 concentration as a heat stress–sensitive indicator and use T4 as a reference indicator (Tao 2003). Liu (2011) found that T3 increased first and then decreased in the first 3 days after heat stress, and T4 first decreased and then increased. Heat stress inhibits the cerebral cortex and suppresses TSH secretion, thyroid hormone declines, and T3 and T4 transform into a negative correlation (Ning 2002). The results of this experiment are similar to those of Liu (2011) and Ning (2002).

4.3 Effects of Taurine on Serum Tissue Injury-Related Indexes in Broilers with Chronic Heat Stress

Most serum enzymes are tissue-specific, and the number of enzymes in serum represents the function of specific tissues and organs. In this experiment, the activity of CK was significantly higher in the heat stress control group than in the normal temperature control group, and it increased significantly with time, indicating that the chickens suffered severe heat stress and muscle tissue damage. Combined with the results of previous studies, it was shown that the myocardium was obviously damaged after heat stress, and the addition of taurine relieved the degree of myocardial injury. Lactate dehydrogenase (LDH) is a key enzyme in glycolysis. Under heat conditions, thermoregulation increases blood flow to the skin causing the liver to be relatively ischemic and hypoxic, and hepatocyte to increase, resulting in increased serum LDH activity (Zhang et al. 2011; Wang 2011). This is consistent with our observations. Simultaneous detection of ALT and AST is commonly used clinically to evaluate liver function and status. Yu (2009) reported that both were significantly increased in the early stage, and the increase in concentration was correlated to liver tissue damage. Obvious particles appeared in the liver after 10 days of heat stress denaturation, steatosis, and even necrotic cell rupture (Yu 2009). The results are consistent with the findings of Chen et al. (2014), who reported that heat stress increased the activity of ALT and AST in mice.

5 Conclusion

The aim of this study was to investigate the effects of taurine on tissue injury, protein metabolism, and basal metabolism of broilers after chronic heat stress by measuring serum physiological and biochemical indices. The results showed that taurine could significantly increase the content of total protein and decrease the content of uric acid in serum of broilers with chronic heat stress. The content of T3 and T4 in serum of broilers with chronic heat stress was significantly increased, while the content of LDH, AST, and ALT in serum of broilers with chronic heat stress was significantly decreased. To provide a theoretical basis for the application of taurine in acute heat stress of broilers.

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Antioxidant Effect of Taurine on Chronic Heat-Stressed Broilers

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Keywords

Broiler · Chronic heat stress · Antioxidant · Taurine

Abbreviations

ALT	Alanine transaminase
AST	Aspartate transaminase
CAT	Catalase
CK	Creatine kinase
GSH-PX	Glutathione peroxidase
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
LDH	Lactic dehydrogenase
MDA	Malondialdehyde
SOD	Superoxide dismutase
T-AOC	Total antioxidant capacity

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1 Introduction

In livestock and poultry production, high temperature stress is a common stressor and an important factor restricting poultry production. The object of the study was to examine the impact of heat stress on broiler chicken and to seek solutions to alleviate the adverse effects of heat stress on livestock and breeding poultry. According to the severity and duration of heat stress, the performance of the antioxidant system and related enzymes is different. Heat stress can damage many organs of broilers (Hai et al. 2006). Normally, after acute heat stress, the activity of antioxidant enzymes (CAT, GSH-Px, SOD) is greatly increased to protect cells from damage by overproduced peroxide. On the other hand, chronic heat stress causes a different result. Long-term heat stress leads to the failure of the antioxidant enzyme system. Different antioxidant enzyme activity changes may originate from heat stress depending on conditions, types, and different tissues. Under heat stress, the content of ROS in broilers increase significantly, resulting in oxidative stress, as the activities of SOD, cat, and GSH-Px decrease significantly, and the liver synthesizes MDA (Yang et al. 2010). Pamok reported that 2 days of constant heat stress of 38 ± 2 °C in 28-day-old chickens lead to an increase in GSH-Px activity until 11 days of heat stress (Pamok et al. 2009). Many chronic heat stress studies have shown that MDA in broiler

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and quail tissues can increase, while antioxidant components (VD, VE, and zinc manganese copper selenium) decrease. In addition, studies have shown that plasma uric acid levels increase in heat stress (Azad et al. 2010). It shows that birds adapt to chronic heat stress by upregulating endogenous antioxidant levels such as GSH and uric acid. Heat stress can also reduce the weight of the bursa of Fabricius, thymus, and spleen, and cause mild and multifocal acute enteritis, increase lymphocytes and plasma cells in the lamina propria of the jejunum, and induce the hypothalamic-pituitary-adrenal axis to respond to heat. High fever can also cause metabolic changes, including promoting the generation of ROS and inducing oxidative damage and high fever (Mujahid et al. 2005; Quinteiro-Filho et al. 2010; Tan et al. 2010; Toyomuzi et al. 2005). It has been reported that the activity of GSH-PX in blood, liver, kidney, and heart decrease during continuous heat stress. Sahin K reported that heat-stressed quail causes reductions in liver SOD, CAT, and GSH-Px activities (Sahin et al. 2010). Lu's research found that taurine can reduce and protect fallopian tube damage induced by acute heat stress and increase the antioxidation and immunity of fallopian tube tissues (Lu 2011).

Taurine is a β-sulfur-containing amino acid, which has a wide range of physiological effects and can participate in the metabolism of nutrients. It also is an effective antioxidant, immune regulator, and source of nerve nutrition (Hayes et al. 1975). Winiarska et al. (2009) added taurine to the drinking water of rabbits and found that the activities of SOD and GSH-Px in the serum were increased, and the accumulation of carboxyl free radicals in the blood and internal organs was reduced. However, if the level of addition was too high, the antioxidant capacity decreased (Winiarska et al. 2009). Studies have shown that adding 0.1% and 0.15% taurine to the diet can significantly increase SOD, GSH-Px, and T-AOC activities of broiler chicks at 1–21 days old and significantly reduce MDA levels (Li et al. 2010; Sahin et al. 2010). Studies by Lu and colleagues found that taurine can effectively inhibit excessive activation of the renin-angiotensin-aldosterone (RAAS) system in stressed hypertensive mice and stabilize blood pressure of rats in the fed group compared with the control group (Lyu et al. 2014). Regarding hormone regulation, He et al. reported that taurine can antagonize the changes in myocardial tissue T3 and T4 levels in a rat stress model induced by isoproterenol and protect the myocardium from damage (He et al. 1998). Taurine not only shows anti-stress effects on experimental animals but also exhibits good anti-stress effects on poultry.

This study combined the damage of chronic heat stress on broilers and the protective effect of taurine on the body. The objective to investigate the protective effect of taurine on chronic heat stress injury in broilers by detecting the content of heat shock protein (HSP) and serum antioxidant index is to provide a theoretical basis for the application of taurine against chronic heat stress of broilers.

2 Methods

2.1 Experimental Design

AA broilers were purchased from Shenyang Yunken Livestock Co., Ltd. 240 1-day-old AA broilers were routinely bred and were randomly divided into five groups at 7-day-old, namely, a normal temperature control group (NT), two heat stress control groups (HS), and three taurine groups, low concentration heat stress group (0.5 g/L taurine +HS added to drinking water), 4 groups taurine medium concentration heat stress group (2 g/L taurine +HS added to drinking water), and high taurine group and concentration heat stress group (add 8 g/L taurine + HS to drinking water). All test chickens were free to drink water and feed freely throughout the day. The taurine-added group started drinking water to add taurine at 8 days of age. The temperature of the normal temperature control group was controlled so that the temperature gradually decreased from 35 °C to 30 °C in the first 7 days and then decreased by 2 °C to 24 °C every week, and then the temperature was maintained at

22–26 °C. The temperature of each group of heat stress was the same as that of the normal temperature group in the first 14 days. The temperature of the 15-day-old group was increased to 34 °C for 2 h, and the temperature was then maintained at 34 ± 2 °C. All groups in the test received normal ventilation.

2.2 Experimental Material

HSP70 and HSP60 kits were purchased from German IBL aliquots; T -AOC, MDA, SOD, and GSH-PX kits were purchased from Nanjing Jiancheng Bioengineering Research Institute; catalase kits were purchased from Beijing Solable Biotechnology Research Institute Co., Ltd.

2.3 Sample Collection

From the age of 15 days, the experiment was carried out formally. 12 hours before the start of each experiment, the broilers could not help drinking water. In the experiment, two broilers were randomly selected for each repetition at 6h, 12h, 7d, 14d, 21d, and 28d after heat stress.

2.4 Testing Indicators and Methods

2.4.1 Determination of Serum Heat Shock Protein

An enzyme-linked immunosorbent assay was used to determine the content of heat shock proteins HSP60 and HSP70 in the serum. The above indicators were tested using kits, and the operation was strictly in accordance with the requirements of the kits.

2.4.2 Determination of Serum Antioxidant Capacity

Serum malondialdehyde content (MDA) was determined by the TBA method; the total oxidative capacity of the serum (T-AOC) was determined by the FRAP method; the superoxide dismutase activity (SOD) was determined by the WST-1 method; and GSH-PX ability to measure serum glutathione peroxidase activity, using CAT catalytic ability to determine the activity of catalase.

2.5 Statistical Analysis

SPSS16.0 statistical software was used for one-way analysis of variance. Multiple comparisons were performed using the DUNCAN method (the difference was significant at $P < 0.05$). The experimental data was expressed as means ± standard error (SE).

3 Results

3.1 Effect of Taurine on Serum Heat Shock Proteins of Chronic Heat Stress Broilers

Figure 1 shows the changes in serum heat shock protein 70 (HSP-70) in broilers. Compared with the NT group, the serum HSP70 content in the HS group was significantly increased at 6h, 7d, 14d, 21d, and 28d ($P < 0.05$), compared with the HS group; the 6th group 3 and 4 were significantly higher than the HS group ($P < 0.05$). The 14th heat stress group 3 and 4 were significantly lower than the HS group ($P < 0.05$). Compared with the NT group, the taurine-added groups were significantly higher than the NT group except for the heat stress group 12h and the heat stress group 14d ($P < 0.05$). The difference between other groups was not significant ($P > 0.05$).

Figure 2 shows the changes of serum heat shock protein 60 (HSP-60) in broilers. Compared with the NT group, the HS group significantly increased at 6h, 12h, 14d, 21d, and 28d ($P < 0.05$). Compared with the HS group, the taurine-treated 7d heat stress, and the heat stress 28d groups 3 and 4 were significantly higher than the HS group ($P < 0.05$). Compared with the NT group, the taurine-treated group contained more HSP70

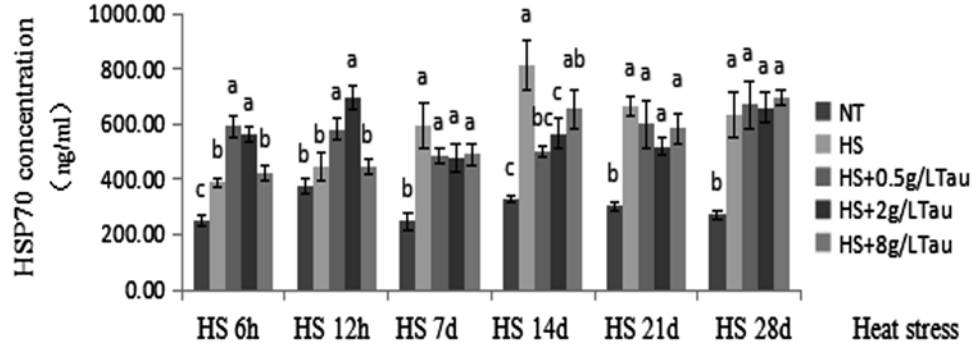

Fig. 1 Effect of taurine supplementation on serum HSP-70 of broilers exposure to chronic heat stress

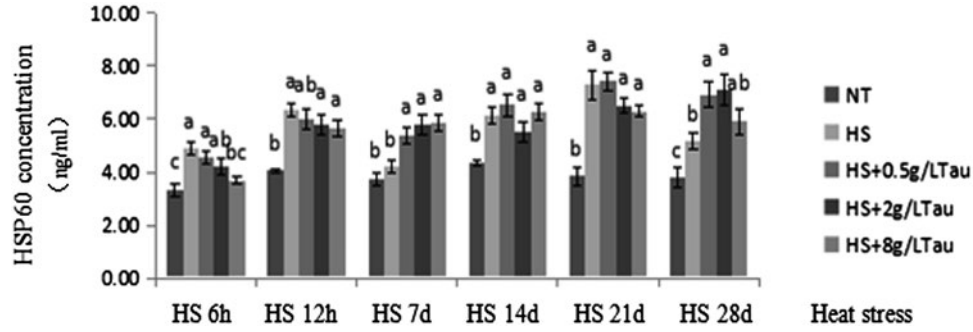

Fig. 2 Effect of taurine supplementation on serum HSP-60 of broilers exposure to chronic heat stress

than the NT group except that the heat stress group 6 and the NT group were not significantly different at 6h ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

3.2 The Effect of Taurine on Serum Antioxidant Capacity of Chronic Heat-Stress Broilers

Figure 3 shows the change of serum malondialdehyde content in broilers. Compared with the NT group, the serum MDA concentration in the HS group increased significantly after 6 hours of heat stress ($P < 0.05$). The taurine treated 6h,7d, and 14d heat stress groups were significantly lower than the HS group ($P < 0.05$). The taurine-treated 12h heat stress groups and the 6h, 7d, 14, and 28d heat stress groups were significantly higher than the NT group ($P < 0.05$). In the taurine-treated groups, heat stress resulted in less malondialdehyde production in the third group than that in the fifth group ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

Figure 4 shows the change in total antioxidant capacity of broiler serum. Compared with the NT group, the 12h, 7d, 14d, 21d, and 28d heat stress groups were significantly lower than that of the NT group ($P < 0.05$). Compared with the HS group, the taurine-treated 12h, 21d group 4, heat stress 7d heat stress groups 4, 5 and heat stress 14d were significantly higher than those of the HS group ($P < 0.05$). On the 28th day of heat stress, the 4th and 5th taurine-treated groups were significantly lower than those in the NT group ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

Figure 5 shows the changes in serum superoxide dismutase activity (SOD) in broilers. Compared with the NT group, the HS group showed a trend with time of first increasing SOD

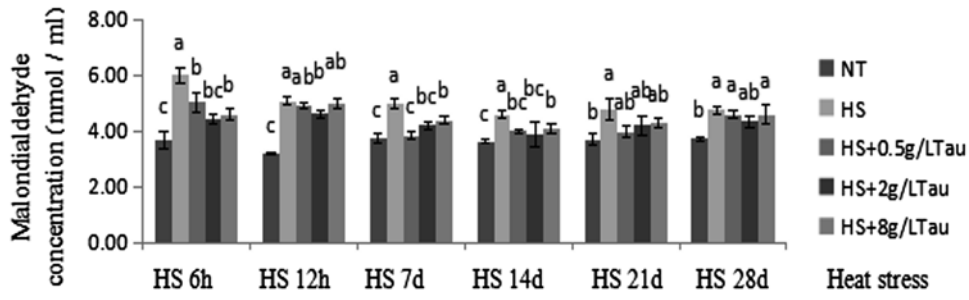

Fig. 3 Effect of taurine supplementation on serum MDA content of broilers exposure to chronic heat stress

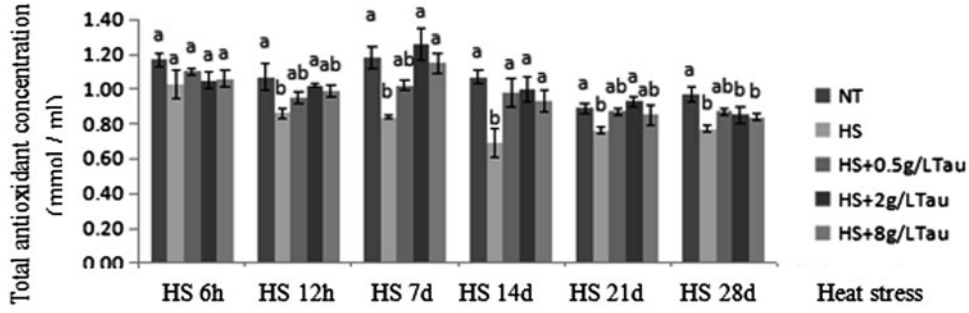

Fig. 4 Effect of taurine supplementation on serum T-AOC of broilers exposure to chronic heat stress

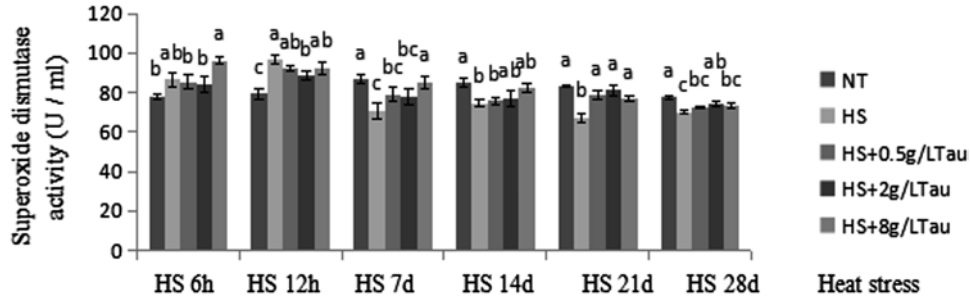

Fig. 5 Effect of taurine supplementation on serum SOD activity of broilers exposure to chronic heat stress

activity followed by decreasing. Compared with the HS group, the 4th heat stress group 4 was significantly lower than the HS group ($P < 0.05$). The 6h and 7d heat stress groups, the 21d heat stress taurine treated group, and 28d groups 3 and 4 were significantly higher than those of the HS groups. The 6h heat stress group 5 and heat stress 12h taurine-treated groups were significantly higher than the NT group ($P < 0.05$). The third group of 7d, 14d, and 28d heat stress group was significantly lower than that of the NT group ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

Figure 6 shows the changes in serum catalase activity (CAT). Compared with the NT group, CAT of the HS group showed a trend of increasing after 6h of heat stress but decreasing after more prolonged heat stress. The heat stress at 6h was significantly higher than that in the NT group ($P < 0.05$), and the heat stress at 12h, 7d, 14d,

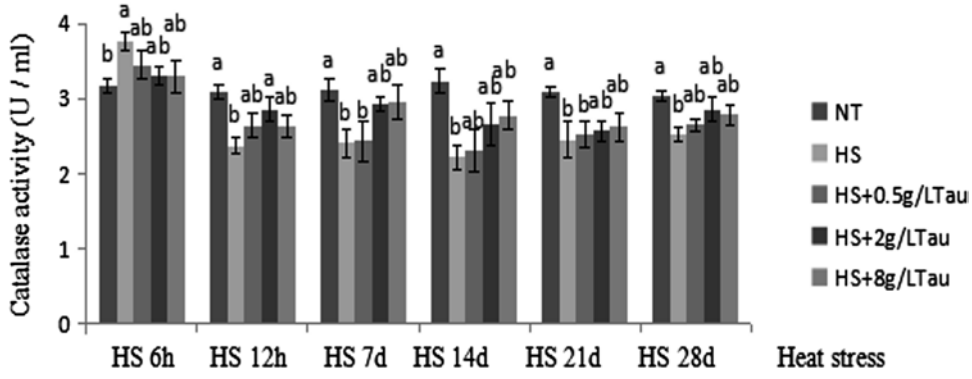

Fig. 6 Effect of taurine supplementation on serum CAT activity of broilers exposure to chronic heat stress

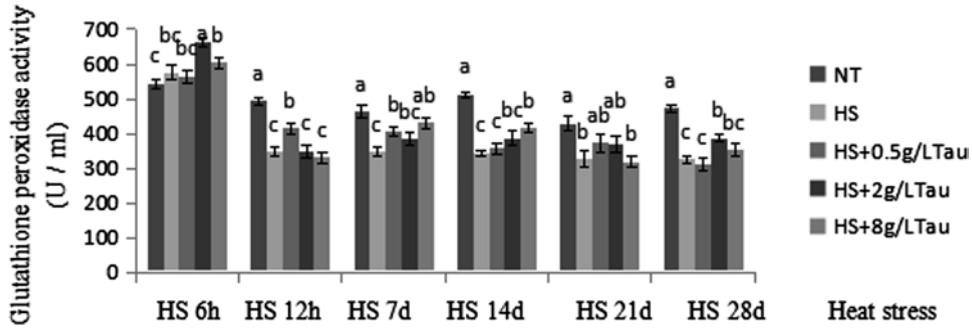

Fig. 7 Effect of taurine supplementation on serum GSH-PX activity of broilers exposure to chronic heat stress

21d, and 28d was significantly lower than that in the NT group ($P < 0.05$). Compared with the HS group, the CAT of the 4th group with heat stress at 12h was significantly increased ($P < 0.05$). The third group of heat stress at 7d and 14d decreased significantly compared to the NT group ($P < 0.05$). The difference between all other groups was not significant ($P > 0.05$).

Figure 7 shows the change in serum glutathione peroxidase activity (GSH-PX) caused by heat stress and taurine treatment. The GSH-PX activity of the HS group at 12h, 7d, 14d, 21d, and 28d was significantly lower than that of the NT group ($P < 0.05$). The heat stress (6h and 28d group 4) and heat stress (12h and 7d group 3) were significantly higher than the HS group ($P < 0.05$). Heat stress (6h in groups 4 and 5) was significantly higher than that in the NT group ($P < 0.05$). GSH-PX activity of the heat stress (12h, 14d, and 28d taurine-treated groups) and heat stress (7d groups 3 and 4) were significantly lower than that of the NT group ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$).

4 Discussion

4.1 Effect of Taurine on Serum Heat Shock Protein of Chronic Heat Stress Broilers

Mitochondria are damaged under the stress of high temperature, which will reduce the oxygen content of cells and increase the production of ROS. At this time, under the stimulation of ROS, the cells produce a group of special protein HSPs. HSP70 is a cytosolic chaperone, which can repair

damaged proteins and degrade irreparable proteins during stress; HSP60 mainly exists in mitochondria and chloroplasts and can fold protein to resist stress. With the occurrence of heat stress, HSP70 and Hsp60 are constantly produced in cells to regulate the body's ability to resist heat stress, and taurine can promote the production of HSP70 and HSP60. After 6 hours of heat stress stimulation, the body's damage is the most serious, and then with the production of intracellular HSPs, the body's ability to resist heat stress is gradually strengthened. Therefore, compared with 6h, the levels of GSH-Px, CAT, T-AOC, and MDA in 12h serum decreased, while the levels of SOD increased.

Heat shock protein 70 (HSP70) is the most important of the group of heat shock proteins. Chickens can cause increased expression of HSP70 under heat stress. Therefore, to a certain extent, it can alleviate the stress damage of a high-temperature environment to the body (Wang 2010). Under the condition of acute heat stress, the expression of HSP70 significantly increases (Xie et al. 2018). Wang's research found that the expression of HSP70 in the intestinal segment after heat stress initially increased and then decreased (Wang 2011). Yan's research found that the expression of HSP60 is specifically elevated after heat stress, while cardiac HSP60 and its genes are elevated at 2 hours of heat stress (Yan 2008). In the present study, HSP60 and HSP70 both increased to varying degrees after heat stress. It may be because taurine reduced the damage of heat stress, reduced the consumption of HSP, and enhanced the regulatory expression of HSP protein.

4.2 The Effect of Taurine on the Blood Antioxidant Capacity of Chronic Heat-Stressed Broilers

There are many reports about the influence of heat stress on the antioxidant content of poultry. Heat stress can affect the balance of oxidative and antioxidant systems in broilers (Lin et al. 2008; Azad et al. 2010; Jin et al. 2010; Zhang et al. 2014). When oxygen-free radical and ROS content is excessive, the balance between oxidation and antioxidant systems is shifted in favor of oxidative damage. At 41 °C, the content of peroxides in the mitochondria and ROS in cells increased, which indicated that heat stress could directly lead to oxidative stress (Motoi et al. 2015). Leading to lipid peroxidation, malondialdehyde (MDA) is the main product of the lipid peroxidation chain reaction, and its content can reflect the body's degree of peroxidation (Zheng 2007; Guo et al. 2018). Mujahid et al. (2005) reported that acute heat stress significantly increases MDA content in skeletal muscle of broilers. Total oxidation capacity (T-AOC) is a comprehensive index used to measure the body's antioxidant system capacity (Mujahid et al. 2005; Zhong et al. 2018). Antioxidant enzymes (SOD, CAT, GSH-PX) reflect the vitality of the body's antioxidant enzyme system. In this study, serum MDA content increased significantly after heat stress and then decreased slightly, but it was still significantly higher than the normal temperature control group. The taurine-treated groups showed an effect at an early stage of heat stress, but there was no significant difference between the latter stage of heat stress (HS21d, 28d) and the heat stress control group, which may be due to the long-term heat stress antioxidant capacity failure. T-AOC decreased significantly after 12 hours of heat stress and maintained a relative balance after 14 days. The taurine treatment group showed a significant effect from 12 hours to 21 days of heat stress and significantly improved the total oxidation capacity. Especially in the 2 g/L treatment group, it performed better. Taurine played a regulatory role to varying degrees; especially the middle-concentration treatment group played an effective role during moderate periods of heat stress.

Heat shock protein can directly release or increase the level of endogenous peroxidase, such as SOD, and increase the synthesis and release of endogenous antioxidants. At 6h, HSP60 and HSP70 in heat stress group were

significantly higher than those in the control group, indicating that HSP was activated first in the heat stress defense mechanism. After 12 hours, SOD, T-AOC, and GSH-Px changed significantly, which may be affected by the expression of heat shock protein. It is suggested that SOD, T-AOC, and GSH-Px may be downstream of HSP60 and HSP70 and regulated by HSP60 and HSP70.

5 Conclusion

Taurine can reduce serum MDA, increase T-AOC, and increase antioxidant enzyme activity. It is suggested that taurine protects broilers from heat shock by regulating metabolism, improving antioxidant capacity, and improving oxidative damage induced by chronic heat stress.

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Part IV

Nutritional Value of Taurine and Consequences of Taurine Deficiency

Grading of Japanese Diet Intakes by 24-Hour Urine Analysis of Taurine and Soy Isoflavones in Relation to Cardiovascular Risks

Mari Mori, Miki Sagara, Hideki Mori, and Yukio Yamori

Keywords

Isoflavones · Sodium · Magnesium · Potassium · Japanese diet · Seafood · Soybean · HDL cholesterol · Cholesterolemia · Blood pressure · Obesity · Metabolic syndrome · Coronary heart disease

Abbreviations

BMI	Body mass index
CARDIAC Study	Cardiovascular Diseases and Alimentary Comparison Study
Cr	Creatinine
HDL	High-density lipoprotein
I	Isoflavone
K	Potassium
Mg	Magnesium
Na	Sodium
T	Taurine

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1 Introduction

Japanese have been enjoying the longest average life expectancy in the world for the last 30 years (WHO 2016; UNDP 2015). Since the *World Health Organization* (WHO)-coordinated Cardiovascular Diseases and Alimentary Comparison Study (CARDIAC) indicated taurine (T) and isoflavones (I), the biomarkers of 24-hour urine (24U) for seafood and soy intakes were commonly excreted among Japanese people in comparison with other populations, 24U T and I were used for scoring the Japanese diet, and the results of this scoring were evaluated for potential associations with cardiovascular risks and the urinary excretion of magnesium (Mg), which is also found in seafood and soy.

2 Methods

In order to estimate nutritional intakes by measuring the biomarkers of nutrients in the 24U samples, a special device to collect 2.5% of the voided urine each time was developed (Yamori et al. 1984, 2018). Urine is voided into the upper compartment of a double-bottomed cup, and just by pushing the rod once, the exact portion of the urine can be collected into the lower compartment. By repeating this simple procedure at each time of voiding urine for 24 h, the daily intakes of salt, vegetables, seafood, soy, and so on can be

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estimated exactly by analyzing their biomarkers such as sodium (Na), potassium (K), T, I, and Mg.

After the development of this simple device for 24U collection for nutritional epidemiology, the CARDIAC Study was started in 1985 (CARDIAC 1990; Yamori 2006; Yamori et al. 2006), for analyzing the biological markers of diet in association with blood pressure and cardiovascular disease mortalities in the world. Thanks to the donations to the WHO from 300,000 companies and individuals, health examinations in 61 populations of 25 countries were carried out successfully (Yamori et al. 2010; Yamori 2005). About 100 males and 100 females aged 48–56 were randomly selected from each study site for anthropological examination, blood pressure measurement, 24U collection, blood tests, and questionnaires regarding diet and medical history, and over 14,000 participants were examined for 20 years ending in 2005. For this worldwide epidemiological study, a standardized automated blood pressure measurement system was used to avoid observer bias. 24U biomarkers to estimate Na, K, Mg, and T intakes were analyzed among 4211 participants (49.7% females) of 50 populations from 22 countries examined from 1985 to 1995. 24U samples from 850 participants (47.8% females) of 18 populations in 12 countries, maintained deep frozen and available for further analysis in 1995, were randomly selected for the detection of the biomarkers for seafood and soy intakes.

Obese subjects were defined as those with body mass index (BMI) $\geqslant$ 30 k/gm^2. Hypercholesterolemic subjects were defined as those with serum total cholesterol $\geqslant$ 220 mg/dl. The markers in 24U are expressed as the ratio of each parameter normalized to creatinine (Cre). 24U T/Cre and I/Cre ratios were categorized in quintiles, T1 to T5 and I1 to I5, and then divided into 25 groups, which were obtained by 5 (T1–T5) $\times$ 5 (I1–I5). Since the Japanese excreted the higher T and I in the urine compared with other populations in the world, all sample were divided into 5 groups, J1–J5, depending on the quintiles of T and I, from the lowest excreters of both to the highest excreters as described in this article. The characteristics of J1–J5 were comparatively analyzed for cardiovascular risks and urinary biomarkers. The proportions of the Japanese within 25 groups were analyzed. General linear models were used to estimate adjusted mean values of BMI, total and HDL cholesterol, atherogenic index (non-HDL/HDL), 24U Na and Na/K, across the five scores (J1 to J5) of the Japanese diet intakes after adjustment for age and sex. Analysis of covariance was conducted to determine a statistical significance between the five groups (J1–J5) on systolic and diastolic blood pressure controlling age and sex. To evaluate the association between the Japanese diet intake and cardiovascular disease risk factors, we estimated adjusted odds ratios for obesity and hypercholesterolemia in relation to the scores of the Japanese diet intakes using logistic regression models, including variables for age and sex.

3 Results

3.1 Salt and Stroke

Our survey with the Maasai in northern Tanzania in 1986 demonstrated their average blood pressure was obviously lower than the average in the world of this study and they had almost no hypertension (CARDIAC 1989). In contrast, the Tibetans living at the foot of Mt. Everest had a higher prevalence of hypertension. We asked them to collect whole-day urine and confirmed that the Maasai used no salt at that time and their daily intake of salt mainly from milk was very low, only 2.5 g a day. The Tibetans consumed 16 g of salt a day on average, and many suffered from severe hypertension (Yamori 2005). Therefore, the mortality rates of stroke, mostly caused by hypertension, were significantly positively related with *24U* salt excretion (Yamori 2006; Yamori et al. 2006). This data indicates that reductions in salt intake down to 7 g a day should greatly contribute to the prevention of stroke. Stroke mortality and bedridden disability and cerebrovascular dementia for which stroke is the major cause are expected to be greatly decreased,

if daily salt intake is reduced down to less than 5 g a day, as recommended by the WHO (2012).

3.2 Soy Isoflavones and Coronary Heart Diseases (CHD)

In this study, we investigated soy I, the structure of which is similar to estrogen, the female sex hormone. Soy I has weak estrogen-like activity, so it is expected to be protective against CHD (Yamori et al. 2018; Ma et al. 2021). Our study demonstrated for the first time in the world that urinary I excretion is inversely related to age-adjusted CHD mortality (Yamori 2006). The data indicate about 20 μmol I is excreted in the urine, corresponding to 40 mg of I contained in 60–75 g of dry soy beans, and is associated with low CHD mortality among Japanese.

3.3 Taurine and CHD

Since our study clearly demonstrated that BMI, systolic and diastolic blood pressure, and total cholesterol levels were significantly higher in people whose 24U T excretions were lower than the average of the T excretion in the world (Yamori et al. 2010); less seafood intake is related to obesity, hypertension, and hypercholesterolemia which are the major risk factors of CHD. Therefore, the age-adjusted mortality rates of CHD were significantly inversely related with 24U T excretion, indicating that the more seafood was consumed, the less the mortality rates of CHD (Yamori et al. 2006; Yamori 2005). The seafood intake of Japanese was the highest, followed by the Mediterranean populations. The consumption of seafood, 80–100 g a day, appears to be associated with low CHD mortality rates of Japanese (Yamori 2005).

3.4 Features of Japanese Diets and the Grading

The average life expectancy is inversely related with CHD mortality rates, and the lowest CHD mortality of Japanese was proven to be due to the highest seafood and soy consumption (Yamori 2006). Therefore, the longest life expectancy of Japanese in the world can be ascribed to the highest seafood and soy intakes. Since Japanese customarily eat seafood and soy containing T or I, our worldwide 24U analysis revealed the obvious characteristics of the Japanese diet. Biomarkers of seafood and soy intakes, I/Cre and T/Cre ratios were divided into five groups (Sagara et al. 2015; Yamori et al. 2015), each from the smallest to the largest quantity of I and T intake (I1–I5, T1–T5), and all participants were divided into 5 by 5, in total 25 groups. Figure 1 indicates the ratio of Japanese in each one of 25 groups (Yamori et al. 2017); the highest seafood and soy consumption group was in the 5th quintile for both T and I (T5/I5), which contained nearly 90% Japanese, and the lowest 1st quintile for both T and I (T1/I1) contained no Japanese, indicating that Japanese are commonly consuming both seafood and soy (Yamori et al. 2017).

Therefore, the features of the Japanese diet can be scored by the quintiles of both T and I excretions in 24U, as shown in Fig. 1a. Then, the tendency of Japanese diet intake was defined as J1–J5, depending on the total of the quintile number of I and T excretions (Fig. 1b). JI, for example, includes the data from T1 and I1 (T1/I1:2), T1 and I2 (T1/I2:3), and T2 and I1 (T2/I1:3), and the other from J2 to J5 include the data from the groups indicated as follows:

J1 = (T1/I2:3) (T1/I2:3) (T2/I1:3)
J2 = (T1/I3:4) (T1/I4:5) (T2/I2:4) (T2/I3:5) (T3/I1:4) (T3/I2:5) (T4/I1:5)
J3 = (T5/I1:6) (T4/I2:6) (T3/I3:6) (T2/I4:6) (T1/I5:6)
J4 = (T2/I5:7) (T3/I5:8) (T3/I4:7) (T4/I4:8) (T4/I3:7) (T5/I3:7) (T5/I2:7)
J5 = (T5/I5:10) (T5/I4:9) (T4/I5:9)

Typical Japanese dietary consumption corresponding to J4 and J5 contains the groups in which the total quintile number of each T and I (T/I:7–10) is over the average of 6. In contrast, the less typical Japanese diets, J1 and J2, contain the groups of the total quintile number of T and I (T/I:2–5) which is below the average of 6.

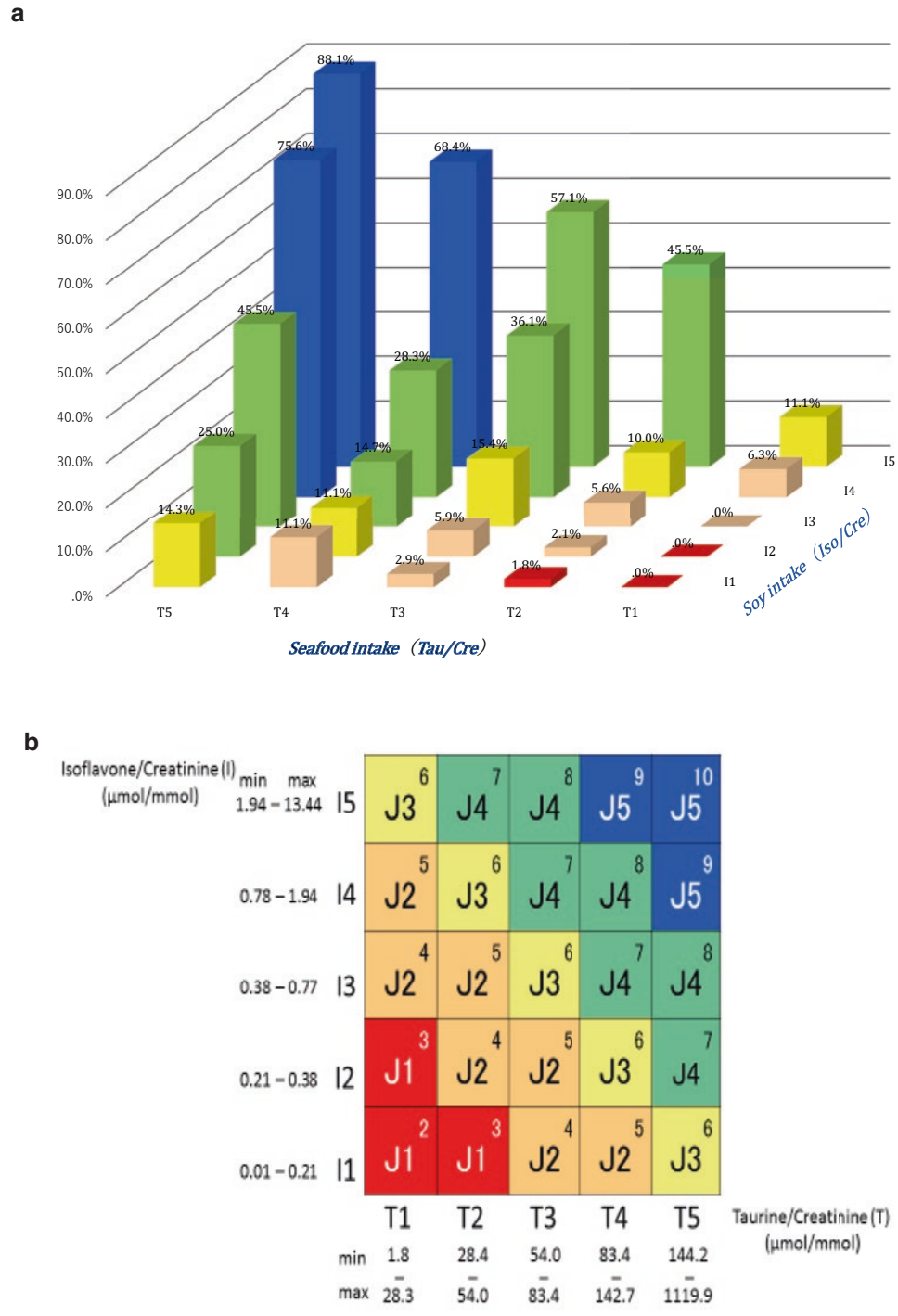


Fig. 1 (**a**) The ratio (%) of the Japanese in the total 25 groups of the people divided by the quintile analyses of 24U biomarkers of isoflavones and seafood taurine excretions. (**b**) The scoring of the Japanese diet intake (J1–J5) depending on the quintile analyses of 24U seafood taurine (T1–T5) and soy isoflavones (I1–I5) biomarker excretions

3.5 Merits of Japanese Diet

Figure 2 indicates the association of obesity or BMI with the scoring of the Japanese diet (Fig. 1b) after the adjustment of age and sex. The typical Japanese diet, shown by J5 and J4, is clearly associated with significantly less obesity and significantly lower BMI. The adjusted odds ratios of obesity associated with the lower scoring of the Japanese diet intake (J≦3) are significantly high relative to J1. Figure 3 indicates the association of hypercholesterolemia or total cholesterol levels in the blood with the grading of the Japanese diet (Fig. 1b) after age and sex adjust-

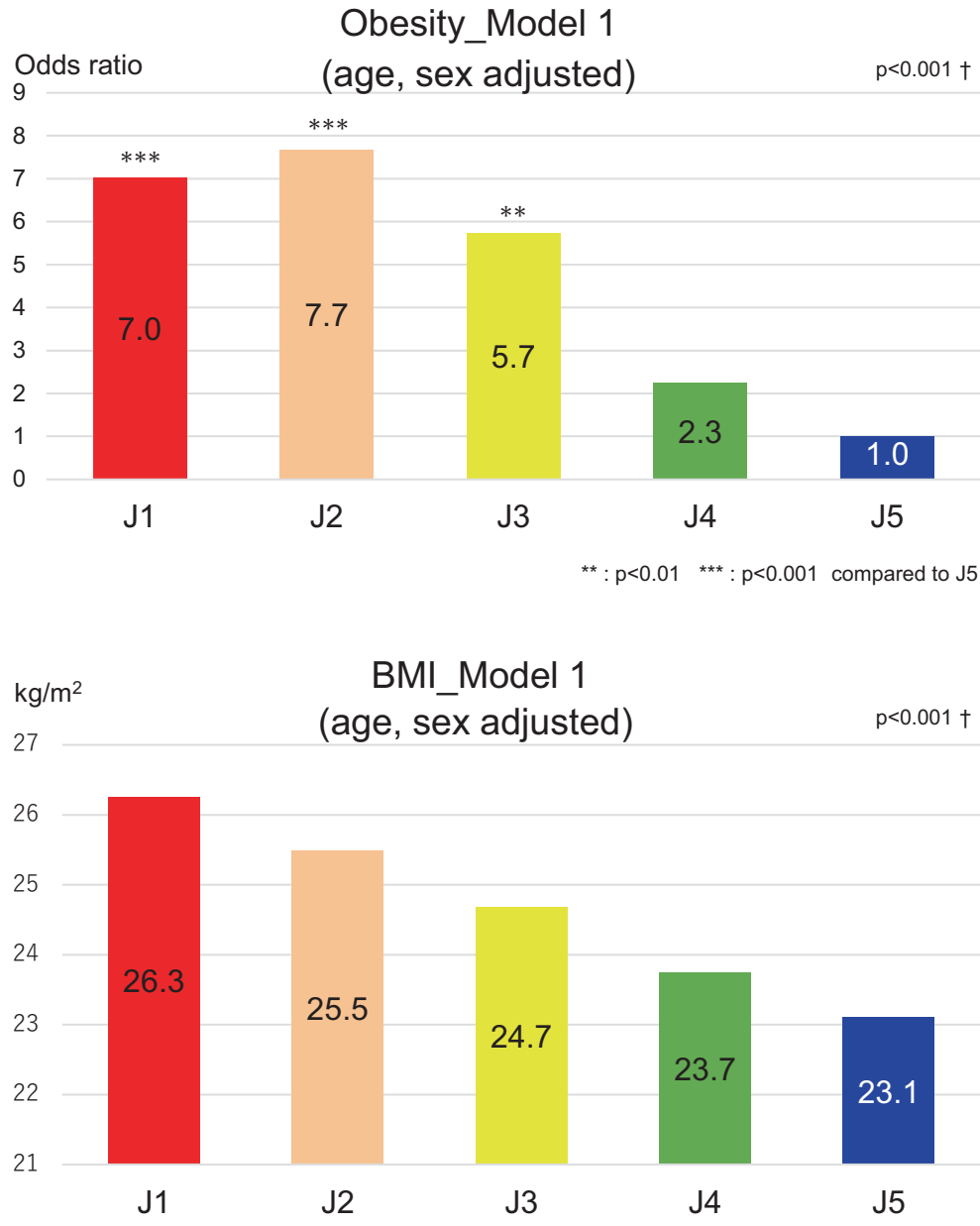
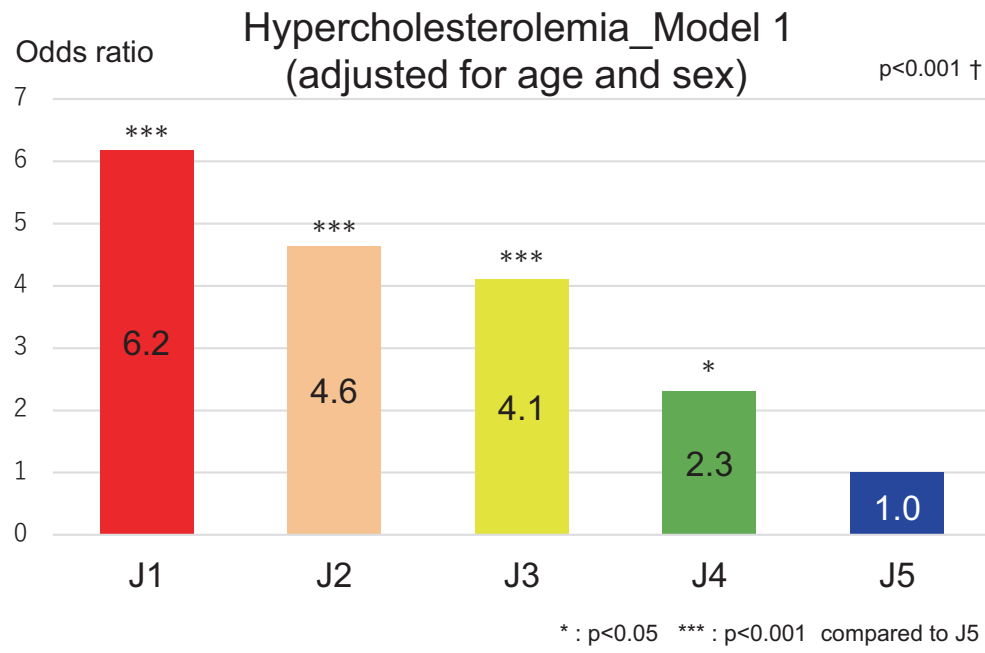


Fig. 2 Significant inverse association of obesity and BMI with the scoring of Japanese diet (J1–J5) after age and sex adjustment (Model 1) († Significance from multiple linear regression)



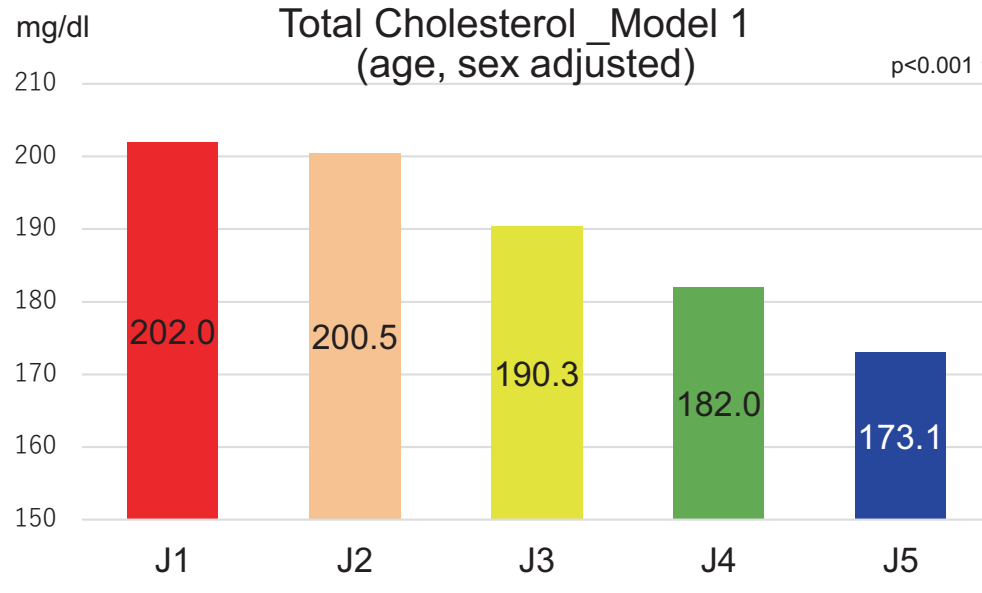


Fig. 3 Significant inverse association of hypercholesterolemia (%) and serum total cholesterol levels with the scoring of the Japanese diet intake (J1–J5) after age and sex adjustment (Model 1) († significance from multiple linear regession)

ment. Figure 3 indicates the positive association of hyper cholesterolemia and total cholesterol level with the scoring of the Japanese diet and the odds ratios in J≦4 are significantly high relative to J1. In Fig. 4 the typical Japanese diet, shown by J5 and J4, are clearly associated significantly with HDL cholesterol and therefore, lower atherogenic index, that is, the ratio of non-HDL to HDL with the scoring of the Japanese diet after age and sex adjustment. The typical Japanese diet, shown by J5 and J4, is clearly related to significantly higher HDL levels, and significantly

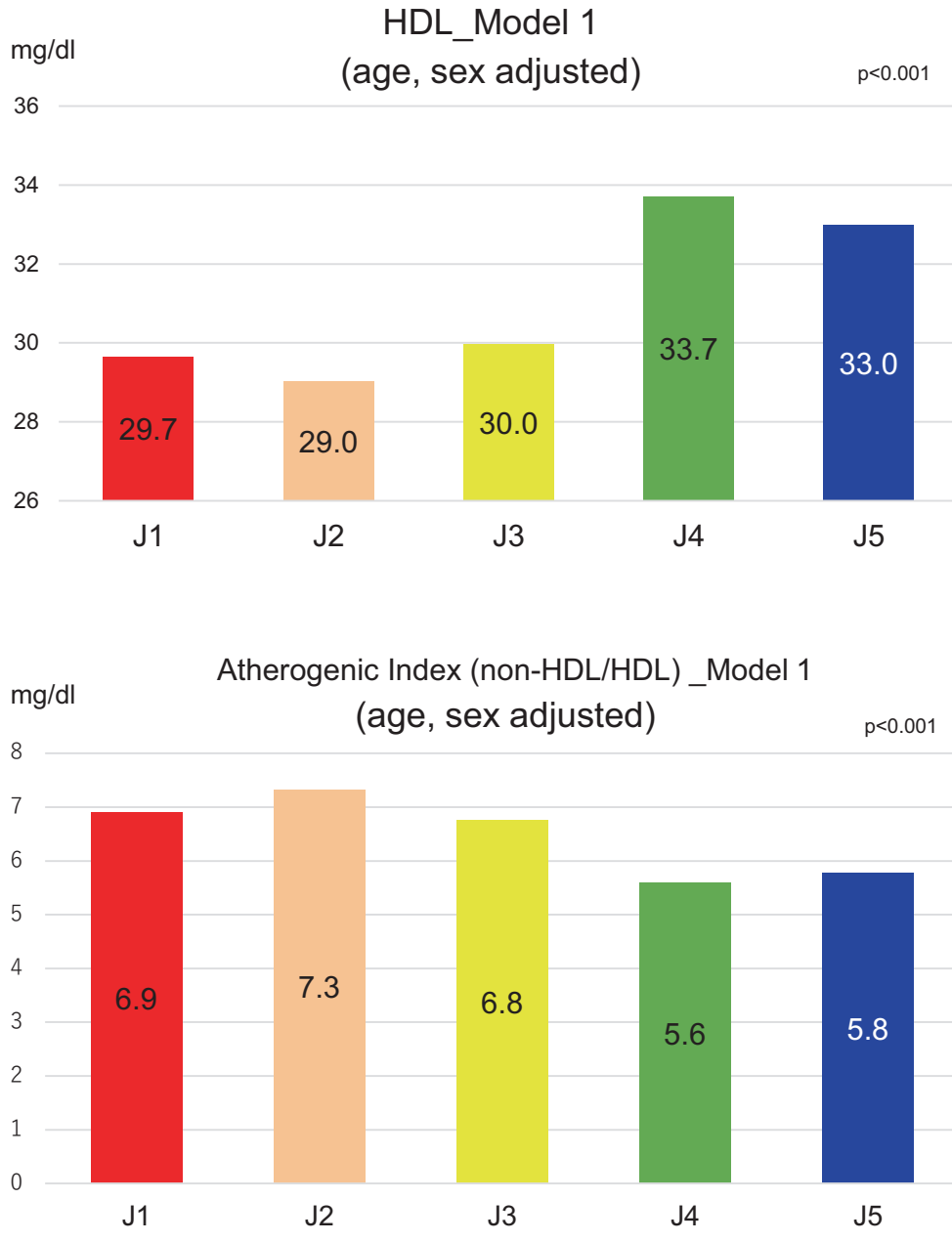


Fig. 4 Significant positive association of HDL cholesterol and significant inverse association of atherogenic index (non-HDL/HDL) with the scoring of the Japanese diet intake (J1–J5) after age and sex adjustment (Model 1) from the multiple linear regession analysis

lower atherogenic index confirming features of the Japanese diet are less dyslipidemic and therefore related to less CHD mortality and morbidity.

3.6 Demerits of Japanese Diet

However, higher Na intake and a higher Na/K ratio are associated with higher scoring of the Japanese diet, J5 and J4, indicating customary higher-salt intake is closely related to the seafood and soy diet (Fig. 5). Therefore, high-salt intake is the obvious demerit of the Japanese diet (Yamori 2006, 2005; Yamori et al. 2006). Accordingly, systolic blood pressure is significantly higher in J5 than in J3, but systolic blood pressure is also higher in J1 and J2 than in J3 (Fig. 6). Diastolic blood pressure is also significantly higher in J1 than in J3 (Fig. 6). Higher Na and Na/K are associated with higher blood pressure, but lower T and I consumption increases blood pressure because of sympathetic nerve activation due to insufficient T intake (Yamori et al. 2010; Mizushima et al. 1996; Nanfang et al. 1996) as well as reduced nitro-oxide synthesis in insufficient I intake (Si and Liu 2008; Rathel et al. 2005) or other mechanisms such as decreased Mg intake (Yamori et al. 2018) and increased obesity (Fig. 2).

Since seafood contains Mg because of seawater and soy contain Mg, the customary Japanese diet containing seafood and soy are rich in Mg (Fig. 7). Mg is an important mineral involved in various biological functions and is related to hypertension, the most common risk factor of cardiovascular diseases (Laurant and Touyz 2000). It is noteworthy that the quintile analysis of 24U Mg/Cre ratios in our worldwide study demonstrated significant associations of the quintile with odds ratios of obesity, hypercholesterolemia, and hypertension indicating higher seafood and soy consumption in the Japanese diet is associated with lower cardiovascular risks not only due to T and I intakes but also to Mg intake (Yamori et al. 2015).

In our worldwide study, 24U T excretion of the total participants was divided into five quintiles. In comparison with the highest 5th quintiles, the lowest 1st quintile showed higher odds ratios for obesity, hypercholesterolemia, and hypertension, respectively (Sagara et al. 2015). T is rich in seafood, and Mg is rich in nuts, seeds, legumes, and dietary fibers. Both T- and Mg-rich diets were customarily consumed by the Japanese as shown by the positive association of Mg with the Japanese diet grading (J1–J5) based on T and I.

4 Discussion

The scoring of the Japanese diet indicated the merits of the Japanese diet which is related to high T, I, and Mg intake. On the other hand, the apparent demerits are high Na and a Na/K ratio which raise blood pressure and can cause stroke, increasing the incidence of bedridden disability and cardiovascular dementia in the elderly. The merits of the customary Japanese diet involving consumption of T, I, and Mg were supposedly consumed even in the prehistoric period (Richards 2002), and the prehistoric Paleolithic diet is regarded to improve metabolism (Frassetto et al. 2009). The ingredients of the ancient diet have been investigated in detail at the well-preserved shell mound (kitchen midden) excavated in Torihama of Fukui Prefecture in Japan (TSMR, Torihama Shell Midden Research Group 1985). It is speculated that according to the ratio of the common food to total intake, Mg-rich nuts and seeds as well as T-rich fish and shellfish were commonly consumed by prehistoric people in the early Jomon period may show the importance of both Mg and T in the evolution of life and nutrition of human beings (TSMR 1985; Hongo 1989; Lucquin et al. 2016).

It is speculated that life was first born in Mg-containing sea 3.8 billion years ago as a single cell, and finally in the process of evolution, our ancestors became hunters and food gatherers. Human ancestors used to consume food containing plenty of Mg and T which supported the physiological function efficiently inside the body, probably gifted with thrifty genes which enabled them to survive with the limited food available.

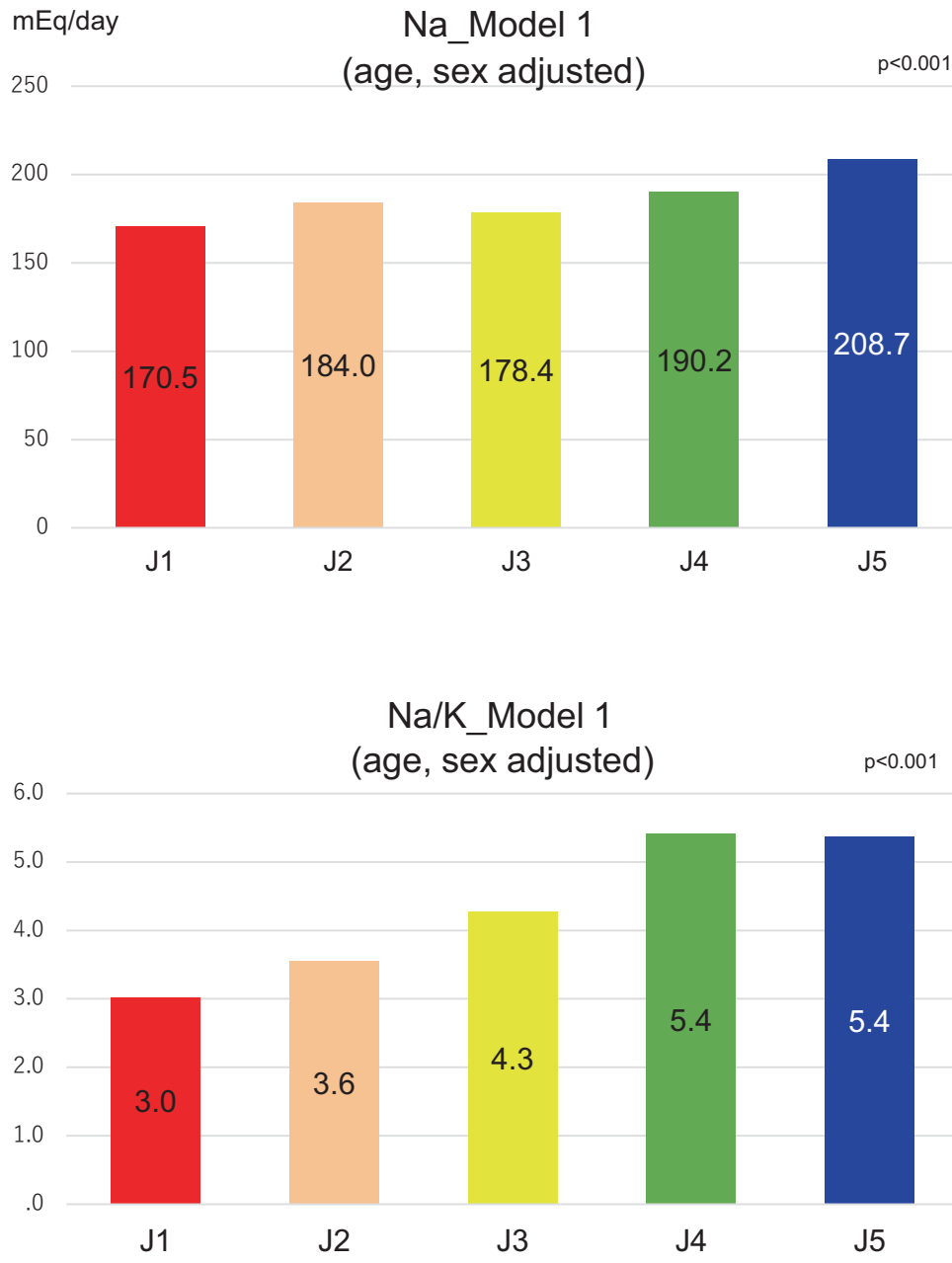


Fig. 5 Significant positive association of 24U Na excretion and Na/K ratio with the scoring of the Japanese diet intake (J1–J5) after age and sex adjustment (Model 1) from the multiple linear regession analysis

Our worldwide data and the grading of the Japanese diet related to Japanese longevity indicate the evolutional concept of human nutrition should be reconsidered for promoting a healthier life without the metabolic syndrome.

5 Conclusion

The Japanese diet which is rich in not only T, I, and Mg from seafood and soy but also vegetables are good for reducing the risks of CHD, thus con-

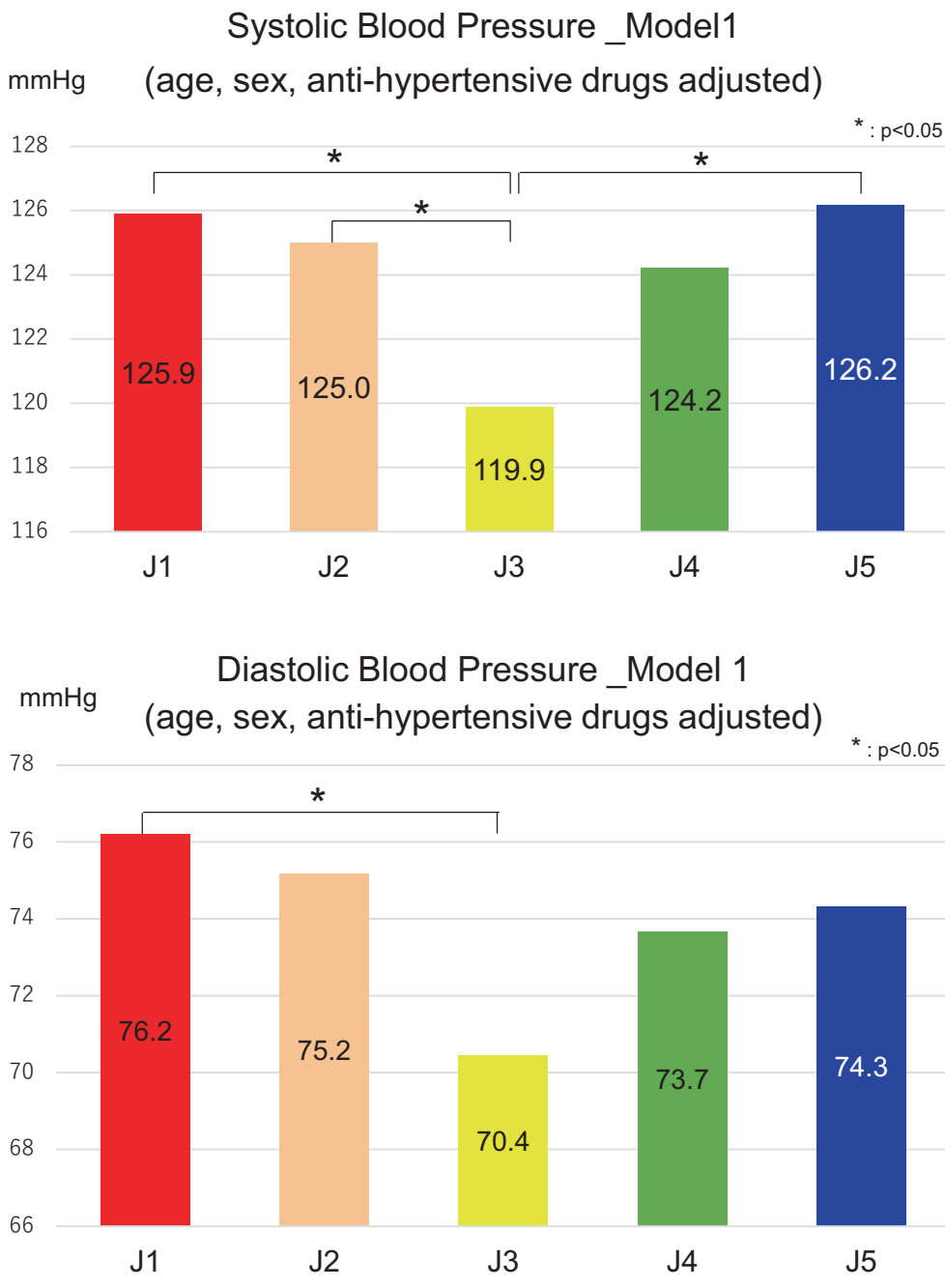


Fig. 6 Systolic blood pressure was significantly higher in J5 and also J1 and 2 than in J3, and diastolic blood pressure was significantly higher in J1 than in J3 (by the analysis of covariance)

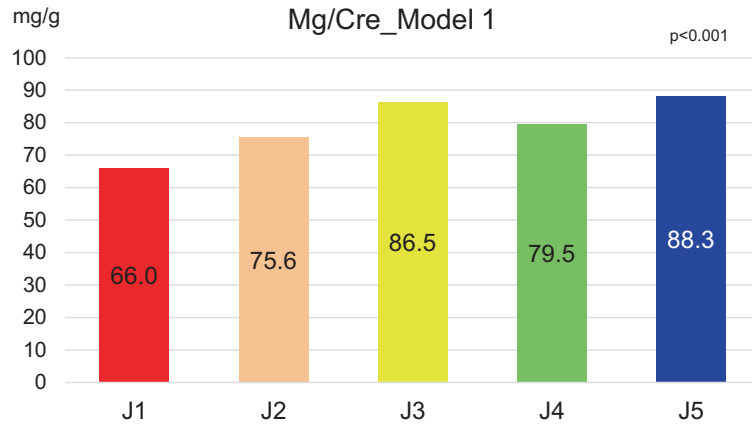


Fig. 7 24U Mg excretion was significantly positively associated with the scoring of the Japanese diet intake (J1–J5) (from multiple linear regession analysis)

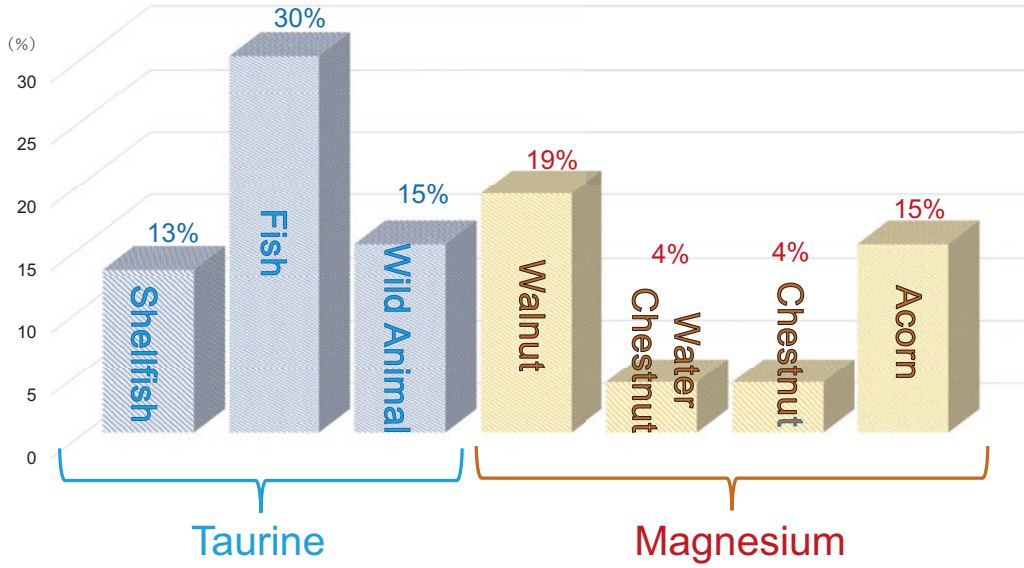


Fig. 8 The speculated ratio of prehistoric taurine and magnesium related to food intake in the prehistoric kitchen midden in Japan. The ratio of the main ingredients of daily food studied at Torihama kitchen midden of the early Jomon period

tributing to the long average life expectancy of Japanese. Also, seaweeds which are rich in Mg and contain T are commonly consumed by the Japanese, and this dietary custom is related to Japanese longevity because both Mg and T are inversely associated with CHD risks (Yamori et al. 2015; Sagara et al. 2015). However, despite these merits, high salt and a high Na/K ratio are the apparent demerits that cause hypertension and stroke, hence shortening the healthy life expectancy of the Japanese. Therefore, we recommend a low-salt diet with soy, seafood, seaweed, and vegetables which counteract the adverse effects of Na. Certain seaweeds which are rich in T in addition to Mg are suitable for vegans and vegetarians to consume as substitutes for seafood. To conclude from our grading of the Japanese diet, a low-salt diet with seafood, soy, vegetables, and seaweeds rich in T and Mg is a recommended diet for everyone in the world.

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Perinatal Taurine Supplementation Preserves the Benefits of Dynamic Exercise Training on Cardiovascular and Metabolic Functions and Prevents Organ Damage in Adult Male Exercised Rats

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Keywords

Arterial pressure · Baroreflex · Exercise · Metabolism · Perinatal taurine · Rat

Abbreviations

BRHR-PHE	Baroreflex sensitivity control of heart rate by phenylephrine
BRHR-SNP	Baroreflex sensitivity control of heart rate by sodium nitroprusside
C	Control
Ex	Exercise training
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
T	Perinatal taurine supplementation
TEx	Perinatal taurine supplementation followed by exercise training

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1 Introduction

Taurine plays many important roles in health and disease from prenatal to adult life. Taurine's physiologic functions include cell volume regulation, growth and development, cell metabolism and control, cardiovascular control, renal excretory function, neurohormonal modulation, and immune function (Lerdweeraphon et al. 2013; Roysommuti and Wyss 2014). For healthy and disease interventions, taurine's actions include anti-oxidation, anti-diabetes, anti-hypertension, anti-dyslipidemia, anti-inflammation, and increased exercise performance. Furthermore, perinatal taurine depletion or excess modulate

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normal and disease status of the adult, particularly in animal models. However, perinatal taurine depletion has been reported to induce more abnormalities during early postnatal and adult life than perinatal taurine supplementation. Thus, taurine supplementation is recommended during pregnancy and lactation to ensure sufficient taurine levels for both mothers and offspring. The taurine requirement during pregnancy and lactation is high, largely due to increased maternal metabolism and fetal and newborn growth (Roysommuti and Wyss 2013). Body taurine content declines with advancing age, which correlates to age-related changes in physiologic functions, including cardiovascular and metabolic functions. Therefore, taurine supplementation and exercise are recommended in the elderly to slow down or prevent these age-related changes (Kurtz et al. 2021).

Adequate and regular exercise is well known to improve health and disease in animal and human models. Effective exercise increases resting cardiac contractility and stroke volume but decreases resting heart rate (Coudert and Van 2000; Kimmerly 2017; Vatner and Pagani 1976). These benefits result at least in part from exercise-mediated decreases in sympathovagal balance to the heart and increased myocardial mass and vascularization. Skeletal muscle hypertrophy is also common among athletes and exercise trainees. The increased muscle mass and subsequent performance are related to exercise-induced protein synthesis, especially related to modulation of cell calcium, protein kinase B (Akt), AMP-activated protein kinase, and targets of rapamycin pathways (Atherton et al. 2005; Horii et al. 2020; Jiang et al. 2013; White et al. 2013). Exercise not only increases protein synthesis but also increases utilization of energy stores, particularly those of fats. Thus, effective exercise decreases body fat and adipocyte size, thereby preventing or improving overweight, obesity, dyslipidemia, and diabetes mellitus in adults (Von et al. 2021). During exercise, oxidative stress increases due to enhanced anaerobic and aerobic energy metabolism. However, oxidative stress at rest among exercise trainees and athletes is lower than that of sedentary subjects (De Carvalho et al. 2021a, b). The low oxidative stress and sympathetic/parasympathetic activity ratio have been reported to underlie hypotensive and other cardiovascular protective effects of exercise. In addition, regular exercise increases immune function by decreasing the levels of pro-inflammatory cytokines (De Carvalho et al. 2021b). However, strenuous exercise increases organ damage, particularly that of the muscle and liver, as supported by increasing serum injury markers after strenuous exercise, e.g., SGPT, SGOT, and creatine kinase (Chung et al. 2020; Nowakowska et al. 2019). Surprisingly, all these benefits of exercise resemble those of taurine action mentioned earlier. Thus, taurine supplementation or diets high in taurine have been used to increase exercise performance and benefits, particularly those related to cardiovascular and metabolic function and control (Kurtz et al. 2021). For example, taurine supplementation decreases oxidative stress and organ damage during and after exercise activity, at least in part via mitochondrial metabolism and cell calcium mobilization (De Carvalho et al. 2021a). Taurine supplementation also decreases sympathetic activity impacting heart and blood vessels, via modulation of the central autonomic pathway (Kimmerly 2017).

Although taurine supplementation is quite safe to use in humans due to its high urinary excretion capacity, perinatal taurine supplementation is reported to underlie some disorders in adult life, particularly in animal models. Perinatal taurine supplementation (3% in drinking water) alters renal excretory function in adult female rat offspring via modulation of the renin-angiotensin system (Lerdweeraphon et al. 2017) and estrogen action (Roysommuti et al. 2017). In male rats, taurine treatment can affect renal nerve activity in response to pulpal stimulation (Khimsuksri et al. 2013). Furthermore, perinatal taurine supplementation combined with a subsequent high-sugar diet after weaning depresses baroreflex sensitivity in adult female rats via estrogen receptors, but not via the renin-angiotensin system (Thaeomor et al. 2013). Prenatal taurine supplementation is also reported to increase postnatal growth and risk of obesity and diabetes mellitus in adult rat offspring (Hultman et al. 2007). Although perinatal taurine supplementation has some adverse effects in adult offspring, the treatment exerts several actions on adult function and

disease. Perinatal taurine supplementation attenuates the adverse effects of high-sugar intake on cardiac and cardiovascular function after cardiac ischemia/reperfusion in adult male rats (Kulthinee et al. 2010). Previously, we have reported that perinatal taurine supplementation in maternal rats with diabetes mellitus or dyslipidemia prevents cardiovascular and metabolic defects in both male and female adult rat offspring (Thaeomor et al. 2017, 2019). As mentioned earlier, regular exercise and taurine supplementation are reported to prevent and ameliorate cardiovascular and metabolic disorders in adult animals and humans. The present study explored the effects of perinatal taurine supplementation followed by dynamic exercise training on cardiovascular and metabolic functions in adult male rats.

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. Pregnant rats were treated with perinatal taurine supplementation (taurine) or without 3% taurine (control) in drinking water. After weaning, male offspring were fed the normal rat chow and water throughout the experiment ($n = 7$ each group). At 4 weeks of age, the male rat offspring were subjected to non-exercise (control without exercise, C; taurine without exercise, T) and swimming exercise training (control with exercise, Ex; taurine with exercise, TEx) until 16 weeks of age. Two days later, blood chemistry and cardiovascular parameters were investigated after an overnight fasting. At the end of the experiment, all animals were sacrificed with a high dose of anesthesia followed by heart, kidney, and visceral fat collection for further investigation.

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 Exercise Training Procedure

Rats were trained to the swimming procedure as previously described (de Souza et al. 2021; Duan et al. 2021). In brief, after 2 days of acclimation (10 min of swimming a day), rats (3 rats each set) were forced to swim in a plastic barrel (45 cm in diameter) filled with water (50 cm in depth, 34–35 °C) for 10–30 min a day and 5 days a week until 16 weeks of age (12 weeks of exercise training).

2.3 Cardiovascular Parameter Measurement

After an overnight fasting, 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-10 fused with PE-50). Each arterial cannula was connected to a pressure transducer and the PowerLab system (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 (Terumo, Terumo Corporation, Tokyo, Japan) for drug infusion. 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). The baroreflex sensitivity control of heart rate 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).

2.4 Blood Chemistry Measurement

Plasma leptin levels were measured by using a commercial kit (Rat Leptin ELISA kit # EZRL-83BK; Merck Millipore, Merck), while plasma triglyceride, plasma creatinine, blood urea nitrogen, SGOT, and SGPT were measured by the

Suranaree Hospital Chemical Analysis Unit (Suranaree University of Technology, Nakhon Ratchasima, Thailand).

2.5 Visceral Adipocyte Size Measurement

Visceral white adipose tissues were fixed in paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (Silverio et al. 2017). The stained sections were obtained with a ×40 objective lens, recorded on a digital camera (DS-Fi2 microscope camera, Nikon, Japan), and displayed on a high-resolution monitor (Eclipse Ci-L microscope, Nikon, Japan). An averaged diameter of adipose tissues was analyzed and measured by Image-Pro Plus 6.0 (ten adipocytes per stained section).

2.6 Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons among groups were performed by using one-way ANOVA followed by the post hoc Duncan's Multiple Range test (Sigma Plot 14.5, Systat Software, Palo Alto, CA 94303, USA). The significant criterion is $p < 0.05$.

3 Results

3.1 Weights

At 16 weeks of age, body weights significantly decreased in Ex and TEx compared to C and T groups (Table 1). Further, kidney weights were not significantly different among the four groups, while heart weights significantly increased in both Ex and TEx compared to C and T groups. In addition, both body weights and heart weights of Ex and TEx groups were not significantly different.

3.2 Blood Chemistry and Visceral Adipocytes

Plasma leptin and triglyceride were not significantly different among the four groups, while visceral adipocyte sizes significantly decreased in both Ex and TEx compared to C and T groups (Table 2). Further, visceral adipocyte size of

Table 1 Body, kidney, and heart weights in adult male rats

Treatment	Bodyweight (g)	Kidney weight (g)	Heart weight (g)
C	359 ± 5	1.31 ± 0.13	1.34 ± 0.07
T	367 ± 7	1.29 ± 0.07	1.29 ± 0.04
Ex	316 ± 2*	1.30 ± 0.05	1.40 ± 0.01*
TEx	314 ± 1*	1.24 ± 0.04	1.41 ± 0.01*

Each value is mean ± SEM
*$P < 0.05$ compared to C groups
C control, *T* perinatal taurine supplementation, *Ex* exercise training, *TEx* perinatal taurine supplementation followed by exercise training, $n = 7$ each group

Table 2 Plasma leptin, plasma triglyceride, and visceral adipocyte size in adult male rats

Treatment	Leptin (ng/ml)	Triglyceride (mg/dl)	Adipocyte size (μm)
C	6.9 ± 0.5	122.1 ± 15.4	410 ± 17
T	6.6 ± 0.5	123 ± 11.7	418 ± 17
Ex	6.5 ± 0.3	111.9 ± 9.7	343 ± 17*
TEx	6.6 ± 0.3	113 ± 6.6	308 ± 13*

Each value is mean ± SEM
*$P < 0.05$ compared to C groups
C control, *T* perinatal taurine supplementation, *Ex* exercise training, *TEx* perinatal taurine supplementation followed by exercise training, $n = 7$ each group

Table 3 Blood urea nitrogen (BUN), plasma creatinine (Cr), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) in adult male rats

Treatment	BUN (mg/dl)	Cr (mg/dl)	SGOT (unit/l)	SGPT (unit/l)
C	25.3 ± 1.5	0.46 ± 0.1	137.9 ± 13.6	34.4 ± 2.6
T	24.4 ± 1.7	0.41 ± 0.1	134.9 ± 12.4	35.1 ± 2.0
Ex	25.7 ± 2.0	0.47 ± 0.1	136.9 ± 11.8	52.7 ± 7.0*
TEx	26.7 ± 1.1	0.37 ± 0.1	148.7 ± 11.0	44.0 ± 5.9

Each value is mean ± SEM
*$P < 0.05$ compared to C groups
C control, *T* perinatal taurine supplementation, *Ex* exercise training, *TEx* perinatal taurine supplementation followed by exercise training, $n = 7$ each group

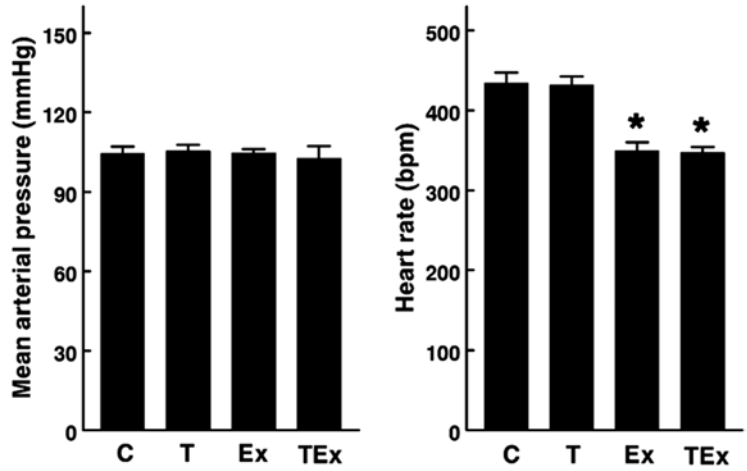

Fig. 1 Mean arterial pressures (left) and heart rates (right) in adult male rats (*$P < 0.05$ compared to C groups, C control, T perinatal taurine supplementation, Ex exercise training, TEx perinatal taurine supplementation followed by exercise training, $n = 7$ each group)

both Ex and TEx groups was not significantly different. While plasma creatinine, blood urea nitrogen, and SGOT were not significantly different among the four groups, SGPT significantly increased in Ex compared to C and T groups (Table 3). Although perinatal taurine supplementation alone did not affect blood chemistry parameters and visceral fats, this treatment attenuated a rise in SGPT in the TEx group.

3.3 Cardiovascular Parameters

Mean arterial pressures were not significantly different among the four groups, whereas heart rates significantly decreased in both Ex and TEx compared to C and T groups (Fig. 1). The heart rates were not significantly different when compared between C and T or Ex and TEx groups. In addition, both BSHR-PHE and BSHR-SNP were not significantly affected by perinatal taurine supplementation and/or exercise training (Fig. 2).

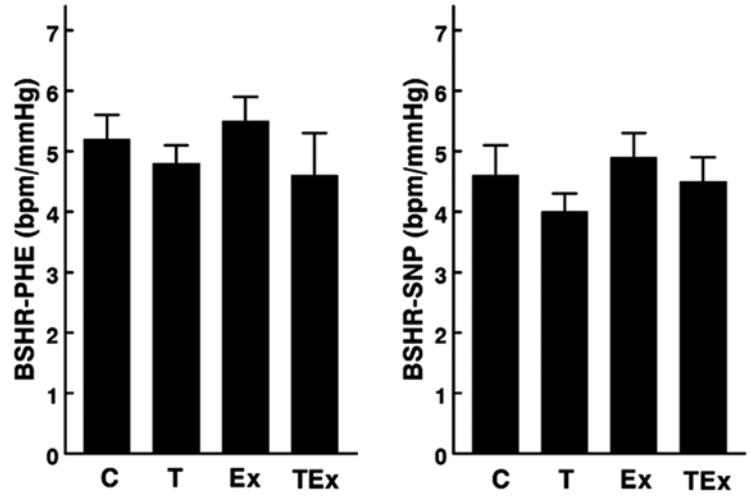

Fig. 2 Baroreflex sensitivity control of heart rate estimated by phenylephrine (BSHR-PHE; left) and sodium nitroprusside (BSHR-SNP; right) infusion in adult male rats (C control, T perinatal taurine supplementation, Ex exercise training, TEx perinatal taurine supplementation followed by exercise training, $n = 7$ each group). No significant differences were observed among groups

4 Discussion

In adults, taurine supplementation or regular exercise is reported to protect or improve cardiovascular and metabolic disorders with advancing age, especially the metabolic syndrome. Further, taurine supplementation can also increase the exercise performance and benefits on heath and disease management (Kurtz et al. 2021). Perinatal taurine supplementation has long-term effects on adult function and disease. This treatment attenuates the effects of high-sugar intake (Kulthinee et al. 2010) and maternal diabetes mellitus (Thaeomor et al. 2017) on cardiovascular and metabolic disorders in adult rat offspring. The present study indicates that perinatal taurine supplementation at a dose (3% in drinking water) had no effect on growth, metabolic, and cardiovascular function but did prevent a rise in plasma SGPT level after 12-week exercise training in adult male rat offspring. The increase in SGPT levels is known to reflect organ damage, especially the liver and muscle, after exercise training (Chung et al. 2020; Nowakowska et al. 2019; Sierra et al. 2019). In addition, perinatal taurine supplementation preserves the benefits of regular exercise on weight loss, decreasing body fats, and decreasing resting heart rate. Thus, the present data support the use of taurine supplements or diets high in taurine during pregnancy and lactation to increase health and physical performance in adult offspring.

Muscle damage and soreness are common after strenuous exercise, as supported by increased muscle injury markers like plasma creatine kinase, SGOT, and SGPT (Chung et al. 2020; Nowakowska et al. 2019; Sierra et al. 2019). The severity of muscle damage after exercise depends on training experience, genetics, age, exercise intensity, hydration, and diets (Fernandez-Lazaro et al. 2020; Mielgo-Ayuso et al. 2020). Further, the high SGPT, but not SGOT, is also a positive index of exercise or physical performance in adults (Chung et al. 2020). Fish intake decreases while fat intake increases exercise-induced muscle damage in marathon runners (Mielgo-Ayuso et al. 2020). Taurine supplementation is reported to reduce exercise-induced muscle damage and soreness, probably related to taurine's anti-oxidative and anti-inflammatory activity (da Silva et al. 2014; Sugiura et al. 2013). Oxidative stress markers and pro-inflammatory cytokines increase during and after exercise training (da Silva et al. 2014; De Carvalho et al. 2017, 2021b; Sugiura et al. 2013; Thirupathi et al. 2018). In contrast, some researchers report no effects of taurine supplementation on exercise-induced muscle damage (Galan et al. 2018; Zembron-Lacny et al. 2009).

The present study is the first to indicate that taurine supplementation during perinatal life has a protective effect on exercise-induced organ damage in adult life. The SGPT level usually reflects liver damage due to its highest concentration in hepatocytes; however, muscle, heart, and kidney damage can also increase SGPT. Liver and kidney damage is observed in swimming exercise training that can be prevented by antioxidant intake (Suarsana et al. 2020). Although the kidney damage can increase plasma SGPT after exercise training, it might not be the case in the present study. Blood urea nitrogen and plasma creatinine were not significantly different among the four groups, indicating no kidney damage. Thus, the high SGPT in the present study might reflect at least liver and muscle damage after swimming exercise. Nevertheless, to identify the specificity and severity of organ damage after exercise training, other blood injury markers and histology of cardiac and skeletal muscle damage should be further investigated. These blood injury markers include creatine kinase, muscle–brain isoform creatine kinase, prohormone of brain natriuretic peptide, cardiac troponin T and troponin I, lactate dehydrogenase, and myoglobin (Mielgo-Ayuso et al. 2020; Nowakowska et al. 2019). Renin-angiotensin system overactivity is reported to underlie muscle damage after exercise training (Sierra et al. 2019; Vaughan et al. 2016). Our previous experiments indicate that perinatal taurine supplementation at the present dose followed by a high-sugar diet alters renal excretory function via renin-angiotensin system dysregulation in adult female rats (Lerdweeraphon et al. 2017). Whether this renin-angiotensin system dysregulation underlies the preventive effect of perinatal taurine supplementation on exercise-induced organ damage or performance needs further studies.

Regular exercise is recommended to control body weight and obesity, by increasing fat metabolism and decreasing fat content. The reduced body weight and visceral adipocyte size in the exercise group supports this beneficial effect of dynamic exercise. However, perinatal taurine supplementation could not alter weight loss and adipocyte size reduction after dynamic exercise training in the present study. In adults, taurine may not directly cause weight loss by increasing body's energy expenditure or calorie-burning ability (Galloway et al. 2008). However, taurine may indirectly increase body fat utilization by increasing exercise performance (Kurtz et al. 2021). This taurine-mediated increase in exercise performance depends on doses and durations of taurine supplementation. The longer duration of taurine supplementation, the higher the physical performance becomes (Ma et al. 2021). This might explain why taurine supplementation only during perinatal life did not increase exercise performance in adult rat offspring in the present study. Cardiac hypertrophy is common in athletes and exercise trainees, which is also supported by the present study. Exercise-induced cardiac hypertrophy is known to increase cardiac contractility; thus, both resting and maximum stroke volume are elevated after regular exercise training (Coudert and Van 2000; Kimmerly 2017; Vatner and Pagani 1976). In adults, taurine supplementation decreases cardiac hypertrophy in animal models via inhibition of the renin-angiotensin system (Schaffer et al. 2000). Together with the absence of an effect of perinatal taurine supplementation on cardiac hypertrophy in TEx group, it is possible that the high cardiac taurine level rather than the programming effect of perinatal taurine supplementation affects exercise-induced cardiac hypertrophy in adults.

Other than increasing muscle strength and cardiac contractility, a decrease in resting heart rate is a good index of exercise efficiency and physical fitness, also supported by the low heart rate in the Ex and TEx groups. The exercise-mediated decrease in resting heart rate is known to result mainly from a decrease in cardiac sympathetic/parasympathetic activity ratio (Coudert and Van 2000; Piras et al. 2021; Vatner and Pagani 1976). The present study further indicates that this low resting heart rate, which was not affected by perinatal taurine supplementation, might not be due to a change of baroreceptor reflex sensitivity. Baroreflex sensitivity usually decreases and resets to a higher arterial pressure during and shortly after exercise activity (Cunha et al. 2015). The present study measured the car-

diovascular and metabolic parameters 2 days after the last exercise activity; thus, the baroreflex sensitivity returned to normal resting function. In adult female rats, perinatal taurine supplementation followed by a high-sugar diet after weaning depresses baroreflex sensitivity via estrogen receptors (Thaeomor et al. 2013). These data suggest that the long-term effects of perinatal taurine supplementation on adult responses to physical activity and diets might be sex dependent. During exercise, the baroreflex sensitivity is lower in men than women, even with similar resting baroreflex sensitivity values (Fu and Ogoh 2019). Whether the effects of perinatal taurine supplementation on exercise efficiency differ between sexes needs further studies.

5 Conclusion

Taurine or diets high in taurine has been recommended for athletes and exercise trainees to increase exercise performance and beneficial effects on health and disease management. Further, perinatal taurine supplementation has been reported to alter cardiovascular and metabolic functions in adult offspring. The present study indicates that, in adult male rats, perinatal taurine supplementation not only preserves the beneficial effects of dynamic exercise training on cardiovascular and metabolic functions but also prevents exercise-induced organ damage.

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Impaired Bile Acid Synthesis in a Taurine-Deficient Cat Model

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Keywords

Bile acids · Oxysterols · CYP7A1 · CYP27A1 · Mitochondria · Cat

Abbreviations

27HC	27-hydroxycholesterol
7DHC	7-dehydroxycholesterol
7αHC	7α-hydroxycholesterol
BAs	Bile acids
C4	7α-hydroxy-4-cholesten-3-one
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CYP27A1	Cholesterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
DCA	Deoxycholic acid
FXR	Farnesoid X receptor
GCA	Glycocholic acid
HPLC-ESI-MS/MS	high-performance liquid chromatography-electrospray ionization tandem mass spectrometry
LCA	Lithocholic acid
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonus epilepsy with ragged red fibers
TBA	Total bile acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
UDCA	Ursodeoxycholic acid
VDAC-1	Voltage-dependent anion-selective channel protein 1
τm5s2U	5-taurinemethyl 2-thiouridine
τm5U	5-taurinemethyluridine

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1 Introduction

Taurine (β-aminoethanesulfonic acid) is an amino acid derivative abundant in most tissues, including the liver, and in the whole body; it is maintained by exogenous intake of animal protein-based foods and endogenous biosynthesis of sulfur-containing amino acids, mainly in the liver (Bella et al. 1999; De La Rosa and Stipanuk 1985). Taurine has been reported to have many physiological and pharmacological actions

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(Huxtable 1980, 1992; Jacobsen and Smith 1968; Miyazaki and Matsuzaki 2014), and the most established action is conjugation with bile acids (BAs) to enhance the water solubility of the BAs for the promotion of their excretion in the bile, micelle formation of the bile, facilitation of lipid absorption in the intestinal tract, and reduction in their toxicity (Danielsson 1963; Sjovall 1959).

Taurine is an essential nutrient for certain species that have low biosynthetic ability, including cats and humans, and serious abnormalities and disorders have been reported in congenital and acquired taurine-deficient states in experimental animal models. In congenital mouse models induced by knocking out a specific gene encoding a transporter of taurine (Ito et al. 2008; Warskulat et al. 2006) and a key biosynthesis enzyme, cysteine sulfinate decarboxylase (Gordon et al. 2015; Mekawy et al. 2021; Park et al. 2014), critical developmental failures and dysfunction have been observed in various tissues including the liver, skeletal muscle, and retina. The acquired taurine deficiency in cats who have low capacity for hepatic biosynthesis and renal regulation of reabsorption is well known (Rentschler et al. 1986; Sturman and Hayes 1980). Serious diseases, including blindness (Hayes et al. 1975; Hayes and Trautwein 1989) and expanded cardiomyopathy (Pion et al. 1987), were caused in cats under the taurine deficiency state induced by insufficient dietary taurine intake (Knopf et al. 1978).

We also evaluated BAs in a taurine-deficient cat model induced by a taurine-deficient diet for 30 weeks (Miyazaki et al. 2019b, 2020). In cats, most BAs in the bile are conjugated with taurine, similar to rodents, while the conjugated ratio of taurine and glycine in humans is known to be 1:3 (Bruusgaard and Thaysen 1970). On the other hand, the ratio of taurine-conjugated BAs in the bile was significantly decreased, to approximately 40%, and in contrast, the level of unconjugated BAs was increased to more than 60% in the taurine-deficient cat model (Miyazaki et al. 2019b), accompanied by taurine body deficiency (see Fig. 1). In addition, total BA (TBA) concentration, as the sum of unconjugated and conjugated BAs, in the bile was significantly decreased in the taurine-deficient cats compared to that of the taurine-containing diet-fed control cats

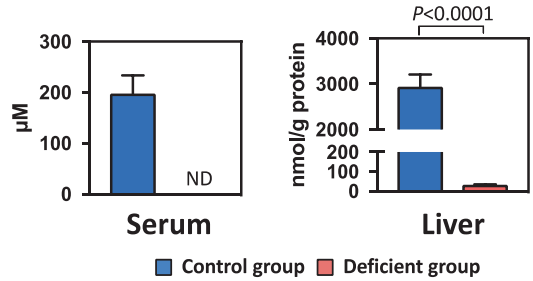

Fig. 1 Taurine concentration in the serum and liver tissue of the control and deficient groups. Abbreviations: *Control group*, taurine-containing diet-fed group; *deficient group*, the taurine-deficient diet-fed group. *ND*, not detectable. The data shown are the means ± SEM. Significant differences were analyzed by unpaired Student's *t*-test

(Miyazaki et al. 2019b). Interestingly, there was a significant difference in the composition of BAs in the bile between the taurine-deficient and control cats; in particular, in the primary BAs, the ratios of cholic acid (CA) and chenodeoxycholic acid (CDCA) were increased and decreased, respectively, in the taurine-deficient cats. After the final steps of BA synthesis, the conjugation of taurine or glycine is realized by two key enzymes: ATP-dependent microsomal bile acid CoA synthase, which converts a bile acid to an acyl-CoA thioester, and cytoplasmic bile acid CoA-amino acid *N*-acetyltransferase, which transfers the acyl-CoA thioester to taurine or glycine (Honda et al. 2004). Therefore, we suggest that taurine plays a role not only in conjugation with BAs but also in BA synthesis.

The present study further investigated the influence of taurine deficiency on the synthesis of BAs in the liver by measuring the intermediates derived from cholesterol in the synthetic pathway using taurine-deficient model cats, as described in a previous volume of *Taurine 11* (Miyazaki et al. 2019b).

2 Methods

2.1 Taurine-Deficient Model Cats

A taurine-deficient cat model was used in the present study. Control (N = 4) and deficient (N = 6) groups of cats were fed a taurine-containing control diet and a taurine-deficient

diet, respectively, for 30 weeks (Miyazaki et al. 2019b, 2020). Thereafter, the serum, bile, and liver tissue were collected from both groups after euthanasia by overdose of pentobarbital. The animal experiment was conducted with the approval of the Animal Care Committee of Ibaraki Prefectural University of Health Sciences (Ibaraki, Japan) (Permit No. 2015-21, 2016-7).

2.2 Taurine Analysis

Taurine in serum and liver derivatized with 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate was measured by a high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) system consisting of a TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography system (Shimadzu, Kyoto, Japan) (Miyazaki et al. 2019a, 2020).

2.3 Bile Acids and Oxysterol Analysis

Bile acids, including unconjugated, taurine-conjugated, and glycine-conjugated forms of CA, CDCA, deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), and oxysterols, including 27-hydroxycholesterol (27HC), 7α-hydroxycholesterol (7αHC), 7α-hydroxy-4-cholesten-3-one (C4), lathosterol, 7-dehydroxycholesterol (7DHC), and desmosterol, were analyzed using the HPLC-ESI-MS/MS system according to previously reported methods (Honda et al. 2008, 2009, 2010; Murakami et al. 2018). The percentages of CA and CDCA, which are the sum of conjugated and unconjugated forms of TBA, were calculated. The free cholesterol concentration in homogenized liver tissue was measured using a commercially available assay kit (Free-Cholesterol E-test Wako; FUJIFILM Wako Pure Chemical Corporation).

2.4 Western Blot Analysis

The protein levels of cholesterol 7α-hydroxylase (CYP7A1) and cholesterol 27-hydroxylase (CYP27A1) in the cytosolic and mitochondrial fractions, respectively, in the liver tissue were evaluated by Western blot analysis. Blots from the cytoplasmic fraction were incubated with primary antibodies against CYP7A1 (1:1000) and β-actin (1:5000), while those from the mitochondrial fraction were incubated with primary antibodies against CYP27A1 (1:1000) and voltage-dependent anion-selective channel protein 1 (VDAC-1; 1:1000). The protein levels of CYP7A1 and CYP27A1 were standardized to the expression levels of β-actin and VDAC-1, respectively.

2.5 Statistical Analysis

Statistical significance was determined by unpaired 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. Statistical analyses were conducted using JMP software (version 14.3, SAS Institute; Cary, NC, USA).

3 Results

3.1 Taurine Concentration in Serum and Liver

Figure 1 shows the taurine concentration in the serum and liver after 30 weeks of supplementations of the taurine-containing or taurine-deficient diets. In the deficient group, the taurine concentration was too low to be detected in the serum, while it was approximately 200 μM in the control group. In the liver tissue, the taurine concentration was significantly lower in the deficient group than in the control group.

3.2 Bile Acid Composition in the Bile

In the control group, more than 99% of the BAs in the bile were in the taurine-conjugated form. In contrast, the taurine-conjugated form of BAs was decreased to be less than 40%, while the unconjugated BA form was increased by more than 60% in the deficient group. As shown in a previous study (Miyazaki et al. 2019b), the TBA concentration in the bile was significantly decreased in the deficient group (172.3 ± 59.4 mM; $P < 0.05$) compared to that in the control group (404.8 ± 58.6 mM, Fig. 2). Figure 2 shows the BA composition in the bile of the two groups. In the control group, more than 80% of the BAs were taurocholic acid (TCA), and the remaining BAs were taurochenocholic acid (TCDCA, 4.7%) and taurodeoxycholic acid (TDCA, 12.9%). Other types of BAs in the control group comprised less than 0.1% of TBA. On the other hand, the percentages of TCA, TCDCA, and TDCA decreased to 34.6%, 2.3%, and 1.5%, respectively. In addition, unconjugated CA accounted for approximately 60% of the TBA, and the glycocholic acid (GCA) concentration was increased to 2%.

3.3 Metabolites and Key Enzymes in the Bile Acid Synthetic Pathway in the Liver

Regarding the basic pathways of BA synthesis from cholesterol in the liver, Fig. 3 illustrates the concentrations of cholesterol and oxysterols and the protein expression levels of CYP7A1 and CYP27A1, which are the key enzymes in the first step involving cholesterol, as well as the percentages of the primary BAs (CA and CDCA) in the bile in the two groups. In the deficient group, the cholesterol level in the liver was significantly lower than that in the control group. The hepatic concentration of the plant sterol sitosterol was also significantly decreased in the deficient group. On the other hand, cholesterol synthesis from acetyl-CoA in the liver was not affected by taurine deficiency because the concentrations of desmosterol, 7DHC, and lathosterol, which are intermediates in the cholesterol biosynthetic pathway, were not different than those in the control group (data not shown).

Among the key enzymes in the BA synthetic pathway (cholesterol catabolic pathway), in the deficient group, the microsomal protein expression level of CYP7A1 was significantly increased,

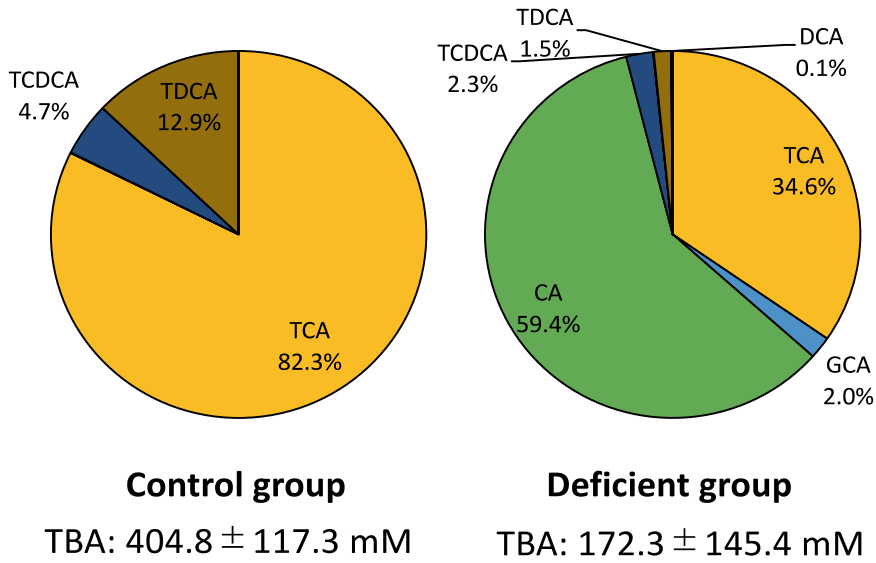

Fig. 2 Composition of bile acids in the bile of the control and deficient groups. Abbreviations: *Control group*, taurine-containing diet-fed group; *deficient group*, taurine-deficient diet-fed group; *CA*, unconjugated cholic acid; *TCA*, taurocholic acid; *GCA*, glycocholic acid; *TCDCA*, taurochenocholic acid; *DCA*, unconjugated deoxycholic acid; *TDCA*, taurodeoxycholic acid

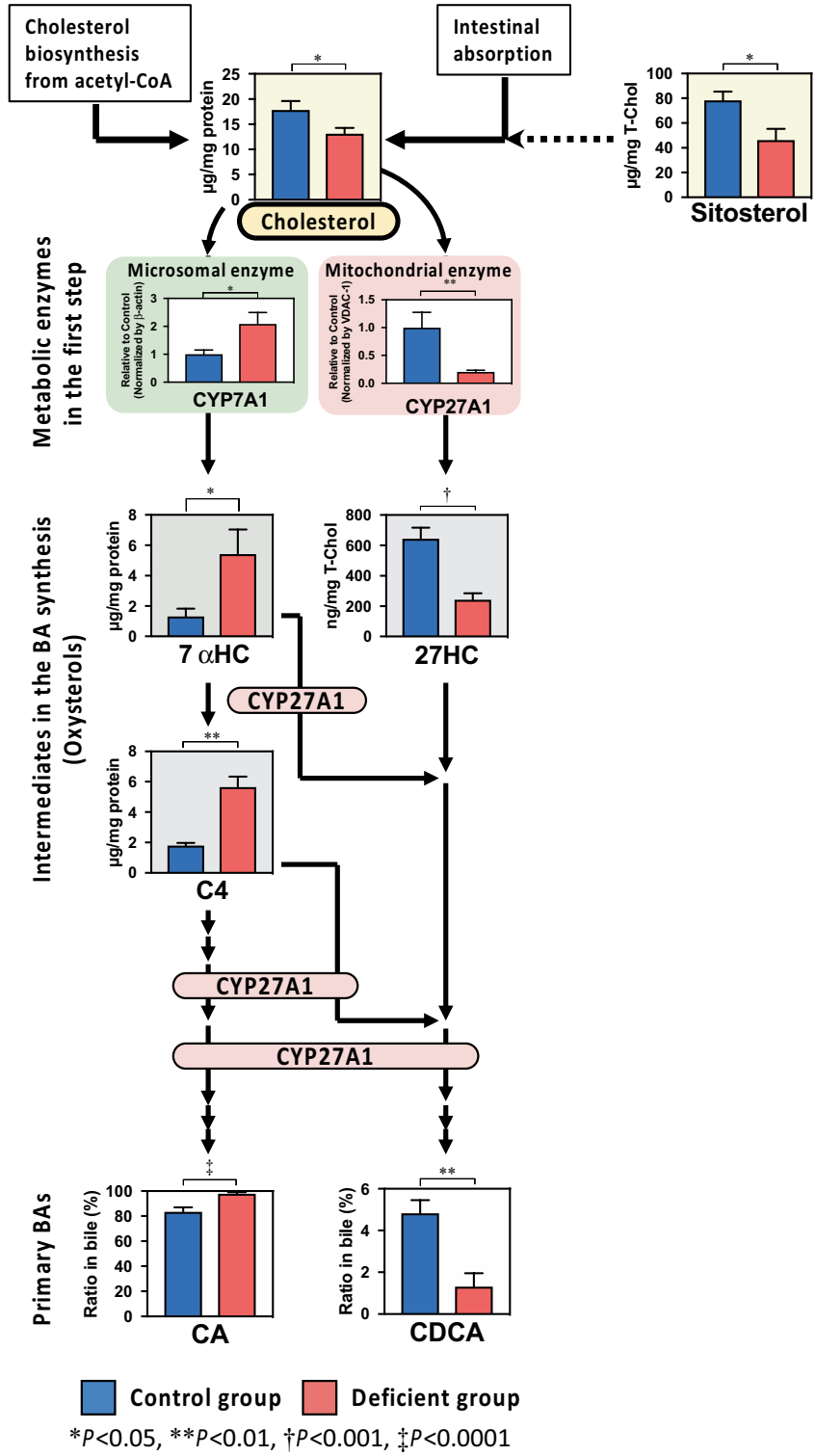

Fig. 3 Cholesterol and oxysterol concentrations and two-key bile acid synthetic enzymes, CYP7A1 and CYP27A1; protein levels in the bile acid synthetic pathway in the liver; and the percentages of the primary bile acids in the bile of the control and deficient cats. The primary bile acids, CA and CDCA, are the sum of unconjugated and amino acid-conjugated bile acids and are shown as ratios to total bile acids. The data are shown as the means ± SEM

while the mitochondrial CYP27A1 expression level was significantly decreased compared to that in the control group (Fig. 3). Along with the significantly increased CYP7A1 expression, the expression of the CYP7A1 protein and its metabolic products, 7αHC and metabolite C4, were significantly higher in the deficient group than in the control group. On the other hand, the level of 27HC, which is a metabolic product of CYP27A1, was significantly decreased in the deficient group compared to that in the control group. Therefore, the ratio of CA to TBA in the bile was significantly increased, while the CDCA ratio was significantly decreased in the deficient group compared with that of the control group.

4 Discussion

In taurine-deficient cats, the ratio of taurine conjugation with BAs in the bile was markedly reduced, and this result was accompanied by taurine deficiency in the whole body (Fig. 1) (Miyazaki et al. 2019b). In addition, the TBA concentration in the bile was significantly reduced to approximately 40%. In the control cats, more than 99% of the BAs were in the taurine-conjugated form, more than 95% were TCA and TDCA, and the remaining BAs consisted of TCDCA. On the other hand, 96% of the BAs in the taurine-deficient cats were conjugated with CA, and unconjugated and glycine-conjugated forms were increased to approximately 60% and 2%, respectively, but TCA was decreased to approximately 35%. TCDCA and TDCA ratios were decreased to approximately 2%. Namely, the BA concentration in the bile was reduced, the balance of primary BAs changed to being predominately CA, and the concentration of the taurine-conjugated forms was deceased. Therefore, taurine deficiency changed both the quality and quantity of BA synthesis, although taurine has been known to conjugate with BA (Danielsson 1963; Sjovall 1959). These observations imply that taurine influences BA synthesis from cholesterol, which is upstream from the BA conjugation reactions (Ferdinandusse and Houten 2006; Solaas et al. 2000).

Bile acid synthesis from cholesterol can be mainly divided into two pathways according to the first metabolic steps: microsomal CYP7A1, which is the rate-limiting enzyme in BA synthesis, and mitochondrial CYP27A1 (Fig. 3). In the former pathway, 7αHC is produced as a direct metabolite of CYP7A1 and then is metabolized to C4, which is a specific marker of CYP7A1 activity (Honda et al. 2004). This pathway mainly leads to CA production following some metabolic steps, including reactions involving CYP27A1, although there are several pathways that diverge from the CDCA production pathway. In the evaluation of the metabolites of the BA synthesis pathway, 7αHC and C4 concentrations were significantly increased, and the expression of CYP7A1 protein was significantly increased in taurine-deficient cats (Fig. 3). In contrast, the latter pathway begins in the mitochondria via CYP27A1, produces 27HC as the first metabolite, and finally produces CDCA following certain steps. In taurine-deficient cats, the 27HC concentration was significantly decreased along with significantly decreased CYP27A1 protein expression. Therefore, the ratio of CA was increased, but that of CDCA was decreased in taurine-deficient cats. The changed balance of the primary BAs in the taurine-deficient cats was due to the differential expression of metabolic enzymes in the first diverging steps that separate the production of CA and CDCA. In particular, the low expression of mitochondrial CYP27A1 was suggested as the main factor because taurine deficiency may cause a low level of functional mitochondria. Taurine modifies mitochondrial tRNAs containing 5-taurinemethyluridine (τm^5U) and 5-taurinemethyl 2-thiouridine (τm^5s^2U) (Suzuki et al. 2002), and impaired taurine modifications have been reported in the inherited mitochondrial diseases, such as mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonus epilepsy with ragged red fibers (MERRF) (Suzuki et al. 2011). In taurine-deficient cats, significantly decreased τm^5U and τm^5s^2U as well as cytochrome c activity were observed in the liver (Miyazaki et al. 2020). Therefore, the decrease in the CDCA synthesis pathway with low CYP27A1

protein expression is likely due to mitochondrial dysfunction caused by taurine deficiency.

On the other hand, the expression of CYP7A1 protein in the liver was enhanced by taurine deficiency. Negative feedback regulates BA synthesis by themselves, which has an endogenous ligand for the farnesoid X receptor (FXR) NR1H4 (Chiang 2004; Ramirez et al. 1994); in particular, CDCA has the most potent ligand activity (Makishima et al. 1999). Therefore, the inhibitory regulation on CYP7A1 gene expression might be attenuated because of the significantly decreased CDCA level.

Accompanied by enhanced CYP7A1 protein expression, the CA ratio was significantly increased in the bile of taurine-deficient cats. However, this ratio is a relative value of BA composition, and the absolute concentration of all BAs in the bile was significantly reduced by taurine deficiency. This outcome is likely a result of the significant decrease in cholesterol concentration in the liver. In taurine-deficient cats, the hepatic concentration of a plant sterol, sitosterol, which is one of the markers for intestinal cholesterol absorption (Miettinen et al. 1990), was significantly decreased (Fig. 2), while the concentrations of the intermediates in the cholesterol biosynthetic pathways were not changed in taurine-deficient cats (data not shown). Therefore, the significantly decreased cholesterol concentration in the liver was likely due to the reduction in intestinal cholesterol absorption. Because the amino acid-conjugated BAs facilitate the absorption of fats, fat-soluble vitamins, and cholesterols in the intestinal tract, intestinal malabsorption of cholesterols was suggested to be caused by the replacement of taurine-conjugated BAs with the unconjugated form of BAs in bile-intestine circulation because of taurine deficiency. This finding is supported by clinical evidence showing that a low serum level of fat-soluble vitamins was observed in patients with BA amidation deficiency (Carlton et al. 2003; Morton et al. 2000; Setchell et al. 2013).

5 Conclusion

In conclusion, taurine deficiency induced by a taurine-depleted diet in cats, which require taurine as an essential nutrient, causes not only the failure of taurine conjugation with BAs but also mediates a decrease in both the quality and quantity of BAs in the bile. The decreased BA content, the imbalance in BAs, the increased CA, and the decreased CDCA ratios, which are caused by liver damage through cholestasis, are likely due to mitochondrial dysfunction with impaired mitochondrial tRNA taurine modifications. The present study results suggest that a reduction in taurine in the body is a risk factor for various diseases caused by abnormal BA metabolism with mitochondrial dysfunction.

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Characterization of Bone Tissue and Bone Morphology in Taurine Transporter Knockout Mice

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Keywords

Taurine · TauT KO mice · BMD · Bone microstructure

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Abbreviations

ALP	Alkaline phosphatase
BMD	Bone mineral density
BS	Bone surface
BV	Bone volume
DEXA	Dual-energy X-ray absorptiometry
TauT	Taurine transporter
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TV	Total volume

1 Introduction

Taurine, a sulfur-containing amino acid, has been shown to have multiple functions, including antioxidative and membrane stabilization activities and roles in modulating the cell volume, ion channel activities, and intracellular calcium levels (Pasantes-Morales and Cruz 1985; Gordon et al. 1992; Huxtable 1992; Camerino et al. 2004; Hoffmann et al. 2009; Kato et al. 2015). It is synthesized primarily by the liver and accumulated in various tissues in the body (Moon et al. 2012). Depending on the species, intracellular concentrations of taurine can vary from 10 to 70 mmol/L in the mammalian heart, brain, neutrophils, skeletal muscle, liver, and retina (Suleiman et al. 1997). By contrast, its extracellular concentrations have been shown to reach only 600–

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800 nmol/L in mice (Shigemi et al. 2011). This steep taurine concentration gradient between the intracellular and extracellular spaces is built up by a sodium-dependent transport system called the taurine transporter (TauT) (Rasgado-Flores et al. 2012), which is expressed in mammalian tissues. Because the ability to synthesize taurine is limited in most tissues, the maintenance of high intracellular concentrations of this amino acid depends upon its uptake from the extracellular space via TauT (Baliou et al. 2020).

In bone tissue, taurine accounts for 0.1% of the body weight, making it an effective element in the regulation of bone metabolism (Gupta et al. 2005; Jeon et al. 2007). Taurine promotes the differentiation of osteoblasts, which also express TauT to maintain a constant intracellular level of the amino acid (Yuan et al. 2006). This amino acid also mediates the activation of alkaline phosphatase (ALP) activity and osteocalcin secretion and the inhibition of osteoclastogenesis (Yuan et al. 2010). Additionally, taurine affects not only bone remodeling but also the expression of connective tissue growth factor and the synthesis of collagen in osteoblast-like cells (Park et al. 2001; Yuan et al. 2007). Choi (2009) has shown that the oral supplementation of taurine could increase the femur bone mineral content in growing rats and may have positive results on bone metabolism in alcohol-fed ovariectomized rats. However, the long-term administration of taurine may have an adverse effect on the bone microstructure. Because of the sulfur-containing characteristic of taurine, its excessive intake could yield increased sulfuric acid production in the body, whereupon the skeleton may need to act as a buffer to neutralize the excess acid (Martiniakova et al. 2019). The effects of taurine on bone may depend on its dose and the subject's general physical condition.

Previous studies have reported the correlation of severe taurine deficiency with the presentation of a variety of disorders in various tissues (e.g., the eye, kidney, heart, and muscle) of TauT knockout (TauT−/−) mice (Warskulat et al. 2004; Ito et al. 2008). However, the exact effects of taurine deficiency or depletion on bone metabolism and bone quality have not been elucidated. Therefore, our aim in this study was to clarify this by comparing the bone mineral density (BMD) and bone microstructure of TauT−/− and TauT+/+ mice.

2 Methods

2.1 Animals and Chemicals

Male TauT−/− and TauT+/+ mice (20 months old) were obtained by mating heterozygous males and females (Ito et al. 2008). The animals were housed in a specific pathogen-free room kept at 22.0 °C and 45–55% relative humidity, under a 12-h light/dark cycle, and given ad libitum access to water and food (MF, Oriental Yeast, Tokyo, Japan). For each experiment, five animals from each mouse group were euthanized, and their tissues were isolated for study. All animal experimental protocols were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Suzuka University of Medical Science, Suzuka, Mie, Japan.

2.2 Histopathological and Immunohistochemical Studies

For each mouse, the right femur was evaluated for its bone microarchitecture and BMD, whereas the left femur was used to prepare a decalcified section for histomorphometric analysis. The femur bones were fixed with 4% formaldehyde in phosphate-buffered saline for 1 day. Then, after their dehydration and paraffin infiltration, the bones were embedded in paraffin blocks, which were subsequently sectioned to 6 μm thickness using a microtome (Leica Microsystems, Wetzlar, Germany) according to routine protocols. After a 48-h heat treatment at 40 °C, the tissue sections were deparaffinized with xylene, hydrated with a gradient series of alcohol, and then washed once with tap water for 5 min. Thereafter, the sections were boiled in 5% urea for 5 min in a microwave oven (500 W) for antigen retrieval. Following

epitope retrieval, endogenous peroxidases were inactivated by incubating the samples with 1% H_2O_2 at 25 °C for 15 min. Then, after blocking the sections with 1% skimmed milk for 20 min, they were incubated with primary antibodies against TauT (1:200 dilution; cat. no. sc-393036; Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 20 °C overnight, followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) at 25 °C for 1 h. The immunocomplexes were visualized using the Peroxidase Stain DAB Kit (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's instructions. The nuclei were counterstained with hematoxylin, and the sections were finally observed and photographed under a microscope (BX53; Olympus, Tokyo, Japan). Immunostaining without primary antibodies was carried out for the negative control sections, which subsequently revealed no positive staining (data not shown).

The histopathological appearance of the bones was evaluated by hematoxylin and eosin staining. The thickness of the femoral growth plate was measured using ImageJ software (version 1.48).

2.3 Measurement of Bone Mineral Density

The BMD of the femur was assessed by dual-energy X-ray absorptiometry (DEXA) using an apparatus for small animals (Dichroma Scan DCS-600; Aloka, Osaka, Japan) according to a previous study. Two femur regions were measured: proximal and mid-diaphysis. The proximal femur represented cancellous bone, whereas the mid-diaphysis of the femur was the cortical bone area.

2.4 Micro-computed Tomography Bone Analysis

The bone microstructure in the proximal femur was assessed by micro-computed tomography (micro-CT) (SMX-90CT; Shimadzu, Kyoto, Japan). Three-dimensional microstructural imaging data were reconstructed, and structural parameters were calculated using TRI/3D-BON software (RATOC System Engineering, Tokyo, Japan). The micro-CT imaging conditions used were as follows: tube current, 110 μA; tube voltage, 90 kV; integration time, 200 ms; and voxel size, 23 μm × 23 μm × 23 μm. The scan was conducted at constant intervals of 23 μm over a region of 920 μm in length from the inferior border of the femur head. The structural parameters measured were the total volume (TV), bone volume (BV), bone surface (BS), bone volume fraction (BV/TV), bone surface to total volume ratio (BS/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp).

2.5 Statistical Analysis

All data are presented as the mean ± standard deviation. Student's t-test (SPSS 21.0) was used to examine differences between the TauT−/− and TauT+/+ mice. Differences in values were considered statistically significant at $P < 0.05$.

3 Results

3.1 External Appearance, Histopathology, and Immunohistochemistry of the TauT−/− Mouse Femurs

The external appearance of the femurs and histological staining of the tissue are shown in Fig. 1. The femurs of the TauT−/− mice showed normal external morphology and appeared similar to those of the TauT+/+ mice (Fig. 1a). There was also no significant difference in femur length between the TauT−/− and TauT+/+ groups (17.0 ± 0.06 and 17.1 ± 0.12 mm, respectively; $P = 0.563$). The TauT−/− mouse femur had the following specific histological features: (1) a small number of trabeculae, (2) thin cortical bone and epiphyseal plate thicknesses, and (3) a wide bone marrow space (Fig. 1b). Next, TauT antibodies were used to localize TauT expression in the bones (Fig. 2). Tissues from the femurs of

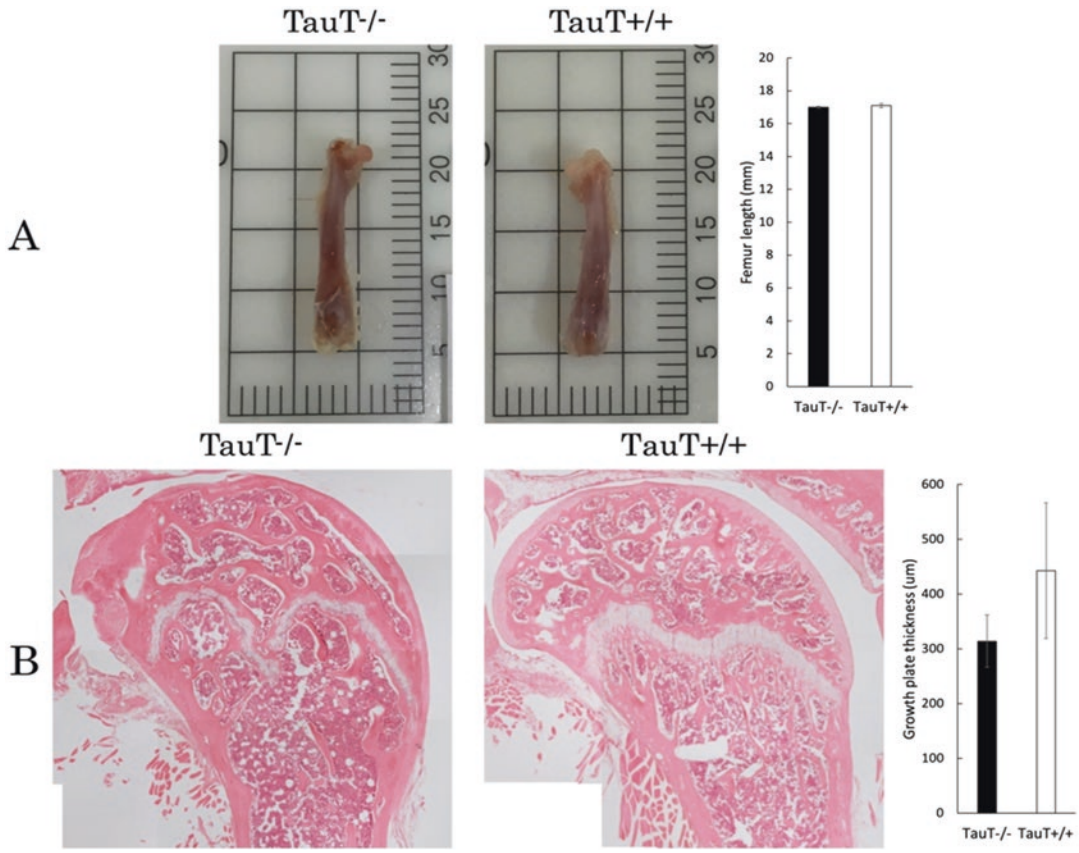

Fig. 1 External morphological and histopathological analyses of the femurs of TauT−/− mice. (**a**) There were no abnormal changes in external appearance of the femurs from the TauT−/− mice, which were similar to those from the TauT+/+ mice. The femur lengths were also not significantly different between the TauT−/− and TauT+/+ mice ($P = 0.563$). (**b**) The specific histological features of the TauT−/− mouse femur were a small number of trabeculae, the thin cortical bone and epiphyseal plate thicknesses, and a wide bone marrow space

TauT−/− and TauT+/+ mice were immunostained using the avidin–biotin complex method. Osteoblasts are normally distributed throughout the surface of a trabecula. Numerous TauT-immunopositive cells were observed in the broad part of the trabecula in all TauT+/+ mice, but not in the TauT−/− mice (Fig. 2). Osteoblasts were observed on the surface of the bone matrix. There was a mixture of cuboidal activated osteoblasts and flattened differentiated osteoblasts in the bone tissue of the TauT+/+ mice, whereas undifferentiated osteoblasts were more frequently observed in that of the TauT−/− mice. Since the thickness of the growth plate affects bone formation, we analyzed the growth plate cartilage in these mice. The ossification zone of the TauT+/+ mouse femur shown in Fig. 1b revealed that the growth plate was of equivalent thickness. By contrast, the growth plate of the TauT−/− mouse femur was of unequal thickness, and some areas were interrupted. However, the difference in growth plate thickness between the TauT−/− and TauT+/+ groups was not statistically significant (314.2 ± 47.8 and 443.0 ± 123.5 μm, respectively; $P = 0.114$).

3.2 Analysis of Bone Mineral Density in TauT−/− Mice

To assess the function of taurine, the BMDs of the TauT−/− and TauT+/+ mice were evaluated

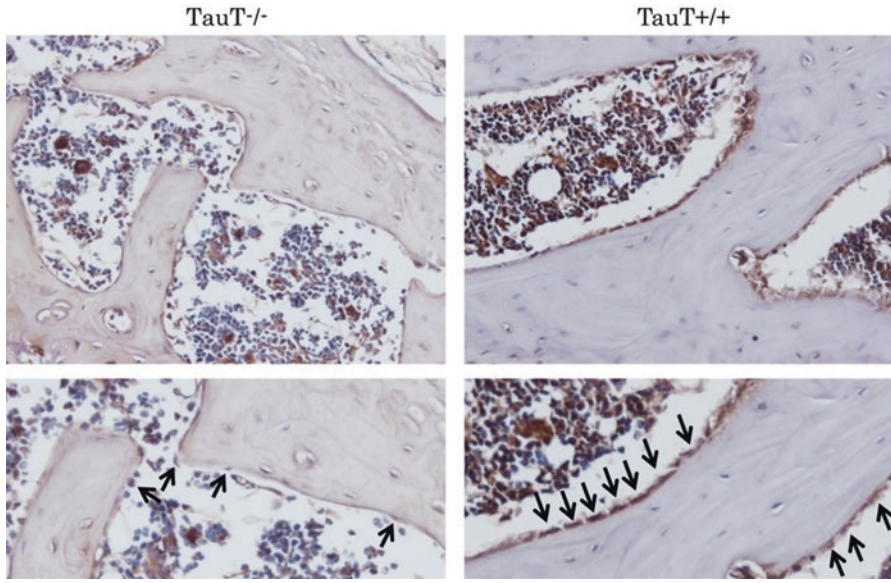

Fig. 2 Localization of taurine transporter (TauT) expression in bone tissue of TauT+/+ and TauT−/− mice. The spatial distributions of taurine and TauT in osteoblasts on the bone surface are shown. Intense TauT immunoreactivity was observed in the osteoblasts of the TauT+/+ mouse femur, whereas weak TauT immunoreactivity was observed in those of the TauT−/− mouse femur

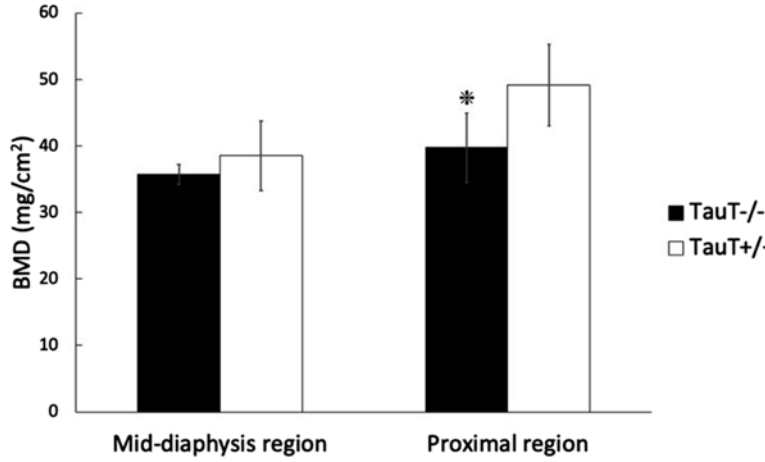

Fig. 3 Comparative analysis of the bone mineral density (BMD) in TauT−/− and TauT+/+ mice. In the mid-diaphysis region, the BMD was not significantly different between the TauT−/− and TauT+/+ groups ($P = 0.261$). In the proximal region, the BMD in the TauT−/− mice was significantly lower ($P = 0.003$)

by DEXA scanning of the proximal and mid-diaphysis regions (Fig. 3). In the mid-diaphysis region (Fig. 3), the BMD of the TauT−/− group was not significantly different from that of the TauT+/+ group (35.7 ± 1.5 and 38.5 ± 5.2 g/cm^2, respectively; $P = 0.261$). By contrast, in the proximal region (Fig. 3), the BMD was significantly lower in the TauT−/− mice than in the TauT+/+ mice (39.7 ± 5.2 and 49.1 ± 6.1 g/cm^2, respectively; $P = 0.003$).

3.3 Structural Analysis of Femoral Trabeculae in TauT−/− Mice Using Micro-computed Tomography

Femurs from the TauT−/− and TauT+/+ mice were analyzed using micro-CT, and the representative 3D reconstructions are shown in Fig. 4a. With regard to the trabecular bone parameters, the BV (0.13 ± 0.03 and 0.18 ± 0.02 mm^3, $P = 0.015$)

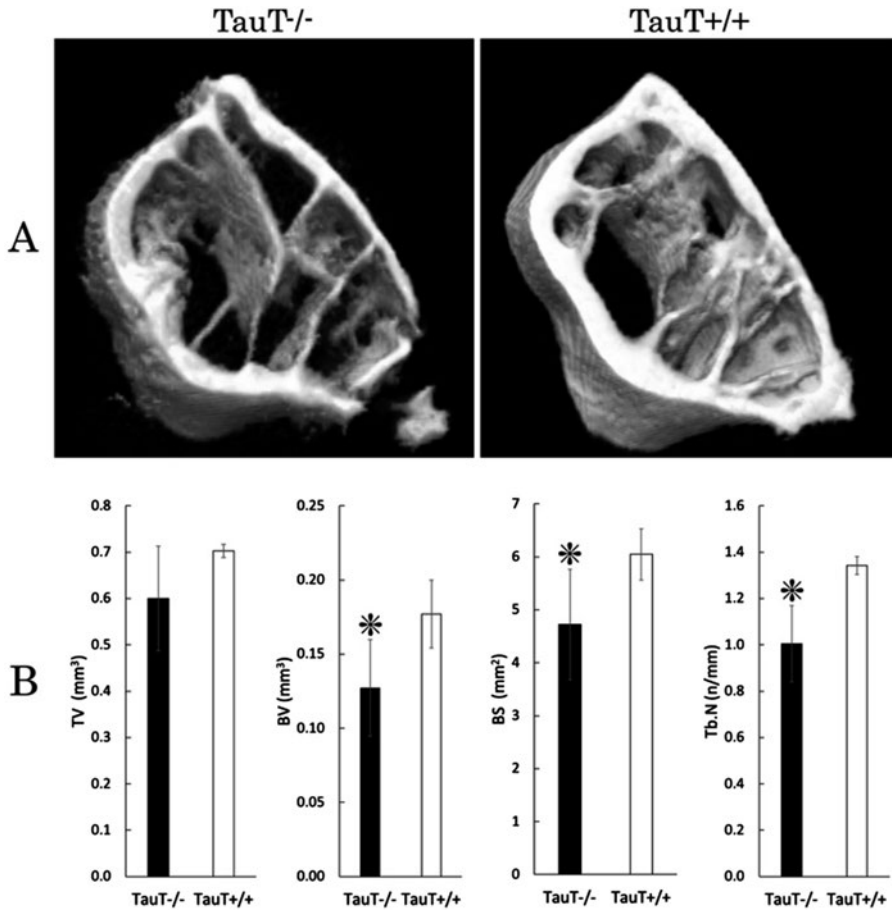

Fig. 4 Structural analysis of femoral trabeculae in TauT−/− mice using micro-CT. The bone microstructure was evaluated in both TauT−/− and TauT+/+ mice. (**a**) Three-dimensional microstructural images of the proximal region of the femurs in both mouse groups. (**b**) The specific micro-CT features of the TauT−/− mouse femur were a significantly smaller bone volume (BV; $P = 0.015$), bone surface (BS; $P = 0.015$), and trabecular number (Tb.N; $P = 0.002$)

and BS values (4.72 ± 1.04 and 6.05 ± 0.48 mm^2, $P = 0.015$) were significantly decreased in the TauT−/− mice relative to the values in the TauT+/+ mice (Fig. 4b). By contrast, there was no significant difference between the two mouse groups in terms of the TV, BV/TV, and BS/TV values. The Tb.N was also significantly lower in the TauT−/− mice (1.00 ± 0.16 n/mm) than in the TauT+/+ mice (1.34 ± 0.04 n/mm). Additionally, no differences in Tb.Th (81.8 ± 14.0 and 80.9 ± 10.9 μm, respectively; $P = 0.146$) and Tb.Sp (175.8 ± 42.5 and 161.9 ± 33.9 μm, respectively; $P = 0.192$) were observed between the TauT−/− and TauT−/− mice.

4 Discussion

In this study, the bone status (e.g., femoral growth plate, BMD, and bone microstructure) in TauT−/− mice was investigated. The results revealed that TauT−/− mice had a lower BMD and poorer bone microstructure than TauT+/+ mice, suggesting that taurine deficiency decreases bone density and quality and therefore increases the risk of fracture. To the best of our knowledge, this is the first study to assess the bone status in TauT−/− mice.

As previously described, TauT, which is also expressed in osteoblasts, maintains a constant

level of taurine in the bone tissue, allowing the amino acid to perform important bone functions and promote osteoblast differentiation. Taurine supplementation has been shown to improve bone formation and differentiation in *ovariectomized* rats (Choi 2017) and in a rabbit model of glucocorticoid-induced osteonecrosis (Hirata et al. 2020). Taurine affects the expression of connective tissue growth factor through cell signaling pathways in osteogenic cells and has been associated with increased ALP activity and collagen synthesis in osteoblast-like UMR-106 cells (Park et al. 2001). It also activates nuclear factor erythroid 2-related factor 2 (Nrf2); induces the expression of the antioxidant enzymes NAD(P)H dehydrogenase [quinone] 1 (NQO1), heme oxygenase 1 (HO1), and glutamate–cysteine ligase catalytic subunit (GCLC); and reduces H_2O_2-induced cell death by activating extracellular signal-regulated kinase (ERK) and the Wnt/β--catenin pathway in osteoblasts. Additionally, the partial reduction in ERK, antioxidant, and ALP activities by a Wnt/β-catenin inhibitor suggests the involvement of other signaling molecules and pathways (Lou et al. 2018). Taurine has been found to mediate the activation of ALP activity and osteocalcin secretion and the inhibition of intracellular reactive oxygen species (Lou et al. 2018). Our findings are consistent with these previous reports. In particular, we found that the TauT−/− mice, which are inherently taurine deficient, had many undifferentiated osteoblasts and a low BMD. Our findings indicate that taurine deficiency may result in osteoblast immaturity and a decrease in the bone density. Considering that oxidative stress is a biomarker of postmenopausal osteoporosis, it is possible that the antioxidative effect of taurine could be involved.

Taurine deficiency leads to poor bone microstructure. Bone strength depends on the intrinsic properties of the materials that take part in bone matrix mineralization, the BMD, and the bone microstructure. The mechanical properties of trabecular bone tissue are determined by the bone microstructure (Fritsch and Hellmich 2007). In normal bone formation, the volume per year turnover rate is 26% for trabecular bone and 3% for cortical bone. Because trabecular bone is more active in remodeling, it is less mineralized than cortical bone (Webster and Jee 1983). The strength of trabecular bone is related to bone fracture and damage, which cause bone remodeling (Lotz et al. 1991). Bone strength is 70% determined by the bone density and 30% by the bone quality (Klibanski et al. 2001). The bone microstructure is the most critical factor for good bone quality. Several reports have been published on the effects of taurine supplementation on the bone microstructure. For example, in vitamin B_{12}-deficient mice, oral taurine administration increased the BV/TV by inducing an increase in insulin growth factor 1 (IGF-1) (Roman-Garcia et al. 2014). Oyster shell powder, which has a high taurine content, increased the femoral BMD, trabecular BV, Tb.Th, and Tb.N in ovariectomized mice, suggesting its potential for the treatment of osteoporosis (Han et al. 2007). By contrast, taurine exposure at the dose of 40 mg/kg bodyweight for 8 weeks was ineffective on the microstructures of both compact and trabecular bone tissues (Martiniakova et al. 2019). In this present study, the decrease in Tb.N in the TauT−/− mouse femur was not consistent with a bigger space between the trabeculae or an increase in Tb.Sp. Collectively, the micro-CT features of the proximal femur of the TauT−/− mouse were a smaller BV, BS, and Tb.N, suggesting that taurine is required for skeletal development of the trabecular bone in mice. The difference from previous studies is that the TauT−/− mice used in this study are unable to take up taurine into the cell. Hence, lifelong taurine deficiency may lead to the degradation of the bone microstructure and a reduction in bone quality.

5 Conclusion

In summary, we found that the bone of TauT−/− mice was characterized by a low BMD and poor bone quality, which decreased the bone strength. These findings support the importance of taurine for bone metabolism. The TauT expressed in osteoblasts ensures the maintenance of a high intracellular taurine concentration, indicating

that taurine can promote osteoblast differentiation during bone formation. Lifelong taurine deficiency may result in the degradation of the bone microstructure and a subsequent reduction in bone quality. Further research is needed to determine the exact role of taurine in the maintenance of bone health.

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Blood Taurine Dynamics in Captive Lions: Relationship with Feed and Bile Acid Composition

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Keywords

Lion · Zoo · Bile acids · Tail blood · Feed

Abbreviations

BAs	Bile acids
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CDO	Cysteine dioxygenase
CSD	Cysteine sulfinic acid decarboxylase
DCA	Deoxycholic acid
LCA	Lithocholic acid
UDCA	Ursodeoxycholic acid

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1 Introduction

Taurine (2-aminoethyl sulfonic acid), which was discovered in bovine bile in 1827, is an abundant free amino acid in mammalian tissues. It reportedly mediates various effects such as cardiotonic action, liver protection, muscle damage prevention, and maintenance of retinal function (Huxtable 1980, 1992; Jacobsen and Smith 1968; Miyazaki and Matsuzaki 2014). Major species differences exist among animals in terms of taurine requirements, synthesis capability, and reabsorption mechanisms (Hayes 1988).

In obligate carnivore felines, taurine is an essential nutrient related to low rates of hepatic biosynthesis and renal reabsorption (Hayes 1988; Pickett et al. 1990). The amino acid is synthesized mainly in the liver in a pathway involving two limiting enzymes: cysteine dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSD). However, hepatic CSD activity in felines is about one-handredth of that in rats (Sturman 1992). Moreover, adaptability of hepatic CDO activity in response to changes in amounts of taurine in the body is also low (Rentschler et al. 1986). In addition, bile acids (BAs) in felines are mainly conjugated with taurine, but little with glycine, which is similar to rodents with a high capacity to synthesize taurine in the liver. Bile salts play important roles in the promotion of bile excretion from the liver and facilitation of lipid absorption in the intestinal tract by formation of

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micelles; felines cannot adequately synthesize vitamin D in the skin (How et al. 1994). Furthermore, they lack the ability to convert provitamin carotenoids including β-carotene into active vitamin A (Schweigert et al. 2002). Bile salts are necessary to promote the intake of fat-soluble vitamins through the diet. Therefore, the ingestion of taurine should be higher for felines than for other animals; felines are prone to taurine deficiency (Markwell and Earle 1995).

Severe symptoms, such as clinical blindness and retinal degeneration, central nerve system dysfunction, deformed spinal skeleton, cardiomyopathy, platelet hyperaggregability, impaired neutrophil function, and liver damage, develop in domestic cats fed a taurine-deficient diet (Hayes 1988; Hayes et al. 1975; Hayes and Trautwein 1989; Knopf et al. 1978; Miyazaki et al. 2019b, 2020; Sturman 1992). Taurine deficiency is not limited to domestic cats, as taurine deficiency-induced symptoms have been reported in captive felines: retinopathy in leopard cats (*Felis bengalensis*) (Howard et al. 1987), central retinal lesions and central visual defects in white Bengal tiger (Pickett et al. 1990), central retinal degeneration in cheetahs (*Acinonyx jubatus*) (Ofri et al. 1996), and rickets in lion cubs (Chesney and Hedberg 2009). Taurine deficiency can be reasonably expected to cause these symptoms in adult lions.

Although wild felines can obtain sufficient taurine from various preys, captive felines housed in zoos may have difficulty in adjusting to the intake of taurine when provided a specific diet. Therefore, information related to taurine dynamics in felines throughout the year is indispensable for improving captive health management. However, because of the difficulty collecting biological samples repeatedly from vital and fierce lions, little information is available to elucidate the relationship between taurine dynamics in blood and its ingestion of food provided to lions. To clarify this issue, we have developed safe methods to collect blood from the tails of felines in zoos. Therefore, the study objectives were to evaluate the status of taurine concentration and BAs in the blood and their relationship to the amount of taurine ingested from feed.

2 Methods

2.1 Subject Animals and Management

This study examined four lions (*Panthera leo*). Lion 1 (male, 6 years old) was born and housed at Hitachi City Kamine Zoo (Kamine Zoo, Hitachi City, Ibaraki, Japan). Lions 2 and 3 were a male (5 years old) and a female (6 years old) born as unrelated at the Kyushu Natural Animal Park (Usa City, Oita, Japan). They were housed at Omuta City Zoo (Omuta Zoo, Omuta City, Fukuoka, Japan). Lion 4 (female, 9 years old) was born at Asahiyama Zoo and was housed at Toyohashi Zoo and Botanical Park (Toyohashi Zoo, Toyohashi City, Aichi, Japan). Blood was collected repeatedly from the tail of each lion for evaluation of taurine and BAs in blood.

Study 1 evaluated the relation between taurine concentration in blood and taurine contents of feed for three lions housed at the Kamine and Omuta zoos: Lion 1, 17 samples from March to December in 2017, and Lions 2 and 3, 14 and 16 samples, respectively, from July 2015 through September 2017.

For Study 2, taurine and BAs in blood samples were evaluated for all four lions: Lion 1, 22 samples from March 2017 through November 2018; Lion 2, 24 samples from July 2015 through December 2018; Lion 3, 26 samples from August 2015 through November 2018; and Lion 4, 7 samples from July 2018 through January 2019. Bile acids were analyzed in all collected samples from Lions 1 and 4, but in nine and ten samples, respectively, for Lions 2 and 3.

2.2 Feeding Schedule and Amounts and Types of Feed

Lion 1 had been fed 6 kg of beef and 1.2 kg of chicken heads (total 7.2 kg meat) routinely once a day at 15:30 or 16:00 on Tuesday, Thursday, Friday, Saturday, and Sunday, with half volumes of them (3.6 kg) given on other days of the week (beef 36 kg/week, chicken 7.2 kg/week). Blood

was collected at 16:30. The total amount of feed was about 43.2 kg per week. In addition, the liquid from thawed meat was refrozen and given with meat in the summer. Then they were fed as is in the winter. Whole chicken, cow's liver, and whole rabbits were fed randomly. However, the additional feed was not given during the 3 days before blood collection.

Lions 2 and 3 were fed twice a day at 9:00–10:00 and 16:00–17:00. Blood was collected between those times (13:00–15:00). Lion 2 was fed a total 25.5 ± 2.4 kg per week: 0.8 ± 0.1 kg of cow kidneys/daily, 3.0 ± 1.1 kg of whole chicken/6 days a week, 0.8 ± 0.3 kg of horse meat and 0.5 ± 0.2 kg of chicken head/once a week, and 0.7 ± 0.5 kg of cow liver/the first 5 days of the month. Lion 3 was fed a total 21.6 ± 3.5 kg per week: 0.9 ± 0.2 kg of cow kidneys/daily and 2.3 ± 1.1 kg of whole chicken/6 days a week, 0.9 ± 0.2 kg of horse meat and 0.5 ± 0.2 kg of chicken head/once a week, and 0.6 ± 0.5 kg of cow liver/the first 5 days of the month.

Taurine content in the main feed was also measured: chicken heads and beef at Kamine Zoo and horse meat and cow kidneys and liver at Omuta Zoo. Three pieces of 50 g of beef, eight chicken heads, and ten pieces of 3–8 g of horse meat and cow kidneys and liver which were fed on the blood sampling days were used for the taurine assay. Except for chicken head samples, all samples were collected from different sites to the greatest degree possible. All feed samples were stored at −60 °C until assay.

2.3 Blood Collection

Three staff members conducted blood collection according to a collection procedure described in an earlier report (Ban et al. 2017) (Fig. 1): (1) a reward meat provider guides a lion to the fence line and lets it lie down; (2) a tail retainer pulls the tail of a lion to the outside of the fence using a hook; (3) a blood collector (a veterinarian) swabs the tail with an ethanol cotton pad (the tail hair was removed with electric hair clippers at Omuta Zoo) and collects blood (1.5–5 mL) from the tail vein. During blood collection, the reward meat provider continued to give meat as a reward for collection procedures. The collected blood was centrifuged immediately to obtain serum at Kamine Zoo or plasma at other zoos because of different equipment from that used usually. Collected samples were kept at −20 °C until analyses of taurine and BAs.

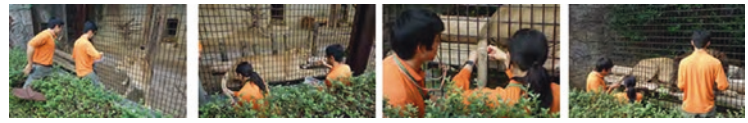
2.4 Taurine Assays in Feed and Blood Samples

Feed, except for chicken heads, was ground using a chopper, and then 50–100 mg of samples were sonicated in tenfold volume of 1% formic acid solution. After centrifugation at 6200 rpm, 4 °C, 10 min, the supernatant was used for analysis. Because of various tissues including bones, crests, hair, and others, the chicken head was first ground with a mincer. Then approximately 1 g was homogenized using a sonicator with the same volume of 1% formic acid solution. Subsequently, the sonicated sample was added with 4 mL of 1% formic acid and was centrifuged at 3600 rpm and 4 °C for 10 min. After supernatant collection, the residue pellet was homogenized further with 1 mL of 1% formic acid. It was centrifuged again. Both supernatants were mixed and were used for subsequent analyses.

Then, 5 μL of serum and plasma and 10 μL of the supernatant of feed samples were mixed with 100 μL of acetonitrile and 50 μL of internal standard solution (APDSTAG® Wako Amino Acids Internal Standard Mixture Solution; Fujifilm Wako Pure Chemical Corp., Osaka, Japan). After derivation with 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate using a method reported earlier in the literature (Miyazaki et al. 2019a), taurine in the samples was measured using the HPLC-ESI-MS/MS system.

2.5 Bile Acid Analysis in Blood Samples

Bile acids in blood samples were measured using the HPLC-ESI-MS/MS system according to a method reported earlier in the literature (Miyazaki



STEP 1 Guiding by a reward meat

STEP 2 Pulling the tail with a hook

STEP 3 Swabbing the tail with ethanol pad

STEP 4 Collecting the blood with keeping of the reward meat

Fig. 1 Process of blood sample collection from a lion tail

et al. 2019b, 2020). In brief, 20 µL of serum or plasma mixed with a mixture solution of internal standards consisting of stable isotope types of BAs dissolved with acetonitrile was diluted with 2 mL of 0.5 M potassium phosphate buffer (*pH* 7.4) and was passed through cartridges (Bond Elut C18, 200 mg; Agilent Technologies Inc., Santa Clara, CA). After washing the cartridge with 1.6 mL of water, BAs 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 was 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. The general HPLC and MS/MS conditions were conducted using a method described earlier (Murakami et al. 2018).

2.6 Statistical Analyses

Averages in multiple subjects were analyzed and compared using the Kruskal–Wallis test. The Steel–Dwass multiple comparison test was applied as a post hoc test when a significant difference was found. Correlation relations of blood taurine concentration to taurine concentrations in feed and compositions of blood BAs were analyzed using Pearson's product moment correlation coefficient. All statistical analyses were conducted using a software (R ver. 3.2.2; The R Foundation for Statistical Computing).

3 Results

3.1 Taurine Content in Feeds and the Relation to Taurine Concentration in Blood (Study 1)

Table 1 presents taurine content in lion feed, including beef, chicken heads, horse meat, cow liver, and cow kidneys fed at Kamine and Omuta zoos. The highest taurine-containing feed was chicken heads (575.0 ± 75.0 mg/kg); the lowest was beef (425.0 ± 135.5 µg/g). Taurine content in whole chickens was estimated from the average content of other feeds. The amount of taurine ingested per month was calculated from the taurine content in feed of each type. The amount of taurine ingestion from feed was higher for Lion 1 (approximately 85.7 g/month) at Kamine Zoo than the others (approximately 63.7 and 55.8 g/month) at Omuta Zoo. However, taurine concentration in blood collected during the same periods with the feeds was higher for Lions 2 and 3 of Omuta Zoo than for Lion 1 of Kamine Zoo (Table 1).

Weights of feed fed the day before blood sample collection were 5.9 ± 0.4, 3.7 ± 0.4, and 3.1 ± 0.4 kg for Lions 1–3, respectively. Figure 2 shows the relationship between the blood taurine concentration and the weight of feed on the day before blood sample collection. For Lion 1, the amount of daily feed was only two cases: 3.6 and 7.2 kg of beef and chicken heads. No correlation was found between taurine concentration in blood and the feed weight (Fig. 2a). Furthermore, no significant correlation was found between the blood taurine concentration and the respective beef and chicken head weights. For Lions 2 and 3 at Omuta Zoo, the daily total amounts of feed

Table 1 Taurine content in feed, estimated amounts of taurine ingestion, and blood taurine concentration in lions

		Lion 1 (Kamine Zoo)	Lion 2 (Omuta Zoo)	Lion 3 (Omuta Zoo)
Sampling period		2017.3–2017.11	2015.7–2017.9	2015.8–2017.8
Type of feed	Taurine content: mg/kg	Estimated taurine ingestion: g/month[a] (feed amount: kg/month[a])		
Beef	425.0 ± 135.5	61.2 (144)		
Chicken head	850 ± 62.5	24.5 (28.8)	1.70 (2)	3.06 (3.6)
Horse meat	575.0 ± 75.0		1.75 (3.2)	1.15 (2)
Cow liver	587.5 ± 12.5		0.41 (0.7[b])	0.35 (0.6[b])
Cow kidney	662.5 ± 25.0		14.8 (22.4)	16.7 (25.2)
Whole chicken	[625][c]		[45.0] (72.0)	[34.5] (55.2)
Estimated total amount of taurine ingestion (g/month)		85.7	63.7	55.8
Taurine conc. in blood (μM)		137.6 ± 9.0	310.6 ± 28.1	239.7 ± 40.5

Footnote: Data [a]Month was calculated as 4 weeks. [b]Cow liver was fed during the first 5 days of the month. [c]Taurine content in the whole chicken was estimated as 625 μg/g (5 μmol/g) from the average values of other feeds

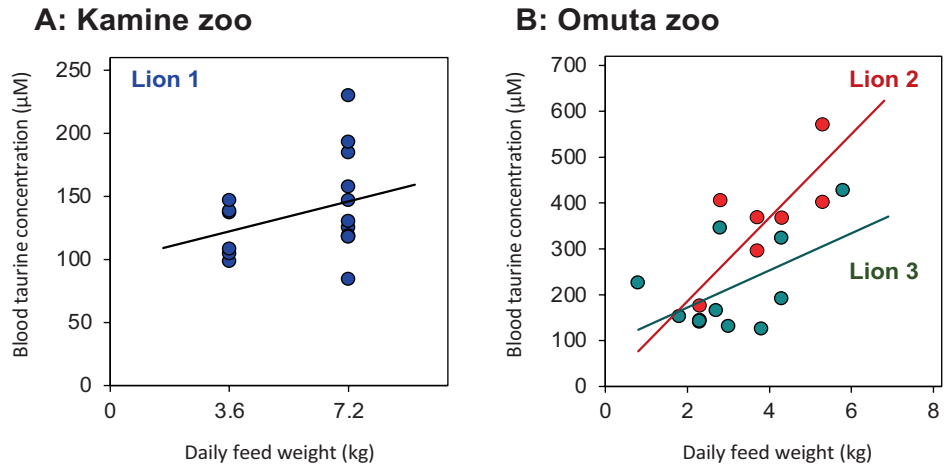


Fig. 2 Relation between amounts of feeds in the day before blood sample collection and the blood taurine concentration. (**a**) Lion 1 at Kamine Zoo ($N = 17$; $r^2 = 0.10$, $P = 0.2172$). (**b**) Lion 2 ($N = 8$; $r^2 = 0.65$, $P < 0.05$) and Lion 3 ($N = 11$; $r^2 = 0.29$, $P = 0.08$) at Omuta Zoo. Correlation coefficients were calculated using Pearson's product moment correlation coefficient

consisting of whole chicken, cow kidney, chicken heads, and horse meat were 3.7 ± 0.4 and 3.1 ± 0.4 kg, respectively. The main feed amounts were whole chicken of 2.8 ± 0.4 and 2.0 ± 0.3 kg, respectively. A positive correlation was found between the blood taurine concentration and the daily total amount of feed. Significance was found for Lion 2 (Fig. 2b). For Lion 2, a significant correlation was found between the blood taurine concentration and feed weight of whole chickens ($r^2 = 0.65$, $P < 0.05$).

3.2 Blood Taurine Levels in the Lions (Study 2)

Figure 3 shows blood taurine concentrations for all four lions. For Lion 1 at Kamine Zoo, the average of blood taurine was 127.2 ± 9.0 μM (55.3–229.9 μM; minimum–maximum). At Omuta Zoo, the averages were 255.4 ± 24.9 μM (76.6–571.8 μM) and 192 ± 28.0 μM (51.0–751.2 μM) for Lions 2 and 3, respectively. The average value for Lion 4 at the Toyohashi Zoo was 83.8 ± 15.2 μM (38.5–139.1 μM). The interquartile ranges (the difference between 75% and 25% percentile) were 54.3, 175.8, 68.0, and 81.2 μM for Lions 1–4, respectively. Taurine concentration was significantly higher for Lion 2 than that of Lions 1 or 4 and also higher for Lions 2 and 3 than that of Lion 4.

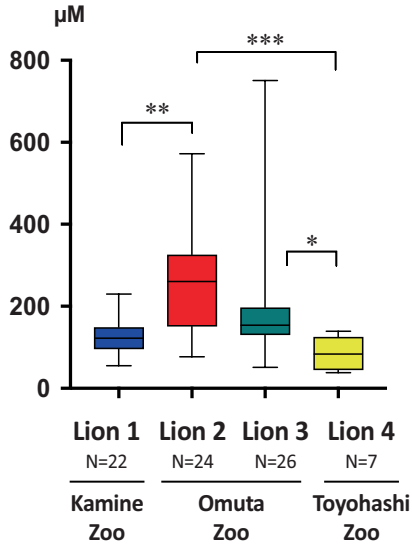


Fig. 3 Blood taurine concentration in the four captive lions in zoos. Data are shown by box and whisker plots as the 10th, 25th, 50th, 75th, and 90th percentiles. *P<0.05, **P<0.01, and ***P<0.001, respectively, denote significant difference with Lion 1 by the Kruskal–Wallis multiple comparison post hoc test

3.3 Bile Acid Composition (Study 2)

The types of BAs in lion blood were cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), but other types such as muricholic acid and hyodeoxycholic acid were not detected (Fig. 4a). The amount of CA was the highest BA (70–80%) in all lions tested. The second most common BA was DCA, which is metabolized from CA in three lions except for Lion 2, in which CDCA was the second highest. The CDCA ratio varied from 4% to 14% among the four lions. The content of LCA and UDCA in all lions was less than 5% and 1%, respectively. No correlation was found between blood taurine concentration and BA composition.

In the composition of the unconjugated and conjugated forms of BAs in blood, the taurine-conjugated forms were around 50% for Lions 1 and 3, but they were more than 70% for Lions 2 and 4 (Fig. 4b). The ratio of the glycine conjugated form was around 1% in all lions. No correlation was found between the taurine concentration and the ratio of the taurine-conjugated form of BAs.

4 Discussion

Taurine is an essential nutrient in felines because of their low ability of synthesize taurine. Although the dietary taurine content has been established for cats maintained on whole prey-based diets for several species of captive felines (Bechert et al. 2002), it remains unknown whether the dietary amount of taurine from prey feed is sufficient to meet the demands of captive lions housed in zoos. The present study repeatedly collected blood samples from the tails of four lions at three zoos fed prey feed including whole animals, meats, and internal organs and evaluated relationships between blood taurine concentration and both taurine ingestion and the compositions of BAs in blood.

Blood taurine concentration was assayed in the 7–26 samples collected repeatedly from four

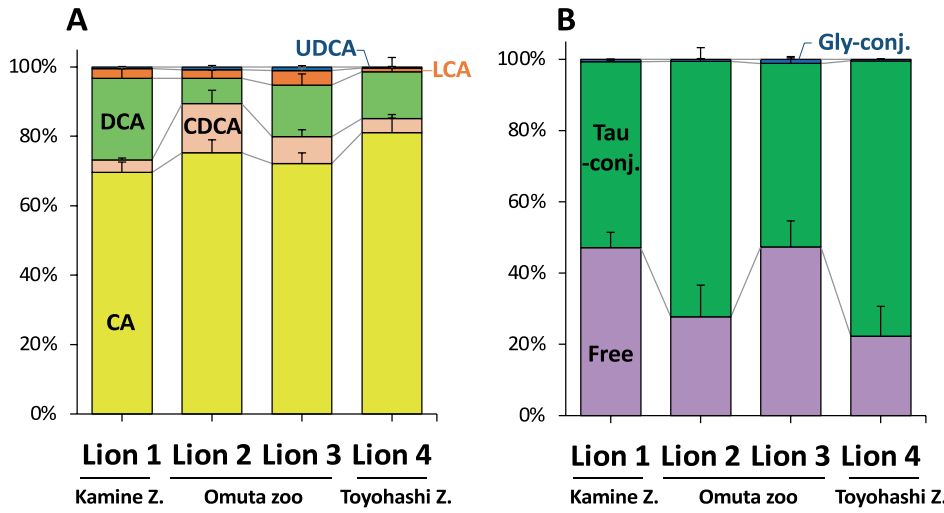


Fig. 4 Compositions of types and conjugated forms of bile acids in blood of lions. (**a**) Composition of bile acid types. Each type of bile acid is the sum of conjugated and unconjugated forms. (**b**) Composition of the taurine-conjugated, glycine-conjugated, and glycine-unconjugated forms of bile acids. Each conjugated and unconjugated form is the sum of all types of bile acids. Data of the compositions are diagrammatized by the mean ± SEM

lions during periods from 7 months to 3 years and 4 months. Results show that the average value of blood taurine concentration of four lions was 183.6 ± 13.8 μM, with individual averages varying from 83.8 to 255 μM. Values at Omuta Zoo were higher than those at the other two zoos. Compared with taurine-deficient felines (Miyazaki et al. 2020; Pickett et al. 1990), values of blood taurine concentration found in this study imply that the four lions examined at the zoos were fed sufficient amounts of taurine in their prey feed.

Many earlier studies have described healthy values of blood taurine concentration for several species of felines, although varying among the reports and species; average values for domestic cats and Bengal tigers varied from 50 to 250 μM and from 120 to 290 μM, respectively (Knopf et al. 1978; Hayes et al. 1975; Miyazaki et al. 2020; Novotny et al. 1994; Pickett et al. 1990; Schmidt et al. 1976). Although the wide variations are attributable to various factors of handling, environments, and conditions, blood taurine concentrations in cats are regarded as depending mostly on dietary taurine content (Sturman 1993). A correlation was found between blood taurine concentration and taurine content in the prey feed for Lions 2 and 3 housed at Omuta Zoo, but not for Lion 1 at Kamine Zoo. The difference was likely to be related to the feeding protocol and the food type and volume. For Lion 1, beef and chicken heads were fed mainly with two patterns per week, consisting of full and half amounts. The reason for a lack of relationship between blood taurine and feed is probably influenced by the non-uniform feeding amounts on the day before blood sample collection. However, the main food was whole chicken with various types of feed for Lions 2 and 3. In particular, the amount of whole chicken feed was higher for Lion 2 than for Lion 3. Therefore, whole chicken amount might be responsible for changes in blood taurine concentration. Taurine concentration was higher for the lions housed at Omuta Zoo than for Lion 1 but was nonetheless lower than the estimated total amount of taurine ingestion. The actual taurine content of whole chicken was higher than the estimated value of 625 mg of taurine calculated for the average value per unit of weight in other prey feeds.

In feed supplied at the two zoos, the highest content of taurine in the feed was 850 mg/kg (over 0.08%) for chicken heads, but the smallest was 425 mg/kg (over 0.04%) for beef. Estimated

total amounts of taurine ingestion were 55.8–85.7 g/month (2–3 g/day) in Lions 1–3 (Table 1). The values are less than 0.1–0.2% of the current recommendations for taurine content in commercial diet supplies for cats (Hedberg et al. 2007) but are higher than the minimum requirements for growing kittens (National Research Council 1986). We have previously reported that a 0.15% taurine-containing diet of 50 mg/kg/day body weight maintained domestic cats in a healthy state over a period of 30 weeks (Miyazaki et al. 2020). Although the body weight of the lions examined in the present study was unclear, the daily taurine ingestion per kg body weight was estimated as 15–20 mg/kg/day, assuming a body weight of at least 150–200 kg. Because the lions were randomly fed with additional feed, the dietary content of taurine for lions examined in the present study was not likely less the recommendation for cats. Therefore, the taurine content in feed provided at the examined zoos fulfilled the demands of the lions, although the possibility exists that dietary requirements for taurine differ between cats and lions.

The most common blood BA of lions examined in this study was CA, which represented 70–80% of total BA content. The taurine-conjugated form was 50–70%, with the rest being unconjugated. The ratios of CA and unconjugated form increased to 90% and 95% in taurine-deficient cats (Miyazaki et al. 2020). Individual differences were found in the types and conjugation rates of BAs among the four lions. No significant correlation was found between blood taurine concentration and the ratios of different types and conjugation status of blood. Further study is warranted to clarify this point.

5 Conclusion

This study, which used blood samples collected repeatedly from lion tails with no anesthesia treatment, evaluated the relationship between blood taurine concentration and feed, as well as the compositions of BAs in the blood of four lions housed at three zoos. According to earlier studies of felines, blood taurine concentration was in the normal range in the four lions housed in zoos, although variations were found among and within individuals. Prey food used in zoos contained an adequate amount of taurine to meet the demands for lions. Because blood taurine was in the normal range, no abnormality was observed in either the blood composition of various BA types or their conjugation status. However, individual variations in BA composition were found among lions. Further investigations must be conducted to clarify the relationship between blood BAs and the health status of lions housed in zoos.

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Part V

Role of Taurine in Diabetes and Obesity

Taurine Ameliorates Apoptosis via AKT Pathway in the Kidney of Diabetic Rats

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Keywords

Taurine · Diabetic nephropathy · Apoptosis · Akt pathway

Abbreviations

caspase-3	Cysteinyl aspartate-specific proteinase-3
DM	Diabetes mellitus
p-Akt	Phosphorylated Akt
STZ	Streptozotocin
tau	Taurine

1 Introduction

Diabetes mellitus (DM) is getting more and more attention in public. According to the report from the World Health Organization, until 2018, there are almost 430 million people who suffered from DM (Alam et al. 2014). Unfortunately, the quantity of DM patients is still growing. In 2040, one out of ten adults is expected to have DM worldwide (Everaert et al. 2021). Being a complicated chronic metabolism disorder, DM is bound up with a variety of complications (Ahmad and Haque 2021; Chatterjee et al. 2017). One of the common complications is diabetic nephropathy. The results of diabetes epidemic showed that about one out of four patients with diabetic nephropathy will progress into end-stage renal disease and lead to an elevated mortality in diabetic patients (Lim 2014). In total, about 30% of patients with type 1 diabetes and 40% of those with type 2 diabetes further develop diabetic nephropathy (Feng et al. 2021). It was indicated that the over-activated oxidative stress and the following apoptosis are the major pathological factors, which resulted in the development of diabetic nephropathy in type 2 diabetes (Gao et al. 2021; He et al. 2021; Rani et al. 2016). Apoptosis may contribute toward the occurrence and development of diabetic nephropathy. Therefore, inhibiting apoptosis was contributed to ameliorate the kidney deficits in diabetes patients.

Taurine, an endogenous amino acid, is a conditionally essential amino acid and exists widely in animal tissues. Taurine plays a diversity of roles in tissues, including antioxidant, neuromodulator, anti-inflammation, regulating the osmotic pressure, and the formation of bile acids (De la

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Puerta et al. 2010; Huxtable 1992). In experimental animal models, it was shown that the protection role of taurine depends on its anti-apoptotic ability in several organs (Baliou et al. 2021; Das et al. 2011; Rashid et al. 2013). A similar protection against apoptosis was also observed in cultured cell model, including glial cells, renal cells, and vascular endothelial cells. Moreover, our group found that taurine inhibited apoptosis in arsenic-exposed mice (Li et al. 2017). We also found that taurine attenuated apoptosis in high glucose-exposed HT-22 cells and hippocampus of diabetic rats (Wu et al. 2020). Therefore, it was speculated that taurine could protect against diabetic nephropathy via blocking apoptosis in the kidney.

In the current study, the STZ-treated T2D in rats was treated with taurine. mRNA expression of cysteinyl aspartate-specific proteinase-3 (caspase-3) and caspase-9 and the activity of caspase-3 were detected as the markers of apoptosis. The expression of Bax, Bcl-2, total Akt, and p-Akt was detected by Western blot. The aim of the study was to explore the protection of taurine against apoptosis in the kidney of diabetic rats and provide some clues for the treatment of diabetic nephropathy.

2 Methods

2.1 Establishment of Animal Model

Male Sprague–Dawley (SD) rats were randomly divided into three groups. Two groups were given high-fat and high-sugar food (1.5% cholesterol, 15% lard, 25% sucrose, 1% sodium cholate, and 57.5% common feed). Four weeks later, the rats were intraperitoneally injected with streptozotocin (STZ) at a dose of 25 mg/Kg of body weight. After 72 h, blood glucose was checked. If the concentration was above 16.7 mmol/L, the diabetes model was considered (Wu et al. 2020). Half of the diabetic rats were given normal water as DM group, and the other rats were treated with 2% taurine solution for 8 weeks as taurine treatment (DM + Tau) group. Control group was fed with normal food and water for the whole period. At the end of the experiments, all rats were sacrificed, and kidneys were harvested for future studies. The animal experiments were approved by the Ethical Committee of Dalian Medical University, China, and performed in accordance with the Animal Handling Guideline of Dalian Medical University.

2.2 Real-Time PCR

Total RNA was extracted from the kidneys using RNAiso Plus (Takara, Tokyo, Japan). RNA was reverse-transcribed using a Reverse Transcription Kit (Takara, Tokyo, Japan). Real-time q-PCR was performed using a SYBR Green PCR kit (Takara, Tokyo, Japan) using the TP800 Real-Time PCR Detection System (Takara, Tokyo, Japan). The following primers were used: caspase-3 (220bp), 5′-TGT CAT CTC GCT CTG GTA CG-3′/5′-AAA TGA CCC CTT CAT CAC CA-3′; caspase-9 (246bp), 5′-AAG ACC ATG GCT TTG AGG TG-3′/5′-CAG GAA CCG CTC TTC TTG TC-3′; and GAPDH, 5′-GGC ACA GTC AAG GCT GAG AAT G-3′/5′-ATG GTG GTG AAG ACG CCA GTA-3′. The PCR programs were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 40 cycles. Data were normalized to GAPDH.

2.3 Determination of Caspase-3 Activity

The caspase-3 activity detection kit (Beyotime, China) was used to assess the activity of caspase-3. In brief, kidneys were homogenized with lysis buffer and then mixed with Ac-DEVD-pNA substrate and reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 10% glycerol). The protein concentration was adjusted to 1–3 mg/ml and measured the absorption at 405 nm with an ELISA reader.

2.4 Western Blot Analysis

Kidney tissues were homogenized with ice-cold RIPA buffer (Beyotime, China) supplemented with 1% proteinase inhibitor and phosphatase inhibitor. The proteins were separated on SDS-PAGE and then transferred to PVDF membrane (Millipore, France). The membrane was incubated with the following antibodies: Akt (1:1000, Proteintech, USA), p-Akt (1:1000, Proteintech, USA), Bax (1:500, Beyotime, China), Bcl-2 (1:500, Beyotime, China), and β-actin (1:500, ZS-Bio, China) for 24 h at 4 °C. Then, the membranes were washed and incubated with HRP-conjugated secondary antibodies (Beyotime, China) at room temperature for 2 h. The immuno-labeling was detected with enhanced ECL reagents (Beyotime, China) and quantified with UVP BioSpectrum multispectral imaging system (UltraViolet Products, USA) and Image J software.

2.5 Statistical Analysis

All results were expressed as mean ± S.D. The statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by LSD test, and performed using SPSS 13.0 statistical software. The p-values less than 0.05 were considered to be significant.

3 Results

3.1 Taurine Treatment Downregulated Caspase-3 and Caspase-9 Gene Expression in the Kidney of DM Rats

The gene expression of caspase-3 and caspase-9 in the kidney of DM rats with or without taurine treatment was examined by real-time PCR. The results showed that caspase-3 gene expression was significantly increased in DM rats; however, the increase was remarkably attenuated with taurine treatment (Fig. 1a). We also found that high-fat and high-sugar food caused a significant upregulation in caspase-9 gene expression. As the expectation, taurine treatment obviously reduced the increase (Fig. 1b). These results implied that taurine inhibited the increase in the gene expression of caspase-3 and caspase-9 which induce apoptosis.

3.2 Taurine Treatment Elevated Caspase-3 Activity in the Kidney of DM Rats

The activity of caspase-3 in the kidney of DM rats with or without taurine treatment was examined. The results showed that caspase-3 activity was significantly increased in DM rats; however, the increase was remarkably attenuated with taurine treatment (Fig. 2), being accordant with the results of caspase-3 gene expression. The results further supported that taurine could inhibit high-fat and high-sugar food-induced apoptosis in the kidney of rats.

3.3 Taurine Treatment Reversed the Bcl-2 and Bax Protein Level in the Kidney of DM Rats

The protein expression of Bcl-2 and Bax in the kidney of DM rats with or without taurine treatment was examined by Western blot. As shown in Fig. 3a, compared with control group, the Bcl-2 level in DM group was evidently decreased, and the reduction was significantly attenuated by taurine treatment. On the contrary, the Bax level in DM group was remarkably increased, and the elevation was significantly inhibited by taurine treatment (Fig. 3b).

3.4 Taurine Treatment Elevated the p-Akt Level in the Kidney of DM Rats

The protein expression of Akt and p-Akt in the kidney of DM rats with or without taurine treatment was examined by Western blot. As shown in

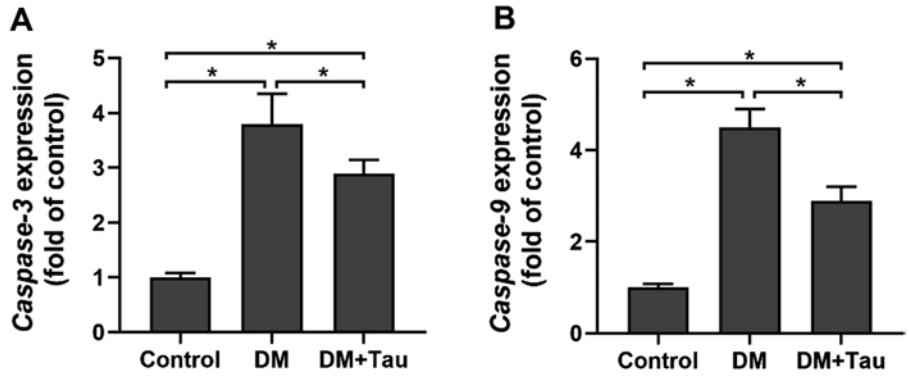

Fig. 1 Effect of taurine treatment on caspase-3 and caspase-9 gene expression in the kidney of DM rats. (**a**) Effect of taurine treatment on caspase-3 gene expression. (**b**) Effect of taurine treatment on caspase-9 gene expression. Values shown represent means + SEM. The asterisk denotes a significant difference between two groups ($p < 0.05$)

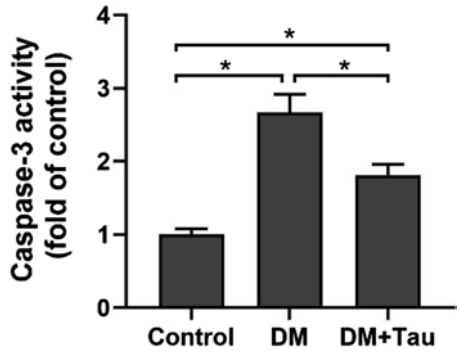

Fig. 2 Effect of taurine treatment on caspase-3 activity in the kidney of DM rats. Values shown represent means + SEM. The asterisk denotes a significant difference between two groups ($p < 0.05$)

Fig. 4a, b, neither high-fat and high-sugar food nor taurine treatment affected the Akt protein level, and there is no difference among the three groups. However, compared with the control group, the p-Akt level in DM group was evidently decreased, and the reduction was significantly attenuated by taurine treatment (Fig. 4a, c).

4 Discussion

Our group previously reported that taurine treatment attenuated neuronal apoptosis in diabetes rat model via NGF pathway (Wu et al. 2020). In the present study, the protective effect of taurine against apoptosis in the kidney of diabetic rats was explored. The results showed that caspase-3 and caspase-9 mRNA expression was decreased in diabetic kidney, which was recovered by taurine treatment. The activity of caspase-3 also increased in diabetic kidney, while the increased activity was significantly attenuated after taurine treatment. We also found that the expressions of Bax and Bcl-2 were disturbed in diabetic kidney, which were reversed by taurine treatment. The decrease of the p-Akt level was also prevented by taurine treatment. These results indicated that taurine-ameliorated apoptosis in diabetic kidney may be through activation of the Akt signaling pathway.

Diabetes, being a metabolic disorder, leads to several complications in organs. Diabetes-induced diabetic nephropathy is the most common pathological damage, characterizing by proteinuria and persistent decline in renal function, and eventually results in the increase of mortality in diabetes patients (McGrath and Edi 2019; Gao et al. 2019). As many other diseases, the development of diabetic nephropathy was involved in the over-activated apoptosis (Liu et al. 2021; Ying et al. 2021). Apoptosis is a highly conserved process with cellular volume reduction, chromatin condensation, and apoptotic body formation (Nowak and Edelstein 2020; Turkmen 2017). The effect of taurine against apoptosis was reported by many studies, includ-

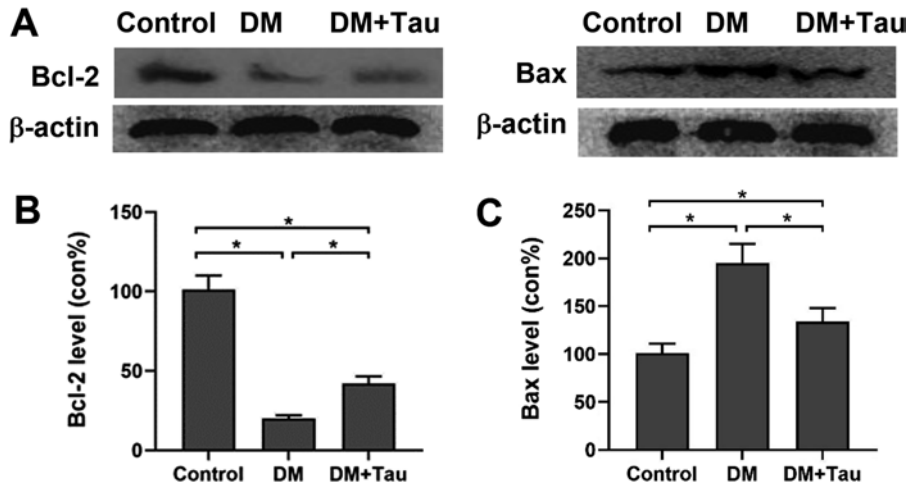

Fig. 3 Effect of taurine treatment on Bacl-2 and Bax level in the kidney of DM rats. (**a**) The expression of Bacl-2 and Bax protein was examined by Western blot. (**b**) Quantification of Bcl-2 protein level. (**c**) Quantification of Bax protein level. Values shown represent means + SEM. The asterisk denotes a significant difference between two groups ($p < 0.05$)

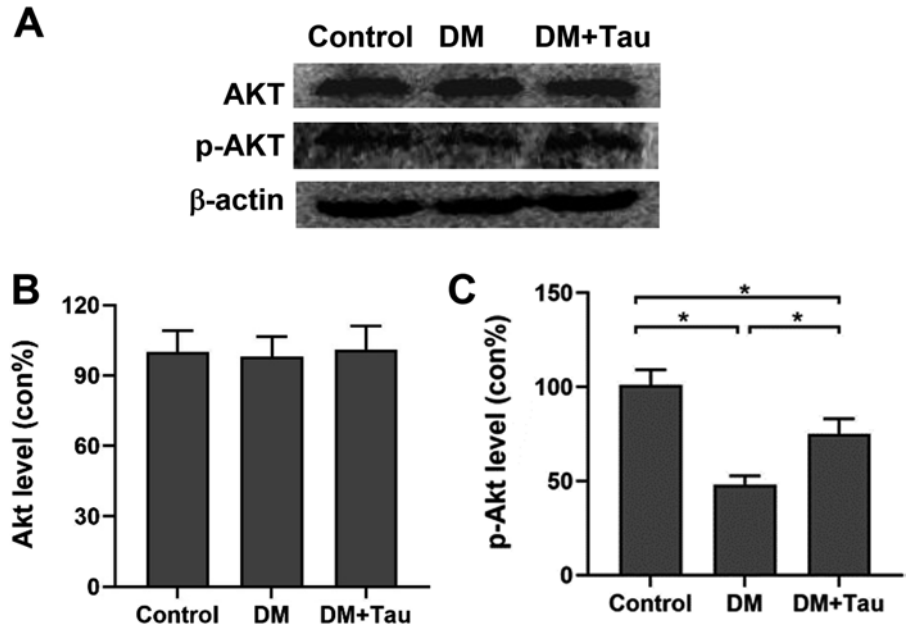

Fig. 4 Effect of taurine treatment on Akt and p-Akt level in the kidney of DM rats. (**a**) The expression of Akt and p-Akt protein was examined by Western blot. (**b**) Quantification of Akt protein level. (**c**) Quantification of p-Akt protein level. Values shown represent means + SEM. The asterisk denotes a significant difference between two groups ($p < 0.05$)

ing in cardiomyocytes (Li et al. 2020), neurons (Wu et al. 2020), liver cells (Wu et al. 2018), and also kidneys (Stacchiotti et al. 2018). Caspases are the main regulators of apoptosis. Our results showed that taurine treatment not only inhibited the increase of caspase-3 and caspase-9 mRNA

expression but also decreased the activity of caspase-3 kidney of diabetic rat, indicating the anti-apoptotic effect of taurine in diabetic nephropathy.

Bcl-2 family is the major regulator for apoptosis, including anti-apoptotic Bcl-2 and pro-apoptotic Bax. Under pathology conditions, Bax would transfer to mitochondria from cytosol and induce mitochondrial cytochrome C release, which in turn trigger the activation of caspases (Xiong et al. 2014). Our results showed that the level of Bcl-2 was significantly decreased and the level of Bax markedly increased in diabetic rat, while the disturbance of Bcl-2 and Bax both reversed by taurine treatment. Akt is a major member of protein kinases, and the phosphorylation modification takes part in the prevention of apoptosis (Wu et al. 2020). It was reported that activated Akt induced phosphorylation of downstream protein and then blocked cellular apoptosis. In the present study, the results showed taurine treatment attenuated the decrease of the p-Akt level in diabetes kidney, indicating the activation of Akt pathway may involve in the anti-apoptotic effect of taurine in diabetic nephropathy.

5 Conclusion

In conclusion, the present study reported the effects of taurine on the diabetic nephropathy. Our results revealed that taurine alleviated apoptosis by regulating the Bcl-2 and Bax level, which was involved in Akt pathway.

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Taurine Ameliorates Oxidative Stress in Spinal Cords of Diabetic Rats via Keap1-Nrf2 Signaling

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Keywords

Taurine · Diabetes · Oxidative stress · Keap1-Nrf2 signaling · Diabetic neuropathy

Abbreviations

DM	Diabetes mellitus
MDA	Malondialdehyde
SOD	Superoxide dismutase
Tau	taurine

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1 Introduction

Diabetes mellitus (DM), a chronic metabolic disorder, is getting worldwide attention. The World Health Organization reported that as of 2018, there are nearly 430 million DM patients suffering from the disease (Alam et al. 2014). In 2040, one out of ten adults is expected to have DM (Everaert et al. 2021). According to clinical research, DM can lead to serious complications, including retinopathy, nephropathy, neuropathy, and autonomic dysfunction (Ahmad and Haque 2021; Chatterjee et al. 2017). One of the most common complications is diabetic neuropathy, whose prevalence among diabetic patients is greater than 50% (Agca et al. 2014). It is reported that the onset and development of diabetic neuropathy are associated with oxidative stress (Hosseini and Abdollahi 2013; Sirdah 2015). Hyperglycemia causes the production of free radicals and a parallel decrease in the antioxidant defense system. Importantly, the nervous system, which requires a large amount of oxygen, is especially susceptible to the damage of oxidative stress (Muriach et al. 2014).

Taurine, a conditionally essential amino acid, exists widely in animal and human tissues. Taurine has various effects and protective actions in tissues, such as antioxidant, anti-inflammation, neuromodulator, regulating osmotic pressure, and the conjugation of bile acids (De la Puenta et al. 2010; Huxtable 1992). Several studies

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showed that the plasma concentration of taurine in DM patients was lower than that of healthy individuals (Franconi et al. 2006). When taurine was administered, the taurine concentration in the nervous system increased and attenuated the severity of DM (Dawson et al. 1999; Zhang et al. 2021). The anti-oxidative stress effect of taurine has been reported by our group and other laboratories (Abdoli et al. 2021; Castelli et al. 2021; Li et al. 2013, 2017). In the present study, the STZ-treated T2D in rats was treated with taurine, and blood glucose was recorded. The expressions of Gap-43 and MBP proteins were detected by Western blot. SOD activity and MDA content were examined as indicators of oxidative stress. The expression of Keap1, Nrf2, and HO-1 gene was detected by real-time PCR. The aim of the study was to explore the protection of taurine against oxidative stress in spinal cords of diabetic rats and provide some clues on the mechanism of action.

2 Methods

2.1 Animal Model

Male 180–200 g SD rats were purchased from Experimental Animal Center of Dalian Medical University. After 2 weeks of acclimation, the rats were randomly divided into three groups (n = 12). The control group was given normal food and water for the entire study period and injected with citrate buffer (STZ control) at the fourth week. The DM group was given a high glucose special diet, 25% sucrose+15% oil +1.5% cholesterol +1% bile acid +57.55% normal diet, for four weeks and later administered with a single dose of streptozotocin (STZ) at 25 mg/kg of body weight. After 72 h, blood glucose was checked. If the concentration was above 16.7 mmol/L, the DM model was considered and given normal diet for another 4 weeks (Wu et al. 2020). Considering the DM + Tau group, the DM model rats were given a normal diet and 2% taurine drinking solution for 4 weeks. At the end of the experiments, animals were euthanized, and organs were preserved for later studies. The research was conducted according to the Animal Guidelines and Agreement with Ethical Committee of Dalian Medical University (Permit number: SCXK (Liao) 2015–2003).

2.2 Weight Gain

Diabetes has a characteristic symptom of sharp weight reduction. Therefore, in order to find the effect of taurine administration on weight gain in diabetic rats, their weight was recorded for the whole treatment period.

2.3 Blood Glucose

The primary characteristic of the diabetic situation is increased or elevated blood glucose for relatively a longer period of time. Fasting blood glucose was checked every 2 weeks by an automatic biochemical analyzer (GA-3 type, SANNUO, China).

2.4 Western Blot

Spinal cords were homogenized with ice-cold RIPA buffer (Beyotime, China) supplemented with 1% proteinase inhibitor and phosphatase inhibitor. The proteins were separated on SDS-PAGE and then transferred to PVDF membrane (Millipore, France). The membrane was incubated with the Gap-43 antibody (1:1000, Sigma, USA), MBP antibody (1:1000, Abcam, USA), or β-actin (1:500, ZS-Bio, China) for 24 h at 4 °C. Then, the membranes were washed and incubated with HRP-conjugated secondary antibodies (Beyotime, China) at room temperature for 2 h. The immuno-labeling was detected with enhanced ECL reagents (Beyotime, China) and quantified with UVP BioSpectrum multispectral imaging system (UltraViolet Products, USA) and Image J software.

2.5 MDA Concentrations

Malondialdehyde (MDA) concentration was assessed with "MDA Assay Kit," a commercial kit

from Nanjing Jiancheng Bioengineering Institute (China), which examined MDA reaction with thiobarbituric acid (TBA) by the end-point assay method. The absorbance was measured at 520 nm. The results were reported as nM MDA/mg tissue.

2.6 SOD Activity

The activity of SOD was assessed with "SOD Assay Kit," a commercial kit from Nanjing Jiancheng Bioengineering Institute (China). Briefly, tissues were homogenized and centrifuged for 15 min. Then, the supernatant reacted with methionine, riboflavin, and nitro-blue-tetrazolium (NBT), and the absorbance was measured at 440 nm. The results were expressed as units (U) SOD/mg tissue.

2.7 Real-Time PCR

Total RNA was extracted from the kidneys using RNAiso Plus (Takara, Tokyo, Japan). RNA was reverse-transcribed using a Reverse Transcription Kit (Takara, Tokyo, Japan). Real-time q-PCR was performed using a SYBR Green PCR kit (Takara, Tokyo, Japan) using the TP800 Real-Time PCR Detection System (Takara, Tokyo, Japan). The following primers were used: Keap1, 5′-ATG TGA TGA ACG GGG CAG TC-3′/5′-AGA ACT CCT CCT CCC CGA AG-3′; Nrf2, 5′-GAT GAC CAT GAG TCG CTT GC-3′/5′-CTG CCA AAC TTG CTC CAT GT-3′; HO-1, 5′-AAC CCA GTC TAT GCC CCA CT-3′/5′-TGA GTA CCT CCC ACC TCG TG-3′; and GAPDH, 5′-GGC ACA GTC AAG GCT GAG AAT G-3′/5′-ATG GTG GTG AAG ACG CCA GTA-3′. The PCR programs were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 40 cycles. Data were normalized to GAPDH.

2.8 Statistical Analysis

All results were expressed as means ± SD. The statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by LSD test, and performed using SPSS 13.0 statistical software. The p-values less than 0.05 were considered to be significant.

3 Results

3.1 Taurine Treatment Improved Weight Gain and Lowered Blood Glucose in Diabetic Rats

As shown in Table 1, the bodyweight of diabetic rats was sharply decreased when compared with the control group. After the 8-week treatment with taurine, the weight markedly recovered and was higher than those of the DM control rats, indicating taurine improved weight gain among diabetic rats. The primary symptom of diabetes is hyperglycemia or high blood glucose. Our results showed that the blood glucose of the control group was relatively constant over the whole period. But diabetic rats showed a significant increase in the blood glucose, which was remarkably reversed by taurine supplementation (Table 1).

3.2 Taurine Treatment Increased Gap-43 and MBP Levels in Spinal Cords of Diabetic Rats

Gap-43, an axon outgrowth relative protein implicated in neurite outgrowth and motility, was examined by Western blot analysis. As shown in Fig. 1a and b, the level of Gap-43 was significantly decreased in spinal cords of DM rats ($p < 0.05$); however, the decrease was significantly reversed after taurine treatment ($p < 0.05$), indicating that taurine protected axons in the spinal cord of DM rats. MBP, the marker of myelin, was also detected by Western blot analysis. Compared with the control group, MBP levels decreased in the DM group (($p < 0.05$, Fig. 1a, c). However, taurine supplementation led to a marked increase in MBP expression ($p < 0.05$), indicating that taurine protects myelinated axons of DM rats.

Table 1 Effect of taurine on body weight and blood glucose of diabetic rats

	Body weight (g)		Blood glucose (mmol/1)	
Group	0 w	8 W	0 w	8 W
Con	465.87 ± 8.331	578.910 ± 9.432	4.890 ± 0.078	4.920 ± 0.103
DM	371.883 ± 10.977[a]	348.610 ± 7.494[a]	22.100 ± 0.238[a]	24.210 ± 0.300[a]
DM + Tau	370.892 ± 6.522	407.140 ± 4.578[b]	22.040 ± 0.359	20.570 ± 0.319[b]

[a]$p < 0.05$, vs control group
[b]$p < 0.05$, vs DM group

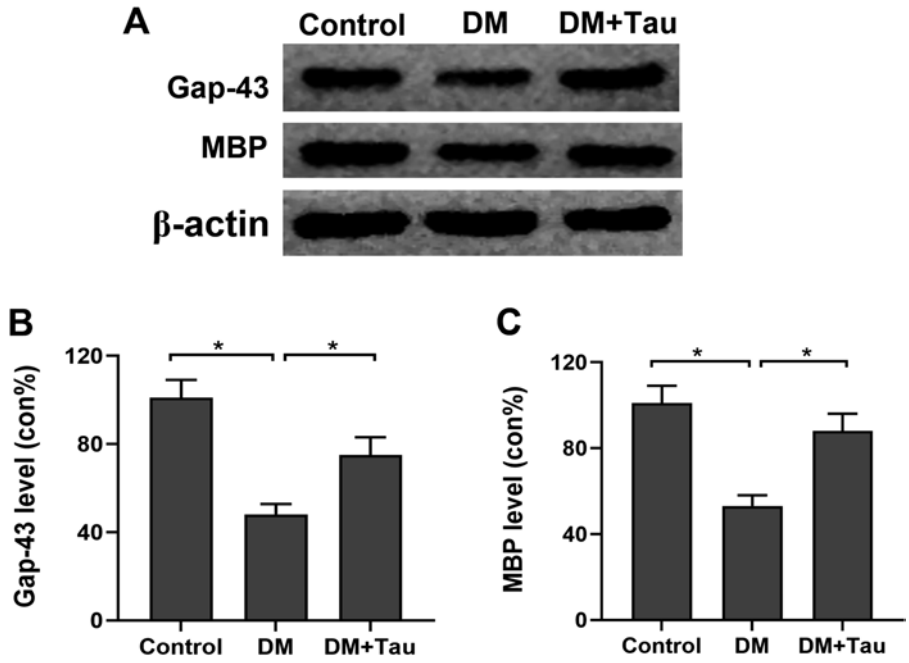

Fig. 1 Effect of taurine treatment on the Gap-43 and MBP level in spinal cords of DM rats. Values shown represent means ± SEM. The asterisk denotes a significant difference between the two groups ($p < 0.05$)

3.3 Taurine Treatment Inhibited Oxidative Stress in Spinal Cords of Diabetic Rats

SOD activity and MDA concentration were detected as indicators of oxidative stress. As shown in Fig. 2a, compared with the control group, the activity of SOD significantly increased in the DM group ($p < 0.05$). However, taurine supplementation led to a markedly decrease in enzyme activity ($p < 0.05$). As expected, in the spinal cords of DM rats, the concentration of MDA was markedly lower than that of the control group, while taurine treatment effectively counteracts these changes ($p < 0.05$, Fig. 2b), indicating taurine treatment significantly inhibited oxidative stress in spinal cords of diabetic rats.

3.4 Taurine Treatment Activated Keap1-Nrf2 Signaling in Spinal Cords of Diabetic Rats

As shown in Fig. 3a, the expression of the Keap1 gene was significantly increased in spinal cords of DM rats; however, the increase was markedly attenuated after taurine treatment ($p < 0.05$). Compared with the control group, the expression of the Nrf2 gene was significantly decreased in the DM group, and the decrease was reversed after taurine treatment ($p < 0.05$). Considering the expression of the HO-1 gene, the gene level was markedly lower than that of the control group, while taurine treatment effectively counteracts these changes

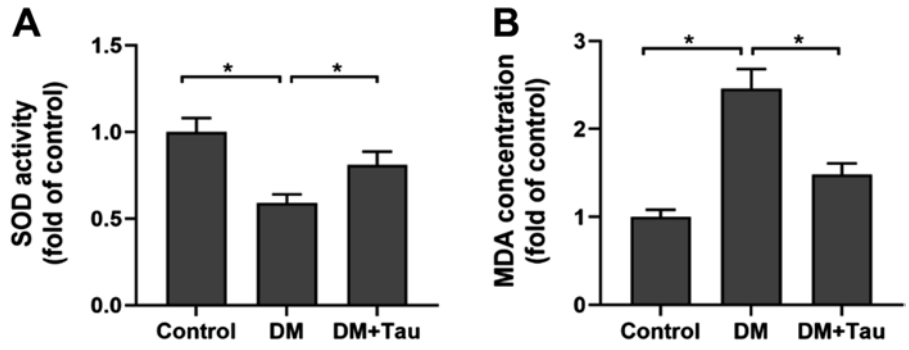

Fig. 2 Effect of taurine treatment on SOD activity and MDA concentration in spinal cords of DM rats. (**a**) SOD activity. (**b**) MDA concentration. Values shown represent means + SEM. The asterisk denotes a significant difference between the two groups ($p < 0.05$)

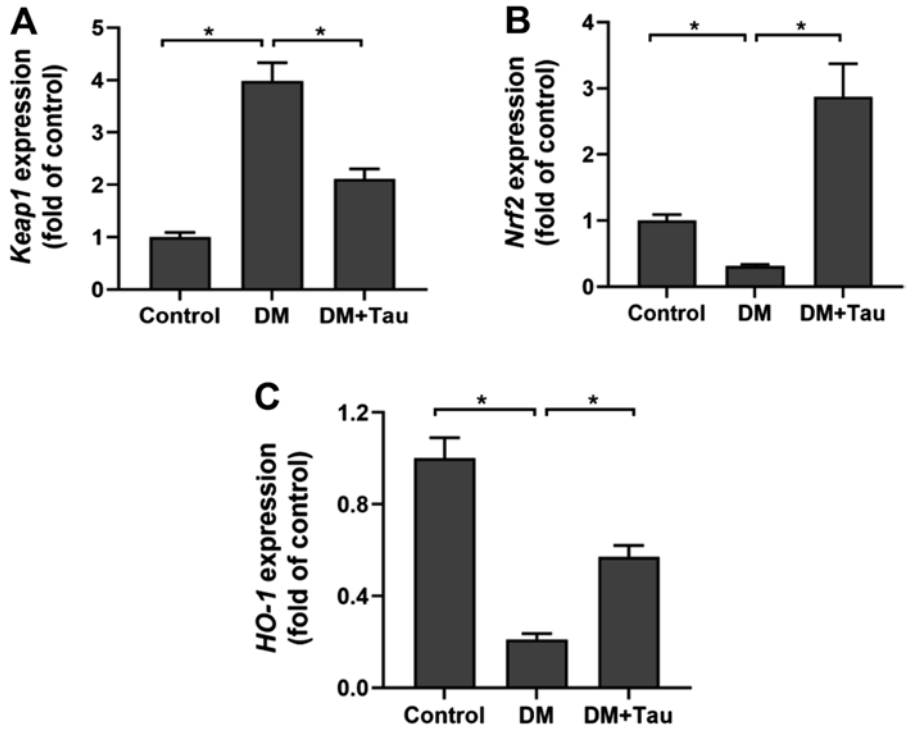

Fig. 3 Effect of taurine treatment on Keap1-Nrf2 signal in spinal cords of DM rats. (**a**) Effect of taurine treatment on Keap1 gene expression. (**b**) Effect of taurine treatment on Nrf2 gene expression. (**c**) Effect of taurine treatment on HO-1 gene expression. Values shown represent means + SEM. The asterisk denotes a significant difference between the two groups ($p < 0.05$)

($p < 0.05$, Fig. 3c), indicating that taurine treatment activates Keap1-Nrf2 signaling in spinal cords of diabetic rats.

4 Discussion

Our group previously reported that taurine treatment attenuates oxidative stress in PC12 cells and C6 cells (Li et al. 2013, 2017). In the present study, the protective effect of taurine against oxidative stress in spinal cords of diabetic rats was explored. The results showed that taurine treatment partially restored body weight and blood glucose in diabetic rats. The expressions of Gap-43 and MBP proteins were decreased in diabetic rats, but this effect was significantly attenuated after taurine treatment. We also found that SOD activity and MDA concentration were disturbed in diabetic spinal cords, which were reversed by taurine supplementation. Moreover, the increase in Keap1 gene expression and the decreases in Nrf2 and HO-1 gene expression mediated by diabetes were remarkably attenuated by taurine treatment. These results indicate that taurine may inhibit oxidative stress in diabetic spinal cords via regulation of the Keap1-Nrf2 signaling pathway.

DM, a kind of metabolic disease, was characterized by higher blood glucose. In this experiment, we found higher blood glucose levels in STZ-induced diabetic rats as compared to the control group, suggesting DM was induced. However, the higher blood glucose was significantly reduced after taurine treatment. Several groups had reported that taurine ameliorates hyperglycemia in DM rats, which is in accordance with our results. These studies indicate that taurine can play an important role in improving hyperglycemia in DM. It was reported that taurine supplementation reduces food intake and increases insulin secretion and action in DM (Carneiro et al. 2009; Kim et al. 2012; Solon et al. 2012; Sarkar et al. 2017). Some studies showed that administration of taurine improves insulin sensitivity, stimulates insulin secretion, and reduces inflammation in DM (Sarkar et al. 2017). The beneficial effects of taurine may be responsible for its hypoglycemic action.

Several groups have reported that exogenous taurine can prevent oxidative stress in diabetic rats (Hosseini and Abdollahi 2013; Sirdah 2015). In this experiment, the indicators of oxidative stress, SOD and MDA, in spinal cords of DM rats with or without taurine treatment were measured. As shown in the results, the activity of SOD in spinal cords of DM rats was significantly decreased, and MDA concentration was significantly increased, which has been attributed to enhanced generation of reactive oxygen species in the mitochondria of diabetic rats oxidizing both glucose and lipids. After 4 weeks of taurine treatment, taurine significantly increased the activity of SOD and reduced the level of MDA, suggesting taurine, as an antioxidant, can significantly reduce oxidative damage in spinal cords of DM rats and lessen the damage of target tissues.

Keap1-Nrf2 signaling is one of the most important antioxidant pathways (Abed et al. 2021; Gao et al. 2021; Xing et al. 2021). Nrf2 is a key transcription factor in the cellular antioxidant stress system. Under oxidative stress, Nrf2 dissociates from Keap1 and translocates from the cytoplasm to the nucleus, then aggregates into heterodimers with MAF protein, and activates the Nrf2 signaling pathway. The Nrf2 pathway triggers the expression of downstream phase II detoxification enzymes (SOD, MDA, GSH, etc.) and the antioxidant enzyme systems (HO-1, Nrf2, NQO1, etc.) and then reduces cell damage caused by reactive oxygen species and electrophilic substances (Abed et al. 2021; Gao et al. 2021; Xing et al. 2021). Keap1 is a negative regulator of Nrf2. Keap1 negatively regulates the function of the downstream antioxidant enzyme Nrf2 and activates the expression of Nrf2. Our results show that after taurine treatment, Nrf2 mRNA in spinal cords was significantly upregulated and Keap1 was significantly downregulated, indicating that the Keap1-Nrf2 signaling is activated in taurine-treated spinal cords of DM rats.

On the other hand, some studies have shown that the mitochondria are the main source of superoxide production, primarily via complex I and complex III of the electron transport chain (Jong et al. 2021). Brownlee's report attributed the increase in the oxidative stress in DM to

superoxide production by the mitochondria (Brownlee 2001). The study by Jong et al. showed that taurine enters mitochondria and increases the biosynthesis of the mitochondrial coding proteins ND5 and ND6, thus enhancing the activities of complexes I and III (Jong et al. 2012). It is revealed that taurine prevents the diversion of electrons into superoxide generation by improving the function of the electron transport chain, indicating the antioxidant effects of taurine in DM may also involve its actions on mitochondria.

5 Conclusion

Above all, DM rats are accompanied by weight loss, hyperglycemia, and oxidative stress, as well as enhanced Keap1 gene expression and suppressed Nrf2 and HO-1 gene expression. Taurine treatment partially restores weight gain, hyperglycemia, and disturbed anti-oxidative defense. Taurine also reduces Keap1 gene expression and increases Nrf2 and HO-1 gene expression and appears to attenuate the severity of oxidative stress via activating Keap1-Nrf2 signaling in spinal cords of DM rats.

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Assessment of In Vitro Tests as Predictors of the Antioxidant Effects of Insulin, Metformin, and Taurine in the Brain of Diabetic Rats

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Keywords

Insulin · Metformin · Taurine · Metformin-taurine · Antioxidant properties · In vitro and in vivo correlations

Abbreviations

AAPH	2,2'-azobis(2-methylpropionamidine) dihydrochloride
CAT	Catalase
DA	Dopamine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRT	Deoxyribose test
Fe(III)	Ferric chloride
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione, reduced form
GSSG	Glutathione, oxidized form
GST	Glutathione S-transferase
HOCl	Hypochlorous acid
HTAU	Hypotaurine
INS	Insulin
LPO	Lipid peroxidation
MDA	Malondialdehyde
MET	Metformin
NAC	N-acetylcysteine
NADPH	Nucleotide adenine dinucleotide phosphate, reduced form
NBT	Nitroblue tetrazolium chloride
NO	Nitric oxide
NO_2^-	Nitrite
PMS	Phenazine methosulfate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine
TBARS	Thiobarbituric acid reactive substances

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1 Introduction

One of the salient features of diabetes mellitus, or simply diabetes, is a generalized state of oxidative stress, characterized by an increase of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and of lipid peroxidation (LPO), and a direct consequence of the hyperglycemic state (Satoh et al. 2005). In turn, oxidative stress is recognized as a leading factor in the development and progression of diabetes and in the development of diabetic complications

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(Babizhayev et al. 2015; Satoh et al. 2005; Wei et al. 2009). The oxidative stress of diabetes is regarded as the results of an imbalance between free radical production and the activity of free radical scavenging systems (Bajaj and Khan 2012). Among major body organs, the brain is characterized by a high vulnerability to oxidative damage owing to a high oxygen consumption rate, an abundant lipid content, and a limited supply of antioxidant enzymes (Muriach et al. 2014). Additionally, oxidative stress in the brain also involves the lowering of the activity of antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD); a decrease of the levels of antioxidant molecules like reduced glutathione (GSH); and the depletion of nicotinamide adenine dinucleotide phosphate (NADPH) needed for the regeneration of GSH from its oxidized (GSSG) state (Muriach et al. 2014).

Antioxidants are at the center of diabetic complications since epidemiological studies reveal a strong association between dietary antioxidant intake and protection against diabetes-related oxidative damage to cells and complications (Rajendiran et al. 2018). In general, these chemical entities can exert their protective function by inhibiting the formation of ROS, by scavenging free radicals, or by enhancing the activity of antioxidant enzymes (Bajaj and Khan 2012). Two compounds that have demonstrated antioxidant properties are metformin (MET) and taurine (TAU). MET, a biguanide widely used by type 2 diabetics as an oral hypoglycemic agent, is reported to reduce the elevated activities of GPx, SOD, and CAT as well as the levels of malondialdehyde (MDA) in the cerebrum of rats exposed to ischemia/reperfusion injuries (Abd-Elsameea et al. 2014). On the other hand, TAU, a conditionally essential amino acid, is endowed with properties that are of benefit to diabetics, including suppression of hyperglycemia in STZ-treated mice (Tokunaga et al. 1979) and alloxan-treated rabbits (Tenner et al. 2003; Winiarska et al. 2009). In addition, TAU is found to reduce the elevation of the serum MDA and of the total antioxidant capacity and to promote increases of the activities of CAT and SOD in erythrocytes of patients with type 2 diabetes (Maleki et al. 2020).

In a previous work by this laboratory, the antioxidant effects of MET and TAU, singly and in combination, were evaluated in the brain of STZ-treated rats and found that these compounds were able to provide significant protection against changes in indices of oxidative stress caused by diabetes, with the magnitude of the effects by each compound being rather similar and with a combined treatment providing only an insignificant improvement (Clark et al. 2017). The present investigation was undertaken with two major objectives. The first objective was to elucidate the intrinsic antioxidant properties of MET and TAU and to compare these properties with those of an established hypoglycemic agent like insulin (INS) and of a proven antioxidant like N-acetylcysteine (NAC). This objective was accomplished by using a battery of six tests for antiradical activity and one test for antioxidant protection of a brain homogenate subjected to LPO. The second objective was to determine the extent to which these results agree with changes in indices of antioxidant activity in the brain of diabetic rats that had been separately treated with INS, MET, TAU, and MET-TAU for six consecutive weeks. In addition to measurements of the blood glucose and plasma INS, brain homogenates were analyzed for enzymatic and nonenzymatic indices of oxidative stress and the results compared with the in vitro data for possible correlations.

2 Methods

2.1 In Vitro Studies

The assay methods used for testing antiradical activity included (a) free radical generation from 2,2′-azobis(2-methylpropanimidine) (AAPH) hydrochloride as described by Nakamura et al. (1993); (b) the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay method according to Cotelle et al. (1996); (c) the deoxyribose test (DRT) for preformed and in situ

generated hydroxyl radicals (HO•) of Gutteridge (1995); (d) the ability to prevent the formation of quinones and free radicals upon the oxidation of dopamine (DA) by ferric chloride (Fe(III)) using the experimental conditions of Dawson et al. (1998); (e) the ability to prevent the oxidation of DA by nitric oxide (NO), generated from sodium nitroprusside (SNP), as described by Biasetti and Dawson (2002); and (f) the ability to scavenge $O_2^{\bullet-}$, generated from phenazine methosulfate (PMS) and using nitroblue tetrazolium chloride (NBT) as an indicator, as described by Ewing and Janero (1995). In addition, protection against LPO of polyunsaturated components in a brain homogenate was assessed by measuring MDA formation according to Buege and Aust (1976). All samples were freshly made as 20 mM solutions using absolute ethanol as the solvent (excepting INS, which was made in water), and all tests were conducted in triplicate. NAC, a known antioxidant and free radical scavenger (Ates et al. 2008), served as a standard of comparison.

2.2 In Vivo Studies

2.2.1 Animals and Treatments

All the experiments were performed on male Sprague-Dawley rats, weighing 200–225 g, and housed for 1 week in a room kept at a constant temperature (23 ± 1 °C) and humidity and on a 12-h light-12-hdark cycle. During this time, the rats had free access to a standard rodent diet (Lab Diet®, PMI Nutrition International, Brentwood, MO) and filtered tap water. All the experimental groups consisted of six rats each. The project received the approval of the Institutional Animal Care and Use Committee of St. John's University, Jamaica, NY, and the animals were cared in accordance with guidelines established by the US Department of Agriculture, Washington DC.

Treatment solutions were prepared just before an experiment in 10 mM citrate buffer pH 4.5 (streptozotocin = STZ) or distilled water (MET, TAU). The INS used was a commercially available intermediate-acting type (Humulin N®, 100 units/mL, from Eli Lilly and Co, Indianapolis, IN). Type 2 diabetes was induced with a single intraperitoneal (60 mg/kg/mL) dose of STZ. Treatments were started on day 15 and continued for the next 41 days, with TAU and/or MET (2.4 mM/kg/mL each, by the oral route) or with INS (4 units/kg, by the subcutaneous route). Rats in the control group received only 10 mM citrate buffer pH 4.5 daily and in a volume equal to that of the STZ solution, and rats in the diabetic control group received only STZ on day 1 and an equivalent daily volume of 10 mM citrate buffer pH 4.5 thereafter.

2.2.2 Samples and Assays

The development and course of the diabetic state was monitored on a weekly basis by measuring the blood glucose level on a drop of tail vein blood with a commercial blood glucose meter and test strips (OneTouch® Ultra® Glucose Meter from LifeScan, Inc., Wantagh, NY). Only animals exhibiting a blood glucose level ≥ 300 mg/dL but not exceeding 500 mg/dL by the third day were used in the study. On day 57 blood samples were collected over heparin sodium, and the corresponding plasma fractions were used for the measurement of INS levels. Immediately thereafter the rats were sacrificed by decapitation, and the skulls were cut open with the help of a Friedman Rongeur with ~2.5 mm cups at the tip (Fine Science Tool, Inc., Foster City, CA) to expose the brains which, after surgical removal by the freeze clamp technique of Wollenberger et al. (1960), were immediately stored at −80 °C in a deep freezer until needed.

Brain samples for the assessment of biochemical indices of oxidative stress were prepared by homogenization of a 500 mg portion of frozen tissue with 10 mL of 0.1 M phosphate buffer saline pH 7.4 solution on an ice bath and using an electric blender (Tissue-Tearor™, BioSpec Products Inc., Bartlesville, OK), followed by centrifugation at 12,000 × g and 4 °C for 30 min and separation of the clear supernatant, which was kept on ice until needed.

The concentration of plasma INS was measured using an assay kit (Insulin ELISA Kit, from

Calbiotech Inc., Spring Valley, CA), and the results were expressed in μIU/mL. The concentration of brain MDA was measured as thiobarbituric acid reactive substances (TBARS) by the end-point assay method of Buege and Aust (1976), and the results were reported as nmol/mg of the tissue. The fluorometric method of Hissin and Hilf (1976) was used to measure the brain levels of GSH and GSSG based on their reaction with *ortho*-phthalaldehyde at pH 8.0 and pH 12.0, respectively. Prior to the measurement of GSSG, any interfering GSH was removed by complexation with N-ethylmaleimide as described by Guntherberg and Rost (1966). The results were reported as μM/g of the tissue. The concentration of NO was measured in terms of its more stable metabolic product nitrite (NO_2^-) using the Griess reagent and the conditions described by Fox et al. (1981). The results were expressed as nM of NO_2^-/g of the tissue. The activity of GPx was measured using the method of Gunzler and Flohé (1985) and was expressed in units/mg of protein. GR was measured as described by Zhu et al. (2006), and the results were reported as μM of NADPH consumed/min/mg of protein. The activity of GST was obtained based on the method of Habig et al. (1974) and expressed as μM of dye-GSH conjugate formed/min/mg of protein, and that of SOD by the method of Misra (1985) with the results being expressed as U/mg of protein. The protein content was measured by the Lowry method (Lowry et al. 1951), using bovine serum albumin to prepare a calibration curve, and expressing the results as μg/mL.

2.3 Statistical Analysis of the Data

The in vitro experimental results are reported as the mean ± SD for experiments performed in triplicate. Results from in vivo experiments are reported as the mean ± SEM for n = 6 rats and were analyzed for statistical significance using unpaired Student's t-test and a computer software (JMP®7 Data Analysis for Windows, JMP USA, Cary, NC). Intergroup differences were considered to be statistically significant at $p \leq 0.05$.

3 Results

3.1 In Vitro Tests

In the first part of this study, the antioxidant/antiradical abilities of TAU, MET, and INS were investigated for their ability to scavenge free radicals and to minimize LPO under in vitro conditions. The first objective was accomplished by using a battery of six different tests (AAPH, DPPH, DRT, DA-Fe(III), DA-SNP, PMS-NBT) and the second objective by inducing LPO of a brain homogenate. To more accurately gauge the intrinsic protective actions of these compounds, they were all tested at an equimolar (20 mM) concentration. The results were compared with those of NAC, a thiol-containing compound with proven antioxidant/antiradical action (Aruoma et al. 1989; Ates et al. 2008).

From the results collectively shown in Table 1, it is evident that the antioxidant/antiradical potency varied quite widely among the various test compounds and that, irrespective of the test used, the degree of potencies for each test agent varied within a narrow potency range. In tests assessing antiradical ability, INS was the test compound providing the strongest lowering effects relative to control specimens free of a test compound (mean 82%, range 73–89%, all at $p < 0.001$ vs. controls), with MET (mean 43%, range 31–49%, $p < 0.001$) and TAU (mean 31%, range 19–43%, $p \leq 0.05$) providing a much weaker effect. Moreover, pairing MET with TAU resulted in only a small enhancement in the antiradical potency seen with MET alone (mean 46%, range 38–54%, $p < 0.001$). Without exceptions, in all these tests, NAC demonstrated the greatest antiradical protection (mean decrease 92%, range 84–95%, all at $p < 0.001$ vs. controls). A similar trend of results was observed in a test assessing the ability to decrease LPO of a brain homogenate by DA-Fe(III) and measured in terms of the mean percentage decrease of MDA formation. Thus, a greater protection was provided by NAC (98%) and INS (55%) in that order than by MET (39%) and TAU (31%). On the other hand, the addition of MET plus TAU led

Table 1 Results of in vitro tests for antiradical and antioxidant properties of test compounds[a,b,c,d]

Compound	AAPH	DPPH	DRT	Fe(III)-DA	SNP-DA	MS-NBT	LPO
Control	0.242 ± 0.006	1.480 ± 0.080	0.838 ± 0.054	0.676 ± 0.019	0.268 ± 0.008	0.037 ± 0.005	0.611 ± 0.001
INS	0.032 ± 0.007***	0.164 ± 0.003***	0.266 ± 0.038***	0.140 ± 0.015***	0.064 ± 0.002***	0.004 ± 0.003***	0.277 ± 0.003***
MET	0.148 ± 0.002***	1.016 ± 0.058**	0.404 ± 0.003***	0.352 ± 0.018***	0.171 ± 0.001***	0.019 ± 0.002***	0.370 ± 0.006**
TAU	0.197 ± 0.009*	1.193 ± 0.230*	0.554 ± 0.037***	0.423 ± 0.013***	0.183 ± 0.002***	0.021 ± 0.001***	0.424 ± 0.002**
MET-TAU	0.137 ± 0.008***	0.914 ± 0.092***	0.357 ± 0.054***	0.310 ± 0.016***	0.160 ± 0.002***	0.016 ± 0.002***	0.302 ± 0.009***
HYTAU	0.098 ± 0.007***	0.514 ± 0.003***	0.258 ± 0.037***	0.232 ± 0.012***	0.084 ± 0.002***	0.023 ± 0.001***	0.285 ± 0.005***
NAC	0.020 ± 0.002***	0.081 ± 0.002***	0.058 ± 0.003***	0.051 ± 0.001***	0.066 ± 0.001***	0.006 ± 0.002***	0.069 ± 0.002***

[a]Values are reported as mean ± SD for n = 3

[b]Control represents a reaction mixture without a test compound, and its result represents no inhibition

[c]NAC served as a reference compound

[d]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0001$

to a greater protection than with either compound alone (51%).

3.2 In Vivo Tests

3.2.1 Blood Glucose

As shown in Table 2, diabetes caused a profound increase in the circulating glucose level (+313%, $p < 0.001$ vs. control), an effect that was markedly decreased by INS (only +23%, $p < 0.001$ vs. STZ) and, to lesser extent, by MET (+90%, $p < 0.001$). In contrast, TAU exhibited a much weaker decreasing effect (+222%, $p < 0.01$) and was without altering the effect of MET when co-administered (+84%). Neither MET nor TAU produced an obvious effect on the basal blood glucose level.

3.2.2 Plasma INS

Diabetes caused a significant decrease in the plasma INS (−76%, $p < 0.001$ vs. control) (Table 2). This effect was reversed by INS (+21%, $p < 0.001$ vs. STZ) and markedly attenuated by MET (−26%, $p < 0.001$) and, to a lesser extent, by TAU (−52%, $p < 0.05$). Providing MET alongside TAU led to only a small increase in the plasma INS relative to MET alone (−18%); and neither MET nor TAU affected the basal INS level.

3.2.3 Brain MDA

The effects of the various treatments on the brain MDA levels are shown in Table 3. Diabetic rats demonstrated a significant increase in MDA formation (+59%, $p < 0.001$ vs. control), an effect that was effectively suppressed by INS (−26%, $p < 0.001$ vs. STZ) and MET-TAU (−10%, $p < 0.001$ vs. STZ), and attenuated to about the same extent by both MET and TAU (+29% and + 28%, $p < 0.01$ vs. control, respectively). None of the treatment agents demonstrated any significant effect on the MDA level of rats in the control group.

3.2.4 Brain NO

The effects of the various treatments on the levels of brain NO, measured as NO_2^-, are presented in Table 3. Diabetes caused a significant elevation of the NO level (+41%, $p < 0.001$ vs. control), but this effect was either suppressed by INS (−16%) or markedly minimized by MET (+12%), TAU (+20%, $p < 0.05$ vs. control), and MET-TAU (+9%). On the other hand, neither MET nor TAU had a measurable effect on the control NO value.

3.2.5 Brain GSH, GSSG, and GSH/GSSG Ratio

As shown in Table 4, the brain GSH was markedly decreased by diabetes (−79%, $p < 0.001$ vs. control), an effect that was effectively

Table 2 Blood glucose and plasma INS levels of diabetic rats receiving a treatment agent on a daily basis for 6 weeks[a,b,c]

Treatment	Blood glucose (mg/dL)	Plasma INS (μU/mL)
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$
STZ	$428.08 \pm 21.77^{***}$	$10.58 \pm 1.33^{***}$
MET	$101.54 \pm 5.57^{+++}$	$44.13 \pm 1.71^{+++}$
TAU	$102.05 \pm 3.33^{+++}$	$42.94 \pm 2.37^{+++}$
INS-STZ	$127.15 \pm 10.71^{*,+++}$	$53.46 \pm 0.97^{*,+++}$
MET-STZ	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 2.35^{**,+++}$
TAU-STZ	$333.27 \pm 6.60^{***,+}$	$20.94 \pm 1.83^{***,+++}$
MET-TAU-STZ	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$

[a]Values are reported as mean ± SEM for n = 6 rats

[b]INS served as a reference compound

[c]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from STZ at $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$

Table 3 Brain MDA and NO levels of diabetic rats receiving a treatment agent on a daily basis for 6 weeks[a,b,c]

Treatment	MDA (nM/mg protein)	NO (nM/mg protein)
Control	$0.157 \pm 0.001^{+++}$	$4.851 \pm 0.014^{++}$
STZ	$0.249 \pm 0.003^{***}$	$6.833 \pm 0.033^{***}$
MET	$0.151 \pm 0.005^{+++}$	$4.634 \pm 0.014^{+++}$
TAU	$0.164 \pm 0.045^{+++}$	$4.721 \pm 0.089^{+++}$
INS-STZ	$0.116 \pm 0.006^{*,+++}$	$4.061 \pm 0.015^{*,+++}$
MET-STZ	$0.202 \pm 0.013^{**,++}$	$5.522 \pm 0.041^{*,+}$
TAU-STZ	$0.199 \pm 0.002^{**,++}$	$5.831 \pm 0.034^{*,+}$
MET-TAU-STZ	$0.141 \pm 0.020^{+++}$	$5.273 \pm 0.025^{++}$

[a]Values are reported as mean ± SEM for n = 6 rats
[b]INS served as a reference compound
[c]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from STZ at $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$

Table 4 Brain GSH, GSSG, and GSH/GSSG ratio values of diabetic rats receiving a treatment agent on a daily basis for 8 weeks[a,b,c]

Treatment	GSH (nM/mg protein)	GSSG (nM/mg protein)	GSH/GSSG ratio
Control	$6.368 \pm 0.966^{+++}$	$13.537 \pm 0.464^{+++}$	$0.472 \pm 0.003^{+++}$
STZ	$1.325 \pm 0.545^{***}$	$5.172 \pm 0.196^{***}$	$0.256 \pm 0.005^{***}$
MET	$6.008 \pm 0.532^{+++}$	$13.788 \pm 0.811^{+++}$	$0.453 \pm 0.002^{+++}$
TAU	$6.301 \pm 0.325^{+++}$	$13.138 \pm 0.232^{+++}$	$0.469 \pm 0.003^{+++}$
INS-STZ	$5.384 \pm 0.045^{*,+++}$	$9.298 \pm 0.703^{**,+++}$	$0.579 \pm 0.001^{*,+++}$
MET-STZ	$4.782 \pm 0.039^{**,+++}$	$10.496 \pm 0.366^{**,+++}$	$0.456 \pm 0.003^{+++}$
TAU-STZ	$4.610 \pm 0.458^{**,+++}$	$11.102 \pm 0.619^{**,+++}$	$0.471 \pm 0.002^{++++}$
MET-TAU-STZ	$5.235 \pm 0.512^{*,+++}$	$10.194 \pm 0.590^{**,+++}$	$0.514 \pm 0.003^{+++}$

[a]Values are reported as mean ± SEM for n = 6 rats
[b]INS served as a reference compound
[c]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from STZ at $^{+++}p < 0.001$

counteracted by all the treatment agents (−16% with INS, −25% with MET, −28% with TAU, −18% with MET-TAU). By themselves neither MET nor TAU exerted an effect on the control value. Table 4 also shows the changes in brain GSSG for the various treatment groups. In parallel with the changes for GSH, diabetes caused a profound decrease of the GSSG (−62%, $p < 0.001$ vs. control). This effect was significantly attenuated by all the treatment agents (−31% with INS, −25% with MET-TAU, −22% with MET, −18% with TAU, all at $p < 0.05$ vs. control). Neither MET nor TAU affected the control values for brain GSH and GSSG. Based on these values, the GSH/GSSG ratio (Table 4) was also found decreased in untreated diabetic rats (−46%, $p < 0.001$ vs. control); however, this effect was reversed by INS- (+25%, $p < 0.001$ vs. STZ) and MET-TAU (+9%, $p < 0.001$ vs. STZ), and brought to within normal by either MET or TAU. As seen with the GSH and GSSG values, neither MET nor TAU had an effect on the control GSH/GSSG value.

3.2.6 Brain Activities of GPx, GR, and GST

The results for the changes in the brain activities of GPx, GR, and GST as a result of diabetes, in the absence or presence of a test compound, are shown in Table 5. First, the diabetic rats showed a much lower brain GPx activity than normal rats (−48%, $p < 0.001$). This effect was effectively attenuated by all the treatment agents, with the effects varying within a narrow range (−15% by INS, −18% by TAU, −24% by MET, all at $p < 0.05$ vs. control). A combined treatment with MET-TAU was slightly more effective than either compound alone (−12%).

Table 5 Brain GPx, GR, and GST activities of diabetic rats receiving a treatment agent on a daily basis for 6 weeks[a,b,c]

Treatment	GPx (U/mg protein)	GR (U/mg protein)	GST (U/mg protein)
Control	$9.910 \pm 0.122^{+++}$	$31.061 \pm 0.069^{+++}$	$0.184 \pm 0.030^{+++}$
STZ	$5.177 \pm 0.105^{***}$	$15.563 \pm 0.040^{***}$	$0.091 \pm 0.040^{***}$
MET	$9.396 \pm 0.140^{+++}$	$31.193 \pm 0.019^{+++}$	$0.184 \pm 0.053^{+++}$
TAU	$10.373 \pm 0125^{+++}$	$30.611 \pm 0.015^{+++}$	$0.185 \pm 0.081^{+++}$
INS-STZ	$8.373 \pm 0.185^{*,+++}$	$24.309 \pm 0.005^{**,+++}$	$0.163 \pm 0.061^{+++}$
MET-STZ	$7.489 \pm 0.168^{**,+++}$	$22.669 \pm 0.040^{**,+++}$	$0.114 \pm 0.067^{***,+++}$
TAU-STZ	$8.094 \pm 0.212^{*,+++}$	$22.187 \pm 0.033^{**,+++}$	$0.124 \pm 0.029^{***,++++}$
MET-TAU-STZ	$8.710 \pm 0.118^{*,+++}$	$23.681 \pm 0.018^{**,+++}$	$0.130 \pm 0.032^{**,+++}$

[a]Values are reported as mean ± SEM for n = 6 rats
[b]INS served as a reference compound
[c]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from STZ at $^{+++}p < 0.001$

Table 6 Brain CAT and SOD activities of diabetic rats receiving a treatment agent on a daily basis for 6 weeks[a,b,c]

Treatment	CAT (U/mg protein)	SOD (U/mg protein)
Control	$3.155 \pm 0.004^{+++}$	$48.387 \pm 0.006^{+++}$
STZ	$1.803 \pm 0.002^{***}$	$16.854 \pm 0.038^{***}$
MET	$2.929 \pm 0.011^{+++}$	$45.455 \pm 0.007^{+++}$
TAU	$2.873 \pm 0.007^{+++}$	$46.876 \pm 0.010^{+++}$
INS-STZ	$2.901 \pm 0.005^{+++}$	$32.258 \pm 0.007^{***,+++}$
MET-STZ	$2.351 \pm 0.002^{**,+++}$	$36.839 \pm 0.002^{**,+++}$
TAU-STZ	$2.320 \pm 0.002^{**,++}$	$32.581 \pm 0.010^{***,+++}$
MET-TAU-STZ	$2.732 \pm 0.006^{*,+++}$	$39.741 \pm 0.018^{*,+++}$

[a]Values are reported as mean ± SEM for n = 6 rats
[b]INS served as a reference compound
[c]Statistical comparisons were significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ and from STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

Second, untreated diabetic rats exhibited a significantly lower GR activity (−50%, $p < 0.001$) than diabetic rats receiving a treatment agent (−22% with INS, −24% with MET-TAU, −27% with MET, −29% with TAU, all comparisons at $p < 0.05$ vs. control). This trend was also verified for the GST activity, which was lowest in untreated diabetic rats (−51%, $p < 0.001$ vs. control) and higher in treated rats (−12% with INS, −30% with MET-TAU, −33% with TAU, −38% with MET). Without exceptions, none of the treatment agents affected the control values to an appreciable extent.

3.2.7 Brain Activities of Brain CAT and SOD

As shown in Table 6, diabetes had a marked lowering effect on the brain activities of both CAT and SOD (−43% and − 65%, respectively, both at $p < 0.001$ and controls), two enzymes that together with GPx protect cells against toxic ROS. All the treatment agents were effective in minimizing these effects, with the protection varying within a narrow range of values. In terms of the CAT activity, the greatest protection was afforded by INS (only −8%, $p < 0.001$ vs. STZ), with MET and TAU afforded a lesser but still significant and almost equivalent attenuating effect (−25% and − 27%, respectively, $p < 0.01$ vs. STZ). Providing diabetic rats with MET-TAU led to a greater protection than with either compound alone (−13%, $p < 0.001$ vs. STZ).

Like for the CAT activity, all of the treatment agents attenuated the loss of brain SOD activity caused by diabetes, its effect, with MET (−24%, $p < 0.001$ vs. STZ) providing a slightly greater protection than either INS or TAU (−33% with both, $p < 0.001$ and STZ). On the other hand, a treatment with MET-TAU resulted in a significant enhancement of the protection attained with MET alone (−8%, $p < 0.001$ vs. STZ).

4 Discussion

The antioxidant potential of the test compounds, i.e., INS, MET, and TAU, was investigated using a set of six colorimetric tests for assessing the ability to trap a specific free radical and one test for LPO based on the generation of MDA in a brain homogenate exposed to DA-Fe(III). To obtain a more accurate idea of the antioxidant potency of each test compound, their responses were compared with those of NAC, a known antioxidant, tested alongside. From the results presented in Table 1, it is evident that the antioxidant power demonstrated by the various test compounds varied in a characteristic and uniform manner, with the highest effects, measured as a decrease relative to a specimen not containing a test compound and representing 100% free radical or LPO, being provided by NAC (mean decrease 92%) and INS (mean decrease 87%), followed distantly by MET (mean decrease 43%) and TAU (mean decrease 31%) in that order. Combining MET with TAU led to results that were only marginally better than with MET alone (mean decrease 46%). While the high antiradical and antioxidant abilities of NAC are associated with its thiol group and take place via nucleophilic reaction with ROS (Ates et al. 2008), in the case of INS, the presence of reactive side chains at residues such as Tyr, Phe, and His that can be attacked or modified by ROS like HO• and $O_2^{\bullet-}$ may account for the observed antiradical effects (Olivares-Corichi et al. 2005). In terms of MET, this biguanide can serve as a trap of HO• at its NH and NH_2 terminals and at the CH_3 end (Badran et al. 2020), but the effect is considered to be weak even at peak concentrations (Esteghamati et al. 2013). Moreover, MET was found not to interact with the $O_2^{\bullet-}$ radical (Khouri et al. 2004).

In a study evaluating the rates of reaction of TAU and of its sulfinic analog hypotaurine (HTAU) with the biologically important oxidants HO•, $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl), it was determined that TAU does not react rapidly with HO•, $O_2^{\bullet-}$, and H_2O_2, and the product of its reaction with HOCl was still sufficiently oxidizing to inactivate α_1-antiproteinase, results that put in doubt an antioxidant role for TAU in vivo. By contrast, HTAU demonstrated an excellent scavenging ability for HO• and HOCl and was found to interfere with the Fe(III)-dependent formation of HO•, although no reaction with $O_2^{\bullet-}$ and H_2O_2 could be detected within the limits of the assay methods used (Aruoma et al. 1988). For the most part, the data shown in Table 1 corroborate these conclusions inasmuch as the replacement of the sulfonic ($-SO_3H$) group of TAU by a sulfinic ($-SO_2H$) group in HTAU led to a greater antiradical (mean decrease 51%, range 60–69%) and antioxidant (mean decrease 53%) effects than TAU. Interestingly, while the suppressing effect of HTAU on the $O_2^{\bullet-}$ radical was rather similar to that of TAU (−38% vs. −31%), the low values seen with both compounds confirm their reported low affinity for this free radical.

At a dose of 60 mg/kg, STZ induced a frank hyperglycemia (>300 mg/dL), raised the brain LPO, measured as MDA (+59%) and NO (+41%), markedly lowered the brain GSH/GSSG ratio (−46%), and led to a marked loss of the brain activities of CAT (−43%), GPx (−48%), GR (−50%), GST (−51%), and SOD (−65). All these changes were significantly attenuated by the various treatment agents, with the strongest effects coming from INS, and, except for the effects on the blood glucose and brain CAT and SOD activities, MET was somewhat more effective than TAU. The lowering effect of TAU on the circulating glucose (−30%) was comparable to that reported by Winiarska et al. (2009) for rabbits made diabetic with alloxan and receiving 1% TAU in the drinking water for 3 weeks. On the other hand, providing TAU with MET led to an insignificant gain on the hypoglycemic and antioxidant actions of MET.

In the case of INS and MET, their intrinsic hypoglycemic ability may play a major role in their protective effects in diabetes since hyperglycemia is recognized as a contributor to the generation of free radicals and, hence, to the oxidative stress associated with the disease (King and Loeken 2004), but such is not the case with TAU since the antioxidant/antiradical features associated with this amino acid are found in this

and other in vitro studies to be suboptimal (Aruoma et al. 1988; Kim et al. 2020). Furthermore, while this study finds that the effect of TAU (−29%) on the diabetic blood glucose to be much more limited than that of INS (−93%) or MET (−70%), other laboratories have concluded that it is ineffective (Lim et al. 1998). Although it is not unexpected to find that a treatment of diabetic rats with INS reversed the hypoinsulenemic state to a point that rose above the control value (by 30%), in the case of MET and TAU, it was determined that they raised the diabetic plasma INS to a lesser extent (by ~3.1-fold and by 1.96-fold, respectively) and with a combined treatment resulting in only a slightly greater effect than with MET alone (3.4-fold increase).

In diabetes, ROS promote the oxidation of cellular molecules, notably long-chain polyunsaturated fatty acids of phospholipids, to generate lipid peroxides that, on further decomposition, can yield toxic aldehydes such as acrolein, 4-hydroxynonenal, and MDA (Slatter et al. 2000). MDA is considered to be a cofactor in the pathogenesis of type 2 diabetes and to be useful as a marker for evaluating glycemic control and the lipid status of diabetic patients (Kamal et al. 2009). Indeed, a direct correlation has been verified between the magnitude of the hyperglycemic state, the plasma MDA, and atherogenic lipid risk factors in patients with type 2 diabetes (Manohar et al. 2013). In the present work, the brain of diabetic rats exhibited a marked increase of MDA compared to normal rats. An interaction between this change and the extent of the prevailing hyperglycemia was evident by finding that a treatment of the diabetic rats with INS attenuated the hyperglycemia and eliminated the formation of brain MDA. Although this trend was also observed in diabetic rats treated with MET, the effect was much smaller. In contrast, TAU was able to reduce the formation of MDA by the diabetic brain to an extent comparable to MET while exerting a much weaker hypoglycemic effect. This finding may indicate that although the degree of the hyperglycemia correlates with the degree of MDA formation, in the case of TAU, the antioxidant action may be taking place by other factors. This assumption is supported by finding that a treatment with MET-TAU lowered the brain MDA to a greater extent than either compound but without affecting the blood glucose level seen with MET alone. Among the various possible mechanisms by which TAU can lower the levels of MDA, there is one suggesting a direct reaction through its amino group to yield a fluorescent 1,4-dihydropyridine adduct and a nonfluorescent enamine derivative (Li et al. 2010).

With regard to the brain NO, the use of animal models of diabetes such as the one used in the present study finds the level to be higher than that of normal animals, an effect that has been related to an stimulatory effect of hyperglycemia on the NO synthase (NOS) activity (Adela et al. 2015) and which has been found to be lowered by a treatment with TAU (Askwith et al. 2012) or vitamin E (Comin et al. 2010). Interestingly, and at variance with the present results, there are also reports indicating a decrease of NOS activity in the brain of diabetic rats (Yazir et al. 2019; Yu et al. 1999). Although all of the treatment agents used in the present study were effective in lowering the diabetic brain NO level, INS was the only one to bring it to a value akin to that of the control group, and, in the case of MET and TAU, a greater effect was achieved when they were co-administered. A previous study from this laboratory has shown that at doses of 1.2–3.6 mm/kg TAU can lower the brain NO of diabetic rats in the cerebellum, brain stem, and spinal cord to values that varied within a narrow range regardless of the dose used (Patel et al. 2016). Moreover, an in vitro study whereby TAU was incubated with SNP, serving as a source of NO, showed that TAU was effective as a NO trap when present at 15–60 mM concentrations (Oliveira et al. 2010).

The massive reduction of the brain GSH by the diabetic state may reflect a decrease synthesis and/or an increased utilization through conversion to GSSG in the face of hyperglycemia (Lutchmansingh et al. 2018; Sekhar et al. 2011). Additional contributory factors for this reduction may be the limited availability of metabolic precursors (glycine, cysteine), a situation that can be remediated through dietary supplementation with the deficient amino acids (Sekhar et al. 2011),

and insufficient levels of NADPH for reconverting GSSG to GSH (Lagman et al. 2015). In the present study, the various test compounds were able to spare the brain GSH and to improve the corresponding GSH/GSSG ratio through their antioxidant properties. In the case of TAU, these compounds have been shown to ameliorate LPO, the oxidation of GSH, and the loss of the activities of antioxidant and of GSH-related enzymes under the prevailing hyperglycemic conditions of diabetes thanks to its ability to curtail the production of free radicals by the mitochondrion. This effect has been related to the improvement of the flux of electrons through complexes I and III of the respiratory chain brought about by TAU with a concomitant increase of oxygen consumption, effects that will curtail the diversion of electrons from the respiratory chain to acceptor oxygen to generate $O_2^{\bullet-}$ (Algire et al. 2012; Jong et al. 2011, 2012). At variance with TAU, information on the effect of MET on the mitochondrial generation of ROS is rather limited and still the subject of ongoing investigation. However, there is sufficient evidence to support a modulatory effect on the production of ROS by mitochondrial complex I and the ability to enhance the mRNA expression of SIRT3, an NAD^+-dependent deacetylase specifically located in the mitochondria that, when overexpressed, reduces ROS production in several tissues (Apostolova et al. 2020). Additionally, the beneficial effects of TAU on the brain activities of GR and GPx may be related to an increased availability of GSH that otherwise would be consumed by interactions with oxidants (Anand et al. 2011). A similar situation may apply to the treatments of diabetic rats with INS (Moreira et al. 2006) or MET (Correia et al. 2012; Vilela et al. 2016).

The present study finds that all the treatment agents were able to protect the brain against losses of antioxidant enzymes such as CAT, GPx, and SOD. Losses of antioxidant enzyme activities in diabetes are ascribed to damage to DNA coding for antioxidant enzymes by ROS and RNS generated as a result of hyperglycemia (Sindhu et al. 2004). Since hyperglycemia is associated with oxidative stress and, thereby, with decreased antioxidant enzyme expression, it is not surprising to find in the present study that a treatment with a hypoglycemic agent such as INS or MET led to significant attenuation of the losses of antioxidant enzyme activity caused by diabetes. Likewise, the daily oral administration of TAU for 8 weeks to patients with type 2 diabetes was found to increase the serum activities of CAT and SOD while lowering the circulating levels of MDA (Maleki et al. 2020), and by using appropriate free radical generating systems, it has been possible to verify the ability of TAU to prevent the loss of SOD as a result of an interaction with ROS and RNS (Oliveira et al. 2010).

5 Conclusion

The present study finds that (a) the extent of antioxidant/antiradical potencies of INS, MET, and TAU uncovered by a battery of in vitro chemical tests varied markedly, being highest with INS and lowest with TAU; (b) the protective actions of INS, MET, and TAU on the plasma INS and on indices of oxidative stress of diabetic rats followed a pattern similar to that noted in vitro, but with the potency differences among the various test compounds varying over a narrower range; (c) while by itself TAU demonstrated a weaker hypoglycemic effect than MET, a combined treatment with MET-TAU can lead to a gain in hypoglycemic and antioxidant effects over one with MET alone; and (d) the value of testing compounds like TAU for antioxidant/antiradical potency under in vitro conditions may not accurately reflect the actual performance of this compound as an antioxidant/antiradical in living organs, tissues, or organisms, namely because such effects are dependent on factors other than chemical features and including physiological changes and/or biochemical mechanisms that are only seen in vivo.

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Potential Binding Sites for Taurine on the Insulin Receptor: A Molecular Docking Study

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Keywords

Insulin receptor · Taurine · Glucose homeostasis · Molecular docking

Abbreviations

AKT	Protein kinase B/thymoma viral proto-oncogene
CR	Region rich in cysteine
ECD	Ectodomain
FnIII-1	Fibronectin type-III domain 1
IR	Insulin receptor
IRα	α subunit of the IR
IRβ	β subunit of the IR
MTORC2	Mammalian target of rapamycin complex 2
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidylinositol 3-kinase
RTK	Receptor tyrosine kinase

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1 Introduction

After a meal, the pancreatic islets of Langerhans release insulin, which is the unique hormone that decreases blood glucose levels. This hormone minimizes hyperglycemia by interacting with the insulin receptor (IR), a receptor tyrosine kinase (RTK), which is expressed on the membrane of all mammalian cells (Menting et al. 2013; Croll et al. 2016; Manning and Toker 2017). The IR structure is constituted of a heterodimer with two α and two β subunits (α2β2). The α subunits (IRα) are located on the extracellular side of the plasma membrane, while the β chain (IRβ) is formed by an extracellular segment, a transmembrane, a juxtamembrane, and the cytoplasmic tyrosine kinase domains. The IRα subunit and the extracellular segment of IRβ are referred to as the ectodomain (ECD) of the IR and contain the insulin-binding sites (Croll et al. 2016; Ye et al. 2017; Scapin et al. 2018; Uchikawa et al. 2019; Gutmann et al. 2020).

Insulin binding to the IR ECD leads to a conformational change, activating the tyrosine kinase activity of IRβ. Subsequently, IRβ autophosphorylates as well as phosphorylates and anchors the IR substrates, which activate the phosphatidylinositol 3-kinase (PI3K). This kinase produces membrane phosphoinositides that anchor and activate the phosphoinositide-dependent protein kinase 1 (PDK1) and the

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mammalian target of rapamycin complex 2 (mTORC2). These proteins phosphorylate and activate protein kinase B/thymoma viral proto-oncogene (AKT), which is an important downstream protein effector of the IR-PI3K signaling pathway, regulating cellular nutrient transport and metabolism, survival, proliferation, and growth (Saltiel and Kahn 2001; Manning and Toker 2017). Disruptions in the activation of IR/PI3K/AKT pathway contribute to cellular damage, leading to impaired glucose homeostasis conditions such as insulin resistance, glucose intolerance, and type 2 diabetes (Shao et al. 2000; Carvalho-Filho et al. 2005; Manning and Toker 2017; Xing et al. 2019).

The sulfur-containing amino acid, taurine (2-aminoethanesulfonic acid), has been shown to regulate glucose homeostasis via regulation of insulin secretion (Carneiro et al. 2009; Ribeiro et al. 2009, 2012) and/or improvement in insulin sensitivity in insulin-target tissues (Ribeiro et al. 2012; Solon et al. 2012; Batista et al. 2013). The mechanism by which this amino acid improves the action of insulin is not yet completely understood; however, a number of studies have provided evidence to indicate that taurine imparts benefits on the physiology of the neurons (Sun et al. 2018), the liver (Ribeiro et al. 2012; Batista et al. 2013), the kidney (Sun et al. 2020), the heart (Takatani et al. 2004), and the vasculature (Baek et al. 2012), possibly via activation of the PI3K-AKT pathway. It has been suggested that taurine activates this signaling pathway via interaction with the IR; Maturo and Kulakowski (1988) reported binding of taurine to purified IR, and Carneiro et al. (2009) demonstrated that intravenous taurine administration increases IRβ phosphorylation. However, the mode by which this amino acid activates IR and insulin signaling and its potential binding sites on the receptor have not been identified, to date. Thus, herein, we aimed to verify whether taurine could be an agonist of IR and also the potential binding modes of taurine on IR, using in silico experiments.

2 Methods

2.1 Identification of Potential IR ECD Pockets for Ligand Binding

The major cavities or connection pockets for ligands on the IR ECD were first calculated using the CASTp web server (Tian et al. 2018). The program identified 317 cavities in the IR ECD. The three largest pockets were chosen as potential binding sites for taurine as they covered the entire IR ECD. Subsequently, all the residues in each pocket were identified using the Swiss PDB Viewer v. 4.1 [SIB, Lausanne, Switzerland (Guex et al. 2009)], the grid center was then determined, and a directed docking in each cavity was performed.

2.2 Taurine and Receptor Preparations for Molecular Docking

The ionization states of taurine were predicted with Chemicalize (ChemAxon, Záhony, Budapest, Hungary), and the ionized form, the major microspecies at pH 7.4, was used for the docking calculations. The 3D structures of this compound were built in Spartan'10 (Wavefunction Inc. Irvine, CA, USA). Firstly, the structures were submitted to conformational analysis using the MMFF force field, followed by geometry optimization with the semi-empirical AM1 method, and then single-point ab initio calculation was carried out using the Hartree-Fock method and the 6-31G* basis set in Spartan'10. The final optimized structures were transferred to Autodock Tools 1.5.7 (Scripps Research Institute, San Diego, CA, USA), and all torsion bonds of the ligands were automatically set to be flexible in the docking studies. The 3D structure of the IR (PDB code 6SOF) (Gutmann et al. 2020) was prepared by removing water molecules and ligands and by adding polar hydrogens and Gasteiger charges, using Autodock Tools 1.5.7.

2.3 Molecular Docking of Taurine on the IR ECD

To evaluate the binding modes of taurine in the IR, molecular docking analysis was carried out using Autodock 4.2 (Scripps Research Institute, San Diego, CA, USA; Morris et al. 2009) and Autodock Tools 1.5.7. The grid box with dimensions of 126 points in the xyz axis and with spacing of 0.375 $Å^3$ was centered in pocket 1 (coordinates x = 152.779, y = 147.322, and z = 156.381). The grid box with dimensions of 104 points in the xyz axis with spacing of 0.375 $Å^3$ was centered in pocket 2 (coordinates x = 137.907, y = 161.699, and z = 207.084). The grid box with dimensions of 98x98x84 points in the xyz axis with spacing of 0.375 $Å^3$ was centered in pocket 3 (coordinates x = 180.846, y = 168.019, and z = 119.423). The lowest energy pose obtained for each docking study was selected and submitted to a visual inspection and analysis of the interactions using Swiss PDB Viewer v. 4.1 [SIB, Lausanne, Switzerland (Guex et al. 2009)], PyMOL v. 2.3 (The PyMOL Molecular Graphics System, version 2.0 Schrödinger, LLC, NY, USA), and Discovery Studio (Dassault Systèmes BIOVIA, Vélizy-Villacoublay, Paris, France).

3 Results

3.1 Potential Major Binding Sites for Taurine on the IR ECD

As previously mentioned, the IR ECD is constituted of two α subunits (IRα), located on the extracellular side of the plasma membrane. Each IRα contains two repetition domains that are rich in leucine (L1-L2), a region rich in cysteine (CR), and two domains of fibronectin type III (FnIII-1-2a). In addition to the IRα, the IR ECD is formed by the extracellular segment of the IRβ, which contains the N-terminus of this protein, and the FnIII-2b and FnIII-3 domains (Menting et al. 2013; Croll et al. 2016; Uchikawa et al. 2019; Gutmann et al. 2020). Figure 1a shows a representation of the full IR structure, highlighting the regions/domains of the IR ECD in different colors.

Insulin binding to IR occurs through four insulin-binding sites present in the IR ECD, denominated binding sites 1 and 2, in one IRα protomer, and sites 1′ and 2′ in the other IRα protomer (Croll et al. 2016; Ye et al. 2017; Scapin et al. 2018). Site 1 is located at the amino acid residues of L1, the carboxy-terminal of IRα and the FnIII-1 segment (Menting et al. 2013; Uchikawa et al. 2019; Gutmann et al. 2020). However, binding site 2 in the IR ECD seems to be located on the back of the major β sheet of the FnIII-1 domain (Uchikawa et al. 2019; Gutmann et al. 2020).

To verify favorable surface regions/cavities for the interaction of potential ligands, the IR ECD was initially directed to the CASTp platform, which demonstrated that this receptor has 317 potential pockets for ligand interactions with different dimensions. Previous studies have indicated that ligand binding sites in receptors usually involve the largest pockets (Rahman et al. 2005; Chowdhury et al. 2014); to determine whether taurine could interact with the IR ECD, we chose the three largest pockets, obtained from the CASTp analysis, to proceed with molecular docking studies of taurine on the IR ECD. In addition, these three largest pockets comprise practically the entire IR ECD (Fig. 1b). Thus, the pocket chosen as pocket 1 is located in the L2, αCT, and FnIII-1,2 regions of both IR ECD protomers (represented in red in Fig. 1b). Pocket 2 comprises the FnIII-2,3 and FnIII-2,3′ regions (represented in green in Fig. 1b). Pocket 3, in turn, contains the L1 and CR regions of one IR ECD protomer, and the L1 and CR regions of the other IR ECD protomer (represented in blue in Fig. 1b). The volume and surface area in the three largest pockets are shown in Table 1. Pocket 1 was considered the largest, followed by pocket 3 and pocket 2. As can be seen in Table 1, the volume of the three largest pockets varies between 14,189 $Å^3$ and 2026 $Å^3$. On the other hand, the surface area of the three pockets varies between 42,768 $Å^2$ and 4288 $Å^2$.

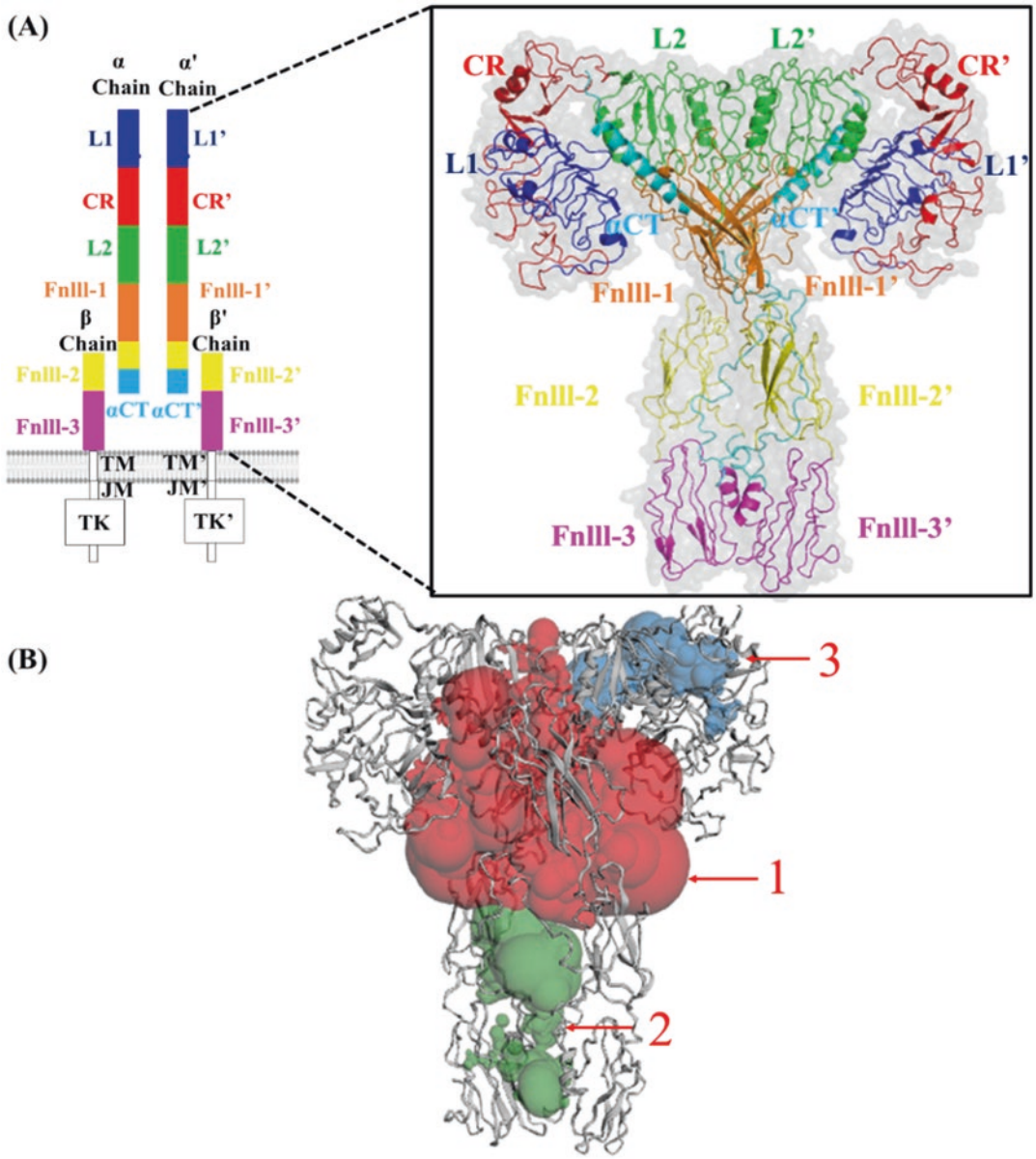

Fig. 1 (**a**) Schematic representation of the full IR structure demonstrating the regions of the ECD (in different colors in the full IR and in the amplified image on the right), containing the leucine-rich repeat domains 1 (L1) and 2 (L2); the cysteine-rich domain (CR); the fibronectin type III domains 1 (FnIII-1), 2 (FnIII-2), and 3 (FnIII-3); and the α C-terminus region (αCT). The transmembrane (TM), the juxtamembrane (JM), and the tyrosine kinase (TK) domains are represented in gray. α chain = IRα; β chain = IRβ. (**b**) Representation of the largest potential binding pockets for ligands in the IR ECD (PDB code 6SOF). Pocket 1 is represented in red, pocket 2 in green, and pocket 3 in blue

Table 1 Volume and surface area of the three largest binding pockets for ligands in the IR ECD (PDB code 6SOF)

Pocket ID	Volume of the pocket (Å^3)	Surface area of the pocket (Å^2)
1	14189.5	42768.2
2	2027.0	4386.2
3	2621.1	4288.3

The binding modes of taurine in the three largest pockets for ligands in the IR ECT are illustrated in Fig. 2a. Molecular docking analysis suggested that taurine has different affinities for the IR ECD pockets. In pocket 1, taurine bound with an energy of −5.23 Kcal/mol (Fig. 2b). In contrast, taurine docking in

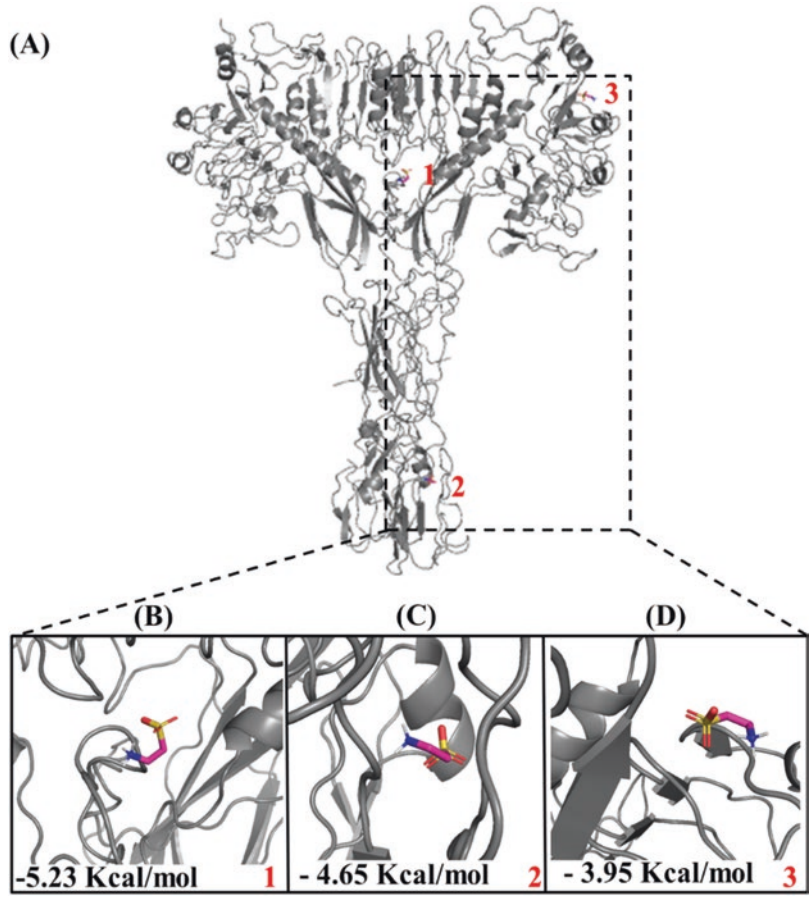

Fig. 2 Binding modes of taurine in the three largest pockets for ligands in the IR ECD. Cartoon of the full IR ECD (in gray, **a**) and representation of the interactions of taurine (in magenta) with pockets 1 (**b**), 2 (**c**), and 3 (**d**) of the IR ECD

pockets 2 and 3 resulted in binding energies of −4.65 (Fig. 2c) and − 3.95 Kcal/mol (Fig. 2d), respectively.

Figure 3 illustrates ionized taurine and its molecular interaction with amino acid residues of the three largest pockets of the IR ECD. The calculation of the ionization state of taurine showed that its ionized form is probably the major microspecies found at pH 7.4, and, therefore, this microspecies was used for the docking studies (Fig. 3a). In pocket 1, binding of taurine to the L2 domain of the IRα and the sulfur group of taurine formed ionic bonds with residues Lys433 and Arg409. In addition, the amino group of taurine formed the same intermolecular bonds with residue Glu517. van der Waals contacts with the residues Phe518, Gln521, and Ala466 were also observed (Fig. 3b). In pocket 2, taurine interacted with the FnIII-3 domain of the extracellular region of the IRβ through hydrogen bonds with residues Arg887 and presented attractive charges with residues Arg887, Arg885, and Glu846 (Fig. 3c). In pocket 3, taurine bound in regions that comprise the L1 and CR domains of the IRα through carbon hydrogen bonding with Val7 residues; van der Waals contacts with the residues Glu30 and Trp251; salt bridges with residues Glu6 and Arg252; and a pi-sulfur bond with the Phe258 residue (Fig. 3d). Notably, findings suggest a better affinity of taurine for pocket 1, due to the greater amount of hydrogen bonds, as well as the formation of other intermolecular bonds with different amino acid residues of the IR ECD.

As previously reported, the IR ECD has four insulin-binding sites (Menting et al. 2013; Croll et al. 2016; Ye et al. 2017; Scapin et al. 2018; Uchikawa et al. 2019; Gutmann et al. 2020). Taurine was observed, herein, to bind to a region

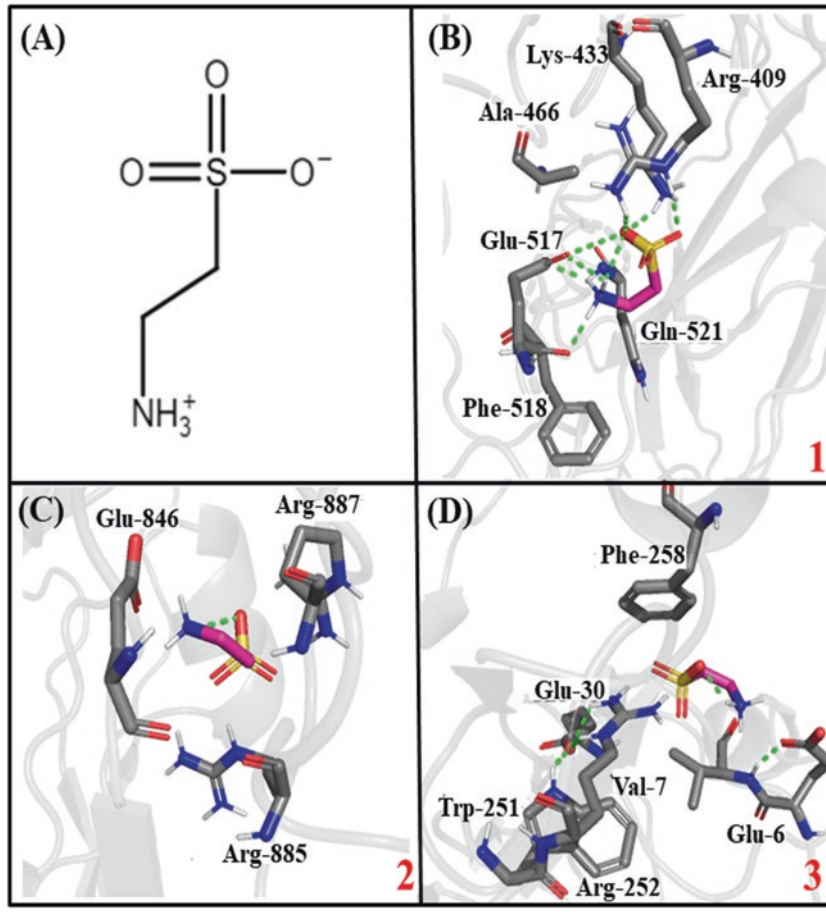

Fig. 3 Docking of taurine in its ionized state with the IR ECD. Chemical structure of taurine in the ionized state (**a**), interactions of taurine in pocket 1 (**b**), pocket 2 (**c**), and pocket 3 (**d**) of the IR ECD. Carbon atoms of the amino acid residues of the IR ECD are represented in grey, taurine is shown in magenta in the three pockets, and hydrogen bonds are shown as dashed green lines

close to site 2 in pocket 1, with which insulin was found to interact (Fig. 4a, b). Taurine binding in the IR ECD was found to be at distances of 35.0 and 38.2 Å from the Tyr14 and Leu13 residues of the A chain of the insulin bound to the IR (Fig. 4d). In addition, a distance of 32.2 Å was observed between taurine and the Phe1 residue of the insulin B chain (Fig. 4d).

In pocket 3, taurine bound to a site close to site 1, at which insulin interacts (Fig. 4a, b). Distances of 27.0, 35.5, and 35.3 Å were observed between taurine and the residues Arg22, Glu21, and Tyr16, respectively, present in the insulin B chain (Fig. 4c). Pocket 2 is located at the end of the IR ECD, representing the possible site of interaction of taurine that is most distant from the sites of insulin interaction. Figure 4a and b and the detail of Fig. 4e demonstrate that there are no insulin molecule residues in this region. Interestingly, our molecular docking results indicate that taurine may interact with sites other than those occupied by insulin, suggesting that this amino acid may activate the IR in sites that are independent of those of insulin.

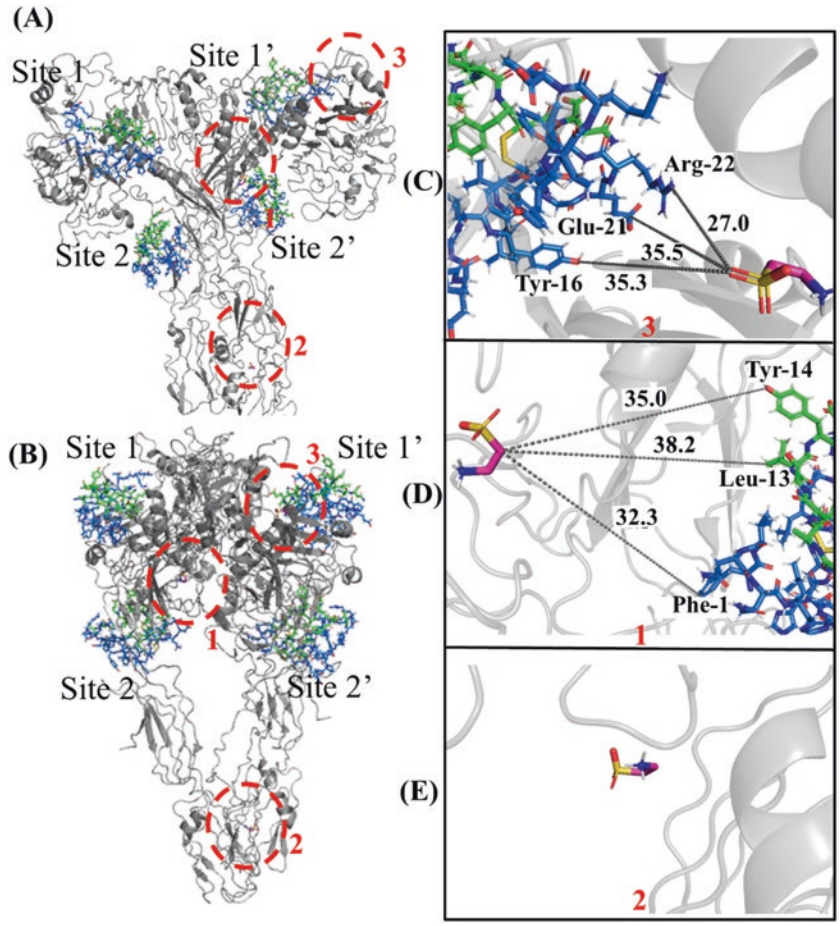

Fig. 4 Binding mode of taurine in the IR ECD. Cartoon of the IR represented in gray, taurine in the three pockets in magenta, insulin A chain in green, insulin B chain in blue, and distance in dashed grey lines. Frontal (**a**) and lateral (**b**) view of IR ECD with the possible sites of the interaction of taurine (within red circles). Distance between taurine and insulin bound in their interactions sites in pocket 3 (**c**), pocket 1 (**d**), and pocket 2 (**e**) of the IR ECD

4 Discussion

Various scientific reports have demonstrated that acute or chronic taurine administration to rodents promotes hypoglycemic actions (Kulakowski and Maturo 1984; Tenner Jr et al. 2003; Leo et al. 2004; Kim et al. 2012). This effect has been associated with a potential binding of the amino acid to the IR (Maturo and Kulakowski 1988; Carneiro et al. 2009). However, the binding sites and mode of interaction of taurine on the IR have not yet been demonstrated.

Our study, using in silico experiments, provided novel evidence that this amino acid may be an agonist of the IR, interacting with the ECD of this receptor and with higher binding energy/affinity to the IR pocket 1. In addition, within the three largest pocket-predicted sites of interaction of taurine on IR, this amino acid bound to the IR ECD in regions near, although outside of, the region in which insulin binds to the IR. This finding is of interest, as a non-peptide IR agonist (known as 4548-G05) that causes conformational change in the IR ECD, leading to insulin signaling activation, also demonstrated binding to the

IR ECD in regions that differ from the region of insulin interaction. Additionally, this agonist interacts in the hinge region between the CR and L2 domains of IRα (Qiang et al. 2014). Thus, despite binding at different sites of the IR ECD to those of insulin, taurine may activate this receptor, because similarly to 4548-G05, this amino acid has high affinity for the L2 domain (present in pocket 1). Furthermore, although taurine bound to different amino acid residues to those reported for the 4548-G05 IR agonist, taurine may induce IR activation, as previous studies have demonstrated that taurine has insulin-mimetic actions. For example, intraperitoneal taurine administration, prior to a glucose tolerance test in Wistar-Kyoto rats, caused a minor increase in blood glucose levels during the test (Kulakowski and Maturo 1984), and intravenous taurine administration to *Swiss* mice increased IRβ phosphorylation in the liver and skeletal muscle to levels similar to those induced by intravenous insulin administration (Carneiro et al. 2009). Therefore, the predicted regions of taurine binding on the IR, identified herein, may contribute to explain, at least in part, the several beneficial organic actions of taurine, potentially mediated by PI3K-AKT pathway activation (Takatani et al. 2004; Sun et al. 2018, 2020).

Although we found lower binding energies of taurine in pockets 2 and 3 of the IR ECD, when compared to pocket 1, it is possible that for taurine to induce IR conformational change, leading to IRβ activation, more than one taurine molecule needs to be bound to the IR. This hypothesis is supported by evidence showing that insulin binding to IR induces a large conformational change in the IR ECD, leading to a structural conversion of the IR from the autoinhibited inverted "V"/"U" shape to the "T" conformation, which approximates the membrane domains of the IR and allows its contact and activation (Scapin et al. 2018; Weis et al. 2018; Gutmann et al. 2018). In addition, Uchikawa et al. (2019) reported that the insulin-IR complex at the "T" activated shape binds with four insulins. These findings, taken together with our molecular docking analyses, indicate various possibilities for the interaction of taurine with the IR. It is also important to emphasize the possibility that taurine could occupy some of these pockets, while insulin docks other sites, which could contribute to the insulin-sensitizing action of taurine that is often reported in pre- and diabetic conditions (Kim et al. 2012; Ribeiro et al. 2012; Solon et al. 2012; Batista et al. 2013). Remarkably, an insulin sensitizer molecule was demonstrated to enhance insulin signaling in the presence of insulin by interacting with the tyrosine kinase domain of the IR, but not with the IR ECD (Manchem et al. 2001). As such, in addition to being an agonist of the IR, taurine may potentially interact with other regions of the IR to improve insulin sensitivity, a proposition that merits further investigation.

5 Conclusion

In summary, our study demonstrated novel information about the effects of taurine on insulin signaling. Molecular docking analyses predicted that taurine may interact with the three largest pocket sites of the IR ECD. Taurine presented the highest binding energy affinity to pocket 1 and lower binding energies to pockets 2 and 3 of the IR ECD. Notably, the sites at which taurine interacts on the IR are near to, but not the same as, insulin's binding sites. The findings of this study indicate that the effects of taurine on glucose homeostasis, namely, a reduction in glycemia and amelioration of the action of insulin, potentially involve an agonist interaction with the IR ECD.

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Dietary Protein Modulates the Efficacy of Taurine Supplementation on Adaptive Islet Function and Morphology in Obesity

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Abbreviations

AKT	Protein kinase B/thymoma viral proto-oncogene
C	Control
CH	C fed with HFD
CHT	CH supplemented with taurine
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase
HFD	High-fat diet
IGF-II	Insulin-like growth factor II
PCNA	Proliferating cell nuclear antigen
R	Restricted
RH	R fed with HFD
RHT	RH supplemented with taurine

1 Introduction

The global burden of obesity increases susceptibility to chronic diseases imposing crippling effects on healthcare systems. This complex pathophysiological state associates with a plethora of metabolic derangements, in which

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insulin resistance and pancreatic β-cell failure play a central role in the development of glucose intolerance and type 2 diabetes. The surge of obesity prevalence has been linked to nutritional transition, in which increased access to relatively inexpensive and calorie-dense food together with reduced physical activity has become the dominant lifestyle. Such transition can be especially harmful in developing countries, where interactions between poor nutrition during in utero development and food oversupply after birth may increase the risk of chronic non-communicable diseases later in life (Shetty 2013; Ssentongo et al. 2021).

Experimental modeling in rodents has demonstrated that exposure of pregnant dams to a protein-restricted diet malprograms glucose homeostasis in the offspring, predisposing them to insulin resistance and pancreatic β-cell dysfunction in adulthood (Ozanne et al. 2005; Bol et al. 2009; Sandovici et al. 2011; Theys et al. 2011; Berends et al. 2018). In addition to in utero development, we and others have investigated the effects of dietary protein restriction on islet function and peripheral insulin sensitivity from weaning until adulthood. In mice, post-weaning protein restriction leads to decreased body weight, reduced insulin secretion from pancreatic β-cells, and increased glucagon release from α-cells, with a concomitant increase in peripheral insulin sensitivity and glucose tolerance that contributes to normoglycemia (Soriano et al. 2010; Marroquí et al. 2012; Vettorazzi et al. 2014). While protein restriction after weaning does not enhance high-fat diet (HFD)-induced expansion of adipose tissue, these mice display catch-up growth and higher shifts toward glucose intolerance and insulin resistance relative to non-obese protein-restricted mice (Batista et al. 2013a; Vettorazzi et al. 2014).

Taurine is a semi-essential amino acid that has been linked to the maintenance of glucose homeostasis in a variety of pathophysiological situations by exerting pleiotropic effects on beta β-cell physiology (Carneiro et al. 2009; Ribeiro et al. 2009, 2010). Under low-protein conditions, taurine supplementation effectively prevents functional impairment of β-cells by restoring expression of cholinergic receptors that potentiate insulin release and proteins involved in insulin granule exocytosis (Batista et al. 2011). On the other hand, protein restriction largely blunts the beneficial effects of taurine on preventing HFD-induced dysregulation of insulin secretion (Vettorazzi et al. 2014; Branco et al. 2017). While resistance to the beneficial effects of taurine supplementation on insulin release caused by protein restriction has been linked to impaired expression of critical regulators of the stimulus-secretion coupling in β-cells (Vettorazzi et al. 2014), whether this also involves changes in islet morphology remains unclear.

Here, we investigated the effects of taurine supplementation on insulin secretion and islet morphology of protein-restricted mice submitted to HFD in adulthood. We find that under normal protein conditions, taurine supplementation prevents obesity-induced insulin hypersecretion in association with increased islet and β-cell areas and increased expression of proliferation markers in pancreatic islets. Conversely, protein restriction blunted the effects of taurine on islet function and morphology. Thus, impairment of islet plasticity by suboptimal nutrition may underlie increased susceptibility to metabolic abnormalities associated with obesity.

2 Methods

2.1 Animals and Diets

All experimental procedures were approved by CEUA UNICAMP (number 1942-1). Male *C57Bl/6* mice weaned at 30 days after birth were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB) at UNICAMP. All animals were kept at 23 °C, with a 12-h light-dark cycle, and had free access to water and food. The mice were randomly distributed into the following groups: control (C) (mice fed on a normal protein diet (14%)) and restricted (R) (mice fed on a low protein diet (6%), n = 3). After 6 weeks, groups C and R mice randomly received, or not, high-fat diet (HFD, 35% fat) for 8 weeks (CH and RH).

Half of HFD-fed mice were supplemented, or not, with 5% taurine in their drinking water since weaning (CHT and RHT). Diets were manufactured according to the American Institute of Nutrition (AIN) guidelines for adult maintenance (Reeves et al. 1993; Batista et al. 2013a) and purchased from Pragsoluções (Jaú, SP). All the experimental procedures were performed after 14 weeks of treatment and are depicted in Fig. 1.

2.2 Pancreatic Islets Isolation

At the end of the experimental period, all mice were weighed and euthanized by decapitation, and a laparotomy was performed to access the liver that was retracted to expose the gallbladder and the proximal portion of the common bile duct. Using a surgical thread, the pancreatic duct was obstructed at the height of Vater's ampulla. In the distal portion of the common bile duct, below the gallbladder, a small incision was made in the duct to introduce an insulin needle, through which 2–3 mL Hanks' solution containing 0.8 mg/mL collagenase type V (Sigma-Aldrich, St. Louis, MO, USA) were injected into the pancreas for digestion of the exocrine pancreatic tissue. The pancreas was removed from the abdominal cavity by dissection and transferred to a 15-mL tube, kept at 37 °C for 11 min. At the end of this period, a brief 30-second agitation was performed to facilitate the breakdown of pancreatic tissue. Tissue digestion was stopped by adding Hank's solution at 4 °C. The tubes were centrifuged, and the supernatant discarded three times for removal of collagenase. The islets, completely separated from the acinar tissue, were collected one by one, under a magnifying glass, by aspiration with a *Pasteur* pipette, previously stretched and siliconized.

2.3 Static Insulin Secretion

Groups of four pancreatic islets from the different experimental groups were transferred to 24-well culture plates containing 0.5 mL Krebs buffer supplemented with 0.3% bovine albumin (m/v) and 5.6 mM glucose. The plates were incubated at 37 °C in a humidified and aerated (95%

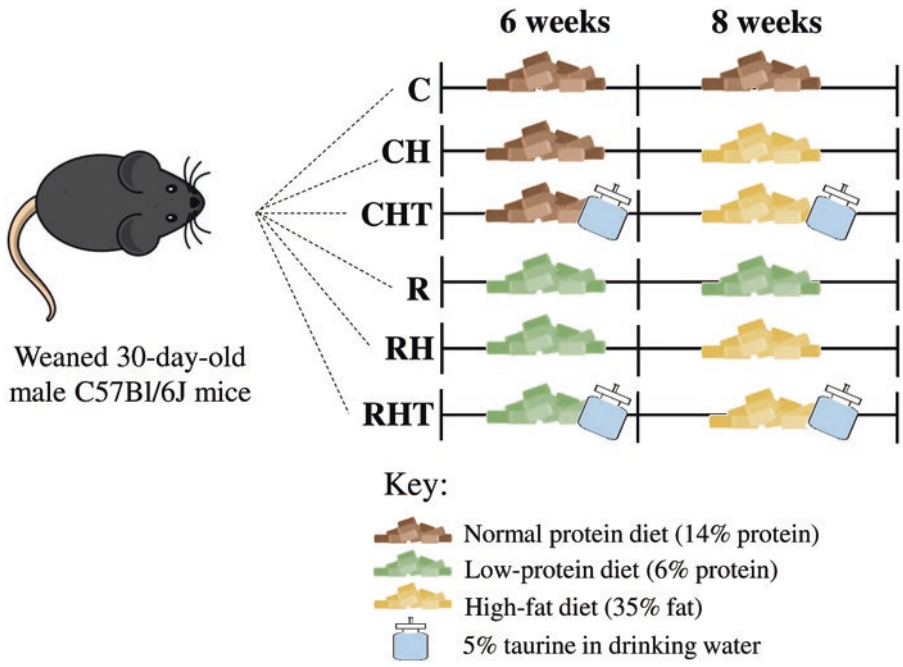

Fig. 1 Experimental animal model design (*for description see "Methods" section*)

O_2/5% CO_2) stove, for 45 min. The solution of pre-incubation was quickly removed and replaced with a new Krebs solution containing 2.8, 11.1, or 22.2 mM glucose. After 1 h of incubation, the plates had the supernatant removed, transferred to the test tubes, and stored at −20 °C for subsequent insulin dosage by radioimmunoassay (Ribeiro et al. 2009).

2.4 Insulin Immunohistochemistry

After euthanasia, the pancreas of all mice was collected and weighed. The pancreas was fixed in Bouin's solution (25% formaldehyde, 75% saturated picric acid, and 5% acetic acid) for 16 h. Then, each pancreas was dehydrated in an ascending series of ethanol, diaphanized in xylene, and embedded in paraffin (Histosec pastilles, Merck). Semi-serial sections of 5 μm were obtained and submitted to insulin immunohistochemistry. For this, the pancreas sections were deparaffinized and hydrated and, after blocking the endogenous peroxidase [with 0.05 M Tris-buffered saline (TBS) pH 7.4 containing 0.3% H_2O_2] and the non-specific binding sites (with 5% skimmed milk and 0.1% Tween 20 in TBS), were incubated with anti-insulin antibody (1:50 dilution in TBS containing 3% skimmed milk; Dako North America, Inc., CA, USA) overnight at 4 °C. Afterward, the pancreas sections were incubated with rabbit anti-guinea pig IgG-conjugated antibody with HRP for 1 h and 30 min. The positive insulin cells were detected with diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) solution (10% DAB and 0.2% H_2O_2 in TBS). Subsequently, the section slides were counterstained with Ehrlich hematoxylin, dehydrated, diaphanized, and mounted with synthetic balm. The islets were photographed with a light microscope (Nikon Eclipse E800) coupled with a digital camera (Nikon FDX-35). Finally, the captured images were analyzed with the Image-Pro Plus for Windows software to analyze the islet, β-, and non-β-cell areas (Ribeiro et al. 2012).

2.5 DNA Fragmentation

Groups of 20 islets isolated from each mice were submitted to intact and fragmented DNA extraction using trizol/triton method (Santos et al. 2011). Both fractions were quantified using a standard DNA curve and the fluorescent dye SYBR green. Total DNA amount per islet was obtained by the sum of the intact and the fragmented DNA extracted. Fragmentation data are expressed as a percentage of fragmented DNA in relation to total DNA.

2.6 Western Blot

Pancreatic islets were homogenized with a sonicator (Brinkmann Instruments, Westbury, NY, USA) in an extraction buffer containing Tris pH 7.5100 mmol/L, sodium pyrophosphate 10 mmol/L, sodium fluoride 100 mmol/L, EDTA 10 mmol/L, sodium orthovanadate 10 mmol/L, PMSF 2 mmol/L and 10 μg/mL aprotinin, and 1% triton-X 100. The samples were centrifuged at 12,000 rpm for 15 min. From the collected supernatant, an aliquot was used for protein quantification by the method of Bradford (1976). The samples were then incubated at 100 °C for 5 min in 25% volume of Laemmli buffer. For electrophoretic running, a biphasic gel was used: stacking gel (4 mM EDTA, 2% SDS, 750 mM Trizma base, pH 6.7) and resolution gel (4 mM EDTA, 2% SDS, 50 mM Trizma base, pH 6.7). The run was carried out at 90 V for approximately 180 min with running buffer containing tris base 200 mM, glycine 1.52 M, EDTA 7.18 mM, and SDS 0.4%. Samples were transferred to a nitrocellulose membrane (Bio-Rad) for 120 min at 120 V on ice, bathed in 25 mM tris base and 192 mM glycine. Afterward, the membranes were blocked with 5% skimmed milk in TBS for 2 h at room temperature or overnight at 4 °C.

Then the membranes were washed three times for 5 minutes and incubated overnight at 4 °C with TBS containing 3% bovine with the following primary antibodies: PCNA (1:1000; Cell

Signaling Technology, Danvers, MA, USA), p-threonine 308 (T308) AKT (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), AKT (1:1000; Santa Cruz), p-ERK1/2 (1:1000; Santa Cruz), or ERK1/2 (1:1000; Santa Cruz). Detection was performed after a 1 h incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution in TBS containing 5% skim milk). The intensity of the bands was evaluated by densitometry with Image J software (NIH, USA), and the values were expressed in fold over the control group. Phosphorylated proteins were normalized to their total form, and PCNA was normalized to the optical density of the band pattern after staining with Ponceau-S solution (Sigma-Aldrich, St. Louis, MO, USA).

2.7 Statistical Analysis

The results were expressed as means ± standard error of the means (SEM). The effect of protein restriction (C vs R) was analyzed by Student's t test and the effects of diet-induced obesity and taurine supplementation was analyzed using one-way (ANOVA) between three groups comprising each dietary protein background (C, CH, CHT or R, RH, RHT) followed by Tukey's post-test using Graph Pad Prism software. The significance level adopted was $P < 0.05$.

3 Results

3.1 Model Characterization

In our model, the low protein diet produced the expected decrease in body weight ($P < 0.001$, C vs R), while HFD induced the expected increases in body weight in both normal protein (C, 26.50 ± 0.08 g; CH, 30.15 ± 1.80 g; $P < 0.01$) and low-protein-fed mice (R, 18.50 ± 0.80 g; RH, 25.70 ± 1.40 g; $P < 0.01$) compared to their respective controls. In mice fed a normal protein diet, taurine supplementation partially prevented HFD-induced obesity, because bodyweight in CHT mice (CHT, 27.4 ± 1.40 g) was not statistically different from CH or C groups. Nevertheless, this protective effect was not observed in low-protein-fed mice, which exhibited body weight (29.00 ± 1.40 g) similar to RH but higher than R group ($P < 0.001$).

3.2 Glucose-Stimulated Insulin Secretion

Freshly isolated islets stimulated with basal, intermediate, and high glucose concentrations (2.8, 11.1, and 22.2 mM) showed a typical dose-response of insulin release (Fig. 2). At all studied conditions, insulin release adjusted for islet DNA content was decreased in R mice by 40% at basal and by 60% at stimulatory conditions, compared to C mice. In normal protein-fed CH mice, diet-induced obesity led to a significant increase of glucose-induced insulin release by 1.5-fold at 11.1 mM compared to C mice and an increasing trend of 1.25-fold at 22.2 mM, though this was not statistically significant. Despite the impairing effects of the low-protein diet on insulin release, in response to diet-induced obesity, islets from RH group showed increases of 2.5-fold and 3.5-fold at 11.1 mM and 22.2 mM conditions, compared to R mice. Notably, taurine supplementation prevented insulin hypersecretion from islets of normal protein-fed CHT mice, but not in RHT mice.

3.3 Pancreatic Islet Morphology

To determine whether the effects of taurine supplementation on insulin secretion in our obesity model were linked to morphological alterations of the endocrine pancreas, we performed insulin immunostaining of pancreas sections for all studied groups (Fig. 3a). Morphometric analysis revealed that pancreas weight from R mice was reduced by 53% compared to C (Fig. 3b), and this persisted even after adjustment to total body weight (C, 0.77 ± 0.02%; R, 0.48 ± 0.2%; $P < 0.001$). Diet-induced obesity led to increased pancreas weight by 30% in CH and by 95% in RH mice, compared to C and R groups, and this was largely unaffected by taurine supplementa-

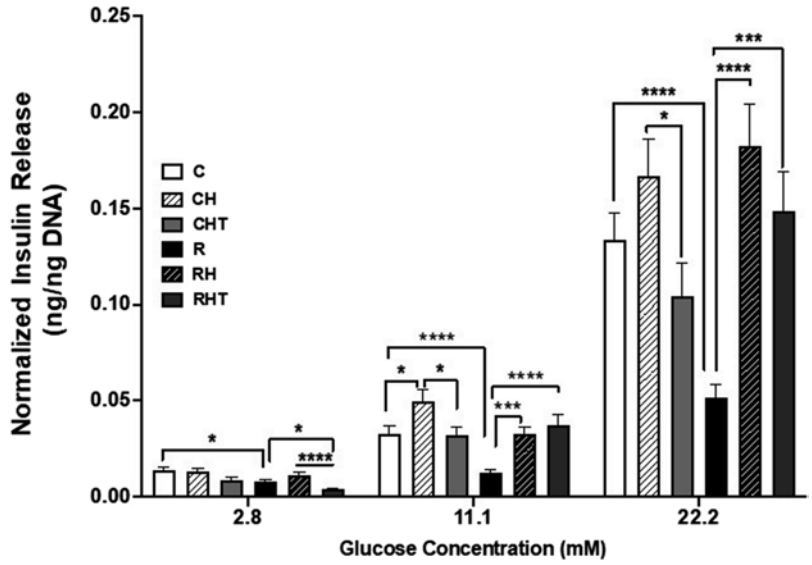

Fig. 2 Glucose-stimulated insulin release from islets of C, CH, CHT, R, RH, and RHT mice. Groups of four islets were pre-incubated in Krebs solution with 5.6 mM glucose for 45 min, following stimulation with indicated glucose concentrations for 1 h. Data are mean ± SEM, n = 27–32 groups of islets per dietary condition from four independent experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$

tion. Body mass-adjusted pancreas weight of HFD-fed normal protein (CH, 0.73 ± 0.05%; CHT, 0.74 ± 0.04%) and low-protein mice (RH, 0.61 ± 0.03%; RHT, 0.52 ± 0.04%) was not significantly different from lean C and R mice. Despite striking reduction of glucose-stimulated insulin secretion and pancreas weight in R mice, total islet (Fig. 3c) and β-cell areas (Fig. 3d) were unchanged. Under normal protein conditions, diet-induced obesity led to a non-significant increase in islet and β-cell areas of CH mice by 14–17%, while in CHT mice, this effect was enhanced to 32% and statistically significant compared to C (Fig. 3c and d). Under low-protein conditions, diet-induced obesity or taurine supplementation did not impose significant alterations on the morphology of the endocrine pancreas. The non-β cell area, which indicates cells within islet boundaries not stained by the anti-insulin antibody, was not changed between groups (Fig. 3e).

3.4 DNA Fragmentation and Proliferation Markers

To gain more insight into the molecular mechanisms that underlie the functional and morphological adaptations of pancreatic islets in our obesity model and its regulation by taurine, we have measured the expression of cell proliferation markers, DNA integrity, and growth factor signaling pathways in freshly isolated islets (Fig. 4).

Expression of proliferating cell nuclear antigen (PCNA) was unchanged in islets of R mice compared to C (Fig. 4a). In normal protein conditions, PCNA expression increased by 2.2-fold in islets of CH mice, although this was not statically significant ($P = 0.3$), while in islets from CHT mice, PCNA expression was significantly elevated by 3.3-fold ($P < 0.05$). No significant changes on PCNA expression were observed in islets from low protein HFD-fed mice.

Since PCNA expression has also been linked to DNA repair (Essers et al. 2005), we measured DNA fragmentation relative to total DNA content (Fig. 4b). In islets from R mice, DNA fragmentation was reduced by 36% compared to C mice. In low-protein HFD-fed mice, DNA fragmentation was increased by 1.36-fold in RH islets ($P < 0.05$), and this was prevented in RHT islets. Under normal protein conditions, no significant effects of diet-induced obesity or taurine supplementation on islet DNA fragmentation were observed. Finally, no changes were observed on basal phosphorylation levels of AKT (Fig. 4c) and ERK (Fig. 4d) proteins.

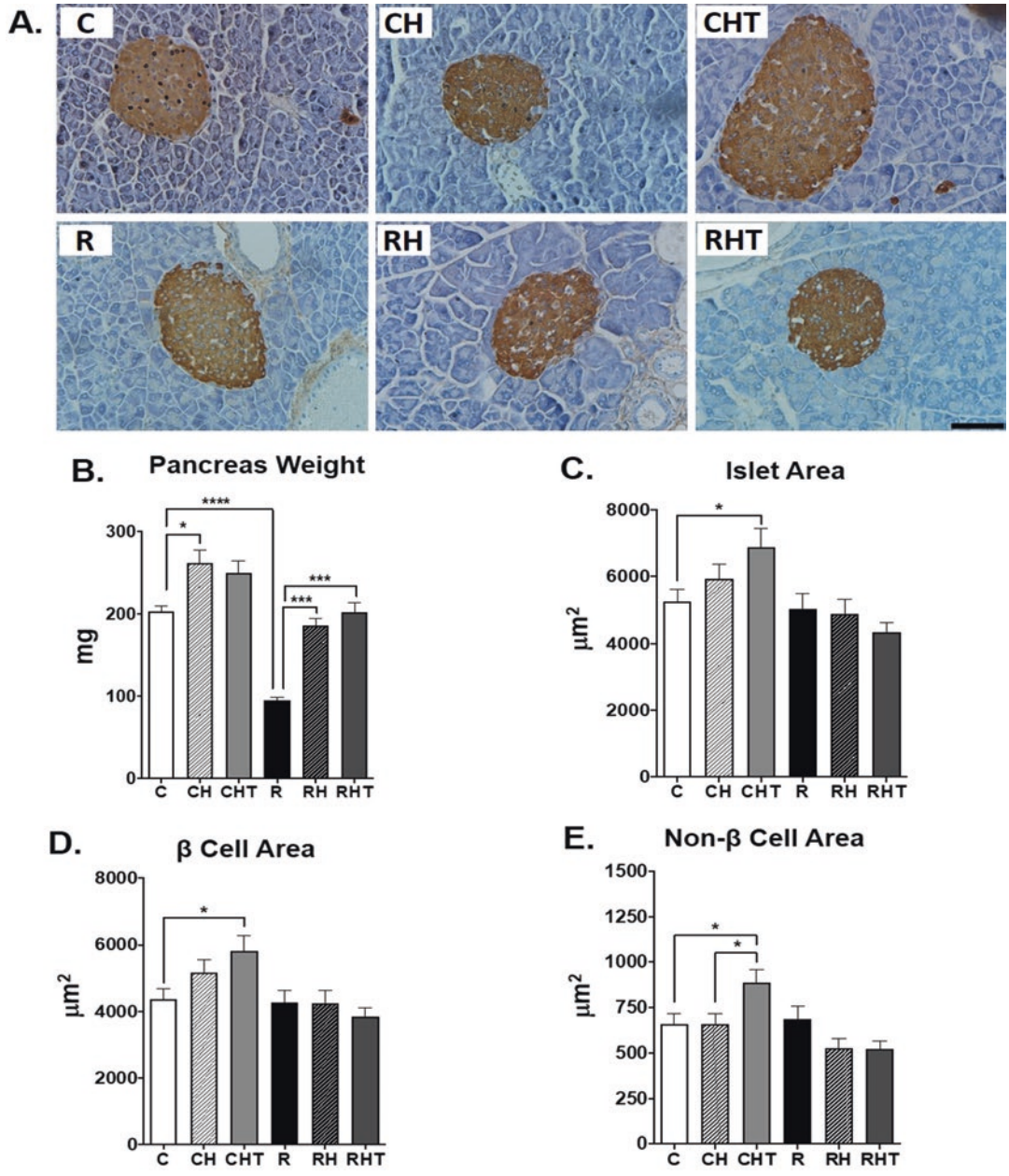

Fig. 3 Pancreatic islet morphology. (**a**) Representative images of islet immunolabeled for insulin (brownish cells = positive DAB staining) in pancreas sections of C (n = 464), CH (n = 507), CHT (n = 574), R (n = 265), RH (n = 490), and RHT (n = 644) mice. Parenthesis indicates the number of islets analyzed. (**b**) Scale bar = 50 µm. Pancreas weight. (**c**–**e**) Area size of islets (**c**), β-cells, (**d**) and non-β cells (**e**). Data are mean ± SEM, n = 3–7 pancreases per group. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$

4 Discussion

Obesity is a complex state characterized by peripheral insulin resistance which, when combined with maladaptive factors that prevent compensatory increases in insulin secretion and expansion of β cell mass, may lead to overt hyperglycemia and type 2 diabetes (Inaishi and

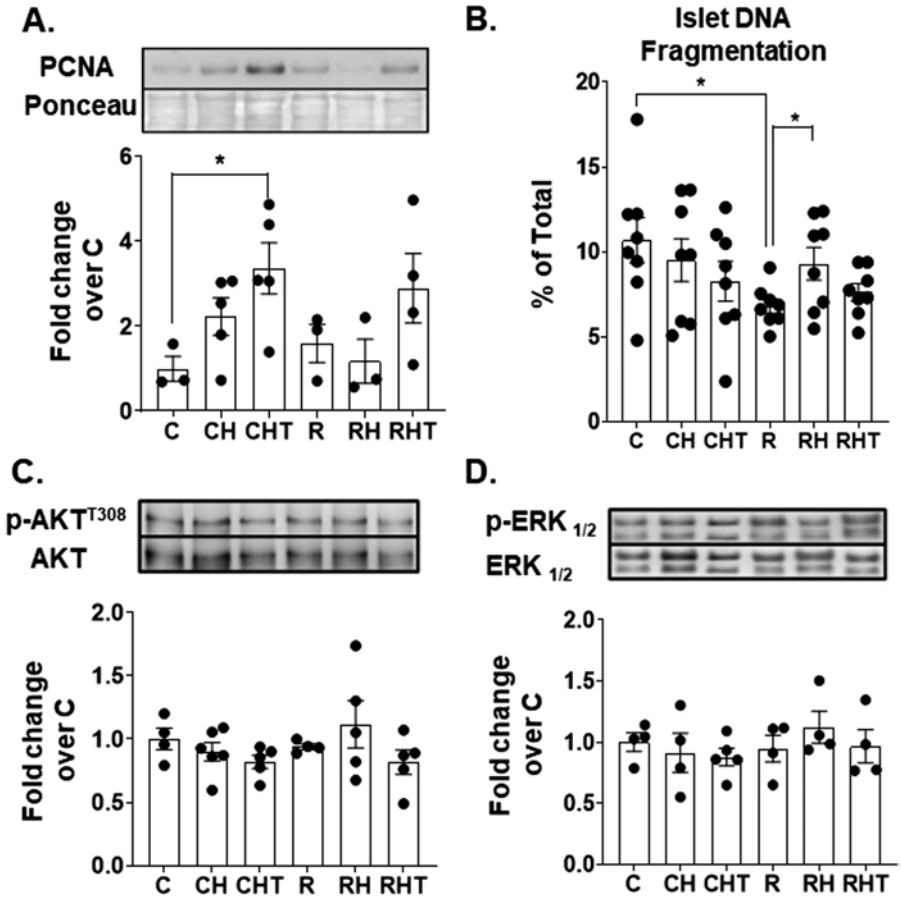

Fig. 4 (**a**) Protein expression of PCNA normalized by total Ponceau-S signal (n = 3–5). (**b**) DNA fragmentation analysis in pancreatic islets expressed as % of total DNA (n = 8 groups of 20 islets per dietary condition). (**c**, **d**) Basal phosphorylation of AKT and ERK1/2 normalized to total protein expression (n = 4–6). Data are mean ± SEM. * $P < 0.05$

Saisho 2020). Here, we have carried functional and morphometric assessment of the endocrine pancreas from protein-restricted mice subjected to diet-induced obesity, with or without taurine supplementation. We find that dietary protein restriction, after weaning, renders mice refractory to the effects of taurine supplementation on islet function and morphology during diet-induced obesity.

At the functional level, consumption of protein-restricted diet leads to reduced glucose-induced insulin secretion in agreement with previous reports in mice and rats (Batista et al. 2011; Vettorazzi et al. 2014; Branco et al. 2017). Despite prominent pancreatic hypotrophy in R mice, pointing to possible alteration of exocrine pancreas and digestive functions, the islet size of these mice remains unaltered. While in contrast with studies using protein-restricted rats, in which the islet and β-cell areas and insulin release are all reduced (Rafacho et al. 2009), this is consistent with previous reports of unchanged total islet DNA content in this model (Branco et al. 2017) and possibly linked to reduced DNA fragmentation shown in this study. Although in our model mice were kept under protein restriction for 6 weeks longer and fed diets designed for maintenance as opposed to the growth diet used by Rafacho et al. (2009), which has more protein and fat (Reeves et al. 1993) and could explain

some of the differences between studies, there is a possibility that the impact of protein undernutrition on islet morphology varies across species.

Insulin resistance is a central feature of pathophysiological states such as diet-induced obesity and imposes complex functional and morphological adaptations on pancreatic β-cells. In this setting, increased β-cell mass and function initially compensate for impaired insulin action but progressively decline due to glucolipotoxicity and increased apoptosis resulting in decreased β-cell mass, islet dysfunction, and hyperglycemia (Hudish et al. 2019; Roden and Shulman 2019). In our study, diet-induced obesity and insulin resistance cause insulin hypersecretion in both dietary protein regimens, but only in RH mice this was associated with higher DNA fragmentation per islet, which was not accompanied by an augmentation in cellular proliferation or DNA repair, since PCNA protein content in RH islets is not changed (Essers et al. 2005). While RH mice develop insulin resistance to a similar extent as normal protein obese mice, it is possible that increased DNA fragmentation in RH islets results from relatively higher functional demand due to a transition from increased insulin sensitivity, such as in R, to impaired insulin action (Batista et al. 2013a). These functional adaptations to insulin resistance, combined with impaired remodeling of islet morphology, could lead to an imbalance between proliferation and apoptosis and represent a point of vulnerability to accelerate β-cell demise, as observed in mice lacking expression of the insulin receptor or downstream effectors specifically in β-cells (Okada et al. 2007; Shirakawa et al. 2017). In addition, insulin-secreting INS-1E cells cultured under low amino acid concentrations show increased ER stress-induced apoptosis (Gonçalves et al. 2019), suggesting that protein insufficiency can also have primary effects on β-cell survival. Interestingly, this effect on DNA fragmentation is prevented in islets from RHT mice in alignment with previously reported roles of taurine in regulating apoptosis and DNA synthesis in islets from protein malnourished rats (Boujendar et al. 2002). While the mechanisms underlying the antiapoptotic action of taurine in the context of obesity and dietary protein restriction remain unresolved, the restoration of mitochondrial metabolism and anti-oxidant functions are likely to be involved (Jong et al. 2021).

In accordance with previous reports (Ribeiro et al. 2012; Vettorazzi et al. 2014; Branco et al. 2017), taurine supplementation prevents HFD-induced insulin hypersecretion at stimulatory glucose concentrations only in isolated islets from CHT mice, but not in RHT group. The lack of beneficial effects of taurine supplementation on islet function in RHT mice is possibly linked to the adverse effects of protein restriction upon HFD-induced taurine transporter expression and taurine concentration in islet cells (Branco et al. 2017), thus impacting intracellular signaling events involved in the regulation of mitochondrial metabolism, β-cell survival, and expansion. Some of the protective effects of this amino acid on pancreatic islets under metabolic stress have been proposed to occur through maintenance of insulin-like growth factor II (IGF-II) expression (Petrik et al. 1999; Arany et al. 2004). In the intra-islet medium, this hormone maintains the normal balance between apoptosis and survival of endocrine cells in the pancreas and also increases insulin secretion upon demand, as occurs in insulin resistance induced by an HFD (Haeften and Twickler 2004; Modi et al. 2015).

Our study also provides novel information about islet morphological adaptations during obesity and its regulation by taurine. Despite obesity-induced increases in pancreas weight, this does not result in altered islet or β-cell areas. In contrast, these morphological parameters are increased in taurine-supplemented CHT, but not RHT mice. Previously, we have demonstrated that in male and female *Swiss* mice fed on a HFD taurine supplementation prevents islet hyperfunction and hypertrophy (Ribeiro et al. 2012). Therefore, our data suggest that such regulatory action of taurine and protein restriction on islet morphology can differ between mouse strains, as a result of distinct genetic backgrounds and propensity to insulin resistance (Parks et al. 2015).

The increased islet size promoted by taurine supplementation in CHT mice may be linked to

increased cell proliferation, since PCNA protein levels are increased without changes in DNA fragmentation, while no evidence of altered proliferation are found in RHT islets. Remarkably, this effect was not accompanied by changes in AKT or ERK1/2, kinases involved in cell multiplication, survival, and growth (Jiang et al. 2018). However, the involvement of these pathways on cell proliferation and islet morphology cannot be ruled out, since their activation was only assessed at basal conditions and without acute stimulation with insulin or taurine (Carneiro et al. 2009; Ribeiro et al. 2012; Batista et al. 2013a, b). These refractory effects to taurine action in RHT mice likely arise from complex interactions between protein malnutrition and obesity, since in lean rat offspring from dams fed on protein-restricted diet, during gestation and lactation, taurine supplementation prevented the reduction in islet area and proliferation (Boujendar et al. 2002). Similar action was observed in the endocrine pancreas of offspring of non-obese diabetic dams that were taurine-supplemented during gestation and lactation (Arany et al. 2004).

While our study forwards the notion of taurine as a bioactive molecule with important antidiabetic properties, to which extent these result from regulation of insulin action in peripheral tissues to modulation of leptin signaling and neurotransmitter release in the brain or to direct actions in β-cells remains a challenging issue to define and should be considered in future studies.

5 Conclusion

In summary, protein restriction in weaned *C57Bl/6* male mice decreases glucose-induced insulin secretion, without modification in islet or β-cell areas possibly supported by reduced DNA fragmentation. Under metabolic stress caused by an HFD, insulin hypersecretion in islets from protein-restricted mice results in increased DNA fragmentation, alerting to an increased susceptibility to accelerate β-cell loss. While taurine supplementation prevents HFD-induced insulin hypersecretion in controls, and this is linked to expansion of the functional β-cell pool, these effects were largely blunted in mice undergoing protein restriction. Thus, in addition to the well-established catch-up growth features imposed by protein restriction, here we provide novel evidence that resistance to agents with therapeutic properties such as taurine represents an additional layer of vulnerability imposed by suboptimal nutrition, especially when individuals are exposed at puberty.

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Taurine and Exercise: Synergistic Effects on Adipose Tissue Metabolism and Inflammatory Process in Obesity

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Keywords

Taurine · Exercise · Therapeutic strategy · Obesity · Adipose tissue

Abbreviations

ACO1	Aconitase 1
ACO2	Aconitase 2
ACOX1	Acyl-CoA oxidase-1
ATP	Adenosine triphosphate
CDO	Cysteine dioxygenase
CIDEA	Cell death activator
CK	Creatine kinase
CRP	C-reactive protein
CPT1a	Carnitine palmitoyltransferase 1a
CSAD	Cysteinesulfinic acid decarboxylase
cycs	Mitochondrial cytochrome c
HOCl	Hypochlorous acid
HSL	Hormone-sensitive lipase
IL	Interleukin
LPL	Lipoprotein lipase
M1	Macrophage type 1
M2	Macrophage type 2
MPO	Myeloperoxidase
mtTFA	Mitochondrial transcription factor A
NO	Nitric oxide
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1α
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PRD1-BF1-RIZ1 homologous domain-containing protein 16
scWAT	Subcutaneous white adipose tissue
TauCl	Taurine chloramine
TNF	Tumor necrosis factor
UCP	Uncoupling protein

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1 Introduction

It is well established that obesity is characterized by a low-grade chronic inflammatory state, which is associated with hypertrophic adipocytes,

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increased infiltration by macrophages, and higher secretion of adipokines and free fatty acids (Murakami 2017). Although the anti-obesity action of taurine is still undefined, taurine seems to be a good strategy for the treatment of obesity through its capacity of membrane stabilization; also its metabolic actions of taurine, such as modulation of energy expenditure, carbohydrate, and lipid metabolism (Murakami 2017); and its anti-inflammatory and antioxidant effects (Schaffer et al. 2010; Schuller-Levis and Park 2003).

In 1968, Jacobsen and Smith (1968) published one of the first reviews that explored the physiological function of taurine and its derivatives in animal and human physiology to clarify the function and fate of the various sulfur-containing compounds that they called as "unfinished mosaic." The review brought new information to the literature regarding taurine levels in animals and humans, highlighting the most crucial effect of taurine in its conjugation with bile acids, regulation of fat absorption and elimination, and osmoregulation capacity. However, they concluded that much remains to be learned about the origin and function of taurine in biological systems (Jacobsen and Smith 1968).

Nowadays, many investigations about taurine's effects in animals and humans have been published so far. Although studies show positive effects for obesity treatment in animals, the therapeutic effects of taurine in the treatment of human obesity are still an "unfinished mosaic," in particular its effects on human adipose tissue.

The comprehension of taurine synthesis and tissue distribution is vital to understand the physiologic roles of taurine in the development and maintenance of organisms. In 2002, Ide et al. (2002) evaluated taurine concentration. The authors quantified the enzymes involved in taurine biosynthesis in different tissues in rats and observed that taurine concentrations were higher in the liver, brain, lung and, kidney but very low in white and brown adipose tissue. Regarding taurine biosynthesis, the study showed that mRNA levels of cysteine dioxygenase (CDO) and cysteinesulfinic acid decarboxylase (CSAD) enzymes were detected in the brain, lung, liver, and kidney, and it was highly expressed in white and brown adipose tissues of rats. However, the level of the activity of the enzyme was high only in the liver. It was lower in the adipose tissue but much higher than the activity observed in the brain, lung, and kidney. Although there was some evidence of taurine biosynthesis in adipose tissue, the results did not confirm a role of adipose tissues in taurine biosynthesis (Ide et al. 2002).

It is essential to consider that taurine action is not necessarily related to the taurine synthesis site. Therefore, it is necessary to explore enzymes involved in taurine synthesis and the distribution of taurine transporters among tissues to understand the mechanism that regulates tissue taurine levels (Ide et al. 2002).

Recently, De Carvalho and colleagues investigated the effects of taurine supplementation on the content of proteins of the taurine synthesis pathway in 3T3-L1 cells and human subcutaneous white adipose tissue (scWAT). It was found that taurine-treated 3T3-L1 cells differentiated in adipocytes (0.25 mM, 0.5 mM, and 1 mM doses of taurine), with increased protein content of the enzyme CSAD but not CDO. However, the supplementation of taurine (3 g) did not increase the protein content of taurine biosynthetic enzymes in the scWAT of obese women (De Carvalho et al. 2021c).

Furthermore, Guo et al. (2019) showed that taurine-treated mice improved insulin sensitivity, alleviated high-fat diet-induced obesity, and upregulated energy metabolism genes in white adipose tissue leading to higher energy expenditure. In humans, taurine supplementation can reduce the plasma concentrations of inflammatory and oxidative markers and increase the plasma concentration of adiponectin (Rosa et al. 2014) and irisin release post-exercise (Batitucci et al. 2019). In the human scWAT, our research group showed that the association of taurine with exercise upregulated the genes involved in mitochondrial function and lipid oxidation (De Carvalho et al. 2021a).

Therefore, the present review sought to explore the effects of taurine supplementation associated with physical exercise as an excellent strategy for treating and preventing obesity.

Exercise is considered a helpful therapeutic strategy for the prevention and treatment of obesity. As such, gaining a better understanding of the molecular pathways activated in response to taurine supplementation when associated with exercise will advance our understanding of potential treatments of metabolic diseases such as obesity and type 2 diabetes.

1.1 Taurine Anti-inflammatory Effects in Adipose Tissue

Metabolic dysfunction promoted by obesity is characterized by increased release of adipokines that activate the pro-inflammatory pathway in the adipose tissue, leading to impaired mitochondrial metabolism and oxidative damage in the adipocytes, which are related to a low-grade chronic inflammatory state and the development of metabolic diseases such as insulin resistance, diabetes, heart disease, and other metabolic disturbances (Fuentes et al. 2013; Mitchell et al. 2013). Both human and rodent obesity are associated with a reduction in mitochondrial content and capacity, which suggests a cause-effect relationship between mitochondrial dysfunction and functional abnormalities of adipose tissue such as disarrangements in the regulation of storage and release of fatty acids, adipocyte hypertrophy and hyperplasia, and production of cytokines, leading to metabolic stress (Heilbronn and Campbell 2008; Huh et al. 2014; Koh et al. 2007).

The impact of taurine in adipose tissue is its requirement for obesity pathogenesis (Murakami 2015, 2017). Low blood levels of taurine are reported in metabolic dysfunctions such as obesity and type 2 diabetes mellitus (Rosa et al. 2014; Zheng et al. 2016). Obesity is associated with taurine deficiency due to a reduction in taurine synthesis in adipose tissue, mainly in preadipocyte differentiation into mature adipocytes in obese individuals with hypertrophic and hyperplasic adipocytes (Murakami 2017).

The physiological role of taurine in adipose tissue under conditions of obesity has been explored, and some studies have shown that taurine can be a strategy for the treatment of obesity in genetically and high-fat diet-induced animal models of obesity (Guo et al. 2019; Kim et al. 2019, 2020) and obese humans (De Carvalho et al. 2021a, b; Murakami, 2017; Wen et al. 2019). Even though the anti-obesity effect of taurine remains unclear, these studies provide critical perspectives for a better understanding of the metabolic outcomes of taurine. Taurine seems to have multiple sites of action, and it is strongly associated with the inhibition of adipocyte inflammation (Murakami, 2017).

The anti-inflammatory effects of taurine are related to its capacity to conjugate with hypochlorous acid. In the inflammatory process, oxidant agents induce the release of chemotactic factors in the cellular membrane, activating the inflammatory response and producing hypochlorous acid (HOCl) via the myeloperoxidase (MPO) pathway by leukocytes. Taurine, the most abundant free amino acid, can scavenge HOCl to form taurine chloramine (TauCl), a more stable and less toxic compound. TauCl will be actively transported into leukocytes and downregulate the release of pro-inflammatory mediators such as nitric oxide (NO) and tumor necrosis factor (TNF), modulating the inflammatory process in the cellular nucleus (Schuller-Levis and Park 2003).

The effects of taurine on obesity-related inflammation had been investigated in mice fed a high-fat diet. The authors found that taurine treatment decreased the infiltration of macrophages and the release of pro-inflammatory cytokines and inhibited the development of hyperglycemia in diet-induced obese mice. Additionally, in vitro tests with bone marrow-derived macrophages indicated that taurine treatment promoted alternative M2 macrophage activation (anti-inflammatory) and inhibited classical M1 macrophage activation (pro-inflammatory) by the taurine chloramines (Lin et al. 2013).

In humans, because of the low-inflammatory grade of obesity, researchers explored taurine effects against obesity. The investigation of Rosa et al. (2014) showed that the supplementation of 3 grams for 8 weeks associated with nutrition counseling was able to increase the levels of the anti-inflammatory marker (adiponectin) and

decrease pro-inflammatory (C-reactive protein – CRP) and lipid peroxidation (thiobarbituric acid reactive substances – TBARS) blood markers. Maleki et al. (2020) showed that taurine (1g, three times/day) decreased inflammatory blood markers (interleukin-6 (IL-6), TNF-alpha, and CRP) after 8 weeks of supplementation in type 2 diabetes and obese individuals.

To verify the anti-inflammatory and antioxidant effects of taurine, a recent investigation supplemented 3 g of taurine for 8 weeks, associated or not with exercise, and evaluated body composition, oxidative stress, and inflammatory markers on blood and scWAT in obese women (De Carvalho et al. 2021b). After 8 weeks of intervention, although no changes were observed for the anthropometric characteristics, taurine blood levels increased. It was observed that taurine supplementation itself decreased interleukin-6 (IL-6), while, when associated with exercise, it increased anti-inflammatory interleukins (IL-15 and IL-10), followed by lower IL-1β gene expression in the scWAT of obese women. Additionally, a histological analysis was performed in scWAT samples. Both taurine and taurine-trained groups showed reduced adipocyte size and increased amounts of connective tissue and multilocular droplets. These findings suggest that taurine, when associated with an exercise protocol, can promote protection against inflammation in obese women (De Carvalho et al. 2021b).

1.2 Taurine Effects in the Regulation of Energy Metabolism in the Adipose Tissue

Another meaningful taurine action is the capacity of regulating energy and lipid metabolism, but it is necessary to investigate its effects on adipose tissue further. Although adipocytes were considered inert in the perspective of energy metabolism, they need functioning mitochondria to produce adenosine triphosphate (ATP) necessary for tissue maintenance (lipogenesis and lipolysis) and the synthesis and release of adipokines (Yin et al. 2014). Several investigations have evaluated the oxidative capacity, biogenesis, and mitochondrial functionality in adipose tissue from rodents and cell culture. Still, it is unclear if these results reflect human adipose tissue (Newton et al. 2011). Few studies describe the effects of obesity and the size of fat cells on mitochondrial oxidative phosphorylation capacity in human adipocytes.

Therefore, taurine can be an alternative to reestablish mitochondrial function and energy metabolism in obese individuals because it can regulate the expression of proteins that modulate thermogenesis and lipid oxidation, such as peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC1α) and peroxisome proliferator-activated receptor (PPAR), which are essential for the regulation of mitochondrial function (Murakami 2015; Tsuboyama-Kasaoka et al. 2006). Additionally, taurine can upregulate the gene expression of acyl-CoA (coenzyme-A) oxidase, acyl-CoA synthetase, acyl-CoA dehydrogenase, and lipoprotein lipase, which are enzymes of lipid metabolism (Murakami 2015).

Taurine anti-obesity effects were investigated in mice fed a high-fat diet associated with taurine supplementation (2% taurine added in the drinking water) compared to mice fed a regular diet or a high-fat diet for 28 weeks. Although no differences were observed among mice fed a high-fat diet and those fed a control diet relative to oxygen consumption and energy expenditure, weight loss and increased expression of adipogenesis-related genes such as PPAR-α, PPAR-γ, C/EBP-α, and C/EBP-β in the brown and white adipose tissues of taurine-supplemented mice were observed. The results confirmed the anti-obesity effects of taurine in animals (Kim et al. 2019).

One more study developed with mice also applied a high-fat diet and performed intraperitoneal treatment with taurine and found that taurine increased energy expenditure, improved insulin sensitivity, alleviated the high-fat diet-induced obesity, and elevated the expressions of PGC1α, uncoupling protein 1 (UCP1), mitochondrial cytochrome C (cycs), and mitochondrial transcription factor A (mtTFA) in white adipose tissue. These results suggest that taurine induces the browning of inguinal white adipose tissue and

can offer potential protection against obesity (Guo et al. 2019).

Furthermore, the increased expression of PGC1α may stimulate the release of irisin, which is a myokine produced during physical exercise that regulates energy metabolism, thermogenesis, and glucose homeostasis, and may also increase the expression of mRNA for UCP1 and consequently upregulate energy expenditure (Boström et al. 2012; Sanchez-Delgado et al. 2016).

To investigate the effects of taurine associated with exercise in irisin release in obese women, Batitucci et al. (2019) evaluated the effects of the supplementation of taurine (3 g, 2 h before training) associated with an exercise protocol (deep water running) for 8 weeks. Although no changes were observed in body weight and composition, the association between taurine and exercise increased resting energy expenditure and blood irisin levels 1-hour post-exercise in obese women. Because irisin is a myokine/adipokines that, under the stimulus of physical exercise, can improve thermogenic capacity in adipose tissue, the higher irisin release promoted by taurine suggests that taurine improves the cross talk between skeletal muscle and adipose tissue, which is a viable strategy for obesity treatment.

1.3 Benefits of the Association Between Taurine Supplementation and Exercise

The association of taurine with physical exercise enhances the benefits of taurine relative to obesity treatment. Exercise can increase energy expenditure, control body weight and composition management, and improve cardiorespiratory conditioning and the inflammatory process (Marion-Latard et al. 2003). The benefits of exercise on chronic inflammation induced by obesity have been deeply reviewed (You et al. 2013; Pedersen and Febbraio 2012). According to You et al. (2013), the potential mechanisms are related to the modulation of cytokine and immune cell release in skeletal muscle and adipose tissue, but also improvements in the cardiovascular system. Exercise training increases the release of IL-6 from skeletal muscle, which inhibits the action of other pro-inflammatory cytokines and increases the levels of anti-inflammatory cytokines in other tissues and systems.

In the immune system, exercise training can reduce the expression of Toll-like receptors and pro-inflammatory monocytes and increase the production of regulatory T cells. Additionally, exercise training can improve angiogenesis and blood flow, thereby reducing hypoxia and associated chronic inflammation. Additionally, exercise can improve adipose tissue lipid oxidation and insulin sensitivity (Pedersen and Febbraio 2012). Considering the cross talk between skeletal muscle and adipose tissue, all these mechanisms contribute to improve adipose tissue blood supply and reduce macrophage infiltration and M1/M2 macrophage ratio, decreasing chronic and systemic inflammation in obese individuals (You et al. 2013; Pedersen and Febbraio 2012).

There is some evidence that the association between exercise and taurine can help in the treatment of the chronic inflammation induced by exercise training in athletes (Kurtz et al. 2021) and induced by obesity (Oharomari et al. 2015; De Carvalho et al. 2021b). In athletes, taurine supplementation reduces creatine kinase (CK) levels, muscle soreness, and muscle damage (Kurtz et al. 2021; Silva et al. 2011; Galan et al. 2018; De Carvalho et al. 2017). According to Kurtz et al. (2021), taurine attenuates excessive inflammation induced by exercise during recovery, not directly by modulating inflammatory markers, but by modulating oxidative stress and other molecular mechanisms of muscle damage repair.

In obese individuals, recently De Carvalho et al. (2021b) investigated taurine's anti-inflammatory effects on blood and adipose tissue inflammatory markers. Taurine supplementation (3 g, 8 weeks) during 8 weeks decreased IL-6 blood levels. When associated with exercise, it increased anti-inflammatory blood interleukins (IL-15 and IL-10) and reduced IL-1β gene expression in the scWAT of obese women. Therefore, the changes in scWAT plasticity mentioned previously and the anti-inflammatory effects observed suggest taurine supplementation

combined with exercise is a promising strategy for modulating the inflammatory process and the regulation of whole-body energy metabolism.

Regarding exercise and energy expenditure modulation, its effects are induced by an increase in UCPs, which are present in the mitochondrial membrane, and uncouple oxidative phosphorylation, resulting in increased heat production, body temperature, and energy expenditure (Syamsunarno et al. 2014). The effects of the UCPs in skeletal muscle have already been explored. However, the impact of exercise in the adipose tissue UCPs needs to be further investigated. It is not clear whether the increase in UCPs occurs due to changes in lipid and energy metabolism or by the increase in the number of mitochondria in adipose tissue (Choi et al. 2014).

Because both exercise and taurine can modulate energy metabolism, expression of UCPs, and thermogenesis, it is expected that the association between taurine and exercise should improve the efficiency of mitochondrial function in adipose tissue and consequently enhance energy metabolism of people with obesity.

The effects of taurine supplementation associated with exercise in the regulation of energy metabolism have been investigated in athletes and physically active individuals (Balshaw et al. 2013; Milioni et al. 2016; Ra et al. 2015, 2016; Rutherford et al. 2010; Silva et al. 2011; Ward et al. 2016; Zhang et al. 2004). Furthermore, for at least 10 years, our research group explored taurine supplementation and exercise effects in animals, obese women, and male athletes. We highlight the following studies that investigated the effects of taurine on trained mice (Martiniano et al. 2015), triathletes (De Carvalho et al. 2017; Galan et al. 2018), and swimmers (Batitucci et al. 2018; De Carvalho et al. 2018).

The first work developed by our laboratory evaluated the effects of taurine supplementation (2%) associated with an 11-week period of physical training in obese rats fed with high-fat diet. In this study, we found that the animals supplemented with taurine had lower visceral and epididymal fat and showed that taurine benefited body composition (Martiniano et al. 2015).

Another study developed with rats (Oharomari et al. 2015) showed the benefits of the association of taurine and exercise for 7 weeks in preventing metabolic disorders induced by a high palatable diet. The authors investigated the effects of 7 weeks of exercise training and taurine supplementation (2% added in ad libitum water). They found that the exercise prevented an increase in body weight and epididymal fat in trained groups and showed a better metabolic profile than the sedentary group, despite taurine supplementation. Although exercise itself enhanced the antioxidant defense system (increased copper-zinc superoxide dismutase and reduced $gp91^{phox}$ oxidase protein expressions), a decrease in superoxide formation in the aorta of the sedentary-taurine supplemented group (75%) was observed, as well as in both trained groups (64% and 77% for trained and trained with taurine supplementation groups, respectively). Therefore, the authors suggested a synergistic effect between taurine supplementation and exercise training relative to the prevention of endothelial dysfunction induced by high palatable diet intake. These studies confirmed a positive effect of the association between taurine and exercise in animals.

In human studies, the combination of taurine and exercise has been investigated in many aspects, such as energy metabolism regulation, antioxidant and anti-inflammatory protection in athletes' performance, and physical fitness in sedentary and obese individuals. Studies developed by our and other research groups have shown promising effects of taurine supplementation on athletes (De Carvalho et al. 2017, 2018; Milioni et al. 2016; Rutherford et al. 2010; Ward et al. 2016), highly active individuals (Carvalho et al. 2020), and obese women with exercise intervention (Batitucci et al. 2019; De Carvalho et al. 2021a, b).

Specifically considering lipid metabolism, to investigate the effects of taurine in athletes' lipid oxidation, the study of De Carvalho et al. (2018), carried out in our laboratory, evaluated the effect of acute supplementation of taurine (6 g) or placebo 120 min before the performance of a maximum swimming performance test. It was observed that taurine induced an increase of 8%

in plasma lipolysis, but no benefits were observed in the athletes' physical performance.

We further explored the effects of taurine on lipid metabolism in active individuals in a fasted state. Carvalho et al. (2020), also carried out in our laboratory, evaluated the effect of acute supplementation of two different doses of taurine (3 g or 6 g) compared to a placebo trial. The subjects were supplemented 90 min before a single bout of fasting aerobic exercise (60% of VO2 max) in three trials with a washout of 7 days. It was found that the supplementation increased lipid oxidation post-exercise, but only the 6 g of taurine dose reduced the respiratory quotient (RQ) when compared to the placebo. The results confirmed the effects of taurine in increasing lipid oxidation post-exercise in healthy young men in the fasted state. Other researchers investigated the effects of acute doses of taurine in the performance of cyclists (Rutherford et al. 2010; Ward et al. 2016) and runners (Milioni et al. 2016), all of which also observed increased fat oxidation.

To further explore the effects of taurine supplementation associated with exercise in the regulation of energy metabolism in the obesity context, we investigated the effects of taurine supplementation (3 g, 2 h before training) associated with exercise (deep water running protocol) during 8 weeks in obese women. We observed that taurine increased irisin release (Batitucci et al. 2019), which is a myokine/adipokine that can improve thermogenic capacity in adipose tissue (Li et al. 2019).

Another investigation explored for the first time the effects of taurine associated or not with a combined aerobic and resistance training protocol on energy metabolism and scWAT metabolism in obese women (De Carvalho et al. 2021a). It was found that taurine supplementation (3g) associated with the training protocol for 8 weeks modulated lipid metabolism by increasing lipid oxidation and improving scWAT mitochondrial respiratory capacity compared to placebo and the only taurine supplementation group. Moreover, the combined intervention increased the expression of genes related to mitochondrial function such as cell death activator (CIDEA), PGC1a, PRDM16, and UCP1 and UCP2 genes, and lipid oxidation such as carnitine palmitoyltransferase 1 (CPT1), PPAR-α, PPAR-γ, lipoprotein lipase (LPL), aconitase 1 (ACO1), aconitase 2 (ACO2), hormone-sensitive lipase (HSL), acyl-CoA oxidase-1 (ACOX1), and CD36 genes. The authors also observed that taurine supplementation itself upregulated genes related to fat oxidation (ACO2 and ACOX1). Therefore, these studies showed that taurine supplementation promotes positive regulatory effects on energy metabolism in obese women, mainly when associated with exercise.

Additionally, we explored the effects of the association between taurine (3 g) and exercise for 8 weeks in obese women. Although no changes were observed for the anthropometric characteristics, taurine supplementation increased taurine blood levels, reduced the size of the adipocytes, and increased the amount of connective tissue and multilocular droplets in samples of abdominal scWAT (De Carvalho et al. 2021b). It is essential to report that the adipose tissue of obese individuals has a majority of unilocular droplets, which are hypertrophic and hyperplasic adipocytes that are associated with a pro-inflammatory environment such as higher adipokine secretion, local hypoxia, and increased fatty acid fluxes, leading to metabolic disorders (Pasarica et al. 2009). Therefore, the study of De Carvalho et al. (2021b) was the first to show the ability of taurine, associated or not with exercise, to induce scWAT plasticity in human samples and reduce adipocytes size in obese women and is a promising strategy to induce browning/beiging in scWAT to combat the epidemic of obesity.

2 Limitations in Studies with Taurine

Although all possible benefits can be reached with taurine supplementation, some limitations must be considered. It is still undefined which dose would be effective for animal and human experimental protocols. It can be observed in the literature doses from 0.5 to 6 g in acute (Balshaw et al. 2013; Carvalho et al. 2020; Fedewa et al. 2018; Rutherford et al. 2010) and long-term sup-

plementation protocols (Batitucci et al. 2019; De Carvalho et al. 2017, 2021a, b; Galan et al. 2018; Martiniano et al. 2015); however, it is unclear which protocol would be adequate for sedentary and physically active individuals.

The interaction between taurine and nutrients provided by food intake has not been explored. The fact that taurine can be synthesized in the body from methionine and cysteine can change taurine utilization (Jacobsen and Smith 1968). An intriguing question is what mechanisms would drive taurine action to a specific function in humans? For example, in obesity, there is a pro-inflammatory scenario that can promote a chronic low inflammatory state. The supplementation of taurine should help the body prevent the anti-inflammatory state, but what about the action of taurine in lipid metabolism regulation? This would explain why taurine promotes no impact on scWAT regarding energy and lipid metabolism, specifically considering gene expression and mitochondrial function when it was not associated with exercise in sedentary obese women (De Carvalho et al. 2021a).

However, when taurine was associated with exercise, the increased energy requirements induced by the exercise training protocol not only modulated the expression of genes related to mitochondrial function and lipid metabolism but also modulated mitochondrial function itself in the scWAT. It is essential to highlight that the primary function of adipose tissue is energy/fat storage, and the improvement in the scWAT mitochondrial respiratory capacity demonstrates the effects of taurine in the interplay between muscle and scWAT tissue metabolism. The effects of taurine in the cross talk between taurine and muscle also demonstrated that taurine associated with deep water running protocol increased irisin release (myokine that regulates energy metabolism) post-exercise in obese women in the study of Batitucci et al. (2019).

Therefore, the association between taurine and exercise promoted "healthier" adipose tissue in obese women investigated in the study of De Carvalho et al. (2021a). The utilization of taurine as a "fat burner" will only be effective if it is associated with increased energy requirements (i.e., induced by exercise) to stimulate the body to drive taurine action to energy metabolism regulation; in other words, when taurine is associated with a physically active or athlete lifestyle, it can modulate metabolism. Without increasing the energy requirements, the body will probably drive the extra available taurine (from taurine supplementation) to biological and physiological functions such as membrane stabilization, inflammatory and oxidative modulation (Schaffer et al. 2010; Schuller-Levis and Park 2003), protein synthesis, cell differentiation in the brain (Li et al. 2017), calcium regulation in muscle cells (De Luca et al. 2015), pancreatic insulin release (Vettorazzi et al. 2014), or elimination of the taurine excess by the urine (Jacobsen and Smith 1968).

The results of taurine supplementation in humans compared to animals are still questionable. The increased energy metabolism, browning effects, and anti-obesity effects of taurine were observed in animals in the study of Guo et al. (2019) and Kim et al. (2019, 2020). In humans, our last published papers showed positive effects in the adipose tissue, such as improved modulation of mitochondrial activity and fatty acid oxidation (De Carvalho et al. 2021a), and changes in adipose tissue plasticity (De Carvalho et al. 2021b), suggesting that taurine supplementation promoted a browning effect in the scWAT of obese women when associated or not with exercise. However, increased energy expenditure and lipid oxidation were observed in humans only when taurine supplementation was associated with exercise (Batitucci et al. 2019; De Carvalho et al. 2021a). According to Murakami (2015), the discrepancies found in the anti-obesity results of taurine in humans compared to animal models can be attributed to the doses used in animal protocols that are not physiologically representative of a human protocol.

The differences in the supplementation method and taurine bioavailability should also be mentioned. In human studies, taurine supplementation can be done by capsules, or the taurine powder can be offered diluted in water. In our case, the 3 g were provided in capsules. On the other hand, in animal studies, taurine supplemen-

tation can be done by gavage, intraperitoneal injection, or added water (ad libitum water). Therefore, the actual amount of taurine obtained can vary in the different supplementation methods mentioned and consequently can change taurine availability in the blood, possibly promoting different outcomes.

Finally, it is crucial to consider the reproducibility of human and animal studies, specifically considering lifespan and period of intervention, because mice have a shorter and accelerated life than humans' lives. As an example, mice are deemed to be aged at 30 months of lifetime, which refers to 120 weeks (Dutta and Sengupta 2016), while for a human to be considered old, according to the World Health Organization classification, it has to have 60 years, which refers to 3360 weeks. Therefore, an intervention for 8 weeks in an animal study refers to ~7% of its lifetime, while with humans, it refers to ~0.2% of a human lifetime. In other words, it is necessary to have a 235-week intervention in a human study to achieve the same 7% of an animal lifetime, and the effects of an 8-week intervention may be more expressive in animals than in human metabolism.

3 Final Considerations

The benefits of taurine supplementation and the importance of adipose tissue to the whole-body energy metabolism are undeniable; however, the impact of the association of taurine and exercise with adipose tissue dynamics remains unclear, especially in the context of obesity. In general, these studies showed that the association of taurine supplementation and exercise could modulate lipid metabolism that also can change adipose tissue morphology in obese individuals and obese animal models, suggesting that taurine can make white adipose tissue metabolically active by the cross talk between adipose tissue and skeletal muscle, in response to increased energy demand promoted by exercise, enhancing whole-body metabolic health. Due to taurine's promising capacity to induce browning/beiging in scWAT, efforts should be continued to elucidate the mechanisms and potential effects of taurine supplementation and exercise on scWAT as a therapeutic strategy to combat the epidemic of obesity.

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Part VI

Metabolic Effects of Taurine

The Taurine-Conjugated Bile Acid (TUDCA) Normalizes Insulin Secretion in Pancreatic β-Cells Exposed to Fatty Acids: The Role of Mitochondrial Metabolism

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Keywords

Taurine-conjugated bile acids · Insulin secretion · Obesity · Mitochondrial metabolism

Abbreviations

DM2	Type 2 diabetes mellitus
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
CS	Citrate synthase
FA	Fatty acids
$FADH_2$	Flavin adenine dinucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IHME	Institute for Health Metrics and Evaluation
INS-1E	Rat pancreatic ß-cell line
JNK	c-Jun N-terminal kinase
NADH	Nicotinamide adenine dinucleotide
NRF1	Nuclear respiratory factor 1
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
PKA	Protein kinase A
TFAM	Mitochondrial transcription factor A
TUDCA	tauroursodeoxycholic acid
UCP2	Uncoupling protein 2
UDCA	Ursodeoxycholic acid

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1 Introduction

The sedentary lifestyle combined with the consumption of energy-dense and high-fat diets has led to an exponential increase in the number of obese individuals, currently accounting for about 600 million adults worldwide (Institute for Health Metrics and Evaluation (IHME) 2018). Thereby, comorbidities associated with obesity have become frequent in the day-to-day life of society. Classically, the first indication of glycemic damage characterized during the development of obesity is insulin resistance, which is associated with glucose intolerance. In this condition, fasting glycemia remains unchanged or slightly increased due to increased insulin secretion, which in a way compensates for the impairment

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in the action of this hormone (Cavaghan et al. 2000). Molecular adaptations are activated to enable pancreatic β-cell hyperfunction; among them, the increase in mitochondrial metabolism stands out (Branco et al. 2017).

Mitochondrial metabolism is involved in cellular homeostasis, having an important role in intracellular signaling, regulation of the redox state, apoptosis, cholesterol metabolism, steroids, and nucleotide biosynthesis. In addition, it stands out as the main oxidative respiration site in the cell, where approximately 90% of cellular oxygen is directed to energy production in the form of heat and ATP (Abu Bakar and Sarmidi 2017; Ritov et al. 2005). Thus, adequate mitochondrial function is essential for energy-demanding processes, including insulin synthesis and secretion (Rovira-Llopis et al. 2017). It is known that chronic exposure of pancreatic islets and β-cell lines to high concentrations of nutrients leads to changes in glucose metabolism, tricarboxylic acid cycle markers, mitochondrial function, and redox balance, culminating in altered insulin secretion (Muoio and Newgard 2008). Furthermore, it has been established that islets isolated from obese mice present with insulin hypersecretion, greater flow, and an oscillatory pattern of $[Ca^{2+}]$ even at low glucose concentrations. All of these changes point to compensatory adaptations of the mitochondrion to alter glucose-stimulated insulin secretion (Gonzalez et al. 2013; Irles et al. 2015). However, these adjustments can also lead to changes in endoplasmic reticular function and oxidative stress, culminating in pancreatic β-cell dysfunction and development of type 2 diabetes mellitus (DM2). Therefore, the discovery of new therapeutic targets that avoid or reverse these alterations is of great importance to prevent the progression and onset of diabetes in obesity.

The use of several bile acids in the treatment of metabolic diseases has proven to be efficient in recent years, among which we highlight TUDCA, formed by the association of ursodeoxycholic bile acid (UDCA) with the amino acid taurine. This bile acid is more common in rodents and bear species; however, TUDCA is also produced in low concentrations in humans (Boatright et al. 2009; Combes et al. 1999). Experimental evidence has emphasized the involvement of TUDCA in protecting against endoplasmic reticular stress, acting as a chemical chaperone, restoring glycemic homeostasis, and thus playing a key role in the pathogenesis of obesity, insulin resistance, and DM2 (Kumar et al. 2012; Özcan et al. 2009; Özcan et al. 2006). Regarding the function and survival of β-cells, works by Zhu et al. (2013) and Engin et al. (2013) demonstrated that TUDCA normalized ATP production and insulin secretion in islet cultures incubated with free fatty acids. Treatment with TUDCA also reduced the expression of reticular stress markers in isolated islets grown for 48 hours in the presence of high glucose concentrations (Malo et al. 2010; Özcan et al. 2006). In addition, it preserved insulin secretion and reduced apoptosis of isolated islets of pigs treated with the sarco−/endoplasmic reticular Ca-ATPase (SERCA) inhibitor thapsigargin (Vang et al. 2014). Acute exposure of mice pancreatic islets to TUDCA potentiates insulin secretion stimulated by high concentrations of glucose, an effect mediated by the cAMP/PKA/CREB pathway (Vettorazzi et al. 2016). However, studies regarding the effects of TUDCA on mitochondrial metabolism during obesity-induced pancreatic β-cells hyperfunction are absent in the literature. Here we show that TUDCA also reestablishes β-cell mitochondrial markers involved on insulin secretion, after exposure to fatty acids.

2 Methods

2.1 Cell Culture

INS1-E cells were maintained in RPMI-1640 medium containing 11.1 mM glucose and supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 g/mL streptomycin, in an oven maintained at 37 °C in a humid environment with a 5% CO_2 support. Palmitic acid and sodium oleate (500 μM) and carnitine (2 mM) were added in maintenance medium, which contained 1% fat-free BSA but no FBS and was subsequently heated at 55 °C for 25 min for the conjugation of fatty acids. The treatment with TUDCA (taurour-

sodeoxycholic acid, sodium salt – CAS 14605-22-2 – Calbiochem) was carried out at a concentration of 300 μM and diluted in growth medium containing the fatty acid mix.

2.2 Experimental Groups

After the cells reached 70% confluency, the following groups were formed:

- Control (C): INS1-E cell line grown in RPMI medium for 48 h.
- Fatty acid mix (FA): INS1-E cell line grown in RPMI medium with the fatty acid mix for 48 h.
- Fatty acid mix + TUDCA (FA + TUDCA): INS1-E cell line grown in RPMI medium with fatty acid mix and 300 μM TUDCA for 48 h.

2.3 Static Insulin Secretion

INS1-E cells were cultured in 24-well plates and treated with different experimental protocols. Afterward, they were pre-incubated with Krebs buffer (115 mM NaCl, 5 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 15 mM HEPES, and 0.3% glucose-free bovine serum albumin (BSA) and balanced with a mixture of 95% O_2/5% CO_2 to a pH of 7.4), at 37 °C, for 1 h. Subsequently, the solution was gently removed and replaced with 1000 μL of the same solution (Krebs) containing different concentrations of glucose (2.8 and 22.2 mM) for 1 h. The supernatant was removed, transferred to test tubes, and stored at −20 °C for a subsequent dosage of insulin by Rat/Mouse Insulin ELISA (Sigma-Aldrich, EZRMI-13 K). The cells were collected using urea/thiourea buffer for later protein measurement and normalization of results.

2.4 RT-PCR

After treatment, INS1-E cells were lysed in 500 μl of TRIzol solution (Ambion by Life Technologies, USA), and total RNA was extracted according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometry, and quality was assessed by the 260/280 nm ratio. The cDNA was synthesized with 1 μg of RNA, using the reverse transcriptase reaction (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA). Quantitative PCR was performed using Fast SYBR® Green PCR Master Mix (Applied Biosystems, USA), on the 7500 Fast Real-Time PCR System (Applied Biosystems) with specific primers, with a focus on mitochondrial metabolism. The sequence of the primers used is as follows: TFAM (Forward CCAAAAAGACCTGGCTCAGC, Reverse GTGACTCATCCTTAGCCCCC), PGC1α (Forward GCACGCAGCCCCTATTCATTG, Reverse AGGATTTCGGTGGTGACA), NFR1 (Forward TGCCCAAGTAATTACTCTGC, Reverse TCGTCTGGATGGTCATTTCAC), and UCP2 (Forward AGCAGTTCTACACCAAGGGC; Reverse TGGAAGCGGACCTTTACCAC).

2.5 Western Blot

For protein expression of citrate synthase (CS) [(G-3): sc-390,693] and c-Jun N-terminal kinase (pSAPK/JNK) [Phospho-SAPK/JNK (Thr183/Tyr185) #9251], INS1-E cells were lysed with 60 μL of Laemmli buffer (0.1% bromophenol blue, 1 M sodium phosphate, 50% glycerol, 10% SDS), after their respective treatments. Afterward, proteins were separated by electrophoresis in a biphasic polyacrylamide gel (SDS-PAGE) and then transferred to a nitrocellulose membrane (Bio-Rad) for 90 min at 120 V on ice, with transfer buffer (25 mM Tris, 192 mM glycine). Subsequently, the membrane was blocked with 5% milk in TBST solution for 2 h at room temperature. Membranes were incubated overnight at 4 °C with the primary antibodies for the study-related proteins, which were detected by HRP-conjugated polyclonal anti-IgG antibody and developed in ChemiDoc MP System (Bio-Rad, Hercules, CA). The intensity and quantification of the bands were assessed by densitometry (ImageTool Software; http://ddsdx.uthscsa.edu/dig/itdesc.html), with the densitometry values of the bands normalized by the densitometry values

of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Anti-GAPDH – G9545 – Sigma-Aldrich] protein.

2.6 Mitochondrial Content by MitoTracker and Mitochondrial Membrane Potential by TMRM

After the respective treatments, INS1-E cells were incubated with the fluorescent probe MitoTracker Green FM (M7514, Invitrogen) at a concentration of 100 nM (diluted in Krebs 11.1 mM glucose) for 15 min at 37 °C to assess mitochondrial density. Then, they were washed three times with PBS, and the fluorescence reading was performed on the SpectraMax M3 fluorimeter plate reader (Molecular Devices), at 490 nm (excitation) and 516 nm (emission) wavelengths.

For mitochondrial membrane potential assays, INS1-E cells were grown in 96-well plates (Falcon), and after the respective treatments, the medium was removed, and cells washed with PBS and subsequently incubated with tetramethylrhodamine methyl ester perchlorate (TMRM – T5428, Sigma) at a concentration of 30 nM (diluted in Krebs 11.1 mM glucose) for 30 min at 37 °C. Next, they were washed three times with PBS, and the fluorescence reading was performed on the SpectraMax M3 plate reader, at wavelengths of excitation at 545 nm and emission at 575 nm.

For normalization of the results, cells were washed with PBS, fixed with 4% formaldehyde for 10 min, washed again with PBS, and then incubated with Hoechst 33342 (Thermo Fisher, H3570; 10 mg/mL) at the final concentration of 1 μg/mL for 15 min. Fluorescence was red on this same equipment at 350 nm (excitation) and 461 nm (emission) wavelengths.

2.7 Reactive Oxygen Species (ROS) Production and Cell Viability

The generation of mitochondrial superoxide radicals was quantified using the MitoSOX™ Red Mitochondrial Superoxide Indicator (M36008, Thermo Fisher). For the quantification of total hydrogen peroxide (H_2O_2), the Amplex™ UltraRed Reagent probe (A36006, Thermo Fisher) was used. Fluorescence was measured using a fluorescent plate reader (SpectraMax M3 fluorimeter, Molecular Devices) according to the manufacturer's recommendations. For normalization of the results, cells were washed with PBS, fixed with 4% formaldehyde for 10 min, washed again with PBS, then incubated with Hoechst (1 μg/mL) for 15 min, again washed with PBS, and then read in this same equipment at wavelengths of 350 nm (excitation) and 461 nm (emission).

Cell viability was assessed from cells incubated with DNA intercalants, Hoechst 33342 (Thermo Fisher, H3570; 10 mg/mL; nucleus labeling) and propidium iodide (Thermo Fisher, P3566; 10 mg/mL; apoptotic cell labeling). For the experiment, INS-1E cells were grown in a 96-well plate (μClear®, Greiner), and after the treatments, 100 μL of the medium from each well was removed, and 100 μL of RPMI-1640 medium containing the two aforementioned reagents were added to a final concentration of 10 μg/mL. After 15 min of incubation at 37 °C, 100 μL of the medium was removed from each well, and 100 μL of RPMI-1640 medium was added. The percentage of apoptotic cells was determined by the High Content Imaging System (ImageXpress, Molecular Devices) using the Live and Dead module of the MetaXpress software (Molecular Devices). To identify viable and dead populations, masks were applied for the DAPI wavelengths (excitation at 350 nm and emission at 470 nm – Hoechst) and Texas red (excitation at 496 nm and emission at 615 nm – propidium iodide).

2.8 Statistical Analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. Data were first analyzed using the Shapiro–Wilk normality test and subsequently submitted to parametric (one-way ANOVA followed by Bonferroni) or nonparametric (Kruskal-Wallis followed by Dunns) unpaired tests, using the GraphPad Prism software, and the level of significance was set at $P < 0.05$. Graphs were also performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 TUDCA Normalizes Insulin Secretion in INS1-E Cells Treated with Fatty Acids

Treatment with fatty acids (FA) for 48 h increased insulin secretion in INS1-E cells by 67%, mimicking the hyperfunction phenotype observed in obese humans. Furthermore, it was demonstrated that the concentration of 300 μM of TUDCA leads to a reduction in insulin secretion stimulated by 22.2 mM of glucose in FA cells (Fig. 1a). Therefore, 300 μM was taken as the lowest concentration of TUDCA with the higher efficiency in normalizing the hypersecretion of insulin, characterized in the groups treated with FA, found to be capable of reducing 123% of insulin secretion stimulated by 22.2 mM of glucose in the FA + TUDCA group (Fig. 1b).

3.2 TUDCA Normalizes Mitochondrial Metabolism in INS1-E Cells Treated with Fatty Acids

Alterations in insulin secretion induced by excessive levels of fatty excess have been shown to correlate with changes in mitochondrial metabolism (Cen et al. 2016). It is known that fatty acids in the pancreatic β-cells can also be converted to

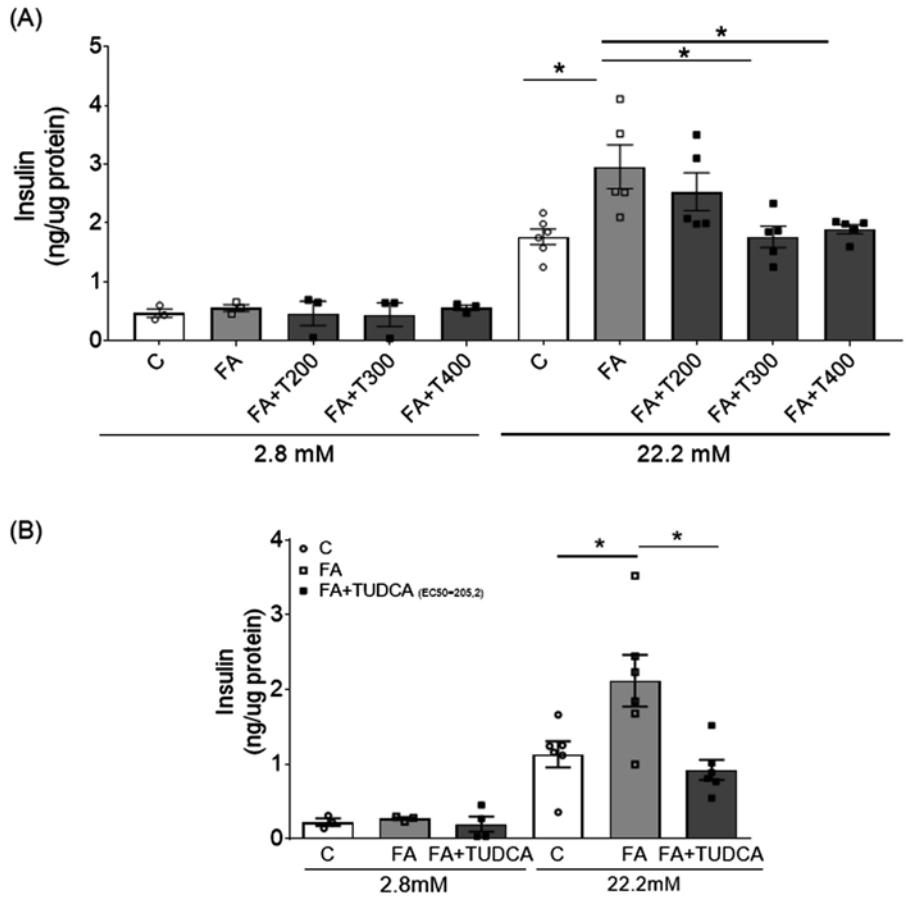

Fig. 1 (**a**) Insulin secretion stimulated by 2.8 mM and 22.2 mM of glucose in INS1-E cells treated with the fatty acid mix containing or not 200, 300, or 400 μM of TUDCA. (**b**) Evaluation of insulin secretion stimulated by 2.8 and 22.2 mM of glucose in INS1-E cells treated with the fatty acid mix with or without 300 μM of TUDCA (EC50 = 205.2). Data are shown as average ± SEM of three to five cell line passages. The asterisk denotes a significant difference between the groups ($p < 0.05$)

acetyl-CoA, which is further metabolized by the citric acid cycle (TCA). Activation of TCA generates reducing equivalents transferred by NADH and $FADH_2$ to the electron transport chain, culminating in the hyperpolarization of the mitochondrial membrane and therefore the generation of ATP, which will be released into the β-cell cytoplasm. The increase in the ATP/ADP ratio causes the closure of ATP-sensitive K^+ channels (KATP), present in the plasma membrane, and the reduction in K+ efflux leads to membrane depolarization which, in turn, causes the opening of the voltage-sensitive Ca^{2+} channels (Cav); the influx of this cation activates the exocytotic machinery of the granules of insulin (Fu et al. 2012, Wollheim and Maechler 2002). Therefore, these alterations modulate mitochondrial biogenesis, TCA enzymes, and mitochondrial membrane potential.

In this study, we found that INS1-E cells treated with the fatty acid mix showed a 2.33-fold increase in PGC1-alpha gene expression (Fig. 2a) and a 42% increase in mitochondria density (Fig. 2b). Furthermore, they presented a higher expression of citrate synthase (41%) (Fig. 2c) and mitochondrial membrane potential (27.6%) (Fig. 2d). These results indicate a strong correlation exists between the boost in insulin secretion and mitochondrial metabolism.

The addition of TUDCA to the FA-containing medium prevented the increase in mitochondrial oxidative metabolism signature, as demonstrated by the reduction of 146% of PGC1-alpha gene expression, 52.5% of mitochondrial content, and 20% of mitochondrial membrane potential. Taken together, these findings support the hypothesis that TUDCA can prevent insulin hypersecretion by controlling mitochondrial metabolism in β-cells.

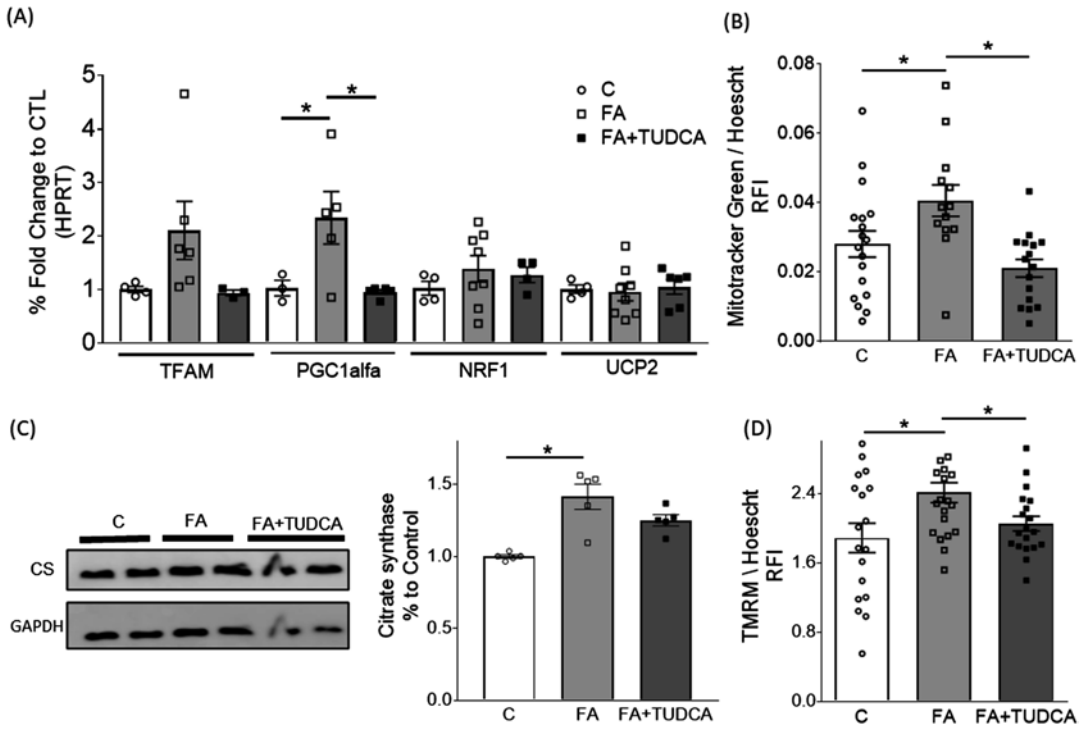

Fig. 2 (**a**) Evaluation of gene expression of mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1α), nuclear respiratory factor 1 (NRF1), and uncoupling protein 2 (UCP2) in INS1-E cells treated with the fatty acid mix with or without 300 μM of TUDCA. (**b**) MitoTracker mitochondrial density, (**c**) citrate synthase protein expression, and (**d**) mitochondrial membrane potential in INS1-E cells treated with the fatty acid mix with or without 300 μM TUDCA. Data are means ± SEM of 3–18 cell line passages. The asterisk denotes a significant difference between the groups ($p < 0.05$)

3.3 TUDCA Reduces Mitochondrial Superoxide Production and JNK Phosphorylation of INS1-E Cells Treated with Fatty Acids

Increased mitochondrial metabolism in pancreatic β-cells during chronic exposure to nutrients has already been shown to contribute to a redox imbalance, increased oxidant production, activation of cell apoptosis pathways, and impaired insulin secretion (Carlsson et al. 1999; Ciregia et al. 2017; Watson et al. 2011). Therefore, β-cell hyperfunction after increased fatty acid intake may precede irreversible cell damage and increase the susceptibility to the development of type 2 diabetes. Thus, we evaluated whether the treatment with fatty acids could be interfering with reactive oxygen species production and cell viability.

As shown in Fig. 3a, b, treatment with the fatty acid mix did not alter the production of H_2O_2 and mitochondrial superoxide in INS1-E cells. Besides, it did not alter protein expression of pSAPK/JNK and the percentage of dead cells. Nonetheless, the treatment with TUDCA reduced mitochondrial superoxide production (50%) and reduced by three times the SAPK/JNK protein content. These effects highlight TUDCA as a possible therapeutic strategy to prevent the main alterations that can lead to the transition from hyperfunction to hypofunction and, consequently, DM2.

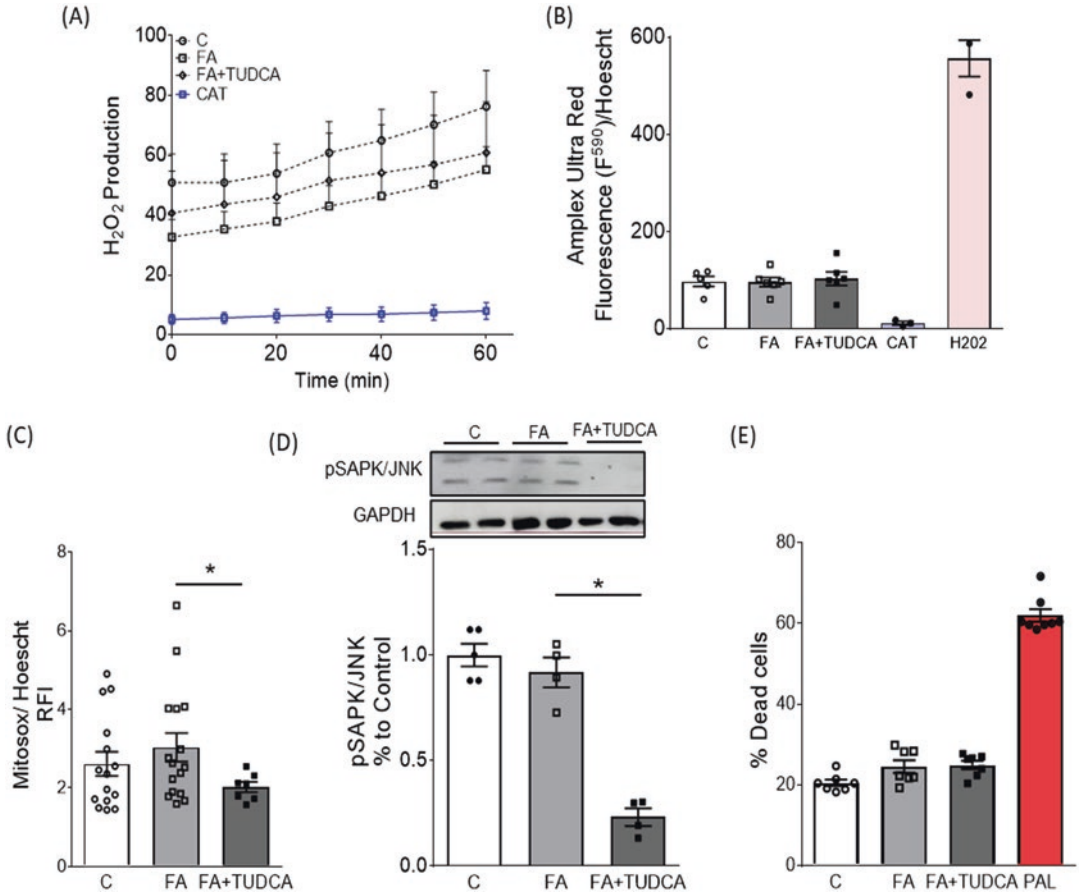

Fig. 3 Evaluation of the production of total hydrogen peroxide (**a, b**) and mitochondrial superoxide (**c**) in INS1-E cells treated with the fatty acid mix with or without 300 μM of TUDCA. Catalase (CAT) was used as a negative control and H_2O_2 as a positive control. Protein expression of phospho-c-Jun N-terminal kinase (JNK) (**d**) and percentage of cells labeled for propidium iodide (**e**) in INS1-E cells treated with the fatty acid mix with or without 300 μM of TUDCA. PAL-500 μM of palmitate was used as a positive control in the cell viability experiment. Data are means ± SEM of 5–19 cell line passages. The asterisk denotes a significant difference between the groups ($p < 0.05$)

4 Discussion

In this study, we showed that treatment withw the fatty acid mix was effective in mimicking pancreatic β-cell hyperfunction in vitro, evidenced by the increase in mitochondrial metabolism and glucose-stimulated insulin secretion. Furthermore, the association of taurine with the bile acid UDCA prevented the increase in mitochondrial metabolism, insulin secretion, and reduced reactive oxygen species (ROS) production as well as JNK phosphorylation in INS1-E cells treated with the mix.

To reach these conclusions, it was necessary to establish an in vitro treatment protocol that would mimic the phenotype of pancreatic β-cells in obese individuals. Through a literature review, it was observed that the use of fatty acids associated with the culture medium of the insulin-producing cell lines is generally focused on the study of mechanisms for maintaining cell viability and on alterations in endoplasmic reticular stress markers. In this sense, Karaskov et al. (2006) found that exposure of INS-1E cells to 500 μM palmitate for 6 hours led to marked apoptosis and increased protein expression of phosphorylated eukaryotic translation initiation factor 2 (eIF2) and activation of ATF4, XBP-1, and CHOP. In contrast, these effects were not observed when they were exposed to oleate. Furthermore, it was shown that treatment with 500 μM of palmitate in human islets for 42 hours led to reduced glucose-stimulated insulin secretion, increased cytochrome C protein expression in the cytoplasm, and positive staining for apoptotic cells (Watson et al. 2011). In this same study, it was found that the addition of oleate to the treatment with palmitate restored insulin secretion, and this effect is directly related to greater viability and less activation of the intrinsic pathway of cellular apoptosis. Like these, other studies have demonstrated the protective effect of oleate use on the functional changes caused by palmitate in the pancreatic β-cell (Biden et al. 2004; Karaskov et al. 2006; Oberhauser et al. 2020). It is also noteworthy that obese children have increased plasma concentration of more than six types of fatty acids, including palmitate and oleate (Okada et al. 2005). Therefore, in the present work, two types of fatty acids are capable of mimicking the phenotype of pancreatic β-cell hyperfunction observed in obese humans.

Among the alterations found in pancreatic β-cells during the development of obesity, cell hypertrophy and hyperplasia stand out, associated with greater oscillations of cytoplasmic calcium, expression of the insulin gene, glucose-metabolizing proteins, and ATP production, in addition to the SNARE complex, which allows the fusion of insulin vesicles with the plasma membrane (Branco et al. 2017; Kusaczuk 2019; Ribeiro et al. 2012; Santos-Silva et al. 2015). In our study, treatment with a fatty acid mix leads to increased insulin secretion, associated with higher PGC1-alpha gene expression, mitochondrial content, citrate synthase expression, and mitochondrial membrane potential. The increased supply of glucose or fatty acid to pancreatic islets has already been shown to induce an increase in the acute expression of PGC1-alpha (Branco et al. 2017). Also, β-cell-specific knockout of PGC1-alpha reduces GSIS modulated by fatty acid oxidation (Oropeza et al. 2015), highlighting the importance of PGC1-alpha for GSIS. There's an intricate network controlling the transcription of the PGC1-alpha gene and the activation of its protein. PGC1-alpha transcriptional activity can be modulated by several nutrient-sensing pathways and hormonal signaling cascades, which control its interaction with a large number of transcription factors by changing its phosphorylation and acetylation state (Besseiche et al. 2018; Yoon et al. 2003).

In our work, treatment with the fatty acid mix did not change ROS content and INS1-E cell viability, even with increased mitochondrial metabolism. However, the literature presents a lot of studies in which chronic exposure to fatty acids reduces mitochondrial metabolism, increases the production of ROS, and impairs cell viability and insulin secretion in pancreatic islets and some insulin-producing cell lines. Thereby, Tian et al. (2015) demonstrated that treatment for 48 h with 500 mM of palmitate led to a reduction in glucose-stimulated insulin secretion, associ-

ated with a lower generation of adenylyl cyclase 9 (cAMP) and induction of exocytosis of insulin granules. Regarding mitochondrial metabolism, Carlsson et al. (1999) verified that treatment with 200 mM of palmitate in mouse pancreatic islets led to reduced insulin secretion, related to lower mitochondrial membrane potential, ATP production, and increased ROS production.

It is known that high concentrations of ROS can initiate intrinsic apoptosis, leading to the release from transition pore formation via BAX or BAD of mitochondrial apoptogenic factors, such as cytochrome c, into the cytosol. Furthermore, cytoplasmic ROS production leads to activation of the ASK1/JNK pathway culminating in caspase 3 cleavage and apoptosis (Buhlman 2016). In this sense, it was demonstrated that INS1-E cells exposed for 48 h with 250 mM of palmitate showed increased mitochondrial superoxide production; greater activation of the IRE1/JNK endoplasmic reticulum stress pathway, with increased caspase activity; and reduced cell viability and insulin secretion (Lin et al. 2012). Together, these data demonstrate that there is a transition from an acute to a chronic state in the treatment of fatty acids in the pancreatic β-cells, which determines the loss of insulin secretion and cell viability. Thus, therapeutic targets that prevent this deterioration are of great importance in preventing the onset of DM2 during obesity.

TUDCA, a molecule that has chemical chaperone activity, has already been shown to improve protein folding and reduce the expression of ERE markers and oxidative stress in muscle, cardiac, auditory, pancreatic, and lung cells (Malo et al. 2010). In addition, it is effective in modulating pancreatic β-cell function, modulating insulin secretion via cAMP/PKA (Vettorazzi et al. 2016), and increasing β-cell mass and insulinemia in mice treated with streptozotocin (Bronczek et al. 2019). Therefore, a highlight molecule for the study on the improvement of pancreatic β-cell survival and function.

In our work, treatment with 300 μM of TUDCA reduced insulin secretion in cells treated with the fatty acid mix. Corroborating our findings, studies such as Zhu et al. (2013) and Engin et al. (2013) have already observed that treatment with TUDCA normalizes ATP production and insulin secretion in islet cultures incubated with free fatty acids. Additionally, TUDCA has been shown effective in reducing the expression of p-ERK (protein kinase RNA-like endoplasmic reticulum kinase (p-PERK) and APMc-dependent transcription factor 6 (ATF-6), markers of reticular stress, in isolated islets cultivated for 48 h in the presence of high glucose concentrations (Özcan et al. 2006; Malo et al. 2010).

In addition to improving the ERE and oxidative stress, treatment with TUDCA has already been shown to be effective in improving the viability of different cell types. In this sense, Xie et al. (2002) demonstrate that human liver-derived Huh7 cell lines treated with thapsigargin, an ERE-inducing drug, and incubated with 100 μM of TUDCA for 18 to 24 h showed reduced expression of proteins involved in ERE and DNA fragmentation via inhibition of activation of caspase 3 and 7 proteins. In our work, TUDCA reduced the production of mitochondrial superoxide and expression of JNK, a protein of the extrinsic pathway of apoptosis, implying a preventive action on cell death that can be induced by chronic treatment with fatty acids.

5 Conclusion

Fatty acid mix treatment led to greater insulin secretion in INS1-E cells by increasing mitochondria content and mitochondrial membrane potential, without altering ROS production and viability. TUDCA prevented the increase of insulin secretion in INS1-E cells treated with fatty acids, in addition to normalizing mitochondrial membrane content and potential while reducing ROS production and JNK phosphorylation. Altogether, these data highlight TUDCA as a therapeutic molecule in preventing the imbalance that occurs between ROS production, mitochondrial metabolism, and insulin secretion in obesity and DM2 progression.

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Effect of Taurine on the Regulation of Glucose Uptake in the Skeletal Muscle

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Keywords

AMPK · Exercise · GLUT4 · TXNIP

Abbreviations

AMPK	AMP-activated protein kinase
GSV	GLUT4 storage vesicle
IRS-1	Insulin receptor substrate-1
OLETF	Otsuka Long-Evans Tokushima fatty
PI3K	Phosphoinositide 3-kinase
TXNIP	Thioredoxin-interacting protein

1 Introduction

There has been a rise in the prevalence of diabetes in Japan over the past 20 years. According to the 2016 "National Health and Nutrition Survey" conducted by Japan's Ministry of Health, Labour and Welfare, it is estimated that there are about ten million pre-diabetic people in Japan who are strongly suspected to have diabetes and in whom the possibility of having diabetes cannot be ruled out.

Skeletal muscle is the largest organ in the human body, accounting for about half of humans' body weight. Because skeletal muscle accounts for up to 85% of the insulin-stimulated glucose uptake (DeFronzo et al. 1981, 1985), maintaining the ability of skeletal muscle to take up blood glucose is important for blood glucose control and the prevention of diabetes. Impairment of skeletal muscle glucose uptake is one of the first steps in the development of type II diabetes.

As blood glucose levels rise, insulin is secreted by the pancreas, and blood glucose is taken up by insulin target organs, such as the skeletal muscle. Specifically, insulin binds to insulin receptors on the cell membrane, and the resulting signals are transmitted inside the cell. GLUT4, one of the glucose transporters, then translocates to the cell membrane from GLUT4 storage vesicle (GSV) and promotes blood glucose uptake (Fig. 1). Exercise (i.e., increased muscle contractile activity) also promotes glucose uptake by translocating GLUT4 through various signals in an insulin-independent way (Fig. 2).

In this review article, I explain the effects of taurine on arguably the two most important factors affecting blood glucose uptake in skeletal muscle: insulin secretion from the pancreas and blood glucose uptake in skeletal muscle.

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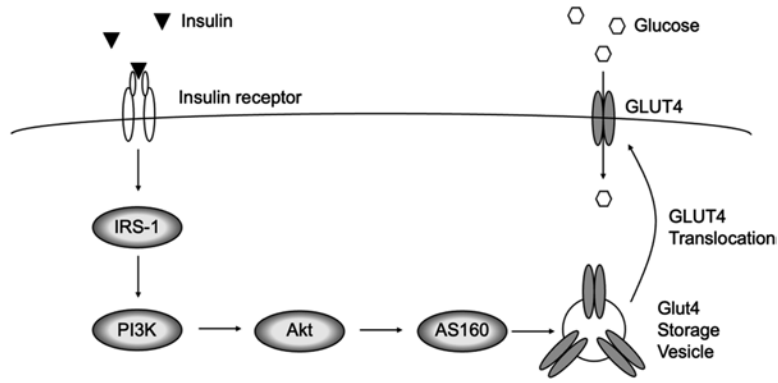

Fig. 1 Insulin-stimulated GLUT4 translocation from a GLUT4 storage vesicle (GSV) via the PI3K/Akt pathway. Taurine increases insulin secretion from the pancreas, leading to glucose uptake in the skeletal muscle

2 Effects of Taurine on Hyperglycemia

Persistent hyperglycemia poses a risk to several end organs, such as the kidney, peripheral nerves, eyes, and brain. When the blood glucose level rises, the beta cells of the pancreatic islets sense this rise and secrete insulin, which causes blood glucose to be taken up by peripheral tissues, such as the skeletal muscle, fat, and the liver, thus lowering the blood glucose level. Several past studies have reported that taurine can lower hyperglycemia by enhancing the secretion of insulin from pancreatic beta cells.

Nakaya et al. reported that Otsuka Long-Evans Tokushima fatty (OLETF) rats, a model rat for type II diabetes, improved their performance on the oral glucose tolerance test when fed 5% taurine in drinking water (Nakaya et al. 2000). Kim et al. also reported that OLETF rats maintained on water containing 2% taurine for 12 weeks showed lower blood glucose levels and less insulin resistance than the control LETO rats (Kim et al. 2012). Furthermore, it was confirmed that insulin secretion from the pancreas was increased in normal mice after administration of 2% taurine solution (Ribeiro et al. 2009). These reports suggest that taurine's improvement of hyperglycemia is directly correlated with its ability to increase insulin secretion from the beta cells of pancreatic islets. The mechanism is believed to involve Ca^{2+}, a key player in several islet cell pathways (Rorsman et al. 2012).

3 Impacts of Taurine on Insulin Signaling and GLUT4 Expression in the Skeletal Muscle

When insulin is secreted by the pancreas, it binds to insulin receptors on the surface of skeletal muscle cell membranes, activating several molecules in the insulin-signaling pathway such as insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), and Akt. This signaling pathway leads to GLUT4 translocation from the cell interior to the plasma membrane, thereby enhancing the cells' blood glucose uptake (Fig. 1). This is one of the mechanisms of insulin-dependent blood glucose uptake in the skeletal muscle.

Zhao et al. (2019) reported that administration of 2% taurine solution to rats fed a high-fat, high-sucrose diet resulted in increased mRNA and protein expression of IRS-1 and GLUT4, as well as improved blood glucose uptake as assessed by the glucose tolerance test. Mikami et al. (2012) reported that taurine combined with fish oil effectively increased GLUT4 protein expression on skeletal muscle cell membranes in KK-A (y) mice, a model of type II diabetes, compared to fish oil alone. These results suggest that taurine administration not only promotes insulin secretion but also increases IRS-1 and GLUT4 protein expression to enhance blood glucose uptake. However, the phosphorylation of Akt, which is downstream of IRS-1 and PI3K, is considered the

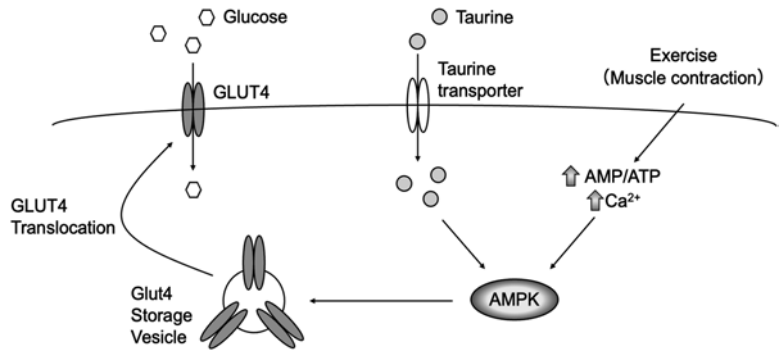

Fig. 2 Schematic overview of the AMP-activated protein kinase (AMPK) pathway in the regulation of skeletal muscle glucose uptake in response to exercise (muscle contraction) and taurine administration. Several studies in rodents have reported that taurine supplementation increases AMPK activity. However, it is still not clear whether taurine increases skeletal muscle glucose uptake via AMPK activation and its downstream signals

most direct indicator of skeletal muscle glucose uptake capacity, and the effect of taurine on skeletal muscle Akt phosphorylation is unknown.

Insulin initiates glucose uptake into most tissues by interacting with the insulin receptor. It has been reported that taurine not only enhances insulin signals but also binds to insulin receptors and enhances skeletal muscle glucose uptake. Maturo and Kulakowski (1988) previously have presented evidence that taurine binds to the purified human insulin receptor. Kulakowski and Maturo (1984) clearly show that pretreatment with taurine (200 mg/kg, i.p.) attenuates the rise in serum glucose levels at 30 min after glucose administration without increasing serum insulin levels. Taken together, these reports suggest that taurine may enhance skeletal muscle glucose uptake either by acting like insulin through interaction with the insulin receptor or by enhancing insulin signaling.

Schaffer et al. (1985) suggest that taurine may enhance the effects of insulin in the heart. Similarly, exposure of hearts to buffer containing taurine (10 mM) led to activation of glycogen synthase activity, resulting in cardiac glycogen accumulation. Because the heart is like the skeletal muscle in many ways, as both increase contractility and insulin enhances glucose uptake, taurine may contribute to the increase in whole-body glucose metabolism by enhancing insulin action in both skeletal and cardiac muscles.

4 Taurine May Increase Skeletal Muscle Glucose Uptake

It is well known that exercise (muscle contraction) increases blood glucose uptake in an insulin-independent manner (Holloszy 2003). Therefore, exercise therapy is recommended for patients with insulin resistance (difficulty lowering blood glucose levels even when insulin is secreted), such as type II diabetes. Takahashi et al. (2014) reported that, in mice, administration of taurine (50 mg/g body weight) immediately after exercise increased blood glucose uptake during a glucose tolerance test. At that time, no difference in insulin concentration was observed between the control group and the taurine-treated group, suggesting that taurine may have the ability to increase blood glucose uptake via a mechanism entirely separate from insulin secretion.

The pathway by which exercise enhances insulin-independent skeletal muscle glucose uptake is known to involve AMP-activated protein kinase (AMPK), at least in part (Mu et al. 2001; Wright et al. 2004). AMPK is activated by exercise (muscle contraction) and participates in the translocation of GLUT4 to increase blood glucose uptake (Fig. 2).

It was reported by Satsu et al. (2019) that AMPK was activated when taurine was added to

Caco-2 cells, an intestinal epithelial model. However, despite the activation of AMPK by taurine supplementation, glucose uptake in the intestine was decreased. Furthermore, mRNA and protein expression of thioredoxin-interacting protein (TXNIP), which has been reported to decrease glucose uptake, were increased by taurine (Gondo et al. 2012; Satsu et al. 2019).

TXNIP has been shown to be a negative regulator of cellular glucose uptake including skeletal muscle (Waldhart et al. 2017; Kawamoto et al. 2018), and recently we found that AMPK activation is one of the mechanisms that counter TXNIP expression in the skeletal muscle (Ra et al. 2020). Ito et al. (2014) reported that mRNA and protein expression levels of AMPK in the skeletal muscle were decreased in taurine transporter KO mice. Borck et al. (2018) reported that administration of 5% taurine solution to ob/ob mice, a leptin-deficient obese diabetic model, increased the activity of AMPK. Furthermore, Ma et al. (2021) found that administration of a 1% taurine solution to SD rats for 21 weeks increased the phosphorylation level of AMPK and the GLUT4 protein expression in the skeletal muscle. The above results suggest that, in contrast to intestinal epithelial cells, taurine can decrease TXNIP expression and increase glucose uptake in the skeletal muscle by activating AMPK in the skeletal muscle.

5 Conclusion and Future Perspectives

This review article outlines the effects of taurine on two aspects of blood glucose regulation: insulin secretion from the pancreas and blood glucose uptake in the skeletal muscle. In many reports, taurine has been shown to improve hyperglycemia such as type II diabetes by increasing insulin secretion from the pancreas. On the other hand, the effect of taurine on blood glucose uptake in insulin target tissues such as the skeletal muscle has not been fully verified. Some reports have shown that taurine can increase the protein expression of IRS-1 and GLUT4 and can activate AMPK as well. We also need to clarify issues such as the phosphorylation of Akt by taurine and its relation to the activation of AMPK in the skeletal muscle. Taurine has been shown to play a role in increasing insulin sensitivity and glucose metabolism in rodents; however, there has been no report on its role in human glucose metabolism, especially in applied physiology and exercise sciences. If the mechanism by which taurine enhances blood glucose uptake in skeletal muscle can be clarified, it would provide valuable new knowledge that can be applied in clinical and sports sciences.

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Differences Between Physiological and Pharmacological Actions of Taurine

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Keywords

Glucose oxidation · Fatty acid oxidation · ATP biosynthesis · Taurine deficiency · Mitochondrial function · Ca^{2+} transport · NADH/NAD^+ ratio · PPARα

Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Na^+]_i$	Intracellular sodium concentration
CrP	Creatine phosphate
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
SERCA	Sarcoplasmic reticular Ca^{2+} ATPase
TauTKO	Taurine transporter knockout

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1 Introduction

Taurine is a β-amino acid that exerts multiple physiological actions, many of which have been uncovered in taurine-deficient animals exhibiting low rates of hepatic taurine biosynthesis. In certain species, such as the cat, taurine is an essential nutrient (Knopf et al. 1978). However, in the rat, taurine is considered a non-essential nutrient, as cellular taurine levels are maintained by active hepatic taurine biosynthesis. Interestingly, rats lacking the taurine transporter develop a pathological condition resembling those of cats maintained on a taurine-deficient diet. Characteristic features of these taurine-deficient animals include cardiomyopathy, retinopathy, immune deregulation, muscle weakness, osmotic imbalances, renal insufficiency, hypertension, atherosclerosis, obesity, neurological abnormalities, hearing loss, and accelerated aging (Rascher et al. 2004; Huang et al. 2006; Sergeeva et al. 2007; Ito et al. 2008, 2014a, b; Kaesler et al. 2012; Schaffer et al. 2014). The involvement of taurine in such a wide range of pathological defects has been attributed to its role as a regulator of several fundamentally important physiological events, including mitochondrial function, survival and death pathways, gene expression, oxidative stress, quality control, calcium homeostasis, and osmotic pressure (Schaffer and Kim 2018). Identifying the biochemical mechanisms underlying the development of each of the

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taurine-related physiological events remains an area of active research.

A common approach to examining the effects of taurine is the exposure of cells, tissues, or animals to high concentrations (mM) of taurine. Normally, the concentration of taurine in excitable tissues, such as the skeletal muscle, the heart, and the brain (5–20 mmol/g wet wt), is approximately 100-fold higher than that in plasma (20–100 mM) (Hayes and Sturman 1981; Huxtable 1992; Graham et al. 1995; Schaffer et al. 2010; DeLuca et al. 2015). This concentration gradient is maintained by a transporter that uses the energy of the Na^+ gradient to promote the accumulation of taurine by these tissues (Schaffer et al. 1982; Huxtable 1992). The Km and Vmax of the taurine transporter in the heart is approximately 100 mM and 32 nmol/min/g dry wt, respectively (Schaffer et al. 1982; Huxtable 1992). Therefore, exposure of the heart to high concentrations of taurine (10 mM) does not increase the rate of taurine uptake by the excitable tissues via the taurine transporter although it may increase taurine uptake via diffusion. This would explain the failure of oral taurine supplementation (1.66 g) in humans to increase muscle taurine content despite a tenfold elevation in plasma taurine concentration (Galloway et al. 2008).

In contrast to pharmacological studies, physiological studies often compare the properties of normal and taurine-deficient tissue. One pathological event that is enhanced in taurine-deficient hearts is elevations in the cleaved form of procaspase 3 leading to increased caspase 3 proteolytic activity and initiation of apoptosis (Jong et al. 2017). In the heart of 3-month-old, taurine-deficient mice, the activation of caspase 3 is preceded by the activation of caspase 9, a mitochondrial-localized protease that initiates apoptosis by activating caspase 3. According to Hu et al. (2013), the activation of caspase 9 is mediated by an apoptosome, which is formed following the release of cytochrome c from the mitochondria. Normally, cytochrome c is bound to phospholipids located in the inner mitochondrial membrane; however, oxidative damage to the phospholipids weakens the electrostatic interaction with cytochrome c, causing its release. According to Jong et al. (2012), the mitochondria of taurine-deficient hearts become oxidatively stressed. Thus, taurine-mediated oxidative stress likely damages key mitochondrial membrane phospholipids leading to the release of cytochrome c, the formation of the apoptosome, and the initiation of a caspase cascade. In an attempt to disrupt this pathway, Jong et al. (2017) treated taurine-deficient mice for 7 days with the mitochondrial-specific antioxidant, MitoTempo. As predicted, decreasing the degree of mitochondrial oxidative stress prevented the activation of caspase 3 in the taurine-deficient heart. However, MitoTempo treatment of wild-type mice containing normal levels of taurine did not affect the levels of active, cleaved caspase 3. Thus, the study of Jong et al. (2017) established a direct link between mitochondrial oxidative stress and the onset of apoptosis in the taurine-deficient heart. It also provided convincing evidence that reductions in intracellular taurine levels contribute to the increased risk of apoptosis. However, pharmacological taurine treatment also protects against apoptosis (Leon et al. 2009; Taranukhin et al. 2010; Das et al. 2012). Therefore, both the physiological and pharmacological effects of taurine share common antioxidative and anti-apoptotic actions. However, it remains to be determined if the mechanisms underlying their actions are identical.

Rodent models of taurine deficiency can be generated by either genetic manipulation of taurine biosynthesis or modulation of taurine uptake by taurine-sensitive cells. It has generally been assumed that defects that develop in these models are caused by taurine deficiency and are therefore considered physiological actions. However, a convincing means of assessing the physiological significance of a specific event is to monitor reversibility of the response upon restoration of cellular taurine content. Although reversibility cannot be established using taurine transporter knockout mice, dietary taurine has been shown to simultaneously restore cellular taurine levels and cellular function of beta-alanine-mediated, taurine deficiency in rodents. Using β-alanine to generate taurine-deficient cardiomyocytes, Jong

et al. (2012) showed that taurine deficiency was associated with reduced rates of respiratory chain function and oxygen consumption and enhanced mitochondrial superoxide production, effects that are reversed by restoration of taurine content. Similarly, normalization of taurine content of isolated taurine-deficient fibroblasts was found to prevent mitochondrial oxidative stress and apoptosis, effects associated with mitochondrial fragmentation (Shetewy et al. 2016). Together, these studies support a physiological role of taurine in the maintenance of mitochondrial structure and function. However, the mitochondrial actions of pharmacological levels of taurine have not been studied. Therefore, a key question that remains unanswered is whether the physiological and pharmacological actions of taurine share the same mechanism. In this study, we tested the hypothesis that the pharmacological and physiological actions of taurine on contractile function and mitochondrial oxidative metabolism involve different mechanisms.

2 Methods

2.1 Model for the Physiological Actions of Taurine

The physiological actions of taurine were assessed by comparing the contractile and metabolic properties of taurine-deficient hearts with those of taurine-repleted hearts (hearts from taurine-deficient rats fed a taurine-rich diet to restore myocardial taurine levels). To produce the animal model of taurine deficiency, male Wistar rats were maintained for 3 weeks on tap water containing 3% β-alanine. This model reduced myocardial taurine content from 98.5 to 59.3 μmol/g dry wt. To restore taurine levels, β-alanine-treated rats were fed a taurine-rich diet (1.5 g taurine added to the regular diet/day) for a period of 3–4 weeks. The physiological actions of taurine were defined as the effects associated with the recovery of intracellular taurine levels (from 59.3 to 99.5 μmol/g dry wt).

Hearts were removed from taurine-deficient and taurine-repleted rats and then perfused with Krebs-Henseleit buffer containing 5 mM glucose, 5 mM acetate, and 2.5 U/l insulin. The hearts were paced at 300 beats/min throughout the experiment. Coronary flow, aortic output, and aortic pressure were measured and used to calculate cardiac work (Schaffer et al. 2016). Perfusate O_2 levels were monitored with a Clark oxygen electrode. Total O_2 consumption was calculated from coronary flow, and the amount of oxygen extracted from the perfusate by the heart. The rate of glucose utilization and oxidation was determined from the rates of [3-^{3}H]-glucose conversion to ^3H_2O, along with lactate and pyruvate production, the latter assayed in coronary effluent samples (Schaffer et al. 2016). The rate of acetate oxidation was determined from the conversion of [2-^{14}C]-acetate to $^{14}CO_2$ (Schaffer et al. 2016). The rate of endogenous fatty acid oxidation was calculated from total O_2 consumption and the rates of acetate oxidation and glucose oxidation (Schaffer et al. 2016). The formation of NADH and $FADH_2$ by the citric acid cycle was calculated from the oxidation rates of acetate, glucose, and endogenous lipids as described in Sect. 2.3. ATP and creatine phosphate content were measured from extracts of freeze-clamped hearts (Schaffer et al. 2016). All data represent a ratio of metabolic parameters for hearts of taurine-repleted rats/taurine-deficient rats. Significance is established when $p < 0.05$.

2.2 Model for the Pharmacological Actions of Taurine

Hearts from normal male Wistar rats were removed and perfused with Krebs-Henseleit buffer containing 5 mM glucose, 5 mM acetate, and 2.5 U/l insulin. The hearts were paced at 300 beats/min throughout the experiment. Each heart served as its own control. Cardiac work was calculated from coronary flow, aortic output, and aortic pressure measurements. After 20 min of control perfusion, perfusate was collected at minute intervals for 3 min and used to assess metabolic properties of the control heart. The hearts were then exposed to medium containing 5 mM

glucose, 5 mM acetate, 10 mM taurine, and 2.5 U/l insulin. This phase of the experiment examined the pharmacological actions of taurine. After 20 min exposure to buffer containing 10 mM taurine, perfusate was collected at minute intervals for a period of 3 min. Total oxygen consumption was calculated from the coronary flow rate, and the amount of oxygen extracted from the perfusate by the heart. The rates of glucose utilization and oxidation, as well as acetate oxidation, were determined as described for the physiological actions of taurine (1.2.1). The rate of change in contractile function and metabolic status of the heart caused by exposure to high concentrations of taurine (10 mM) represents the pharmacological actions of taurine.

2.3 Calculation of Metabolic Rates of Taurine-Deficient and Control Hearts

Acetate oxidation was calculated from the generation of $^{14}CO_2$ from [2-^{14}C]-acetate. Endogenous lipid oxidation, which we call fatty acid oxidation, refers to O_2 consumption not related to glucose and acetate oxidation. Glucose oxidation was calculated from glucose utilization, lactate generation, and pyruvate production (Schaffer et al. 2016). The ratio of ATP produced to O_2 consumed, which is used in the calculation of ATP synthesis are as follows: glucose, 3.17; acetate, 2.5; and palmitate, 2.8. The net yield of ATP from lactate output was assumed to be 2 μmol ATP/μmol of glucose, whereas pyruvate output was assumed to be 8 μmol/μmol of glucose (2 for glycolysis and 6 for oxidation of cytoplasmic NADH).

3 Results

3.1 Pharmacological Actions of Taurine on Contractile Function

In 1866, Carl Ludwig developed the first isolated perfused heart preparation. The most commonly used preparation, referred to as the Langendorff preparation, was created by Oscar Langendorff in 1895. These pioneering studies showed that hearts beat spontaneously if perfused with buffer containing a substrate capable of generating ATP, an appropriate concentration of key cations, such as Na^+, K^+, and Ca^{2+}, a source of oxygen and an appropriate pH. Since the development of the perfusion system, many constituents of blood have been found to influence contractile function, with taurine being one of them. Nonetheless, the concentration of taurine found in normal plasma (20–100 μM) has no influence on contractile function of the isolated heart. Yet, normal blood taurine is essential for maintaining the intracellular taurine pool of the heart, which in turn ensures normal contractile function of the heart in vivo. However, pharmacological concentrations of taurine (mM) stimulate contractile function, in part through a Ca^{2+}-dependent mechanism by delaying the loss of myocardial Ca^{2+} and contractile function of hemodynamic hearts perfused with medium containing low concentrations of Ca^{2+} (Chubb and Huxtable 1978; Chovan et al. 1980; Franconi et al. 1982).

Figure 1a shows the relationship between perfusate Ca^{2+} concentration and cardiac work in hearts perfused with Krebs-Henseleit buffer containing or lacking 10 mM taurine. In the absence of taurine, the heart perfused with medium containing 0.6 mM Ca^{2+} barely generates sufficient pressure to eject fluid against an afterload pressure of 110 cm H_2O. As the Ca^{2+} concentration of the perfusate is increased from 0.6 to 1.0 mM, cardiac work increased fourfold, reaching a workload of 0.33 kg-m/g dry wt/min. Raising the Ca^{2+} concentration of the perfusate from 1.0 mM to 1.25 mM resulted in a further 30% increase in cardiac work. Addition of 10 mM taurine to the perfusion medium increased cardiac work over the entire [Ca^{2+}] range of 0.4 mM to 1.25 mM, with the largest effects seen in hearts perfused with buffer containing the lowest concentrations of Ca^{2+}. As expected, hearts generating the greatest workloads consumed the most oxygen. Ventura-Clapier et al. (2011) established that a strict relationship exists between cardiac performance (energy utilization) and O_2 consumption (energy production). Indeed, the relationship in Fig. 1b between cardiac work and oxygen consumption follows a defined curve, with the

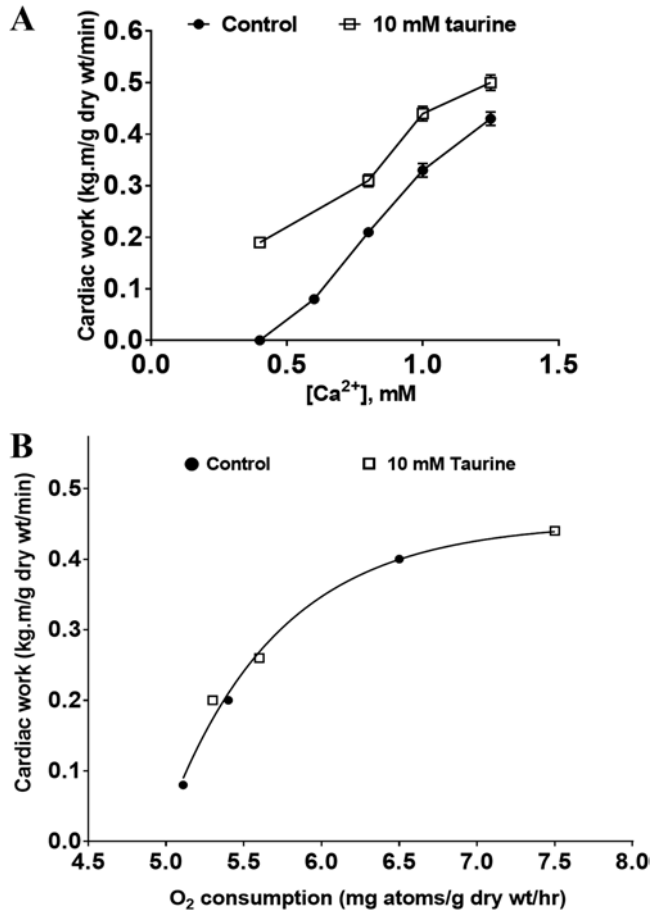

Fig. 1 The effect of pharmacological taurine (10 mM) on the Ca^{2+} dependence of myocardial contractility. (**a**) Hearts from normal rats were perfused with Krebs-Henseleit buffer lacking taurine but containing various concentrations of Ca^{2+}. Cardiac work was determined after 20 min of controlled perfusion. The hearts were then switched to medium containing 10 mM taurine. After 20 min, cardiac function was determined. Data shown represent means ± SEM of five hearts. Taurine significantly increased cardiac work at all Ca^{2+} concentrations examined ($p < 0.05$). (**b**) Relationship between energy production (O_2 consumption) and energy utilization (contractile function). Hearts were perfused according to description in A. Oxygen consumption was calculated from the rate of coronary flow and the amount of O_2 extracted from the perfusate by the heart, which was monitored with an O_2 electrode placed in the perfusate. Taurine increased both contractile function and O_2 consumption, yielding points that fall on the upper portions of the energy production vs. energy utilization curve

taurine-treated and taurine-untreated hearts fitting on the upper and lower portions of the curve, respectively.

3.2 Pharmacological Actions of Taurine on Metabolic Flux and ATP Biosynthesis

The heart consumes ~1 mmol ATP/sec, which means that the entire high-energy phosphate pool of the heart must be renewed every 20 s to maintain normal high-energy phosphate levels. When ATP is cleaved by myocardial ATPases to provide energy for contraction, ADP is produced, which in turn stimulates the generation of ATP by the electron transport chain and oxidative phosphorylation. Indeed, O_2 consumption of hearts perfused with medium containing 10 mM taurine increased 9.2 ± 0.4% and was accompanied by a 11.2 ± 0.6% increase in ATP biosynthesis (Fig. 2b). A balance is maintained between ATP

biosynthesis and ATP utilization of hearts exposed to 10 mM taurine, resulting in no change in the high-energy phosphate content of the heart; the CrP/ATP ratios of the control and taurine-treated hearts were 1.6 ± 0.1 and 1.7 ± 0.1, respectively (Fig. 2b).

Pharmacological levels of taurine stimulate substrate flux through all of the major metabolic pathways of the heart. The pathway most affected by taurine exposure is glucose oxidation, whose flux rose 23.9 ± 1.0% after exposure of the heart to medium containing 10 mM taurine (Fig. 2a). Pyruvate dehydrogenase is the initial enzyme involved in glucose oxidation. Therefore, evidence that pyruvate output decreased by 4%, while both glucose utilization and lactate output increased 19%, suggests that pharmacological levels of taurine divert pyruvate into acetyl-CoA (Fig. 2c). The rate of flux through the other two sources of acetyl-CoA, fatty acid oxidation and acetate, were stimulated after taurine exposure by 12.8 ± 0.6% and 5.8 ± 0.4%, respectively (Fig. 2a). Together, the three sources of acetyl-CoA combined to enhance citric acid cycle flux by 8.8 ± 5.1%, with taurine addition increasing the rate of ATP biosynthesis from glucose, acetate, and fatty acids by 22.7%, 5.5%, and 12.8%, respectively.

3.3 Physiological Actions of Taurine on Contractile Function

Taurine deficiency leads to the development of dilated cardiomyopathy associated with diminished systolic and diastolic function (Pion et al. 1978; Novotny et al. 1991; Ito et al. 2008). One unique feature of taurine-deficient cardiomyopathy is the development of cardiac atrophy. Although the mechanism underlying the reduction in cardiomyocyte size in the taurine-deficient heart remains to be determined, there are several factors that may contribute to diminished contractility. Like other models of heart failure, the taurine-deficient heart becomes ATP deficient (Schaffer et al. 2016). However, taurine deficiency develops a unique mechanism for regulating contractile function. In most models of heart failure, the expression of the sarcoplasmic reticular Ca^{2+} ATPase (SERCA2a), a Ca^{2+} transporter that regulates $[Ca^{2+}]_i$ during the contraction-relaxation cycle, is downregulated. Instead, taurine deficiency modulates $[Ca^{2+}]_i$ and the Ca^{2+} dependence of the sarcoplasmic reticular Ca^{2+} ATPase rather than decreasing SERCA2a expression (Ramila et al. 2015). The activity of sarcoplasmic reticular Ca^{2+} ATPase is regulated by the phosphorylation state of the phosphoprotein, phospholamban. In the taurine-deficient heart, the phosphorylation state of phospholamban is reduced, which is known to increase the ability of the phosphoprotein to decrease sarcoplasmic reticular Ca^{2+} ATPase activity (Ramila et al. 2015). This series of events reduces the amplitude of the Ca^{2+} transient and contributes to the development of the cardiomyopathy (Schaffer et al. 2000). Cardiac work of the taurine-deficient and taurine-repleted hearts was 0.34 ± 0.02 and 0.4 ± 0.02 kg-m/g dry wt/min, respectively.

3.4 Physiological Actions of Taurine on Metabolic Flux and ATP Biosynthesis

A common chemical marker of energy deficiency in the heart is the creatine phosphate/ATP ratio (Neubauer et al. 1997). Although both creatine phosphate (CrP) and ATP are considered high-energy phosphate compounds, ATP is the predominant high-energy phosphate used in maintaining contractile function, while CrP serves as a high-energy phosphate reserve that is utilized by creatine phosphokinase to form ATP from ADP. The taurine-deficient heart is energy deficient, as its CrP/ATP ratio is 23% lower than that of the normal heart. The effect of taurine deficiency on the energy state of the heart is reversible, as taurine supplementation restores the normal CrP/ATP ratio (Fig. 2a).

The restoration of the CrP/ATP ratio by taurine repletion is largely mediated by an increase in oxidative metabolism, with glucose and acetate oxidation rising 33.5% and 24.7%, respectively (Fig. 2a). Yet, the most prominent effect of

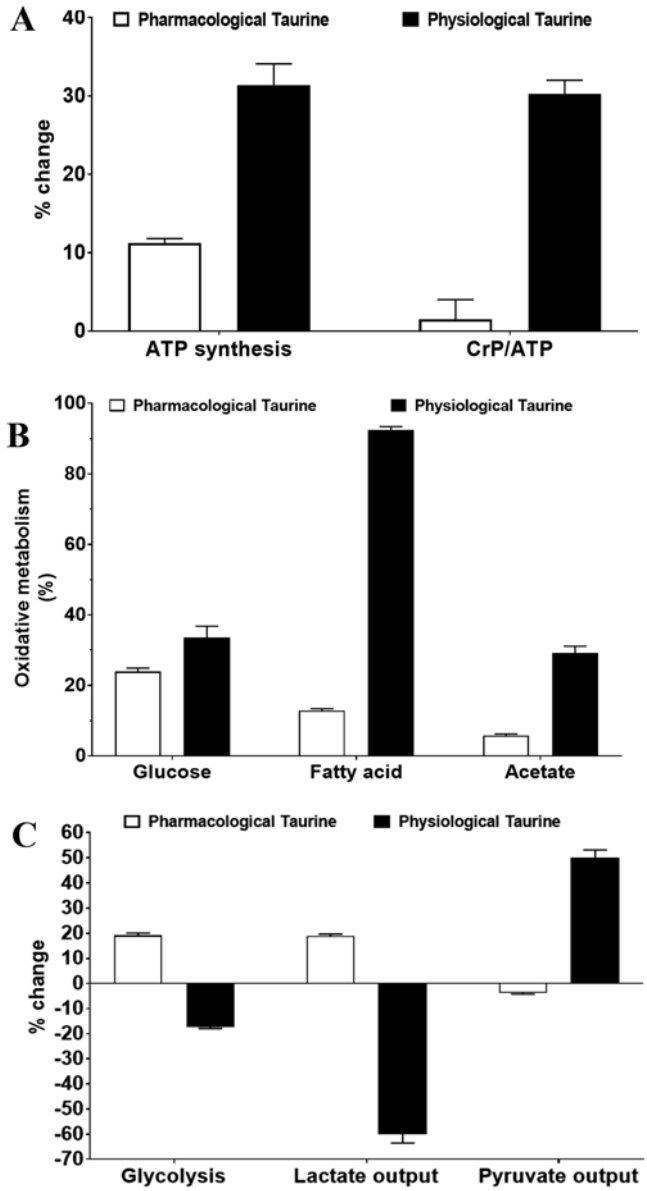

Fig. 2 The effect of physiological and pharmacological taurine on high-energy phosphate metabolism. Hearts from normal, taurine-deficient, and taurine-repleted rats were perfused with buffer containing 5 mM ^{3}H-glucose, 5 mM acetate, and 1 U/l insulin. Pharmacologically treated hearts were subjected to perfusion with buffer containing 10 mM taurine, 5 mM ^{3}H-glucose, 5 mM acetate, and 1 U/l insulin. Values shown for pharmacological taurine represent the ratio of the metabolic parameters for taurine-treated hearts/normal hearts, while the parameters for physiological hearts represent the ratios of metabolic parameters for taurine-repleted hearts/taurine-deficient hearts. (**a**) The rate of glycolysis was determined from the rate of $^{3}H_2O$ generation from ^{3}H-glucose. To calculate the rate of glucose oxidation, the rates of lactate and pyruvate production were subtracted from the glycolytic rate. Acetate oxidation was derived from the conversion of 2-^{14}C acetate to $^{14}CO_2$. The rate of endogenous fatty acid oxidation was calculated from the rates of O_2 consumption, acetate oxidation, and glucose oxidation. All values shown represent means ± SEM of five hearts. (**b**) The rate of ATP synthesis was calculated from the rates of glucose, acetate, and fatty acid oxidation. The values shown for the CrP/ATP ratio are derived from the content of creatine phosphate and ATP measured in the extract of isolated hearts perfused as described in A. (**c**) Glycolytic flux is derived from the rate of $^{3}H_2O$ generation from ^{3}H-glucose. Lactate and pyruvate output was calculated from the lactate and pyruvate content of the perfusate. Values shown represent means ± SEM of five hearts

taurine repletion is the 92.5% increase in fatty acid oxidation (Fig. 2a). All three oxidative pathways feed into the citric acid cycle, whose flux is elevated 8.8% by taurine feeding. Overall O_2 consumption rose 32.6% during taurine repletion, an effect attributed to taurine-mediated regulation of the electron transport chain.

The acceleration of electron transport also impacts glucose metabolism. In the taurine-deficient heart, the activity of complex 1 of the respiratory chain is significantly decreased, resulting in the accumulation of NADH and an elevation in the NADH/NAD^+ ratio (Jong et al. 2012). In order to prevent excessive accumulation of NADH by the heart, the metabolic pathways involved in NADH generation are regulated by the NADH/NAD^+ ratio. In the case of glucose metabolism, elevations in the mitochondrial NADH/NAD^+ ratio inhibit pyruvate dehydrogenase, diminishing the oxidation of pyruvate by the citric acid cycle (Hansford 1976). In the cytosol, elevations in the NADH/NAD^+ ratio favor the reduction of pyruvate to lactate (Schaffer et al. 2016). Treating taurine-deficient rats with a taurine-rich diet prevented this diversion of pyruvate away from the citric acid cycle. Hence, physiological elevations in intracellular taurine content led to a 33.5% increase in both glucose oxidation and pyruvate dehydrogenase flux but a 60.0% decrease in lactate generation (Fig. 2b, c).

4 Discussion

The present study supports the hypothesis that the physiological and pharmacological actions of taurine are entirely different. To examine the physiological actions of taurine, taurine-deficient rats were fed a taurine-rich diet to restore intracellular taurine levels. Differences in the properties of the taurine-deficient and taurine-repleted hearts define the physiological function of taurine. By contrast, the pharmacological actions of taurine were evaluated in normal hearts initially perfused with buffer lacking taurine, followed by a 20-min perfusion with buffer containing 10 mM taurine.

Increases in contractile function were associated with both the physiological and pharmacological actions of taurine. Although both actions appear to alter myocardial Ca^{2+} handling, the specific mechanisms underlying each action are different. In the case of the pharmacological actions, taurine-mediated elevations in contractile function were dependent on the Ca^{2+} concentration of the perfusion buffer, a response linked to the stimulation of an inward Ca^{2+} current via the L-type Ca^{2+} channel (Satoh and Horie 1997; Sawamura et al. 1990). The characteristics of the inward Ca^{2+} current are complex, perhaps because they relate to the importance of the taurine/Na^+ transporter and its indirect effects on the Na^+/Ca^{2+} and Na^+/H^+ exchangers (Suleiman et al. 1992; Chapman et al. 1993). For example, the trans-sarcolemmal entry of Na^+ during taurine transport might favor Ca^{2+} influx via the Na^+/Ca^{2+} exchanger (Bkaily et al. 1998). It has also been suggested that the sarcoplasmic reticulum might contribute to the pharmacological actions of taurine; however, recent evidence suggests that those actions of taurine are physiological.

The concentration of taurine normally found in the plasma has no effect on contractile function of the isolated rat heart, an observation attributed in part to regulatory mechanisms that restrict the amount of taurine that can accumulate in the heart. Normally, the intracellular myocardial taurine concentration is approximately tenfold higher than that located in the plasma, a pattern that forms a steep concentration gradient across the sarcolemmal membrane. Hence, taurine influx occurs against a concentration gradient that only becomes thermodynamically favorable when the transporter simultaneously catalyzes the influx of Na^+ down its concentration gradient, providing the energy for taurine influx. However, the accumulation of Na^+ via the taurine/Na^+ symporter is limited, as increases in $[Na^+]_i$ drive the reversal of the taurine/Na^+ symporter, leading to taurine extrusion (Suleiman et al. 1992). Moreover, the effect of taurine on contractile function depends on the properties of sarcoplasmic reticular Ca^{2+} transport, which regulates the $[Ca^{2+}]_i$ in the vicinity of troponin and the other muscle proteins that determine the force of contraction. According to Steele et al. (1990), taurine modulates Ca^{2+} uptake and release by the sarcoplasmic reticulum, with the maximal effect occurring at a taurine concentration of 5 mM. In

the TauTKO (taurine transporter knockout) mouse, taurine deficiency is associated with a relative dephosphorylation of the regulatory phosphoprotein, phospholamban, which enhances the capacity of the phosphoprotein to inhibit the sarcoplasmic reticular Ca^{2+} ATPase (Ramila et al. 2015). Although the properties of the key Ca^{2+} transporters and muscle proteins were not examined in the present study, it is noteworthy that normal contractile function is restored in both beta-alanine-treated rats fed a taurine-enriched diet for 3–4 weeks (in the present study) and nutritionally deficient cats fed a taurine-enriched diet (Pion et al. 1978).

Physiological and pharmacological taurine mediate strikingly different metabolic changes. Pharmacological taurine is associated with an increase in flux through the catabolic pathways of all the major metabolic substrates, with glucose oxidation increasing more than fatty acid or acetate oxidation. Because myocardial glucose utilization and oxidation both increase a comparable 20–25% after exposure to pharmacological taurine, it is logical to assume that increased glycolytic flux is largely responsible for the acceleration in glucose oxidation. The rate-limiting steps in the glycolytic pathway of hearts exposed to pharmacological taurine appear to be glucose uptake and phosphofructokinase, whose activities are sensitive to alterations in contractile function, increases in ATP utilization, and decreases in the ATP/ADP ratio (Neely and Morgan 1974). Similarly, the positive inotropic effect of pharmacological taurine appears to be responsible for the elevations in fatty acid and acetate oxidation. Together, the oxidation of all three substrates combines to cause a modest 11.2% increase in ATP biosynthesis.

The transition from taurine deficiency to normal taurine content is associated with major alterations in high-energy phosphate metabolism. Underlying most of those metabolic changes are the restoration of normal electron transport chain activity and the subsequent decrease in the $NADH/NAD^+$ ratio (Jong et al. 2012; Schaffer et al. 2016). Among the regulatory enzymes inhibited by a high $NADH/NAD^+$ ratio are glyceraldehyde-3-phosphate dehydrogenase (glycolysis), pyruvate dehydrogenase (glucose oxidation), alpha-ketoglutarate dehydrogenase (citric acid cycle), isocitrate dehydrogenase (citric acid cycle), citrate synthase (citric acid cycle), pyruvate dehydrogenase kinase (glucose oxidation), and 3-hydroxyacyl CoA dehydrogenase (fatty acid oxidation). Thus, the decrease in the $NADH/NAD^+$ ratio during taurine repletion plays a major role in the recovery of the CrP/ATP ratio during taurine repletion. Also contributing to the improvement in the high-energy phosphate state of the heart during restoration of the intracellular taurine pool is an increase in PPARalpha, a transcriptional regulator of fatty acid metabolism (Lopaschuk et al. 2010). Among other actions, PPARalpha increases both the uptake of long-chain fatty acids by the mitochondria and the activity of enzymes involved in fatty acid beta-oxidation. Because of the upregulation of PPARalpha, taurine repletion preferentially accelerates fatty acid oxidation relative to glucose and acetate oxidation.

Another characteristic feature of taurine repletion is the downregulation of both glucose utilization and lactate output. These effects are attributed to taurine-mediated regulation of respiratory chain complex I activity (Jong et al. 2012). In the taurine-deficient heart, complex I activity is severely depressed, resulting in an elevation in the $NADH/NAD^+$ ratio. This is analogous to hypoxia, in which electron transport is also inhibited, leading to an increase in the $NADH/NAD^+$ ratio. Pyruvate metabolism is highly dependent on elevated $NADH/NAD^+$ ratios, which promote the phosphorylation and inhibition of pyruvate dehydrogenase, thereby diverting pyruvate away from the citric acid cycle (Hansford 1976). Instead, pyruvate is diverted into the cytosol, where it is converted to lactate via lactate dehydrogenase. Taurine repletion unravels this sequence of events, leading to a 33.5% increase in glucose oxidation, a 50% increase in pyruvate output, and a 60% decrease in lactate output. Interestingly, taurine supplementation also depresses glucose utilization, an effect likely related to an elevation in citrate content, an inhibitor of the rate-limiting enzyme of glycolysis, phosphofructokinase (Mozaffari et al. 1986).

5 Conclusion

The present study shows that both the metabolic and contractile effects of increased intracellular taurine levels (physiological action) are entirely different than those of elevated extracellular taurine levels (pharmacological action). The physiological actions of taurine are mediated by an increase in complex I activity of the electron transport chain and the activation of sarcoplasmic reticular Ca^{2+} ATPase activity. Taurine repletion restores taurine levels, reduces lactate output, and increases the energy state of the heart, which is clinically relevant because patients that develop congestive heart failure are at risk of death from impaired mitochondrial function and reduced energy levels. Moreover, the formation of 5-taurinomethyluridine $tRNA^{Leu(UUR)}$, whose levels increase with taurine repletion, is defective in patients suffering from the mitochondrial disease, MELAS. These patients develop cardiomyopathy and myopathy, which are associated with impaired mitochondrial function, reduced energy levels, and enhanced lactic acidosis. In contrast to the physiological functions of taurine, the strictly pharmacological actions of taurine appear to have limited clinical benefit.

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Part VII

Role of Taurine in the Brain

Taurine and the Brain

Simon S. Oja and Pirjo Saransaari

Keywords

Brain · Development · Trophic actions · Volume regulation · Receptors · Taurine as drug

Abbreviations

GABA γ-aminobutyrate

1 Taurine in Development

Taurine is a widespread constituent in various animal tissues being enriched in the brain in most species. The level varies among different species, being, for example, low in the guinea pig but high in the rat (Oja et al. 1968). It significantly diminishes during the development (Oja et al. 1990; Dawson et al. 1999). All cells in the brain contain taurine, but it is not significantly more enriched in any brain nuclei. Taurine serves a wide variety of functions in the central nervous system, though it is not a constituent of proteins (Ripps and Shen 2012). The extracellular concentrations in the rat hippocampus have been reported to be 20.6 μM (Lerma et al. 1986) and in the striatum 25.3 μM (Molchanova et al. 2004).

Dr. John Sturman has made with his cat colony a number of significant observations on the significance of taurine in the development of the central nervous system. Offspring born to mothers subjected to taurine deprivation show a disarray of the tapetum lucidum in the eyes (Sturman et al. 1982). Many of them are stillborn, and their survival rate is significantly reduced during the postnatal development (Sturman et al. 1984). The morphology of the cerebellum is abnormal with a delayed migration of cells from the external granule cell layer (Sturman et al. 1985). In contrast to cats, rats tolerate remarkably well taurine deficiency even during early developmental stages. Primates and human occupy an intermediate position between cats and rats (Tochitani 2017). Some children who have been fed by parenteral nutrition lacking taurine have shown ophthalmological and electrophysiological abnormalities (Geggel et al. 1984). All infant formulas prepared from taurine-poor cow milk are therefore, nowadays, fortified with added taurine.

2 Trophic Actions of Taurine

Taurine has a role in neuronal and stem cell differentiation (Li et al. 2017). Taurine has been shown to be involved in neural stem/progenitor cell proliferation in the developing rat brain

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(Shivaraj et al. 2012). In both vertebrate and invertebrate species, taurine promotes neurite outgrowth, synaptogenesis, and synaptic transmission during the early stages of brain development (Mersman et al. 2020).

Taurine apparently has also some beneficial actions in the adult brain. It promotes hippocampal neurogenesis in aging mice and increases the survival of newborn neurons (Gebara et al. 2015). The decline of brain taurine levels has been associated with cognitive deficits, whereas chronic administration of taurine seems to ameliorate age-related deficits such as in memory acquisition and retention (Suárez et al. 2016). Solid evidence supports taurine cytoprotective actions, directly or indirectly related to an antioxidant effect in the brain development (Pasantes-Morales and Hernández-Benítez 2010). In addition to the trophic actions, taurine has been proposed to have further physiological functions, including membrane stabilization, cell volume regulation, mitochondrial protein translocation, antioxidative activity, neuroprotection against neurotoxicity, and modulation of intracellular calcium levels (e.g., Tochitani 2017).

3 Cell Volume Regulation

The basal release of taurine at least partially reflects leakage from the cells. Cell-damaging conditions, especially hypoosmotic media, have markedly increased the release in various brain preparations. For example, taurine release from cerebral cortical slices (Oja and Saransaari 1992), cultured neurons (Schousboe et al. 1990), and astrocytes (Pasantes-Morales et al. 1990) is enhanced in vitro by hypoosmotic incubation media. Hypoosmotic solutions in brain microdialysis have likewise markedly increased the levels of extracellular taurine in vivo (Solís et al. 1988). Taurine has therefore been suggested to act as an organic osmolyte, contributing to the cell volume regulation, which is particularly critical in the brain (Pasantes-Morales 2017).

However, taurine is not the only amino acid released by hypoosmotic media. For instance, glutamate and aspartate are released as well (Haskew-Layton et al. 2008). In addition, to hypoosmotic media, other damaging conditions also evoke taurine release, for example, ischemia (Oja and Saransaari 2011). Moderate concentration of K^+ evokes osmosensitive release of taurine, but higher concentrations trigger the release by an osmoresistant mechanism in the rat hippocampus in vivo (Rodríguez-Navarro et al. 2009). Consistent with this, we have shown that mouse cerebral cortex slices release taurine in addition to hypoosmotic media also in hyperosmotic media when stimulated with high potassium ion concentrations (Oja and Saransaari 1992). The participation of taurine in cell volume regulation is well documented, but it is not its only function.

4 Taurine and Amino Acid Receptors

In many studies, taurine has been shown to inhibit neuronal firing, causing hyperpolarization of excitable membranes affecting permeability of ions (Saransaari and Oja 2008). These effects are likely to stem from the actions on membrane receptors. The γ-aminobutyrate (GABA) and glycine receptors may be involved, but in certain cases, the effects do not seem to result from their activation (Cañas et al. 1992; Frosini et al. 2000). There is thus reason to look for different receptors specific for taurine itself (Kilb 2017). Taurine receptors may exist in the frog spinal cord (Kudo et al. 1988) and lobster olfactory organ (Sung et al. 1996). Taurine has been found to modulate GABA binding to $GABA_A$ benzodiazepine complex in solubilized rat brain membranes (Malminen and Kontro 1986). It likewise interferes with sodium-independent glycine binding to brain membranes (Kontro and Oja 1987). Taurine also binds sodium independently to purified synaptic membranes (Kontro and Oja 1985).

Taurine seems to act as a ligand of glycine receptors in the rat inferior colliculus and is involved in the information processing of the central auditory system like the neurotransmitter glycine (Xu et al. 2006). Glycine receptors are activated by taurine at low concentrations in the

immature rat hippocampus (Wu and Xu 2003) and preoptic hypothalamic neurons (Bhattarai et al. 2015), whereas both glycine and GABA receptors are activated at high concentrations. Taurine may even have higher efficacy than glycine to glycine receptors in substantia gelatinosa neurons in the rat (Wu et al. 2008). In the rat anteroventral cochlear nucleus, taurine can serve as a neuromodulator to strengthen both GABAergic and glycinergic neurotransmissions (Song et al. 2012).

Taurine may also modulate orofacial pain through the activation of glycine and/or extrasynaptic $GABA_A$ receptors in the substantia gelatinosa neurons (Nguyen et al. 2013). The effect on the extrasynaptosomal receptors is pronounced in the thalamus (Jia et al. 2008). The antinociceptive effect of taurine is antagonized by strychnine indicating the involvement of glycine receptors (Pellicer et al. 2007). The neuroprotective effect of taurine against focal cerebral ischemia in rats is mediated by activation of both $GABA_A$ and glycine receptors (Wang et al. 2007). Taurine has also been suggested to be a physiological gliotransmitter (Choe et al. 2012). Taurine prevents the ammonia-induced accumulation of cGMP by acting as both glycine and $GABA_A$ receptors in the rat striatum (Hilgier et al. 2005). The target of taurine shifts from glycine receptors to GABA receptors during mouse postnatal development (Yoshida et al. 2004; Tang et al. 2008).

Taurine is a full agonist of the glycine receptors in the ventral tegmental area of young rats (Wang et al. 2005). Taurine efficiently gates glycine α1 receptors (Schmieden et al. 1992), but both human and rat α2 and α3 glycine receptors show only weak responses to taurine (Kuhse et al. 1990). Relatively minor modifications in the structure of glycine receptors may dramatically alter their sensitivity to taurine. For instance, isoleucine in position 111 and alanine in 212 are important for activation in the mammalian α1 subunits, and valine in these positions in α2 reduces the taurine efficacy (Schmieden et al. 1992). The homomeric zebrafish α1 receptor exhibits exceptionally high sensitivity for taurine, almost comparable to glycine (David-Watine et al. 1999). The cloned homomeric glycine receptor α4 subunit from chicks containing valine in positions 111 and 212 is also potently activated by taurine (Harvey et al. 2000). The efficacy of taurine has likewise been shown to be variable at different types of recombinant $GABA_A$ receptors (Jia et al. 2008; Kletke et al. 2013).

The investigations have convincingly shown that taurine interacts with glycine and GABA receptors. The possible existence of independent taurine receptors still remains open.

5 Taurine as a Drug

The actions of taurine are predominantly inhibitory. It is thus natural that exogenous taurine has been tested in situations when inhibitory effects are desired. The physiological properties of taurine may offer potential for the design of new pharmacological strategies (Ochoa de la Paz et al. 2019). Taurine administration has been assumed to be an attractive option for treating various neurological disorders (Jakaria et al. 2019). The possible mode of action and clinical applications in neurological diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease has been discussed by Menzie et al. (2014).

Taurine protects against oxidative stress, apoptosis, and inflammation in injured brain cells (Niu et al. 2012). It has been shown to prevent mitochondrial dysfunction and protect neurons against endoplasmic reticulum stress associated with neurological disorders (Kumari et al. 2013). Taurine supplementation likewise reduces neuroinflammation and protects against white matter injury after intracerebral hemorrhage in rats (Zhao et al. 2018). In rats, taurine improves cerebral blood flow after traumatic brain injury which may alleviate edema and elevation in intracranial pressure (Wang et al. 2016). It lessens the corpus callosum damage, attenuates the neuronal cell death in the hippocampus, and improves neurological functions (Gu et al. 2015). Furthermore, taurine can aid cognitive impairment and may inhibit Aβ-related damages in Alzheimer's disease (Kim et al. 2014). Taurine ingestion enhances

the performance in energy-demanding and challenging situations in athletes and young subjects (Sajid et al. 2017). In contrast to studies on young rats, no neuroprotective effects of taurine were seen on functional impairment or tissue loss in aged rats after traumatic brain injury (Gupte et al. 2019). Taurine reduces ischemia-induced caspase-8 and caspase-9 expression in the hypothalamic supraoptic and paraventricular nuclei, the key inductors of apoptosis (Taranukhin et al. 2008). It protects cerebellar neurons of the external granular layer against ethanol-induced apoptosis in 7-day-old mice (Taranukhin et al. 2012).

The simultaneous administration of high amounts of taurine and ethanol kills mice (Taranukhin et al. 2013). Taurine also differently modulates ethanol-induced anxiolytic- and anxiogenic-like behaviors depending on the concentration in the zebra fish (Fontana et al. 2020). During the last years, the consumption of energy drinks has been continuously increasing. Today, taurine is present in almost all energy drinks, mostly together with caffeine (Bkaily et al. 2020). In particular, children and adolescents do not benefit from this combination and may suffer ill effects (Curran and Marczinski 2017). Caffeine and taurine inhibit proliferation of immature oligodendrocytes, dendritic branching, and axonal integrity of hippocampal neurons (Serdar et al., 2019). This combination also induces pyknosis and chromatolysis of the cerebral and medullary neurons and degeneration and abnormal Purkinje cells in the cerebellum (Al-Basher et al. 2018). A young man has been reported to show a generalized epileptic seizure after energy drink consumption (Calabrò et al. 2012). Recently, a dramatic increase in the use of energy drinks and alcohol in combination may bring forth more adverse effects (Manchester et al. 2017). However, after a short-duration and low-intensity exercise, energy drinks and taurine were able to produce only minor electrocortical activity changes in the frontal regions in men (Paulucio et al. 2017).

Taurine reduces epileptiform activity induced by removal of Mg^{2+} in combination with rat entorhinal cortex-hippocampal slices (Kirchner et al. 2003). It also potentiates the anticonvulsive effect of the $GABA_A$ agonist muscimol (Winkler et al. 2019). High doses of taurine attenuate recurrent epileptiform discharges in the immature hippocampus partially mediated by glycine receptors (Chen et al. 2014). However, the efficacy of taurine in epilepsy is reduced after a few months and is manifest only in about one-third of human epileptic patients (Oja and Saransaari 2013).

As a whole, taurine is not generally harmful for people. Its possible potential in clinical use has not yet been adequately explored, and more research on this aspect would be very welcome.

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Electrophysiological Evidence for Anti-epileptic Property of Taurine

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Keywords

Taurine · Kainic acid · Isoniazid · Seizures · Excitability · Cortical ripples · Electrophysiology

Abbreviations

EPSP	Excitatory postsynaptic potential
GAD	Glutamic acid decarboxylase
HFO	High-frequency oscillation
ISO	Isoniazid
KA	Kainic acid
LFO	High frequency oscillation
PSD	Power spectral densities

1 Introduction

Seizures are one of the most common pathological conditions encountered in humans and are more common in children than adults. The highest incidence of seizures occurs during the first year of life (Chagnac-Amitai and Connors 1989). The increased excitability in the developing brain appears to be secondary to a developmental imbalance between maturation of excitatory and inhibitory circuits.

Epileptogenicity is characterized by chronic hypersensitivity to sensory stimuli and is intrinsically dependent on hyperexcitability. Alterations in the GABAergic system have been associated with the onset of hyperexcitability. Possible mechanisms that can contribute to increase neuronal excitability and seizure propensity include functional alterations in mediators of inhibitory and excitatory neurotransmission, ion homeostasis, ion pumps, hormones, and neuromodulators.

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The neonatal brain contains high levels of taurine (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 (Wang et al. 1998) (stroke, injuries, hypoxia), suggesting that taurine may play a vital role in neuroprotection. A possible mechanism of taurine's neuroprotection lies in its calcium modulatory effects and agonistic role on $GABA_A$.

The considerable pool of taurine in the brain has important functional implications. 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 bicuculline-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 $GABA_A$ 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 $mg.kg^{-1}$) reduce 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.

2 Methods

2.1 Animals

A total of 12 mice were used in this study. The mice were 6-month-old C57BJ6X129 males. All mice were housed in groups of 3 to 4 in a pathogen-free room maintained on a 12-hr light/dark cycle and given food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY and were in conformity with the National Institutes of Health Guidelines (IACUC # 20–005).

2.2 Drug Administration

Taurine, kainic acid, and isoniazid were dissolved in isotonic saline (at 43, 5, and 10 mg/ml, respectively). Animals received the indicated drugs through a stationary butterfly needle inserted subcutaneously before the start of the recordings and attached to a 20-cm catheter to avoid any electrostatic artifacts during the injections. After each injection, the catheter was flushed with a small volume of saline so the whole dose of the drug could be delivered.

2.3 Intracerebral Recordings of Local Field Potentials

Mice were anesthetized with ketamine/xylazine mix (90/10 $mg.kg^{-1}$ i.p.) and scalps were shaved and then fixed on a stereotaxic apparatus. Right-side craniotomies were made at AP 2.5 mm from the bregma and L 0.5 mm (medial prefrontal cortex). Extracellular recordings were obtained with tungsten electrodes with impedances of 1–2 MΩ. Electrodes were placed in infragranular layers (0.5 lateral and 1.0–1.2 mm deep in prefrontal cortex). Local field potential (LFP) from the prefrontal cortex was recorded. LabChart-8 (ADInstruments, Colorado Springs, CO, USA) was used for LFP recording. This includes both frequency-domain and time-domain features that have been extracted: the low-frequency oscillations (LFO) (delta 0.4–4 Hz, theta 5–7 Hz, alpha 7–12 Hz, beta 13–25 Hz, and gamma 26–80 Hz) and high-frequency oscillations (HFO) (slow ripples 125–250 Hz and fast ripples 250–500 Hz). All recordings were passed through a preampli-

fier connected to the electrode, amplified using model 1700 Differential AC Amplifier (ADInstruments), and digitized at 10 kHz.

2.4 Statistical Analysis

Electrophysiological results are shown as the mean ± SEM of each parameter, as mentioned above, and significance values were determined by one-way ANOVA with repeated measures and a post hoc Dunnett test with $p < 0.05$. Peak amplitude of the response was calculated using LabChart software (ADInstruments, Colorado Springs, CO, USA).

3 Results

3.1 Pharmacology of Isoniazid- and KA-Induced Epileptiform Discharge

Kainate receptors are densely present in hippocampal and cortical interneurons and other principal neurons (Netopilova et al. 1997; Noebels 1994). Kainate receptors are channels that regulate K^+ and Ca^{++} conductance, responsible for fast synaptic transmission through generation of excitatory postsynaptic currents (EPSCs) (Richards et al. 1995; Samoriski et al. 1997; Snead 1994). Activation of these receptors by kainic acid (KA), which trigger a cascade of events resulting in permanent damage and seizures, is the basis for the temporal lobe epilepsy model (El Idrissi and Trenkner 2003). At a dose of 5 $mg.kg^{-1}$, kainate first produced seizures with no motor expression and recorded as regular EEG spiking, varying from 60 to 80/s (Fig. 1). Occasionally, these seizures ended with rapid tail shakes and could be followed by a recurrent seizure with high-frequency spikes and no behavioral concomitants. In this study, we used a combination of non-convulsive doses of isoniazid and KA. We injected a low dose of isoniazid to partially inhibit GAD activity and subsequently injected low doses (10 mg/kg) of KA, a depolarizing agent with preferential binding to limbic structures. Injection of 10 mg/kg of isoniazid partially inhibited GAD activity in all brain regions with no visible convulsions exhibited by mice. However, intracerebral recording revealed epileptiform discharges shortly after isoniazid injection. Subsequent addition of KA acid further increased the firing rates as shown in Fig. 1. By combining sub-threshold dose of isoniazid with a non-convulsive dose of KA, we could selectively and reproducibly induce epileptiform discharges without the onset of convulsions. As shown in Fig. 1c, d, there was a significant increase in the slow and fast ripple oscillations post-KA injection, indicative of an epileptogenic brain.

3.2 Taurine Suppresses Epileptiform Discharges and Ripple Oscillations

Using the same paradigm of partial GAD inhibition and depolarization with KA, we tested the anti-epileptic properties of taurine. We found that taurine injection (43 $mg.kg^{-1}$, s.c.) before isoniazid and KA injection significantly reduced the amplitude of population spikes (Fig. 2a), the firing rates of neuronal circuits (Fig. 2b), and the firing rates of both slow and fast ripple oscillations (Fig. 2c, d). These data indicate that injection of taurine significantly increases the threshold for seizure onset as indicated by reduced high-frequency brain oscillations, a hallmark for epileptogenicity and hyper-synchronous firing.

3.3 Taurine Suppresses KA-Induced Epileptiform Activity and HFO Brain Waves

We have previously shown both in vitro and in vivo that taurine has a neuromodulatory role on both excitatory and inhibitory signals (El Idrissi and Trenkner 2004). Taurine works concomitantly with GABA to activate $GABA_A$ receptors, thus enhancing neuronal inhibition. Taurine also acts downstream of glutamate receptor activation through the regulation of cytoplas-

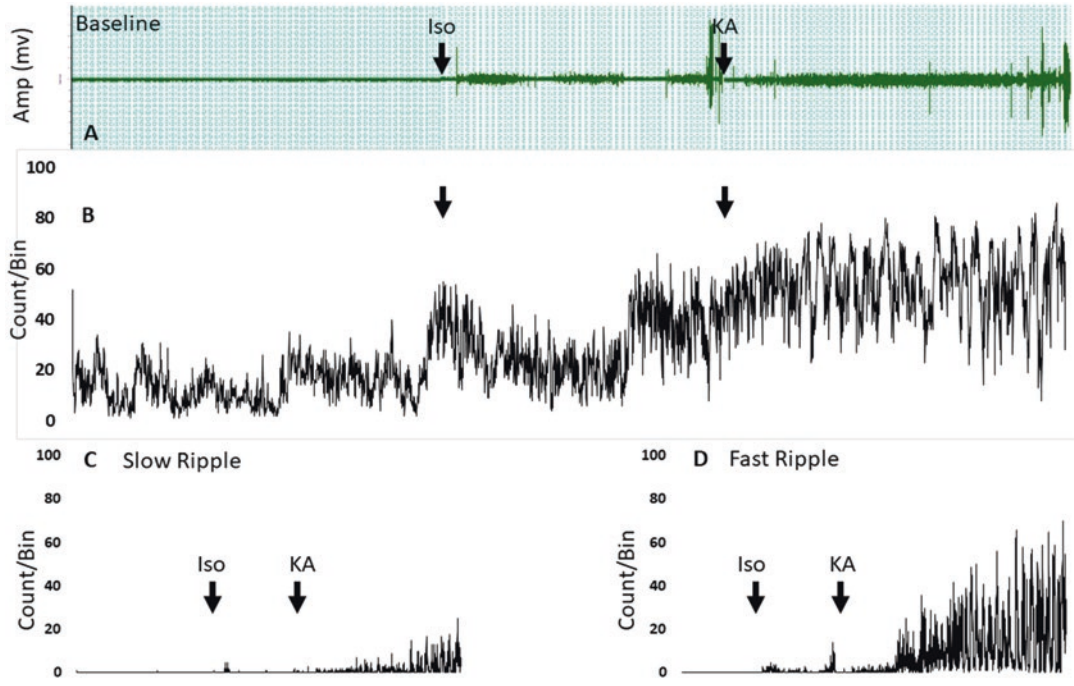

Fig. 1 (**a**) Selective enhancement of cortical ripples after isoniazid and KA injections. Firing rate analysis with a detection threshold of 100 μv shows a significant increase after isoniazid injection and a further drastic increase post KA injection (**b**). Spike histograms of filtered frequencies show a significant increase in both slow ripples (125–250 Hz) and fast ripples (250–500 Hz) post KA injection (**c** and **d**, respectively). Bin size was set to 1 s

mic and intra-mitochondrial calcium homeostasis (El Idrissi et al. 2009), therefore preventing neuronal hyperexcitability. To further investigate the anti-epileptic properties of taurine in the KA seizure model, we injected taurine (43 $mg.kg^{-1}$, s.c.) to mice exhibiting epileptiform discharge in response to KA injection (Fig. 3). Within 10 min post taurine injection, we observed a significant reduction in the frequency and amplitude of bursts of population spikes (Fig. 3a). Concomitant with this cessation of burst of population spikes, there was a drastic reduction in the HFOs (slow and fast ripples, Fig. 3b, c, respectively). PSD analysis of field potentials, slow ripples and fast ripples, revealed a drastic reduction in response to taurine injection (Fig. 3d, e, f, respectively). These data strongly suggest that taurine, acting through $GABA_A$ receptors, increases inhibitory drive rendering neuronal circuits more resistant to seizure induction, therefore leading to desynchronization and cessation of bust population spikes.

3.4 Taurine Suppresses Ripple Events in Mice Post-Seizure Induction

Spike histogram analysis of recorded field potentials and HFOs show a significant reduction in the firing rate after addition of taurine. Within 10 min post taurine injection, we observed a significant reduction in the number of burst population spikes (Fig. 4a) with near-complete cessation of HFOs (slow and fast ripples; Fig. 4b, c, respectively). Representative traces of 1 s recordings of field potentials from the frontal cortex during an epileptiform discharge after KA addition show the onset of burst of population spikes which are mainly due to HFOs (Fig. 4d). Within 10 min of taurine addition, there was almost a complete elimination of epileptiform discharges and HFOs (Fig. 4e). These data provide clear evidence for an anti-epileptogenic role for taurine mediated through activation of $GABA_A$ receptors and enhancement of the inhibitory drive.

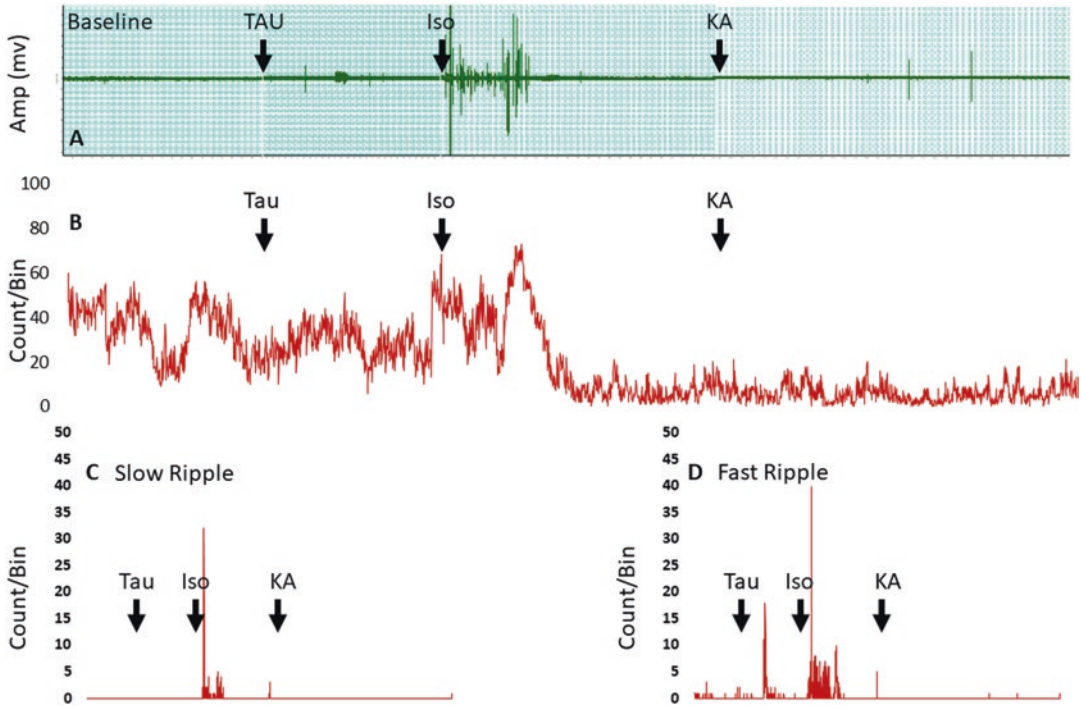

Fig. 2 Suppression of cortical ripples with taurine injection. (**a**) Firing rate analysis with a detection threshold of 100 μv shows a significant decrease in the amplitude of isoniazid and KA-induced field potentials in the presence of taurine. (**b**) Spike histogram analysis of recorded field potentials show a significant reduction in the firing rate in the presence of taurine. (**c** and **d**) are spike histograms of filtered frequencies showing a near-complete suppression of both slow ripples (125–250 Hz) and fast ripples (250–500 Hz) post-KA injection in the presence of taurine. Bin size = 1 s

4 Discussion

In the adult brain, inhibitory GABAergic interneurons modulate the activity of principal excitatory cells via their GABAA receptors and thus adjust excitatory output of neuronal circuits. GABA synthesis by the rate-limiting enzyme GAD also plays a central role in the regulation of GABA-mediated inhibition and hence neuronal excitability. The expression of GAD has been shown to be activity-dependent (Nishimura et al. 2001; Ramirez and Gutierrez 2001) and to be influenced by the effectiveness of GABAergic inhibition (Freichel et al. 2006; Kang et al. 2001; Riback et al. 1988, 1993).

In this study, we used a combination of pharmacological agents that target the excitatory or inhibitory system and electrophysiologically determined seizure susceptibility in the presence or absence of taurine. The rationale was to create a condition where the inhibitory system is pharmacologically weakened and challenge the excitatory system with KA, a depolarizing/convulsive agent. Unlike isoniazid, which causes a global inhibition of GAD, KA is more selective for limbic structures, where the hippocampus, the dentate gyrus, and the entorhinal cortex are the structures most affected by KA (Ben-Ari 1985; Bruton 1993). Isoniazid was injected (10 mg.kg^{-1}) and followed by KA injections (5 mg.kg^{-1}). This allowed isoniazid to partially inhibit GAD and weaken the inhibitory system prior to challenging the brain with KA. By combining these sub-threshold non-convulsive doses of isoniazid and KA, we could selectively and reproducibly induce ictal activity without noticeable convulsions (Fig. 1). Control mice showed a significant increase in the slow and fast ripple oscillations

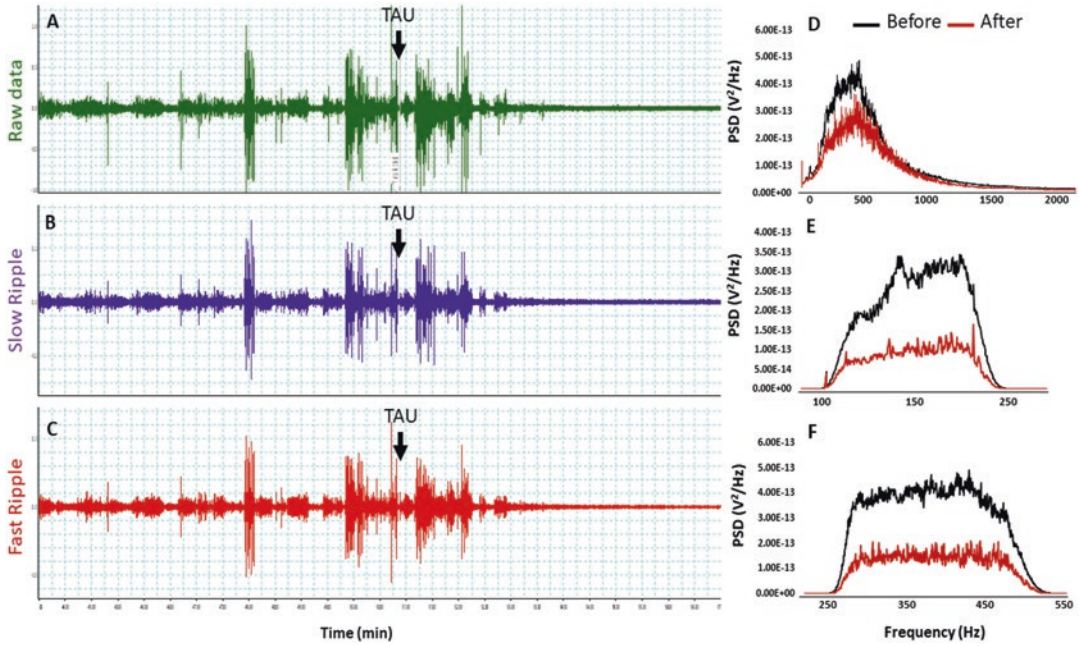

Fig. 3 Taurine suppresses KA-induced population spikes and HFOs of brain waves. (**a**) Representative traces of 40 min recordings of field potentials from the frontal cortex during an epileptiform discharge and after taurine addition. (**b** and **c**) represent the traces of the filtered HFOs before and after taurine injection. (**d**, **e**, and **f**) represent PSDs of field potentials, slow ripples (125–250 Hz), and fast ripples (250–500 Hz), respectively, before and after taurine injection showing a drastic reduction in the peak PSDs

post-KA injection, indicative of an epileptogenic brain. However, taurine injection before isoniazid and KA injection significantly reduced the amplitude of population spikes (Fig. 2a), the firing rates of neuronal circuits (Fig. 2b), and the firing rates of both slow and fast ripple oscillations (Fig. 2c, d). These data indicate that injection of taurine significantly increases the threshold for seizure onset as indicated by reduced high-frequency brain oscillations, a hallmark for epileptogenicity and hyper-synchronous firing. Interestingly, when injecting taurine into a brain undergoing epileptiform discharge, we observed a significant reduction in the number of burst population spikes (Fig. 4a) with near-complete cessation of HFOs (slow and fast ripples, Fig. 4b, c, respectively). Within 10 min of taurine addition, there was almost a complete elimination of epileptiform discharges and HFOs (Fig. 4e). These data provide clear evidence for an anti-epileptogenic role for taurine mediated through activation of GABAA receptors and enhancement of the inhibitory drive.

The findings of the current study reinforce the role of GABA inhibition in the maintenance of functional neuronal circuits characterized by a critical balance between excitatory and inhibitory inputs and suggest the potential complexity of changes in this system that result from alterations in the expression or activity of key proteins. Most importantly, the identification of specific alterations in neural excitability induced by hyperexcitability will enhance our understanding of the basis for the long-lasting altered cellular and synaptic properties that contribute to chronic hypersensitivity to normal stimuli, characteristic of epileptogenicity. Using this paradigm of pharmacological manipulation, one could test for the potency of the inhibitory system in other models where the effectiveness of this system is shown to be altered. These data also strongly suggest that taurine, acting through GABAA receptors,

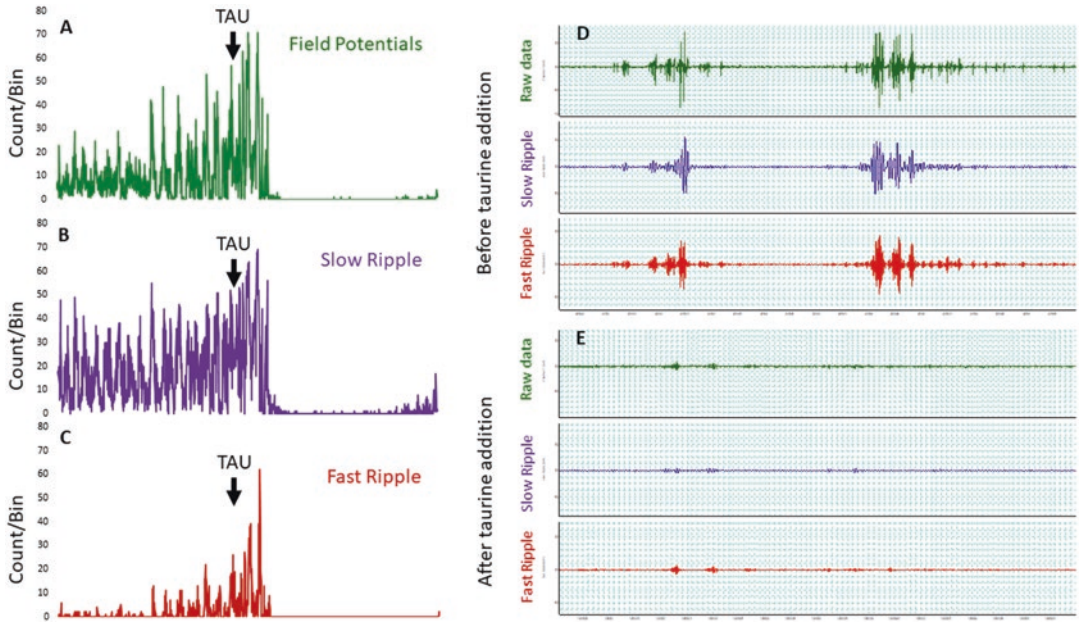

Fig. 4 Suppression of burst of population spikes and of cortical ripples after taurine injection. Firing rate analysis with a detection threshold of 100 μv shows a significant decrease 10 min after the addition of taurine (**a**). Similar decrease was observed in the firing rates of HFOs (**b** and **c**). (**d**) Representative traces of 1 s recordings of field potentials from the frontal cortex during an epileptiform discharge after KA addition showing the onset of burst of population spikes. (**e**) Same time frame after the addition of taurine showing the complete absence of any ictal activity

increases inhibitory drive rendering neuronal circuits more resistant to seizure induction, therefore leading to de-synchronization and cessation of bust population spikes.

5 Conclusion

Increasing taurine concentration in the brain renders neuronal circuits resistant to seizure induction through inhibition of GAD and activation of KA receptors. Conversely, injection of taurine to a brain undergoing ictal activity leads to a complete cessation of population spikes and ripple activity that typifies epileptogenic brains. These data provide clear evidence for an anti-epileptogenic role for taurine mediated through activation of GABAA receptors and enhancement of the inhibitory drive.

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Observation of Acupuncture Effects on the Expression of Taurine Transporter and Taurine in the Senescence-Accelerated Mouse Brain: A Pilot Study

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Keywords

Taurine · Taurine transporter · Acupuncture · Acupoint · Cerebellum · Hippocampus

Abbreviations

SAMP8	Senescence-accelerated prone mice
SAMR1	Senescence-accelerated resistant mice
TauT KO	Taurine transporter knockout mice
TauT	Taurine transporter

1 Introduction

Taurine, which is present almost throughout the human body, is known to exhibit anti-inflammatory and antioxidant effects and has been reported to contribute to the suppression and prevention of symptoms in various diseases by maintaining cell functions (Niu et al. 2018; Jakaria et al. 2019). Moreover, animals with taurine deficiency have been confirmed to experience dysfunction of the skeletal muscle, cardiac muscle, retina, and neurons (Ito et al. 2014). Ito et al. (2008) found that in taurine transporter knockout (TauT KO) mice, cardiac output was reduced due to structural changes in the heart, and there was an increase in the expression of heart failure marker genes. They also showed that TauT KO mice have structural defects in the skeletal muscle, resulting in decreased exercise endurance (Ito et al. 2008). Taurine is also

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thought to play an important role in the brain. It regulates the volume of astrocytes and neurons and the excitability of neurons in the central nervous system (Oja and Saransaari 2017). Taurine increases the production and secretion of insulin in the pancreas. They also found that taurine enhances insulin receptor expression and excitability in the hippocampus (El Idrissi 2019). Taurine and TauT have been reported to be localized in the Purkinje cells and granular layer of the cerebellum and in the CA1 region of the hippocampus (Pow et al. 2002). Taurine can protect the cerebellum and hippocampus in arsenic-exposed mice (Piao et al. 2019). The effects of long-term taurine administration in aging mice have shown that taurine increases the survival of new neurons and increases hippocampal neurogenesis in adult mice (Gebara et al. 2015). These reports indicate that taurine plays an important role in neuronal protection in the brain.

Previous reports have indicated an important relationship between human dementia and taurine excretion. The urinary excretion of taurine was higher in elderly patients with dementia than in healthy elderly individuals (Gao et al. 2017). It has also been reported that a group of dementia patients who received dietary taurine showed great improvement in language and executive abilities (Gao et al. 2019). These reports suggest that taurine is present in the brain and that taurine administration is an important treatment for neurological disorders, such as dementia.

Acupuncture, a traditional medicine technique, has long been used to treat pain and psychological disorders and has also been reported to be effective in treating many kinds of diseases in the nervous, digestive, and respiratory systems, as well as being a treatment method with few side effects (Kaptchuk 2002). The effects of acupuncture have received much attention, especially in the treatment of neurological disorders. For example, acupuncture has been reported to be effective in the treatment of ischemic stroke, cognitive disorders, Parkinson's disease, and epilepsy (Jiang et al. 2016; Kluger et al. 2016; Li et al. 2018). It has been reported that acupuncture stimulation increases the expression of TauT in the cerebral cortex and hippocampus of mouse models of epilepsy (Jin et al. 2005; Li et al. 2005; Yang et al. 2006). These reports suggest that increased TauT expression may be one of the mechanisms underlying the suppression of neurological symptoms, such as epilepsy and cognitive disorders. Several studies have reported that acupuncture improves dementia, which draws attention to the possibility that acupuncture stimulation is effective against neurological diseases (Zeng and Zhao 2016; Yu et al. 2018; Sun et al. 2018; Lee et al. 2014). We have shown that simultaneous acupuncture stimulation of the head acupoints "Bai-Hui" (GV 20) and "Yintang" (Ex-HN3) in rats produced strong antidepressant effects similar to those of antidepressant drug therapy (Tanahashi et al. 2016). Based on these findings, we hypothesized that simultaneous acupuncture stimulation of the GV 20 and Ex-HN3 acupoints should not only have an antidepressant effect but also have an effect on other neurological disorders.

To examine the effects of acupuncture stimulation on the aging of the central nervous system, senescence-accelerated prone (SAMP8) mice, an animal model of accelerated aging, were used. In this study, we focused on the expression of taurine and TauT in SAMP8 mice and examined the effects of acupuncture stimulation of two acupuncture points, GV20 and EX-HN3.

2 Methods

2.1 Experimental Animals

All protocols were approved by the Animal Experiment Ethical Review Committee of the Suzuka University of Medical Science (permission number 298). Male senescence-accelerated resistant (SAMR1) and SAMP8 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were maintained at a room temperature of 22 ± 3 °C, 55 ± 5% humidity, and a 12-h light/dark cycle. The mice were given free access to commercially available animal feed (CE-2; CLEA Japan, Inc.) and water. In this study, male mice were used to eliminate the effects of the estrous cycle. Six animals per group were used

for the experiment. The animals were divided into three groups: SAMR1 (n = 6), SAMP8 (SAMP8 control group; n = 6), and SAMP8Acp (SAMP8+ acupuncture group; n = 6). The body weights of all animals were measured from 23 to 27 weeks. Acupuncture stimulation was performed in mice from 24 to 27 weeks of age.

2.2 Acupuncture Stimulation

SAMP8Acp mice were stimulated with GV 20 and Ex-HN3 acupuncture points of the mice using acupuncture needles (Fig. 1). Acupuncture was performed on a pair of acupoints of "GV20" and "Ex-HN3" Yintang. GV20 "Bai-Hui" is located at the inferior border of the occipital protuberance on the vertical midline of the posterior part of the head. Ex-HN3 "Yintang" is located at the midpoint of the glabella between the inner and medial ends of the eyebrows on the face. Mice were inserted at a depth of 5 mm for 20 min at these acupoints. The acupuncture needle was 0.25 mm in diameter and 15 mm on length, made of stainless steel (D-type; SEIRIN Co, Ltd. Shizuoka, Japan). The method of acupuncture stimulation was based on a previous report (Tanahashi et al. 2016; Takagi et al. 2017). During acupuncture stimulation, the mouse was fixed with adhesive tape on its tail in an overturned cage. The other groups were fixed at the same time as the P8. These acupuncture interventions were performed 5 days/week for 4 weeks on mice from 24 to 27 weeks of age.

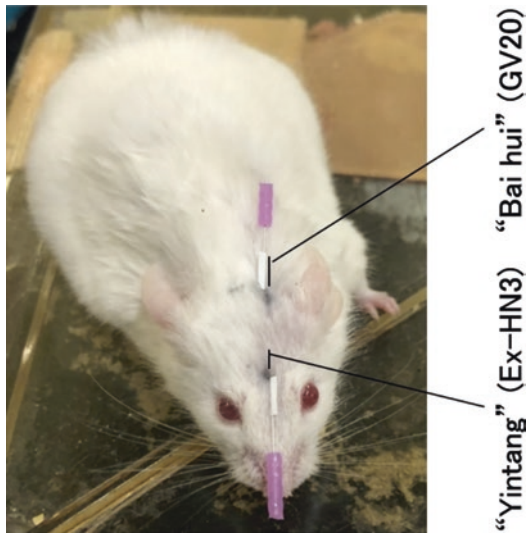

Fig. 1 Stimulations of acupuncture were performed at GV20 "Bai Hui" and Ex-HN3 "Yintang" acupoints

2.3 Sample Collections

Mice were euthanized using intraperitoneal injections of a barbital sodium salt solution (120 mg/kg) after the last intervention at 27 weeks. Brains were immediately removed and stored at −80 °C, and the brains were used for molecular biological and pathological analyses.

2.4 Analysis of mRNA Extraction by Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the brain tissue using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). The amount of RNA was determined spectrophotometrically. cDNAs encoding for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TauT were evaluated by reverse transcription-PCR (RT-PCR) using SuperScript III (Invitrogen, Carlsbad, CA, USA).

To quantify the expression, mRNA levels of TauT were also analyzed by real-time RT-PCR using PowerUp SYBR Green Master Mix and ABI Prism 7000 SDS according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). The forward and reverse primers for mouse GAPDH were 5′-ATG GGA GTT GCT GTT GAA GTC A-3′ and 5′-CCG AGG GCC CAC TAA AGG-3′, respectively. The forward and reverse primers for mouse TauT were 5′-GCA CAC GGC CTG AAG ATG A-3′ and 5′-ATT TTT GTA GCA GAG GTA CGG G-3′, respectively.

2.5 Immunohistochemical Methods

2.5.1 Preparation of Mice Brain Samples for Immunohistochemistry (IHC)

Mice were weighed and anesthetized by an intraperitoneal injection of 50 mg/kg sodium pentobarbital. After the mice had fallen asleep, the abdominal cavity was opened with a midline incision to the sternum. A diagonal cut was made to each side of the sternum through the rib cage, extending to either side of the neck. The sternum could be clamped back with hemostatic forceps to provide access to the heart. An intravenous catheter was inserted into the left ventricle. For one mouse, 20 ml saline was infused over 2 min. Switch perfusion to 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde. It was common to see turgor and twitching of the upper extremities at the initial flow of the fixative into the mouse. For a mouse, approximately 50 ml over 7 min could be infused. After completion of the procedure, the arms and tip of the nose were stiff. The mouse was dissected from the perfusion apparatus, and the brain was carefully removed to avoid tissue damage. After carefully removing the brain from the body, the brain was fixed at room temperature overnight and stored in PBS at 4 °C. The mouse brain for IHC was paraffin-embedded and sectioned at 6 μm thickness onto silanized glass slides. Slides were stored at ambient temperature in slide cases until use.

2.5.2 Immunofluorescent Staining Procedure

Paraffin sections of the mouse brain were deparaffinized in xylene for 5 min with frequent shaking in a glass box. The sections were then treated with xylene in another glass box for 3 min, followed by treatment with 100, 90, 80, 70, and 50% (v/v) ethanol for 120 s. To ensure complete removal of the paraffin, the sections were soaked in PBS for 30 min. To retrieve the antigens, the sections were heated in 5% (w/v) urea for 5 min in a microwave oven and then left in 5% urea until the temperature was reduced to room temperature. Rinse sections in PBS at room temperature three times for 5 min each. Sections were blocked for 30 min in PBS containing 5% (v/v) normal goat serum antibody buffer. Double immunofluorescence labeling was performed. The sections were incubated with the primary antibody, rabbit polyclonal anti-taurine antibody (2 μg/ml), and anti-TauT antibody (1:400, Santa Cruz Biotechnology, Inc., sc-39,306) overnight at room temperature. The sections were washed with PBS at room temperature three times for 5 min each and were incubated with the secondary antibody, Alexa 594-labeled goat antibody, against rabbit immunoglobulin G (IgG) (1:400) for 3 h at room temperature. Sections were treated with Alexa 488-labeled goat antibody against mouse IgG (1:400; Molecular Probes, Eugene, OR, USA), which was mixed with Alexa 594-labeled goat antibody against rabbit IgG and this mixture. The sections were washed three times with PBS over 30 min. Pipet DAPI-Fluoromount-GT™ Mounting Medium (SouthernBiotech, USA) was placed onto the section and covered with a cover glass. The samples were dried overnight at 4 °C in a refrigerator. The stained sections were examined under an Olympus BX53 fluorescence microscope and photographed using an Olympus DP14 microscopic digital camera.

2.6 Statistical Analysis

Statistical significance was assessed using the one-way analysis of variance with the Tukey–Kramer post hoc test using PRISM (version 5.0; GraphPad Software, La Jolla, CA, USA). The threshold for significance was set at $P < 0.05$.

3 Results

3.1 Comparison of Body Weight

SAMP8 mice were significantly underweight compared to SAMR1 mice. Furthermore, simultaneous acupuncture stimulation of GV20 and

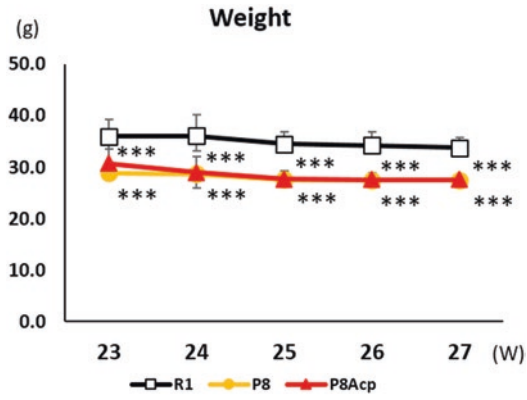

Fig. 2 Comparison of the body weights of senescence-accelerated resistant (SAMR1) (R1) and senescence-accelerated prone (SAMP8) (P8) mice at 23 to 27 weeks of age. Values are presented as mean ± standard deviation (n = 6). ***P < 0.001 versus SAMR1 mice. P8Acp: SAMP8 + acupuncture group

EX-HN3 had no effect on the bodyweight of SAMP8 mice (Fig. 2).

3.2 Acupuncture Stimulation Increases the mRNA Expression of TauT in SAMP8 Mice

Acupuncture stimulation of GV20 and EX-HN3 for 20 min was performed in SAMP8 mice. Acupuncture stimulation of these acupuncture points significantly increased the expression of TauT mRNA in the cerebella of mice in the P8Acp group, which was approximately nine times higher than those in the SAMP8 group (Fig. 3a, $P < 0.01$). Similarly, the expression of TauT mRNA in the cerebella was significantly increased in mice of the SAMP8Acp group, and the expression of TauT mRNA in the cerebella of mice of the P8Acp group was approximately sixfold higher than that in the hippocampi mice of the SAMP8 group (Fig. 3b, $P < 0.05$). There was no significant difference in the expression of TauT mRNA in the cerebella and hippocampi between mice of the SAMR1 and SAMP8 groups.

3.3 Effects of Acupuncture on TauT and Taurine Expression in the Cerebella of SAMP8 Mice

Immunohistochemical staining (Fig. 4) was performed to confirm the expression of TauT and taurine in the cerebella of mice of the SAMR1, SAMP8, and SAMP8Acp groups. SAMP8 mice showed decreased expression of TauT in the Purkinje cells and stratum granulosum cerebellum compared to the normal-aging model of SAMR1 mice. Acupuncture stimulation increased TauT expression in the cerebellar Purkinje cells and granule cells in the granular layer, which was reduced in SAMP8 mice. Taurine expression was decreased in the Purkinje cells in SAMP8 mice compared to SAMR1 mice. This decrease in expression was restored by acupuncture stimulation, as well as by TauT. The expression of taurine in the granule cells was not different between SAMR1 and SAMP8 mice but was enhanced by acupuncture stimulation.

3.4 Effects of Acupuncture on the Expression of TauT and Taurine Immunoreactivities in the Hippocampi of SAMP8 Mice

We further confirmed the expression of TauT and taurine immunoreactivity in the hippocampus by immunofluorescence (Fig. 5). The expression of TauT and taurine immunoreactivity in the hippocampus was weak in the SAMR1 and SAMP8 groups, whereas the expression of TauT and taurine immunoreactivities in the SAMP8Acp group increased significantly.

4 Discussion

Taurine is abundant in the cerebellum and hippocampus (Pow et al. 2002). In the brain, taurine plays a role in inhibitory neurotransmission and

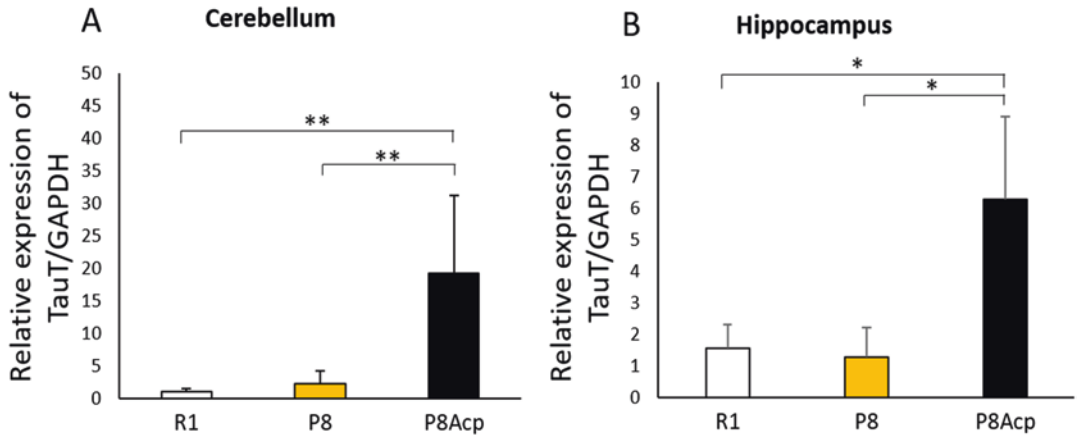

Fig. 3 mRNA expressions of taurine transporter (TauT) under the manual acupuncture stimulation in the brain. Values are mean ± SD (n = 5). *$P < 0.05$; **$P < 0.01$ versus senescence-accelerated resistant (SAMR1) mice. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase; R1, SAMR1 group; P8, SAMP8 group; P8Acp, SAMP8+ acupuncture group

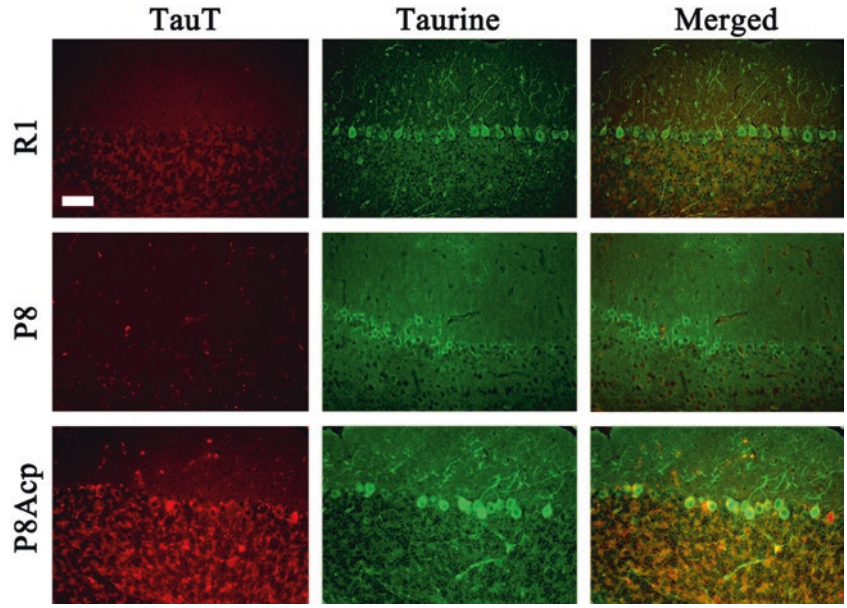

Fig. 4 Expression of taurine transporter (TauT) and taurine immunoreactivities in cerebella of mice of each experimental group (SAMR1, R1; SAMP8, P8; SAMP8Acp, P8Acp). The expression of TauT immunoreactivities in the SAMP8Acp group was found to be higher than that in the SAMP8 and SAMR1 groups. Scale bar = 100 μm. *SAMR* senescence-accelerated resistant mice, *SAMP* senescence-accelerated prone mice

maintains a constant cell size in the nervous system (Oja and Saransaari 2017). Taurine deficiency in the brain results in delayed cell differentiation, and taurine can improve neuron formation in a kitten (Palackal et al. 1986; Maar et al. 1995). Taurine has also been reported to promote neural development in the adult brain and may be important for neurogenesis (Hernández-Benítez et al. 2012). One way of supplying taurine into the body is through the

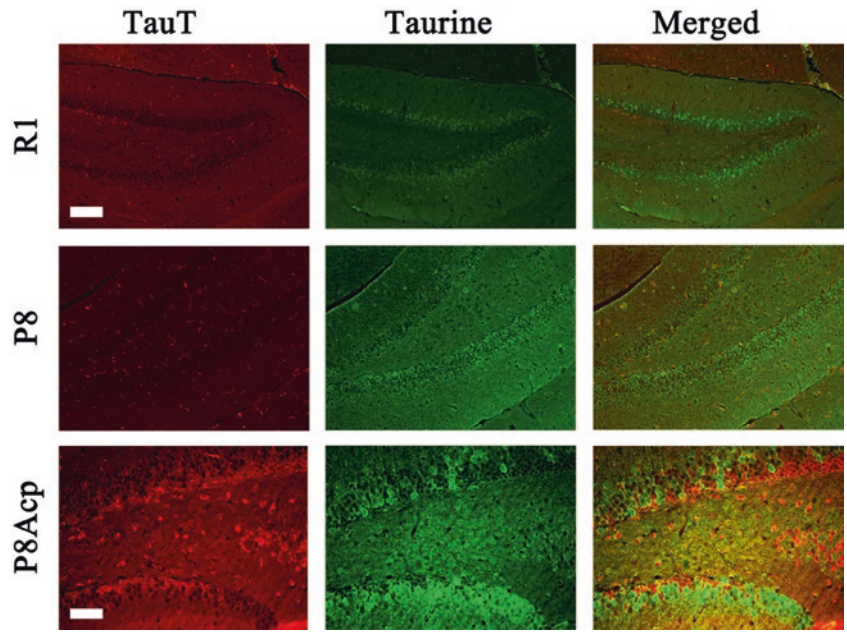

Fig. 5 Expression of taurine transporter (TauT) and taurine immunoreactivities in hippocampi of mice in each experimental group (SAMR1, R1; SAMP8, P8; SAMP8Acp, P8Acp). The intensity of TauT and taurine immunoreactivities in the SAMP8Acp group was found to be higher than that in the SAMP8 group. R1 and P8, scale bar = 100 μm; P8Acp, scale bar = 200 μm. *SAMR* senescence-accelerated resistant mice, *SAMP* senescence-accelerated prone mice

pathway of cellular uptake by TauT in the small intestine when taurine is ingested. TauT is an important transporter of taurine in cells and regulates taurine transport by means of ions, pH, and temperature (Voss et al. 2004). The reduction of taurine in the body of TauT KO mice resulted in functional disorders, such as muscle atrophy, decreased motor skills, and retinal abnormalities (Ito et al. 2008). The therapeutic effects of taurine administration have been shown to improve neurodegenerative diseases, such as Parkinson's disease, stroke, and epilepsy (Jiang et al. 2016; Menzie et al. 2013). The mechanism of improvement indicates that taurine can increase neonatal neuron survival, resulting in neurogenesis, and inhibits neuronal inflammation by decreasing inflammatory cytokines (Jakaria et al. 2019).

There have been some reports on the relationship between taurine and TauT by acupuncture stimulation. Jin et al. (2005) administered beta-alanine to a rat model of kainic acid-induced epilepsy to induce a state of inhibited taurine absorption and found increased neuronal cell death in the hippocampi of rats treated with kainic acid and beta-alanine compared to the kainic acid-induced rats, and electroacupuncture (EA) stimulation was applied to the acupuncture points called "water groove" (GV26) on the face and "spring" (K1) on the sole of the foot at an energizing frequency of 6 Hz and an energizing time of 30 min. The results showed that EA stimulation suppressed epilepsy scores and neuronal cell death in the hippocampus and increased the expression of TauT in the hippocampus (Jin et al. 2005). To examine different animal models of epilepsy, Li et al. created a rat model of penicillin-induced epilepsy and applied EA stimulation to the head and neck acupuncture points of "Bai-Hui" (GV20) and "Feng Fu" (GV16) under the conditions of a total of 30 min of current flow, 100 Hz frequency, and 6 mA current intensity. The results showed that rats in the EA and taurine groups had improved epileptic symptoms and reduced electroencephalogram frequency and

amplitude compared to the model rats. The expression level of the taurine transporter in the hippocampus and cerebral cortex, which decreased during epileptic seizures, was increased by the administration of taurine or EA stimulation. They also showed that taurine administration or EA stimulation increased TauT expression levels in the hippocampus and cortex, which were decreased during epileptic seizures. This study demonstrated that the combination of taurine administration and EA stimulation improved epileptic symptoms and that the expression level of the taurine transporter increased more synergistically with taurine administration plus EA stimulation than with EA stimulation alone (Li et al. 2005).

We used SAMP8 mice to investigate the effects of manual acupuncture stimulation of GV20 and EX-HN3 points on the expression of TauT and taurine in the cerebellum and hippocampus. SAMP strain mice develop aging-related pathologies, such as immune dysfunction, learning and memory deficits, and colitis. Among them, SAMP8 mice exhibit learning and memory impairments, sarcopenia, weight and brain weight loss, and muscle atrophy (Takeda et al. 1994). SAMP8 mice are often used as a model for Alzheimer's disease, which exhibits learning and memory impairments, and sarcopenia, which is a loss of skeletal muscle mass associated with aging (Jia et al. 2020). The results showed that the mRNA of TauT in the cerebellum and hippocampus of the SAMP8Acp group was markedly increased in our study. Immunostaining analysis showed that the expression of TauT in the Purkinje cells and the neuron in the granule layer of the cerebellum were markedly increased after acupuncture stimulation on GV20 "Bai Hui" and Ex-HN3 "Yintang" acupoints in the SAMP8Acp group, and the expression of taurine was also markedly increased in the Purkinje cells of mice in the SAMP8Acp group. Acupuncture stimulation increased the contents of taurine and TauT in the cerebellum and hippocampus, which may improve learning ability and motor skills. Moreover, manual acupuncture stimulation, rather than EA stimulation, showed sufficient effects, suggesting that the stimulation of the GV20 and EX-HN3 acupoints contributed to the increase in taurine and TauT expression. Since we have previously reported that acupuncture stimulation of the double acupoints, GV20 and EX-HN3, simultaneously exerts antidepressant effects (Takagi et al. 2017), it is likely that manual acupuncture stimulation of both GV20 and EX-HN3 acupoints is necessary in this study. Through this study, we were able to show that acupuncture stimulation could be one of the mechanisms by which acupuncture improves neurological symptoms by increasing TauT and taurine in the brain by acupuncture stimulation. Although behavioral evaluation and detailed mechanistic studies are needed in the future, we provide vital evidence for a new pathway between taurine and acupuncture through the taurine transporter, which showed the possibility of acupuncture and moxibustion treatment being a specific role strategy in neurological disease therapy.

5 Conclusion

The role of taurine in the brain is to act as an inhibitory neurotransmitter during the development of the neonatal brain. Acupuncture stimulation has been reported to improve epilepsy and psychiatric disorders; however, the detailed mechanism of this improvement is unknown. In the present study, manual acupuncture stimulation of GV20 and Ex-HN3 in SAMP8 mice increased the expression of TauT in the cerebellum and hippocampus. Taurine expression increased in the Purkinje cells of the cerebellum. These results suggest that acupuncture stimulation of GV20 and Ex-HN3 might affect cerebral neurological diseases through a mechanism mediated by TauT and taurine. We propose a new mechanism for the relationship between taurine and acupuncture while hoping that our findings will provide a potential means of preventing neurological diseases.

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The Regulatory Effects of Taurine on Neurogenesis and Apoptosis of Neural Stem Cells in the Hippocampus of Rats

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Keywords

Neurogenesis · Apoptosis · Proliferation · Glutamate · Taurine

Abbreviations

AD	Alzheimer's disease
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CUMS	Chronic unpredictable mild stress
DG	Dentate gyrus
ECL	Enhanced chemiluminescence
HRP	Horseradish peroxidase
MDA	Malondialdehyde
NSCs	Neural stem cells
SGZ	Subgranular zone
SOD	Superoxide dismutase
TUNEL	TdT-mediated dUTP nick-end labeling

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1 Introduction

Depression, a syndrome of abnormal mood and a widespread mental disorder that affected about 300 million people worldwide, is the second major cause of death in 15–29 years old individuals and brought more than one trillion dollars of the economic burden each year (Kessler et al. 2003). The inhibition of neurogenesis in the hippocampus, which occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) throughout life, has been discovered to be the underlying pathogenesis of depression (Ernst et al. 2006; Tang et al. 2016). Blocking neurogenesis negatively affects hippocampal control of mood states such as depression and responses to stress and fear (Aimone et al. 2014; Farioli-Vecchioli et al. 2008; Santarelli et al. 2003). However, the inhibition of adult hippocampal neurogenesis has been demonstrated to lead to cognitive deficits with negative effects on memory and learning in hippocampal-dependent tasks (Clelland et al. 2009; Revest et al. 2009). Therefore, the promotion of neurogenesis from neural stem cells (NSCs) in the hippocampus will be the most fundamental and effective therapy for depression.

Taurine, one of the most abundant amino acid in mammalians' brain (Chen et al. 2014), was well known to exert important effects in the development of the central nervous system (CNS) (Taranukhin et al. 2009), such as improving

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neurogenesis in the hippocampus of rats, repairing cognitive impairment of Alzheimer disease (AD) mouse, and increasing cell proliferation and the survival of newborn neurons in the DG (Gebara et al. 2015; Wu et al. 2013) and cultured adult neural stem cells (Hernandez et al. 2012). Besides, the protective effect of taurine on glutamate-induced neural damage has been reported and discussed from the aspects of the activation of GABAA receptors, inhibition of membrane depolarization, and maintenance of Ca^{2+} homeostasis and the consequently indirect inhibition of calpain activation and all downstream events (Leon et al. 2009). Our previous studies also proved the antidepressive effect of taurine on rats and improved expression of neurotrophic factors regulating neural proliferation, differentiation, and apoptosis (Wu et al. 2017). Toyoda and Iio (2013) have reported that the antidepressant effect of taurine may due to the phosphorylation of ERK1/2, Akt, and CREB, but the exact mechanism remains to be further studied.

The principle of glutamate-induced excitotoxicity has been well established experimentally in both in vitro and in vivo systems after neuronal exposure to glutamate. In the present study, glutamate, the abnormally high concentration of which is a risk factor damaging neurogenesis from NSCs and was usually used as a stimulator of depression, was administered to induce injury in NSCs in vitro (Mao et al. 2010). Meanwhile, chronic unpredictable mild stress (CUMS) was applied to rats to establish depressive animal models. Taurine was administered both in vitro and in vivo to investigate the regulation mechanism of taurine on neurogenesis and apoptosis of NSCs, to provide a new target for the prevention and treatment of depression using taurine.

2 Methods

2.1 Animals

SPF male Wistar rats (120 ± 20 g) were obtained from Chang Sheng Biotechnology Co., LTD, Liaoning province, China, kept at a temperature under 22 ± 2 °C in Laboratory Animals of Shenyang Agricultural University. Animal handling and experimental procedures followed the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shenyang Agricultural University (201706018).

2.2 Experimental Procedure

The rats were divided into four groups randomly. Rats in taurine groups (T, T + CUMS) were intraperitoneally injected (i.p.) daily with 500 mg/kg taurine dissolved in sterile saline, while rats in the control group (N) and CUMS stress group (CUMS) were i.p. the same volume of saline. Taurine was administered to rats 1 week before the commencement of model establishment, which lasted for 35 consecutive days.

2.3 Preparation of CUMS

The procedure of CUMS was applied according to our previous study (Wu et al. 2017). Rats were kept in a single cage and underwent 28 days of CUMS. The following stressors were applied in a completely random order to produce unpredictable mild stress every day: food deprivation (24 h), water deprivation (24 h), inversion of day/night light cycle (light on in the night and light off in day time), wet bedding (200 ml of water added to 300 g sawdust bedding), clipping the tail with forceps (1 min, the upper 1/3 of the tail), forced swimming (4 °C cold water for 6 min), and electrical stimulation with a voltage of 30–60 V, current of 1 mA, and frequency of 2 Hz.

2.4 Immunohistochemistry of Ki67

At the end of the experiment, the rats were anaesthetized and perfused with PBS followed by 4% paraformaldehyde. The brains were collected and fixed in 4% paraformaldehyde at 4 °C for 48 h and successively dehydrated in different concentrations of ethanol followed by transparentize in xylene. The tissues were then embedded in paraffin and cut into 5 μm. Immunohistochemistry of

Ki67 was performed in every 10 sections through the whole hippocampus. The slices were mounted and incubated in primary antibody rabbit anti-Ki67 (1:80, Abcam, ab15580, USA) solution overnight at 4 °C, following intervening rinses with 0.03% Triton-X PBS, incubated with secondary antibody for 25 min at room temperature. DAB was applied for 50 s. Positive cells located in the SGZ were counted bilaterally through the DG.

2.5 TdT-Mediated dUTP Nick-End Labeling (TUNEL)

Every 10 sections through the whole hippocampus were selected for TUNEL staining. The paraffin sections were dewaxed and rehydrated by heating at 60 °C followed by washing in xylene and rehydration through a graded series of ethanol and double dist. The tissues were then incubated with -proteinase K working solution for 20 min at room temperature. After rinsed with PBS and dried, the sections were incubated with TUNEL reaction mixture (Roche, Cat.NO.11684817910, Germany) at 37 °C for 1 h in a humidified atmosphere followed by incubation with converter POD for 30 min at 37 °C. Finally, DAB substrate (ZSGB-bio, ZLI-9017, China) was added and incubated for 10 min at room temperature. Mount the slides under glass coverslip and analyze under the microscope. Positive cells located in the SGZ were counted bilaterally through the DG.

2.6 Primary Cell Culture

SD fetal rats born within 24 h were purchased from Liaoning Chang Sheng Biological Co. Ltd. (Benxi, China). The brain of a fetal rat was dissected, and the hippocampus was separated by mechanical digestion to extract hippocampal NSCs. The procedure was depicted in Fig. 1a; 1×10^5 cells/flask were seeded and cultured with DMEM/F12 medium (HyClone, SH30023.01, America) supplemented with 2% B27 (Gibco, 17,504,044, America) and 20 ng/mL FGF-2 and EGF (PeproTech, 400-29, 400-25, America) in 37 °C incubator (5% CO_2). Half of the culture medium was renewed on the other day, and NSCs were allowed to proliferate for another 4–5 days before the neurospheres could be observed. NSCs are then allowed to differentiate in DMEM/F12 culture medium supplemented with 10% FBS (BI, 04-001-1A, Israel) (Fig. 1).

2.7 Drug Treatment

The second generation of neurospheres was collected and digested into single-cell suspension by mechanical digestion. NSCs were then divided into four groups: Cell in the normal control group (Control) was cultured in DMEM/F12 containing 2% B27, 20 ng/mL FGF-2, and EGF; cells in the taurine control group (Taurine) were cultured in the same medium as the normal control group except for supplementation with 10 mM taurine; cells in the glutamate group (Glutamate) were cultured in the same medium as normal control group except for supplementation with 10 mM glutamate for 1 h; and cells in the taurine treatment group (Tau+Glu) were pre-cultured with 10 mM taurine for 96 h followed by 10 mM glutamate for 1 h.

10 μM U0126 (MCE, HY-12031, America), a highly selective inhibitor of mitogen-activated protein (MAP) kinase 1 and 2 (MEK1/2), were dissolved in DMSO and added in the culture medium for 1 h to block the phosphorylation and activation ERK1/2. Cells in the DMSO group were cultured in the same medium as the control group except for supplementation with 0.1% DMSO; cells in the U0126 + Glu group were cultured in the same medium as the glutamate group except for supplementation with 10 μM U0126; and cells in the U0126 + Tau+Glu group were treated the same as taurine treatment group except for supplementation with 10 μM U0126.

2.8 Cell Counting Kit-8 (CCK8) Assay

Cell viability was assayed by CCK-8 (Dojind, CK04, Japan). The second generation of neurospheres was collected and mechanically dissociated into single-cell suspension, followed by seeding on 96-well plates coated by poly-D-Lysin (Sigma, P7886, America). 5×10^4 cells per

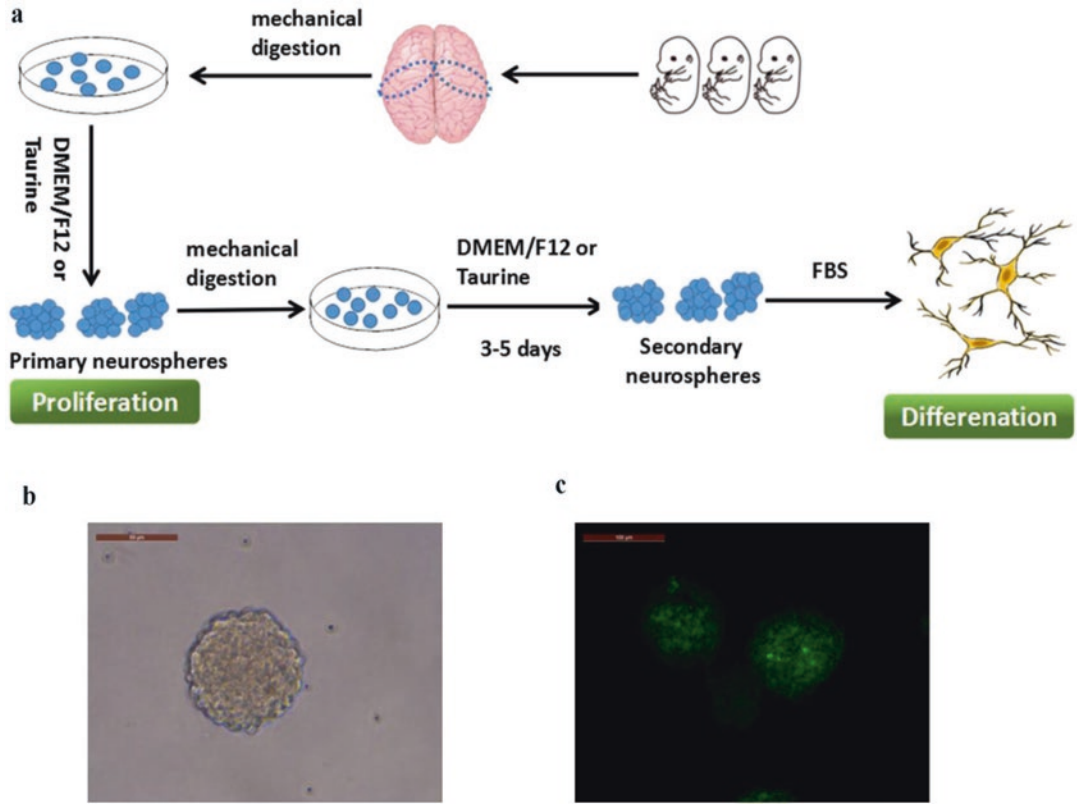

Fig. 1 Photomicrographs of NSCs in neurospheres. (**a**) Schematic drawing of culture NSCs in vitro. (**b**) Phase-contrast photomicrograph of a neurosphere (×400). (**c**) Immunofluorescence staining of NSCs for Nestin (green, ×400). Scale bar: 50 μm

well were cultured in 100 μl DMEM/F12 for 24 h. 10 μl CCK8 were added in each plate and incubated for 4 h at 37 °C. The optical density was measured by a microplate reader (Infinite® M200 PRO, TECAN) at 570 nm. There were five repetitions in each group. Cell viability = (As-Ac)/(Ab-Ad)×100%. As: experimental hole; Ac: experimental blank hole; Ab: control hole; Ad: control blank hole.

2.9 Immunocytochemistry

The second generation of neurospheres was collected and mechanically dissociated into single-cell suspension followed by seeding on poly-D-Lysine-treated cell climbing sheet at 12-well plates (5×10^5 cells) for the immunostaining of nestin to identify the cultured cells (Hu et al. 2018a, b; Imayoshi et al. 2011). 10 μM 5-Bromo-2-deoxyuridine (BrdU) (Sigma, B5002, America) were supplemented in each well and incubated for 4 h at 37 °C for immunostaining of BrdU to detect cell proliferation. The second generation of a single cell (5×10^5 cells) was cultured in DMEM/F12 with 10% FBS for 5 days followed by immunostaining of GFAP and β-tubulin III as previously described to assay cell differentiation of NSCs. The single-cell suspension was seeded on a poly-L-Lysine-treated cell climbing sheet, and the double immunofluorescence analysis was performed followed by fixing the cells with 4% paraformaldehyde (PFA) for 30 min at room temperature. The cells were then blocked for 1 h with 5% goat serum (Solarbio, SL038, England), permeabilized with 0.3% Triton X-100 (Solarbio, T8200, England), and incubated for 2 h with combinations of the primary antibodies at 4 °C (mouse anti-nestin:1:200, Santa Cruz, SC-23927, America; mouse anti-BrdU:1:200, Santa Cruz,

SC-32323, America; rabbit anti-GFAP: 1:200, Santa Cruz, sc-9065, America; rabbit anti-β-tubulin III: 1:200, Abcam, ab52901, England); a second antibody (Alexa Fluor 488 goat anti-mouse IgG: 1:400, Thermo, A11029, America; Alexa Fluor 594 goat anti-rabbit IgG: 1:400, Thermo, A11037, America) was incubated for 1 h. Subsequently, the cells were counterstained by DAPI (Sigma, D9542, America). There were five repetitions from each group, and five visual fields per slide were observed using a fluorescence microscope (DMI4000B, Leica, Germany) and analyzed by LAS version 4.4.0 software (Leica Microsystems, Switzerland). Data were presented as the percentage of BrdU, β-tubulin III, and GFAP-positive cells in the total number of cells (DAPI-stained cells).

2.10 Determination of Cell Apoptosis

Apoptotic cell death was determined by TUNEL Bright Green Apoptosis Detection Kit (Vazyme, A112, China) following the manufacturer's instructions. Briefly, NSCs were seeded on poly-D-Lysine-coated glass coverslips and fixed in 4% paraformaldehyde for 20 min at room temperature followed by being permeabilized in 0.1% Triton X-100 for 25 min. The cells were then equilibrated in 1 × equilibration buffer for 20 min at room temperature and incubated with 50 μL TUNEL reaction mixture for 1 h at 37 °C before further counterstained with DAPI (1 μg/mL). Data were presented as the percentage of TUNEL-positive cells in the total number of cells (DAPI-stained cells). Fifteen random fields on each sample were counted using a fluorescence microscope (DMI4000B, Leica, Germany) and analyzed by LAS version 4.4.0 software (Leica Microsystems, Switzerland) under 40× magnification.

2.11 Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Assay

Cells were broken by ultrasonic treatment (SB-500DTN, SCTENTZ, Ningbo, China); intracellular MDA concentration and SOD activity were detected by WST-1 and TBA according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The optical density was measured by a microplate reader (Infinite® M200 PRO, TECAN) at 532 and 450 nm respectively.

2.12 Western Blotting

Cells were harvested and lysed in cell lysis buffer (Pplygen, C1050, China) to get protein samples, the concentration of which was determined by BCA protein assay kit (Pplygen, P1511, Beijing, China). The same quantity (25 μg) of protein from each group was resolved on 10% SDS/PAGE gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Millipore, USA). Transferred membranes were blocked with 5% skim milk (or with BSA for phosphorylated protein) for 1 h, incubated overnight at 4 °C with primary antibodies: mouse anti-beta Actin antibody (dilution 1:1000, Abcam, ab8226, Cambridge, UK); rabbit anti-TrKB (dilution 1:1000, Cell Signaling Technology, #4603, America); rabbit anti-ERK1/2 (dilution 1:1000, Cell Signaling Technology, #4695, USA); rabbit anti-CREB (dilution 1:1000, Cell Signaling Technology, #9197, USA); rabbit anti-brain-derived neurotrophic factor (BDNF) (dilution 1:1000, ab108319, Abcam, Cambridge, UK); rabbit anti-P-ERK1/2 (dilution 1:1000,Cell Signaling Technology, #4370, USA); and rabbit anti-p-CREB (dilution 1:1000, Cell Signaling Technology, #9198, USA), followed by the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (dilution 1:5000, Proteintech, SA00001–1, SA00001–2, USA) for 1 h at 37 °C. Immunoreactive bands were detected with enhanced chemiluminescence (ECL) (Pierce, USA). The optical density of the protein bands was scanned by DNR bioimaging system (Gel Capture Microchemi 4.2, Israel) and analyzed using GelQuant v12.3 software (DNR Bio-Imaging Systems, Israel). The relative expressions were normalized to β-actin.

2.13 Statistical Analysis

Data are presented as means ± SEM. Differences were considered significant at $p < 0.05$. Comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test and were performed using SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA).

3 Results

3.1 Effect of Taurine on Proliferation and Apoptosis of Neurons in the DG of the Hippocampus

Ki67 staining in Fig. 2c and d showed that the positive rate of Ki67 in the DG area of the hippocampus in CUMS group was only 9.6%, which was significantly lower than 21.2% in the control group ($p < 0.01$), while the positive rate of Ki67 in DG area of hippocampus in T + CUMS group was 18.4%, which was much higher than that in the CUMS group ($P < 0.01$). There was no significant difference between the control and the taurine control groups ($P < 0.05$). The results showed that CUMS could reduce the expression of Ki67 in the DG region of the hippocampus, and the administration of taurine could promote the proliferation of neurons.

As was shown in Fig. 2a and b, the TUNEL assay showed that the apoptotic rate in the DG area of the hippocampus of rats in CUMS group was 63.54%, which was 1.71 times higher than 37.06% in the control group ($p < 0.01$). The apoptotic rate in the DG area of the hippocampus in the T + M group was 39.77%, which was significantly lower than that in CUMS group ($p < 0.05$). There were no significant differences between the T and the control groups ($P > 0.05$). The results indicated that CUMS could induce apoptosis of cells in the DG of the hippocampus, and the administration of taurine could significantly inhibit the abnormal apoptosis of neurons induced by chronic stress.

3.2 Identification of NSCs

Undifferentiated NSCs formed round neurospheres that were suspended in the culture medium as was shown in Fig. 1b. Almost all the cells in neurospheres expressed nestin protein, which is a specific protein marker for neural stem cells appeared as green staining (Fig. 1c), suggesting the NSCs isolated from the hippocampus were neural stem cells.

3.3 Protective Effect of Taurine on NSCs from Injury by Glutamate

The protective effect of taurine on glutamate-injured NSCs was investigated by its role on cell viability. As Fig. 3 depicted, glutamate reduced cell viability to 52.16%, which was remarkably lower than the control group ($p < 0.01$). Taurine administration significantly blocked the harmful effects of glutamate on cell viability to 86.04% ($p < 0.01$). There was also an obvious effect of taurine on cell vitality in the absence of glutamate ($p < 0.01$), indicating taurine administered in a culture medium can also exert positive effects on the survival and viability of NSCs.

3.4 Effects of Taurine on the Proliferation of NSCs

To demonstrate the effect of taurine administration on the proliferation of NSCs, BrdU which can be incorporated into the newly synthesized DNA of replicating cells was commonly used to label and detect proliferating cells. Furthermore, to detect the possible role of the BDNF/ERK/CREB pathway in the effect of taurine on glutamate-injured NSC proliferation, U0126 was administered to block the phosphorylation of ERK1/2. As was illustrated in Fig. 4, $Brdu^+$ cells in the U0126 group were remarkably decreased; compared to the control group, $Brdu^+$ cells in the taurine group were increased ($p < 0.05$), which was also higher than that in the U0126 + Tau group ($p < 0.01$), manifesting BDNF/ERK/CREB

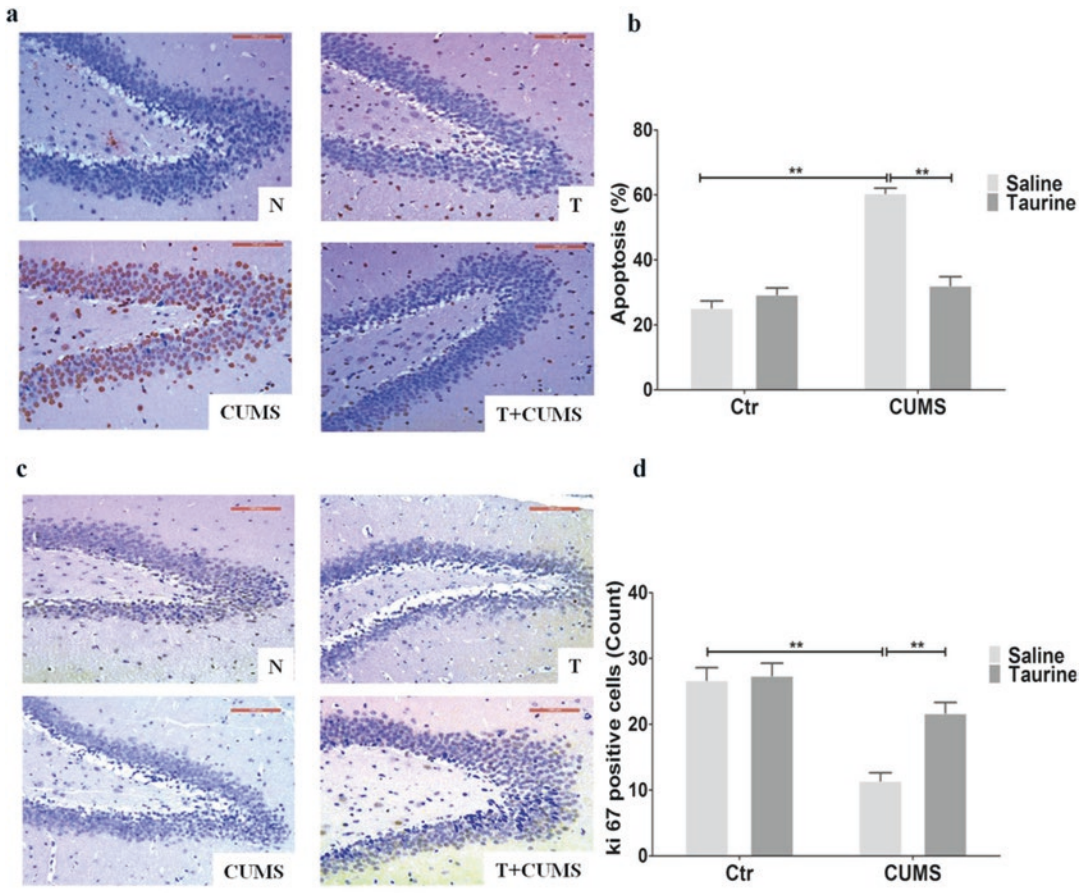

Fig. 2 Effect of taurine on proliferation and apoptosis of neurons in the DG of the hippocampus. (**a**) Immunohistochemistry of TUNEL. (**b**) Rate of TUNEL positive cells. (**c**) Immunohistochemistry of Ki67. (**d**) Rate of Ki67 positive cells. The cells were stained with DAB (Ki67 or TUNEL positive cells) and hematoxylin. The total cells were hematoxylin-positive cells. Ctr: rats without CUMS. CUMS: rats received CUMS every day. Every 10 sections through the whole hippocampus were collected. Data are presented as the means ± SEM. n = 6. *p < 0.05; **p < 0.01. One-way ANOVA plus post hoc analysis

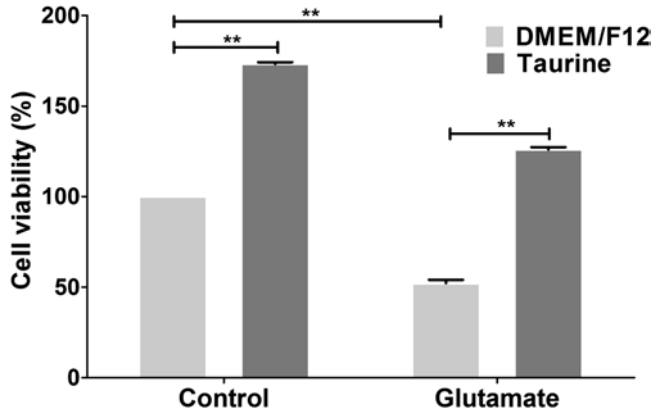

Fig. 3 Effects of taurine administration on the viability of NSCs injured by glutamate. Cells were cultured in DMEM/F12 for 24 h followed by incubation in CCK8 for 4 h at 37 °C. Then the optical density was measured by a microplate reader (Infinite® M200 PRO, TECAN) at 570 nm. Cell viability = (As-Ac)/(Ab-Ad) × 100%. As: experimental hole; Ac: experimental blank hole; Ab: control hole; Ad: control blank hole. n = 5; *p < 0.05, **p < 0.01

pathway plays an important role in taurine's promotion effect on cell proliferation. But when compared between the U0126 and the U0126 + Taurine group, Brdu$^+$ cells were higher in U0126 + Taurine group, suggesting the effect of taurine was partly diminished by the blockade of ERK signaling pathway, further indicating that taurine could take effect on cell proliferation partly through the BDNF/ERK/CREB pathway. A reduction in the number of Brdu$^+$ cells in the glutamate group was observed compared to the control group ($p < 0.01$), suggesting glutamate exerts a toxic effect on cell proliferation, especially in the U0126 + Glu group. Brdu$^+$ cells were lower than the glutamate group ($p < 0.05$), illustrating the blockade of ERK signaling pathway aggravating the toxic effect of glutamate. Brdu$^+$ cells were higher in Tau+Glu group than the Glu group, suggesting taurine can prevent the toxic effect of glutamate on cell proliferation. Under the presence of U0126, Brdu$^+$ cells in U0126 + Tau+Glu group were lower than Tau+Glu group, indicating the effect of taurine was through the BDNF/ERK/CREB pathway. However, since there were still differences in the U0126 + Glu group and U0126 + Tau+Glu group, indicating the effect of taurine on the proliferation of NSCs injured by glutamate was partly mediated by BDNF/ERK/CREB pathway.

3.5 Effects of Taurine on Differentiation of NSCs

After being induced by FBS, NSCs are differentiated into neurons which are marked by β-tubulin

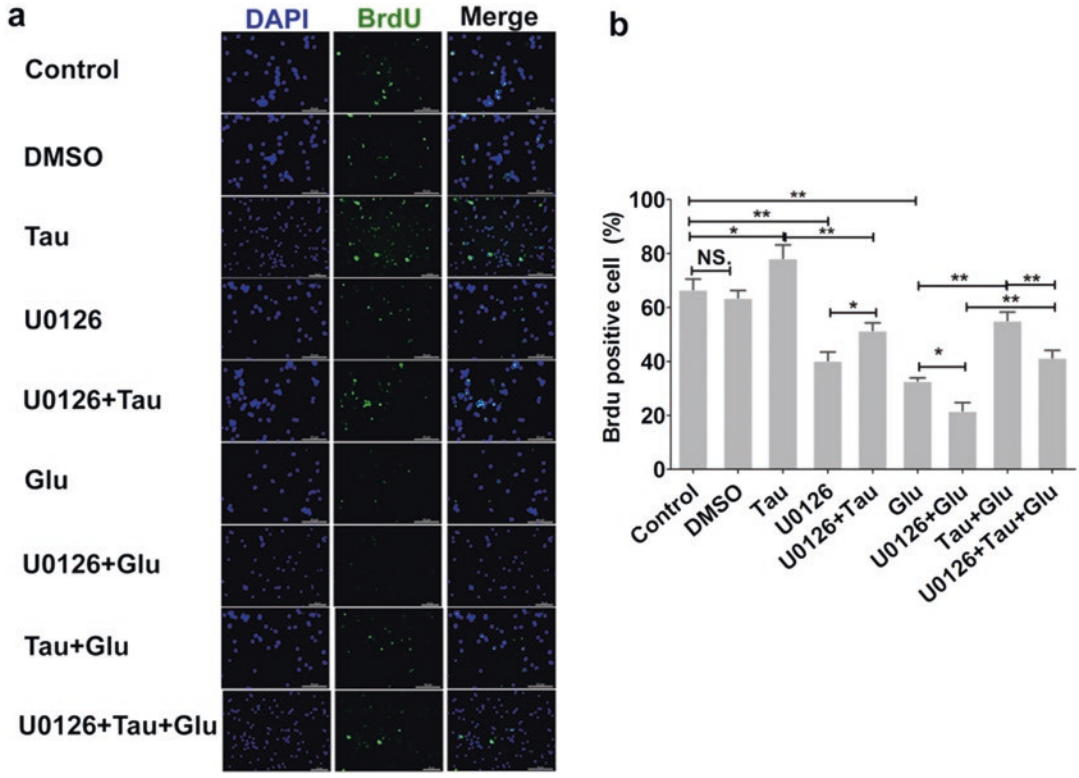

Fig. 4 Effect of taurine on the proliferation of NSCs injured by glutamate. (**a**) Immunofluorescence staining of BrdU and DAPI in NSCs cultured with taurine and glutamate. The second-generation neurospheres were collected and mechanically dissociated into single-cell suspension followed by seeding on poly-D-Lysine-treated cell climbing sheet at 12-well plates (5×10^5 cells); U0126 was used to block the phosphorylation and activation of ERK1/2. 10 μM BrdU were supplemented and incubated for 4 h at 37 °C for immunostaining of BrdU (green). The cells were then counterstained by DAPI (blue). ×400, scale bar = 50 μm. (**b**) The proliferation rate was presented as the percentage of BrdU-positive cells in the total number of cells (DAPI-stained cells). Data are presented as the means ± SEM. n = 25. *$p < 0.05$; **$p < 0.01$. One-way ANOVA plus post hoc analysis

III and astrocytes which were marked by GFAP. As was shown in Figs. 5 and 6, the rate of β-tubulin III$^+$ and GFAP$^+$ cells was significantly lower in the glutamate-treated group compared with those of the other groups ($p < 0.05$ or $p < 0.01$). Treatment of taurine can stimulate the differentiation of NSCs into both neurons and astrocytes, which was represented as increased β-tubulin III$^+$ and GFAP$^+$ cells compared to those of the control group ($p < 0.05$ or $p < 0.01$). The impediment of glutamate on NSC differentiation was also rectified by taurine administration ($p < 0.05$ or $p < 0.01$).

3.6 Effects of Taurine on Oxidative Stress in NSCs Induced by Glutamate

As was illustrated in Fig. 7, 10 mM glutamate treatment for 1 h induced a significant increase in the accumulation of intracellular MDA and an obvious reduction in SOD activity compared with those of the control cells ($p < 0.01$). However, treatment of NSCs with taurine remarkably counteracted the negative effect of glutamate on antioxidative capacity, as manifested by a significant decrease in MDA levels and an increase in SOD activity ($p < 0.01$).

3.7 Effects of Taurine on Apoptosis of NSCs Induced by Glutamate

TUNEL staining was used to detect apoptosis of NSCs induced by glutamate. The results were shown in Fig. 8, glutamate treatment significantly induced apoptosis of NSCs, which was higher than that in the control group ($p < 0.01$). Meanwhile, the stimulation of apoptotic cell death was modified by taurine ($p < 0.01$), suggesting an inhibitory effect of taurine on glutamate-induced NSC cell apoptosis.

3.8 Effects of Taurine on Protein Expression of Key Factors Involved in BDNF/ERK/CREB Pathway in NSCs

Finally, since the mechanism underlying the actions of taurine remains unclear, we investigated the possible involvement of the BDNF/ERK/CREB pathway, one of the proposed mechanisms by which taurine might act, by using a selective inhibitor of ERK. As seen in Fig. 9a and b, glutamate inhibited the protein expression of BDNF and its downstream target-

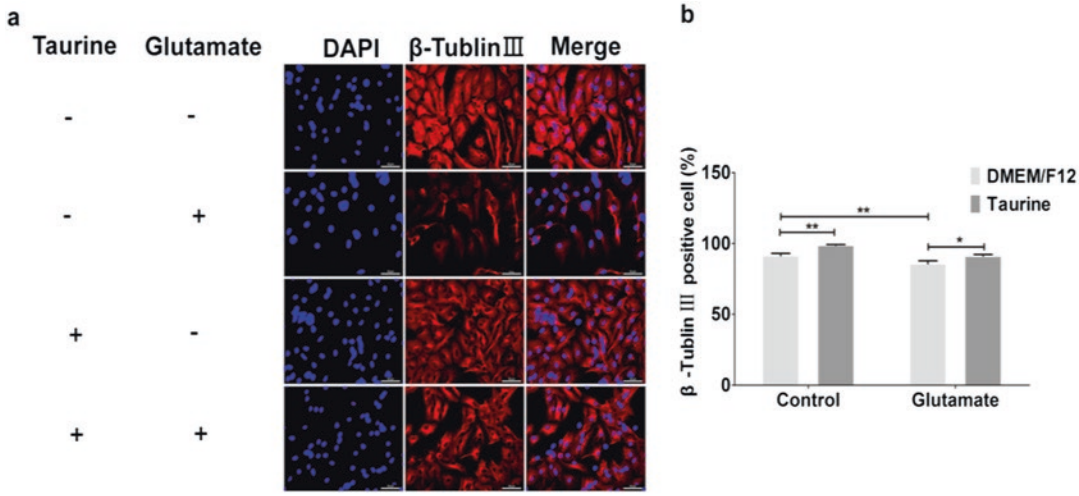

Fig. 5 Effect of taurine on the differentiation into neurons of NSCs. (**a**) Immunofluorescence staining of β-tubllin III. (**b**) β-Tubulin III positive rate. The second generation of single cell (5×10^5 cells) was cultured in DMEM/F12 with 10% FBS for 5 days followed by immunostaining of β-tubulin III. The cells were then counterstained by DAPI (blue). ×400, scale bar = 50 μm. The differentiation rate was presented as the percentage of β-tubulin III$^+$ cells in the total number of cells (DAPI-stained cells). Data are presented as means ± SEM. n = 25. *$p < 0.05$; **$p < 0.01$. One-way ANOVA plus post hoc analysis

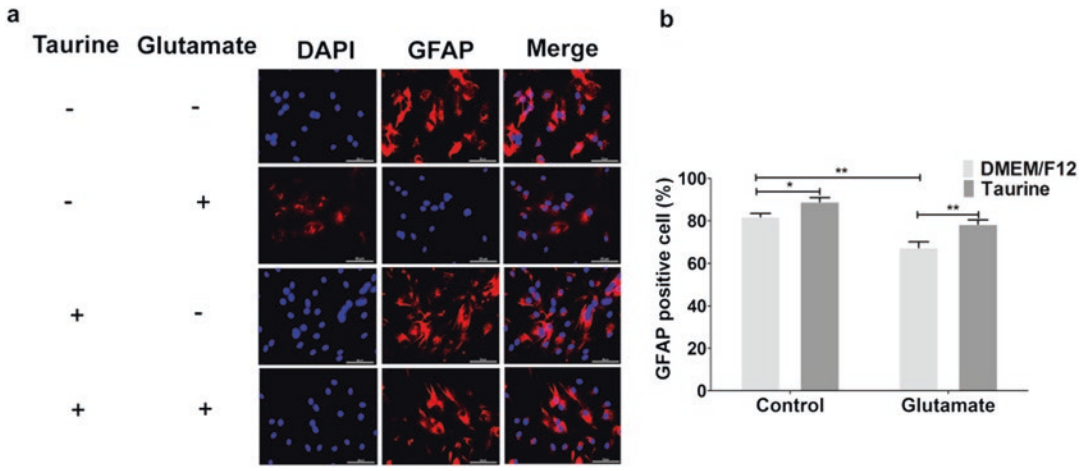

Fig. 6 Effect of taurine on the differentiation into astrocytes of NSCs. (**a**) Immunofluorescence staining of GFAP and DAPI. (**b**) GFAP positive rate. The second generation of single cell (5×10^5 cells) was cultured in DMEM/F12 with 10% FBS for 5 days followed by immunostaining of GFAP which was used as a marker of astrocytes (red). The cells were then counterstained by DAPI (blue). ×400, scale bar = 50 μm. The differentiation rate was presented as the percentage of GFAP$^+$ cells in the total number of cells (DAPI-stained cells). Data are presented as means ± SEM. n = 25. *p < 0.05; **p < 0.01. One-way ANOVA plus post hoc analysis

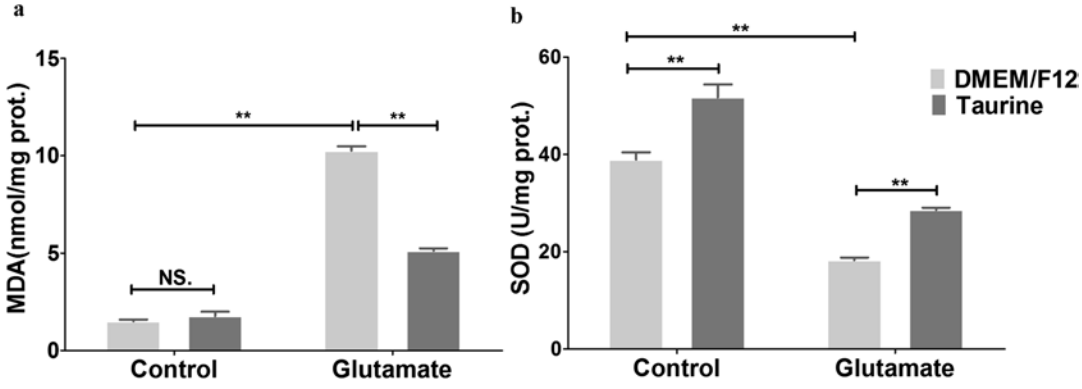

Fig. 7 Effects of taurine on oxidative stress of NSCs induced by glutamate. (**a**) MDA levels of NSCs. (**b**) SOD viability of NSCs. Data are presented as the means ± SEM. n = 5, *p < 0.05; **p < 0.01; One-way ANOVA plus post hoc analysis

ing protein TrkB, as well as the phosphorylation of ERK1/2 and CREB, which were all promoted by taurine administration ($p < 0.01$ or $p < 0.05$). Also, in this case, Fig. 10a and b showed that the protein expression of p-ERK1/2 was blocked, and glutamate-hindered CREB phosphorylation was aggravated in the presence of U0126, a selective inhibitor of ERK1/2 ($p < 0.01$), indicating that the inhibition of ERK1/2 activation significantly affects the expression and phosphorylation of its downstream protein CREB in NSCs injured by glutamate. Because taurine-induced phosphorylation of CREB in glutamate-injured NSCs was restrained by U0126 ($p < 0.01$), taurine administration appears to affect the BDNF/ERK/CREB pathway of NSCs injured by glutamate, which were partly blocked by U0126.

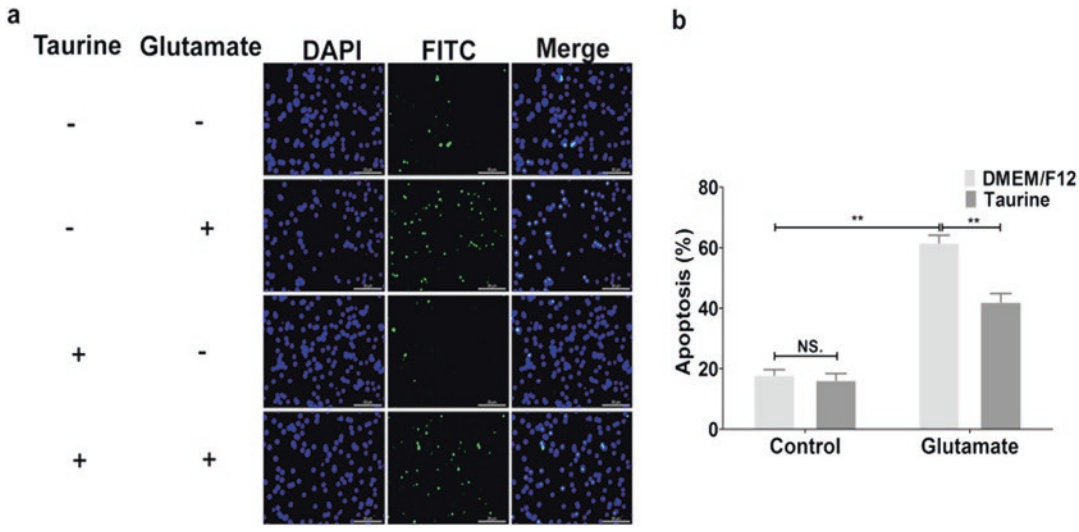

Fig. 8 Effects of taurine administration on glutamate-induced apoptosis of NSCs. (**a**) TUNEL staining for NSC apoptosis in vitro. NSCs were incubated with a TUNEL reaction mixture (green) and counterstained with DAPI (blue). Merged cells were apoptotic cells. ×400 magnification, Scale bars = 50 μm. (**b**) Quantification of TUNEL+ ratio to the total number of cells (DAPI-stained cells). Data are presented as the means ± SEM. n = 15. *p < 0.05; **p < 0.01; NS. p > 0.05. One-way ANOVA plus post hoc analysis

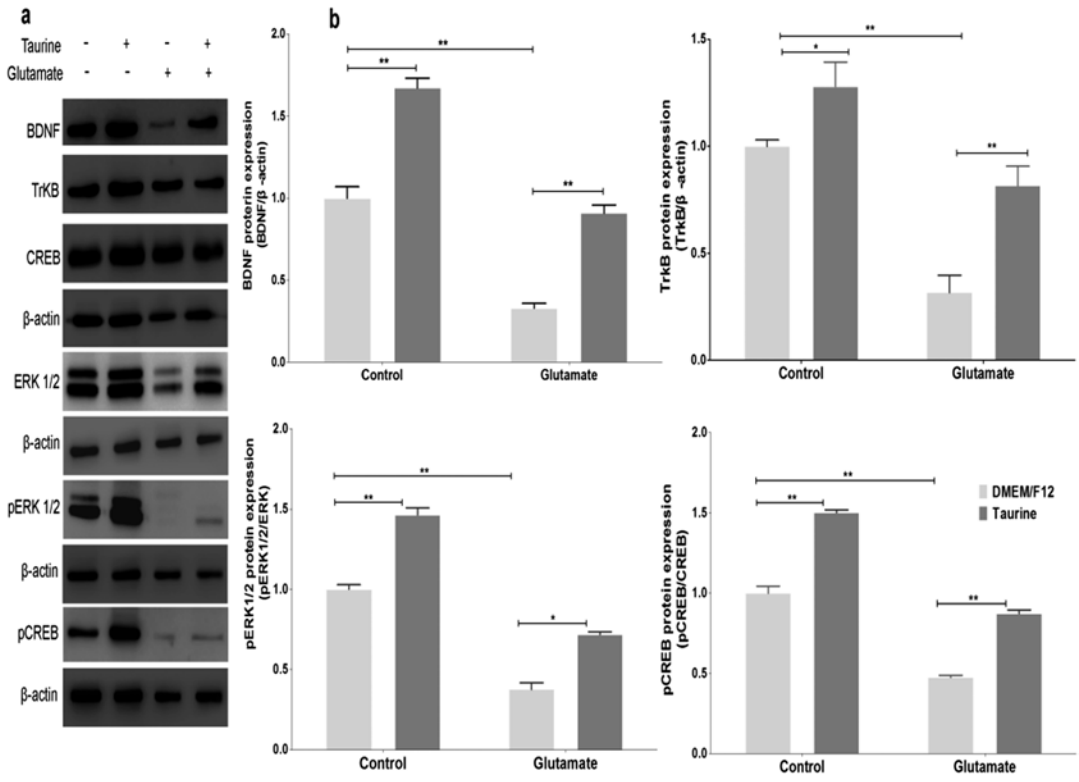

Fig. 9 Effect of taurine on protein expression of key factors involved in BDNF/ERK/CREB pathway in NSCs injured by glutamate. (**a**) Protein expression of key factors involved in BDNF/ERK/CREB pathway in NSCs cultured with taurine and glutamate. (**b**) Quantification of the relative BDNF, TrkB, p-ERK1/2, and p-CREB protein levels was normalized to the control group. Data are presented as the means ± SEM. n = 5, *p < 0.05; **p < 0.01. One-way ANOVA plus post hoc analysis

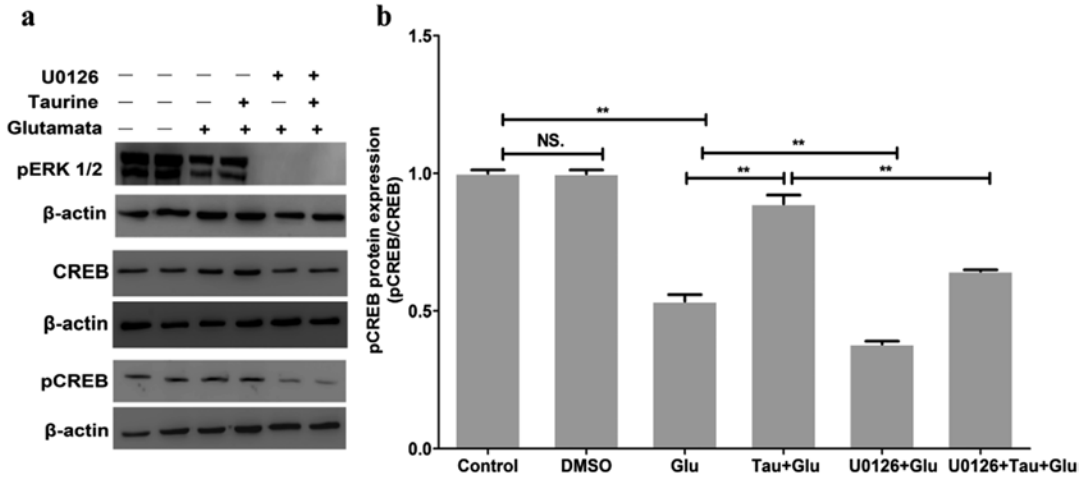

Fig. 10 Effect of taurine on protein expression of p-ERK1/2 and p-CREB in NSCs injured by glutamate cultured with U0126. (**a**) Protein expression of p-ERK1/2 and p-CREB in NSCs injured by glutamate cultured with U0126. (**b**) Quantification of the relative p-CREB protein levels was normalized to the control group. Data are presented as the means ± SEM. n = 5, *p < 0.05; **p < 0.01, NS. p > 0.05. One-way ANOVA plus posthoc analysis

4 Discussion

The major depressive disorder is a serious threat to people's physical and mental health. New neurons generated by NSCs in the adult mammalian hippocampal dentate gyrus are implicated in emotional regulation (Eisch and Petrik 2012), learning, and memory. Decreased hippocampal neurogenesis has been demonstrated in depressed patients (Huang et al. 2010) and animal models induced by chronic psychosocial stress (Kempermann and Kronenberg 2003). Moreover, chronic antidepressant treatment was found to relieve depressive symptoms by stimulating hippocampal neurogenesis (Anacker et al. 2011). In our previous study, taurine was found to prevent depressive behavior in CUMS-induced depressive rats (Wu et al. 2017). In the present study, taurine was further found to promote the proliferation and prevent apoptosis of cells in the DG of the hippocampus in CUMS rats. The results were inconsistent with Xu and Ayuob (Xu et al. 2019; Ayuob et al. 2020), who found that modulation of apoptosis and neurogenesis contributes to the treatment of depression. To further illustrate the mechanism of taurine on neurogenesis and apoptosis, hippocampal NSCs were first isolated from the hippocampus of newborn rats utilizing a mechanical digestion method and were then cultured in vitro with excessive glutamate to induce NSC injury as was reported previously (He et al. 2017; Narimatsu et al. 2013).

In adult mammals, emotional regulation is involved in neuron and neuroglia cell regeneration originated from NSCs that are persisted in the walls of the lateral ventricles and SGZ of the hippocampus (Hayashi et al. 2018). Neurons are the major structural and functional elements of the nervous system, the primary function of which is to transmit and receive information through nerve impulses and electrochemical signals. Gliacytes, which are highly abundant throughout the CNSCentral nervous system (CNS), can nourish the growth of neurons and other cells, participate in neurotransmitter metabolism, and play a pivotal role in glutamate uptake to prevent excitotoxicity (Di Meo et al. 2016). It has been demonstrated that both survival and proliferation of hippocampal-derived NSCs decrease in depressive animal models (Miguel et al. 2014; Ryu et al. 2018; Wang et al. 2014) and MDD patients (Epp et al. 2013), while antidepressant drugs mediate effects through activat-

ing NSC differentiation (Di Meo et al. 2016; Hayashi et al. 2018), indicating impaired hippocampal neurogenesis by decreased proliferation or differentiation of NSCs was related to depressive disorder disease (Chen et al. 2015). In the present study, in vivo results showed that ki67-positive cells were decreased in CUMS rats, while in vitro results examined cell viability of NSCs and positive expression of proliferation marker BrdU, immature neuron marker β-tubulin III, and the astrocyte marker GFAP, which were significantly reduced by glutamate treatment, suggesting that CUMS and glutamate can hinder the survival, proliferation, and differentiation of NSCs, an observation consistent with previous studies (Hu et al. 2018a, b; Shah et al. 2016; Zhang et al. 2016a, b). Taurine has been proved to promote the development of the fetal brain by improving the proliferation and differentiation of NSCs (Huang et al. 2018; Li et al. 2017a, b, c, d; Liu et al. 2013; Pasantes et al. 2015) in murine and human and counteract the suppressive effect of lipopolysaccharide on the proliferation in the hippocampus of rats (Wu et al. 2013); similar results were also observed in cultured NSCs obtained from the SVZ of adult mice (Hernandez-Benitez et al. 2012), showing that taurine can be considered not only as a neurotransmitter but also as an important neurodevelopmental modulator (Kilb and Fukuda 2017). The above results are similar to the present study that taurine administration significantly increases ki67-positive cells in CUMS rats and increases cell viability and cell proliferation of NSCs impaired by glutamate. Notably, we also found that the positive expression levels of β-tubulin III and GFAP were increased in NSCs treated by both taurine and glutamate, illustrating that taurine promotes NSC differentiation into both neurons and astrocytes. The results are consistent with our previous findings that taurine can alleviate depression-like behavior induced by chronic mild unpredictable stress in rats though elevating neurogenesis, neuronal survival, and growth in the hippocampus (Wu et al. 2017).

Depression has been proved to be accompanied by oxidative stress arising from an imbalance between oxidant and antioxidant compounds in favor of excessive free radical generation (Li et al. 2017a, b, c, d). Excessive reactive oxygen species (ROS) production is well-known to induce cellular damage which ultimately affects neurogenesis or neurodegeneration in the brain (Yi et al. 2018). During depression, a high level of glutamate accumulates in the hippocampus (Wu et al. 2017), which has been reported to induce cellular damage that eventually triggers cell apoptosis or cell death via increases in the amount of intracellular free Ca^{2+} (Ludka et al. 2017; Song et al. 2017). Besides, accumulation of Ca^{2+} in mitochondria will further lead to an increase in the production of ROS that triggers a breach in the mitochondrial membrane's permeability and finally leads to the release of pro-apoptotic factors that ultimately trigger cell apoptosis (Prentice et al. 2015). This is supported by our observations in the present study that the increased ROS, MDA, and decreased SOD activity were found in NSCs cultured with glutamate, accompanied by the high rate of apoptosis. The results were similar to studies in cells cultured in vitro and in the hippocampus of adult rats exposed to glutamate, which altered the levels of SOD, GSH, and MDA, caused the generation of ROS, resulted in oxidative stress, and ultimately induced apoptosis (Lv et al. 2017; Motaghinejad et al. 2017; Sadeghnia et al. 2017). It has been well discussed that taurine can protect neurons from glutamate-induced injury (Bhat et al. 2020). On the one hand, it can enhance the activity of sarcoplasmic Ca^{2+}-ATPase associated with the maintenance of cytosolic Ca^{2+} homeostasis via the uptake of cytosolic Ca^{2+} (Ramila et al. 2015). On the other hand, taurine has been found to exert antioxidant activity in various tissues including the hippocampus (Rodriguez-Martinez et al. 2004) and inhibit apoptosis through the regulation of factors on the mitochondrial cell apoptotic pathway including Bcl-2, Bax, and Caspase 9 (Leon et al. 2009). This effect of taurine was also found in 2,5-hexanedione-damaged PC 12 cells (Li et al. 2017) and in the cortex of epilepsy model mice that taurine can decrease glutamate levels in the brain and exhibits antioxidant properties through normalizing GSH and SOD levels (El-Abhar and El Gawad 2003).

Moreover, taurine has been reported to attenuate oxidative damage in the rat brain caused by dibromoacetonitrile, which was manifested by decreases in MDA concentration in the brain (Sayed et al. 2012). In diabetes-induced oxidative stress in the brain, taurine was found to increase GSH, CAT, GSH-Px, and SOD activities (Patel and Lau-Cam 2017). Meanwhile, it has been reported that taurine supplementation markedly ameliorates As-induced apoptosis by a mitochondria-related pathway in the mouse hippocampus (Li et al. 2017) and prevents anesthetic isoflurane-induced cognitive impairment by inhibiting ER stress-mediated apoptosis in the hippocampus of aged rats (Zhang et al. 2016a, b). Combining the above results, the present data illustrate that taurine can decrease apoptosis in the hippocampus of CUMS rats, increase SOD activity, decrease MDA levels, and inhibit apoptosis of NSCs cultured with glutamate, indicating the protective effect of taurine on CUMS and glutamate-induced NSC injury are partly dependent on its antioxidant capacity to reduce ROS accumulation and further protect mitochondria and the cell against apoptosis.

BDNF, the most ubiquitous member of the neurotrophins family in the CNS, is not only vital to neurogenesis and neurodevelopment regulation including proliferation, differentiation, maturation, and synaptic plasticity in the hippocampus (Sun et al. 2018) but is also involved in many neurological diseases, such as cognitive disorder, depression, and psychopathy (Leal et al. 2017; Thompson et al. 2011). Moreover, accumulating studies show that both mRNA and protein expression of BDNF decrease in patients and animal models of depression (Molendijk et al. 2013; Pilar-Cuellar et al. 2013), and many antidepressive drugs exert effects by elevating BDNF concentration in the hippocampus and prefrontal cortex (Ghosal et al. 2018; Santarelli et al. 2003). In the brain, the effects of BDNF are mediated by the activation of intracellular signaling pathways upon high-affinity binding to its receptor, tropomyosin-related kinase B receptor (TrkB) (Serra et al. 2017), which triggers ERK cascades that activate and phosphorylate cAMP response element-binding protein (CREB) at Ser133, resulting in the secretion of neurotrophic factors including BDNF and promoting neuronal survival, differentiation, regeneration, axonal growth, dendritic maturation, and use-dependent synaptic plasticity (Lesiak et al. 2013). Therefore, the BDNF-regulated neurotrophic pathway was considered as a potential target for depression treatment and antidepressant selection. In this study, we examined the effects of taurine on BDNF/ERK/CREB signaling in glutamate-damaged NSCs. The results show that glutamate administration significantly downregulates the expression of BDNF, TrkB, Erk1/2, and CREB and inhibits the activation of ERK 1/2 and CREB. Meanwhile, BrdU-positive cells were decreased by glutamate treatment. However, U0126, the inhibitor of p-ERK 1/2, aggravates the downregulation of p-CREB and BDNF expression, and decreases the number of BrdU-positive cells compared with cells that were only treated with glutamate, indicating NSCs were impaired by glutamate, an effect associated with the BDNF/ERK/CREB signal pathway. The present results are consistent with previous findings that glutamate can induce cytotoxicity by affecting ERK expression in PC12 cells (Liu et al. 2017; Yue et al. 2015). It is worth noting that the protein expression of BDNF, TrkB, ERK 1/2, p-ERK 1/2, CREB, and p-CREB is increased in NSCs cultured with taurine. Moreover, taurine inhibits the downregulatory effect of glutamate on the BDNF/ERK/CREB pathway and increases the number of BrdU-positive cells. Inhibition of the ERK pathway by U0126 diminishes the effect of taurine on the protein expression and activation of CREB in NSCs injured by glutamate. Besides, a number of BrdU-positive cells in the taurine and glutamate pretreated group are higher than those of the cells treated with U0126. The results are in agreement with the previous reports that taurine increases ERK, BDNF, and CREB expression in rats (Caletti et al. 2015; Jia et al. 2016; Martinez-Lopez et al. 2013), and taurine supplementation has been shown to induce proliferation in neural progenitor cells through activation of ERK 1/2 phosphorylation and its pathway (Shivaraj et al. 2012). The results indicate that taurine protects NSCs against injury by glutamate partly through activating the BDNF/ERK/CREB signal pathway.

5 Conclusion

The present results indicate that taurine protects neurogenesis and apoptosis in the DG of the hippocampus in CUMS rats and against glutamate-induced NSC injury. On the one hand, taurine can increase the survival, proliferation, and differentiation of NSCs injured by CUMS or glutamate, the mechanism of which maybe through the upregulation of the BDNF/ERK/CREB pathway. On the other hand, taurine can also inhibit the abnormal apoptosis of NSCs, the mechanism of which was partly through its antioxidant activity.

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Brain-Derived Neurotrophic Factor Potentiates Entorhinal-Dentate but not Hippocampus CA1 Pathway in Adult Male Rats: A Mechanism of Taurine-Modulated BDNF on Learning and Memory

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Keywords

BDNF · Dentate gyrus · Entorhinal cortex · Field potential · Hippocampus · Power spectral density

Abbreviations

AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	Brain-derived neurotrophic factor
CA1	Hippocampus CA1
fEPSP	Field excitatory postsynaptic potential
fIPSP	Field inhibitory postsynaptic potential
GABA	γ-aminobutyric acid
NMDA	n-methyl-d-aspartate
TrkB	Tyrosine kinase B

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1 Introduction

Taurine is present in the brain, where it contributes to many functions from conception onward. Other than its role as a cell osmolyte, taurine promotes growth and development during early life and affects neural activity and function in adults (Roysommuti and Wyss 2015). During prenatal and early postnatal life, taurine acts as a major inhibitory neurotransmitter/modulator in the brain, whereas during the postnatal period, GABA gains dominance along with glycine as central inhibitory transmitters. Like other organs, brain taurine content declines with advancing age, correlated to a high risk of learning and memory disorders and other brain diseases in the elderly. Although the effects of taurine on learning and memory in healthy subjects have not been fully assessed, taurine supplementation prevents or improves learning and memory performance in many disorders. These include AD (Javed et al. 2013; Saumier et al. 2009), learning and memory disorders related to perinatal taurine

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deficiency (Zicker et al. 2012), and Fragile X syndrome (El et al. 2009). Accumulated data indicate that taurine supplementation ameliorates abnormalities in brain growth, behavior, and memory, at least in part, via the action of BDNF in both human and animal models, e.g., Fragile X syndrome (Erickson et al. 2013), depressive-like behavior (Kim et al. 2020), corticosterone-triggered cellular damage (Lee et al. 2020), repeated restraint stress-induced behavioral deficits and hippocampal anomalies (Jangra et al. 2020), and hexabromocyclododecane-decreased spatial learning and memory (Zhang et al. 2017).

BDNF is a family of nerve growth factors that play several physiological roles in neural growth, differentiation, maturation, adaptation, and apoptosis (Gonzalez et al. 2019; Lin and Huang 2020; Miranda et al. 2019). BDNF increases synaptogenesis, synaptic strength, and immune function in the central nervous system (Golia et al. 2019; Kowianski et al. 2018). In addition, it also affects non-neural tissues, particularly cardiomyocytes (Kermani and Hempstead 2019; Pius-Sadowska and Machalinski 2017). Thus, BDNF is synthesized by several cell types, including neural, glial, and myocardial cells. BDNF can diffuse across blood-brain barrier, and therefore, plasma BDNF levels are often used as an indicator of brain BDNF content and function (Balietti et al. 2018). Notably, high plasma BDNF levels improve learning and memory and increase BDNF content in hippocampus and entorhinal cortex, but the same maneuver decreases BDNF in some other brain areas (Marinus et al. 2019; Mrowczynski 2019; Pedersen 2019). Mature BDNF (m-BDNF or BDNF) affects target cells via TrkB receptors, and abnormal TrkB receptor density and activity are present in some types of cognitive disorders, including AD and other dementias (Kowianski et al. 2018; Miranda et al. 2019).

AD is an age-related neurodegenerative disease affecting people around the world. The longer life span of people makes AD an increasing worldwide, economic, and social burden on society (Matej et al. 2019; Sun and Alkon 2019). Abnormal increases in amyloid-β and Tau protein in neural tissue in the learning and memory pathway (particularly in hippocampus and entorhinal cortex) appear to play a significant role in this disorder (Naseri et al. 2019); however, the available drugs currently used to treat AD, e.g., acetylcholine esterase inhibitor and NMDA receptor blockers, do not significantly alter neural amyloid-β or Tau content or metabolism. This suggests that other mechanisms contribute to the pathogenesis of AD. Further, it is well known that learning and memory is a complex, integrated, neurological process involving many brain areas and several external factors, including sensory inputs and physical activity. Recently, many neurotrophic factors have been reported to play important roles in the process of learning and memory, and their abnormal levels or functions may influence the pathogenesis of AD, in both human and animal models (Fang et al. 2019; Miranda et al. 2019; Sun and Alkon 2019; Xiang et al. 2019). In addition, neural stem cell implantation has recently been reported to be a novel treatment for AD, and it involves neurotrophic factor synthesis and function in the brain (Bali et al. 2019; Elia et al. 2019; Hayashi et al. 2020). Examples include BDNF and neuregulin-1β in the dentate gyrus, CA1, and entorhinal cortex.

Increases in learning and memory are dependent on neocortical and entorhinal-hippocampal pathways, particularly entorhinal-dentate and entorhinal-CA1 pathways (Miyashita 2019; Yavas et al. 2019). These pathways develop short- and intermediate-term memory, which is then stored as long-term memory in the neocortex. Activation of memory from the neocortex is also dependent in part on the entorhinal-hippocampal pathways (Ryan et al. 2008). Further, these pathways are modulated by some neurotrophic factors (Alkadhi 2019). Previously, we reported that neuregulin-1β promotes neurogenesis, synaptogenesis, and growth of hippocampal neurons (Gerecke et al. 2004). In addition, neuroregulin-1β increases long-term potentiation of entorhinal-dentate but decreases the entorhinal-CA1 communication (Roysommuti et al. 2003). BDNF is also a neurotrophic factor that potentiates and inhibits long-term potentiation in different brain areas (Miranda et al. 2019). The present study tests the hypothesis that BDNF differentially modulates long-term potentiation of entorhinal-hippocampal pathways. Both peak slopes of fEPSP and power spectral analysis of

dentate gyrus and CA1 field potentials were studied during repetitive stimulation of entorhinal cortex in mature rats.

2 Methods

2.1 Animals

Male Sprague-Dawley rats (9–10 weeks of age) were obtained from Harlan Sprague Dawley, Inc. and were maintained at constant humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (0600–1800 h). Food and water were available ad libitum throughout the experiment. All experimental protocols were approved by the University of Alabama at Birmingham's Institutional Animal Care and Use Committee and were in accord with the *Guide for the Care and Use of Laboratory Animals* (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf).

2.2 Experimental Protocol

Entorhinal cortex stimulation electrodes and hippocampal field potential recording electrodes were positioned as previously reported (Roysommuti et al. 2003) and the method description partly reproduces their wording. In brief, after the animal was anesthetized with methohexital (Brevital; 50 mg/kg), the midline skin above the head of each rat was cut, and two small burr holes were drilled through the right-side skull for insertion of field potential recording electrodes (AP +2.8 mm., ML −1.0 mm.) and entorhinal cortex stimulation electrodes (AP +4.8 mm., ML −5.3 mm.). First, a paired, stainless steel electrode (0.1 mm. outer diameter) was stereotaxically placed into the entorhinal cortex (dorsoventral −8.3 mm.), which was used to continuously stimulate the cortex with paired pulses (duration 0.15 msec, interval 50 msec, frequency 0.10 Hz; Grass S48 stimulator and photoelectric stimulus isolator; Grass, Quincy, MA, USA).

Second, a stainless steel recording electrode (12 MΩ; A-M Systems, Sequim, WA, USA) attached to a microinjection tube (0.008″ OD × 0.004″ ID) connected to polyethylene tube number 20 (PE20) was slowly, stereotaxically lowered into the brain to record hippocampal field potentials in the CA1 or the dentate gyrus (DV −3 to −5 mm.). Placement was confirmed by the change in the field potentials while lowering the electrode (Messaoudi et al. 1998). The microinjector was cemented to the electrode such that the tip of the micropipette was 0.4 mm. above the tip of the recording electrode. The recording electrode was connected to a DAM-80 differential amplifier (WPI, Sarasota, FL, USA) to amplify (500) and filter (500–1000 Hz) the analog signal. The output signal was displayed on an oscilloscope (Nicolet, Madison, WI, USA), digitized (at 10,000 Hz), and stored on a computer using the BIOPAC Systems (BIOPAC Systems, Santa Barbara, CA, USA).

Third, following surgery (< 30 min), the entorhinal cortex stimulation was started and continuously performed to the end of experiment. Anesthetic conditions were maintained by α-chloralose (0.1 g/kg body weight), and body temperature was maintained at 37 ± 0.5 °C, using a heated water jacket connected to a temperature controller (YSI, Yellow Spring, OH, USA). Animals recovered for at least 1 h or until the field potential slopes were consistent for the least 15 min. Thereafter, BDNF (8 μM m-BDNF, Sigma-Aldrich) or an equimolar solution of cytochrome C (horse heart, Sigma-Aldrich) in artificial cerebrospinal fluid (ACSF, 0.5 μl each) was slowly infused either in CA1 or dentate gyrus over 2 min, and the stimulation and field potentials were continuously recorded for at least 1 hour. At the end of the experiment, electrode locations were marked by injection of DC current (1.0–1.5 mA) for 15 s. Finally, all rats were sacrificed, and the brain was removed, stored at −70 °C, sectioned, stained with crystal violet, and microscopically examined for electrode placement. Only rats in which the three electrodes were correctly placed were selected for further review.

A sample micrograph of similar electrode placements and field potentials has been previously reported (Roysommuti et al. 2003). For showing all positive and negative waves of each field potential, the original typical tracing in the

present study was amplified (after noise filtering and line smoothing) and redrawn for better resolution (see Figs. 1b and 2b).

2.3 Peak fEPSP Analysis

A fEPSP slope of each field potential was calculated as the slope of the rising phase between 25 and 75% of the peak positive wave (from zero baseline), by using Acknowledge software (BIOPAC Systems). Six slopes within 2 min were averaged (Roysommuti et al. 2003). Changes in slopes after chemical infusion were expressed as a percent of the last 2-min baseline slope.

2.4 Power Spectral Analysis of Field Potential

Power spectral analysis of local field potentials recorded from surface electrodes is widely used to elucidate the specific function, modulation, and disorder of different brain areas. The power spectral density of hippocampal field potential at a certain frequency range can help clarify the long-term potentiation relationship to the peak fEPSP. The field potentials selected for peak slopes measurement were also power spectrally analyzed by using fast Fourier transformation (Acknowledge software, BIOPAC Systems) as previously reported (Rakmanee et al. 2017), and the method description partly reproduces their wording. In brief, each total field potential was selected (all upward and downward waves of each field potential, see Figs. 1b and 2b) using the Acknowledge software (BIOPAC Systems) and selected fast Fourier transformation, Hanning window, pad with zeros, removal of mean, trend, and linear magnitude, yielding the final power spectrum, and then measured for selected integral under curve areas in the frequency ranges 0–70 Hz and 70–200 Hz. For each period (2 min), data of six fields were averaged, while each group's power spectral pattern (see Figs. 3a and 4a) was averaged from 4 to 7 rats by using Acknowledge software (BIOPAC Systems).

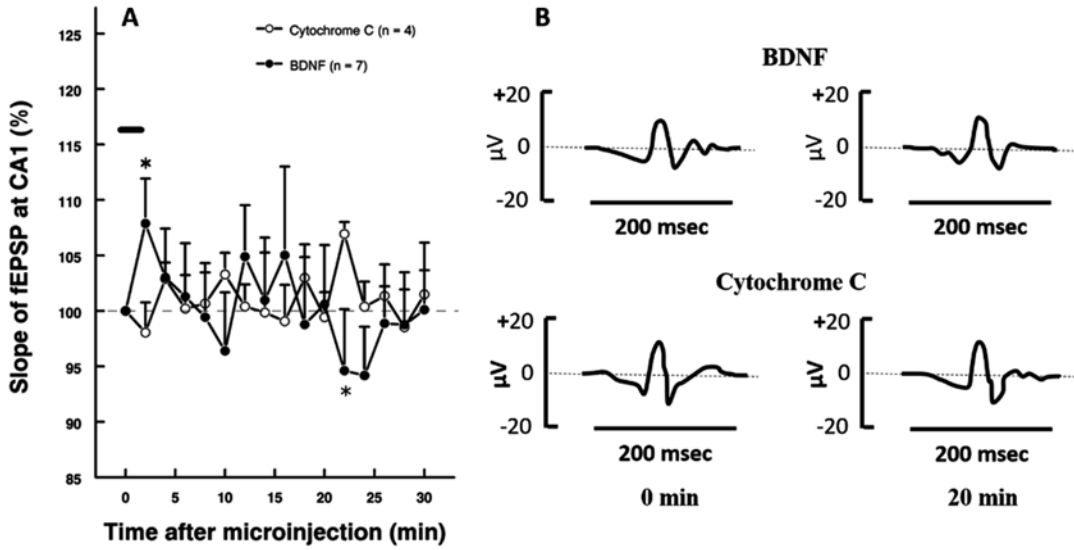

Fig. 1 (**a**) Changes in the peak slopes of field excitatory postsynaptic potentials (fEPSP) during continuous stimulation of entorhinal cortex after microinjection of brain-derived neurotrophic factor (BDNF) and cytochrome C (control) into CA1 (bar strip) in anesthetized rats (*P < 0.05 compared between groups). (**b**) The typical fEPSPs (redrawn from original tracings after amplifying, noise filtering, and line smoothing) before (0 min) and 20 min after initiated BDNF (upper two panels) or cytochrome C microinjection (lower two panels). It is noteworthy that the peak slope of each field was calculated from the rising phase between 25 and 75% of the peak positive wave (from zero baseline) by using the Acknowledge software (BIOPAC Systems)

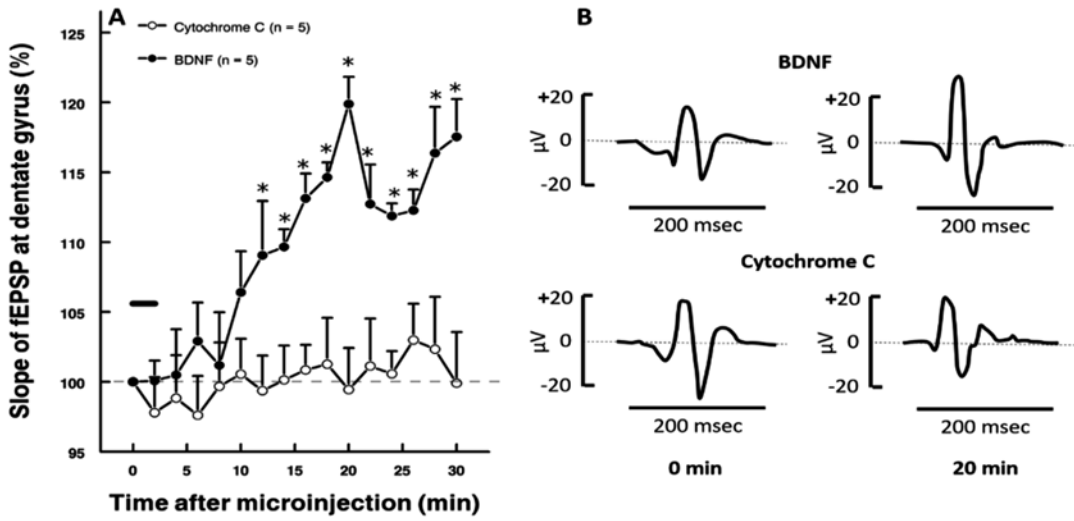

Fig. 2 (**a**) Changes in the peak slopes of field excitatory postsynaptic potentials (fEPSP) during continuous stimulation of entorhinal cortex after microinjection of brain-derived neurotrophic factor (BDNF) and cytochrome C (control) into dentate gyrus (bar strip) in anesthetized rats (*P < 0.05 compared between groups). (**b**) the typical fEPSPs (redrawn from original tracings after amplifying, noise filtering, and line smoothing) before (0 min) and 20 minutes after initiated BDNF (upper two panels) or cytochrome C microinjection (lower two panels). It is noteworthy that the peak slope of each field was calculated from the rising phase between 25 and 75% of the peak positive wave (from zero baseline) by using the Acknowledge software (BIOPAC Systems)

2.5 Statistical Analysis

All data are expressed as mean ± SEM and were statistically evaluated by one-way ANOVA followed by the post hoc Duncan's multiple range tests for peak fEPSP slopes and the Student's unpaired t-tests (one-tail) for power spectral densities (StatMost32 version 3.6, Dataxiom, CA, USA). The significant criteria were $p < 0.05$.

3 Results

3.1 Peak fEPSP Slopes

In the CA1 region, either cytochrome C or BDNF microinjection induced similar fluctuations in peak fEPSP slopes above and below the baseline response (about ±5% from baseline; Fig. 1a). Significant differences between groups were observed 2 and 22 min after initiated BDNF or cytochrome C microinjection. In dentate gyrus, cytochrome C microinjection did not alter the peak fEPSP slopes compared to the baseline, while compared to the cytochrome C group, the BDNF microinjection slowly and significantly increased the peak fEPSP slopes which reached a peak at ~20 min after initiated BDNF microinjection ($P < 0.05$). These field potential responses were sustained for more than 30 min post-injection (Fig. 2a).

It is noteworthy that equimolar sucrose or heated neuregulin-1β in artificial cerebrospinal fluid was previously injected into the CA1 and dentate gyrus, and their peak fEPSP slopes responded similarly to those of cytochrome C injection in the present study (Roysommuti et al. 2003).

3.2 Power Spectral Density and Pattern of Field Potentials

To find the frequency components of field potentials affected by BDNF, we measured significant peaks of power densities following BDNF perfusion, determining power spectral densities of field potentials at 20 min after initiated BDNF or cytochrome C injection (based on the peak fEPSP slope response after BDNF in the dentate gyrus). We relied on frequency investigation using fast Fourier analysis. The data indicate that both CA1 (Fig. 3a) and dentate field potentials (Fig. 4a) displayed an averaged power spectral density pat-

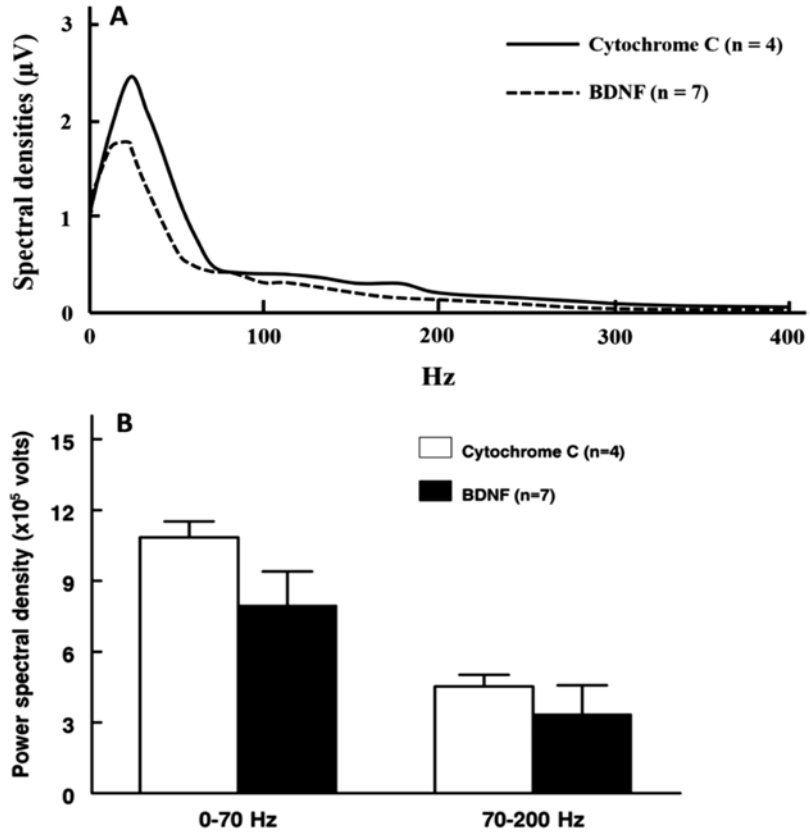

Fig. 3 (**a**) Patterns of power spectral densities of field excitatory postsynaptic potentials (fEPSP) during continuous stimulation of entorhinal cortex at 20 min after initiation of cytochrome C (control) and brain-derived neurotrophic factor (BDNF) microinjection into CA1 in anesthetized rats. (**b**) Power spectral densities the y-axis unit is ($x10^{-5}$ volts) between frequencies 0–70 and 70–200 Hz of above fEPSP power spectrum. Data are averaged from 4 to 7 rats each group. No significant differences of power spectral densities were observed between groups of the same frequency ranges

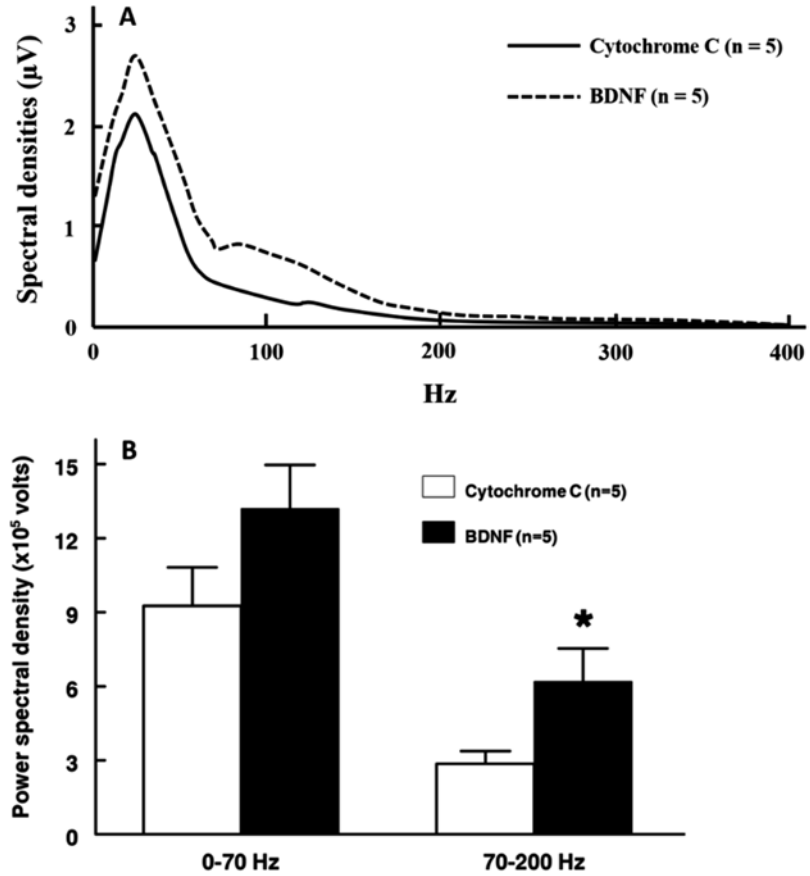

Fig. 4 (**a**) Patterns of power spectral densities of field excitatory postsynaptic potentials (fEPSP) during continuous stimulation of entorhinal cortex 20 minutes after initiation of cytochrome C (control) and brain-derived neurotrophic factor (BDNF) microinjection into dentate gyrus in anesthetized rats. (**b**) Power spectral densities the y-axis unit is ($x10^{-5}$ volts) between frequencies 0–70 and 70–200 Hz of above fEPSP power spectrum. Data are averaged from 5 rats each group (*$P < 0.05$ compared between groups of the same frequency ranges)

tern with two peaks of spectral densities between the frequencies 0–70 and 70–200 Hz. In CA1, neither cytochrome C nor BDNF caused a significant change in the power spectral densities at either 0–70 Hz or 70–200 Hz (Fig. 3b). In contrast, in the dentate gyrus, 0–70 Hz were not significantly different between groups, but the power spectral densities of frequencies between 70 and 200 Hz were significantly higher in the following BDNF compared to the cytochrome C microinjection (Fig. 4b). Thus, BDNF (versus control) increases the 70–200 Hz (but not the 0–70 Hz) power spectral densities of local field potentials in the entorhinal-dental synaptic pathway.

4 Discussion

Entorhinal inputs to the dentate gyrus and CA1 play an important role in learning and memory (Yavas et al. 2019). Long-term potentiation of synaptic transmission by repetitive stimulation of entorhinal cortex and peak fEPSP slopes have been widely used to explore altered learning and memory in animal models (Levy et al. 2019; Penasco et al. 2019; Roysommuti et al. 2003). The present data indicate that local infusion of BDNF into the dentate gyrus significantly increases peak fEPSP slopes in the dentate gyrus, but not in the CA1. These data differ from the effects of neuregulin-1β, in that neuregulin-1β increases the peak fEPSP slopes in dentate gurus but decreased the slopes in CA1 (Roysommuti et al. 2003). This potentiation effect of BDNF in the dentate gyrus, but not in the CA1, is consistent with a significant increase in power spectral density of dentate field potentials at 70–200 Hz following BDNF injections into the dentate gyrus but not significantly altering frequencies below 70 Hz. These results support the location-specific action of different neurotrophic factors within the hippocampus and may provide insight into learning and memory processes (Miranda et al. 2019). In addition, the present data suggest that, within the hippocampus, the taurine-amelioration effect on learning and memory impairment via BDNF might act through the entorhinal-dentate rather than entorhinal-CA1 pathway.

The positive effects of BDNF on the dentate gyrus but not CA1 field potential agree with other reports (Alkadhi 2019; Miranda et al. 2019). It is well known that the dentate gyrus contains mainly the small granule cells, while the CA1 contains mostly pyramidal cells and other neurons. Further, the granule cells are more capable to neurogenesis than other hippocampal cells (Lee and Son 2009; Pawluski et al. 2009; Sawada and Sawamoto 2013), and several neurotropic factors including BDNF affect the dentate gyrus neurons more than they affect neurons in other brain areas.

BDNF protein has two isoforms, pro-BDNF and m-BDNF. In mature animals, the ratio of m-BDNF to pro-BDNF is high compared to that observed in young animals (Kowianski et al. 2018). The pro-BDNF acts via p75NTR (p75 neuro trophin receptor) and sortilin (sortilin-related vacuolar protein sorting 10 protein (Vps10p)-domain sorting receptor 2). The present study used m-BDNF, which acts via TrkB receptors on the cell membrane of target cells. Although the TrkB receptors and BDNF are found both in dentate gyrus and CA1 (Di et al. 2019; Miranda et al. 2019), in the present study, entorhinal activation of the dentate gyrus (but not activation of the CA1) increased field potentials in animals microinjected with BDNF. The present data suggest that the BDNF may play an important role in modifying the input from entorhinal cortex to the dentate gyrus, thereby affecting the main gateway to the hippocampus. Further, in the CA1, BDNF may play other roles rather than the modulation of entorhinal-pyramidal cell synapses. In hippocampal slices, BDNF added to the bath fluid modulates inhibitory, but not excitatory, transmission within CA1 (Frerking et al. 1998). Thus, a transient rise of peak fEPSP slope 2 min after BDNF injection in CA1 might reflect an acute suppression of the inhibitory synaptic transmission within the CA1.

Axons from the entorhinal cortex excite the granule and pyramidal cells mainly via glutamate release (Takeda and Tamano 2016; Tamminga et al. 2012). Postsynaptic NMDA receptor GluN2a subunits are found in higher density in dentate gyrus versus CA1, but NMDA receptor

GluN2b and AMPA receptor GluA2 subunits are in higher density in CA1 (Alkadhi 2019). The lower AMPA receptor GluA2 subunit presence in the dentate gyrus suggests that the AMPA receptors in the dentate gyrus have higher calcium permeability than those in CA1. The BDNF-TrkB receptor complex activates phospholipase C-γ to increase cell Ca^{2+} (Kowianski et al. 2018). Thus, BDNF could potentiate glutamate synaptic transmission by postsynaptically increasing a transient calcium inward current more in the dentate gyrus than in CA1. However, BDNF may potentiate glutamate release by presynaptically increasing calcium uptake during depolarization, directly or indirectly via presynaptic glutamate facilitation (Savtchouk et al. 2019). While these data support an increased effect of BDNF on long-term potentiation induced by entorhinal cortex input to the dentate gyrus rather than the CA1, we note that the threshold for entorhinal cortex stimulation to induce long-term potentiation in the dentate gyrus is higher than that in CA1 (Abraham et al. 1996; Trieu et al. 2015). To exclude this difference, the current study used the same maximal stimuli to continuously induce baseline field potentials in both the dentate gyrus and CA1.

An electrical field potential is a summation of excitatory and inhibitory postsynaptic potentials recorded from dentate gyrus or CA1. Although the peak slope of fEPSP or fIPSP has been used to indicate neural activity of certain brain areas, it is invasive and is not easily applied to use in humans or conscious animals. To date, power spectral analysis of local field potentials measured on the surface of the head has been widely used to specifically explain brain area functions, particularly those related to external stimuli. A specific frequency or a certain range of frequencies of local field potential has been reported to relate to the specific brain area or function (Barbieri et al. 2014; Burns et al. 2010). The present study indicates that the power spectral densities between the frequencies 70 and 200 Hz and the peak fEPSP slopes of the dentate gyrus and CA1 field potentials consistently showed the effect of BDNF on entorhinal-hippocampal synaptic transmission. The physiological significance of this frequency range and its power density is like the power spectral analysis of externally recorded hippocampal field potentials (including dentate gyrus and CA1) in conscious, 4–9 months old Fisher344-Brown Norway rats (Sheremet et al. 2018). The power densities of frequencies 70–400 Hz increase with increasing exercise intensities in these rats.

Exercise or physical activity has been used to increase cognition in animals and patients with cognitive impairment (Cefis et al. 2019; Lippi et al. 2020), and its beneficial effect is increased by taurine supplementation (Chupel et al. 2021). The input signal for this involves neocortex to entorhinal cortex and then entorhinal cortex to dentate gyrus. BDNF administration is also reported to increase positive effects of physical activity on cognition in both animals and humans with cognitive disorders, including AD (Cefis et al. 2019; Lippi et al. 2020). Thus, these data support the value of clinical and physiological application of non-invasive local brain field potential recording and power spectral analysis in conscious animals and humans, to better understand underlying causes of cognitive disorders.

5 Conclusion

Direct hippocampal field potential recording and measurement of peak slopes of fEPSP and fIPSP have been used to explain the cognitive function of hippocampus. Their changes refer to long-term potentiation for learning and memory. This study demonstrates that BDNF increases peak slopes of fEPSP and long-term potentiation in the dentate gyrus (but not CA1) in response to continuous entorhinal cortex stimulation. Further, the present data also indicate that the power spectral density of the local field potential at frequencies 70–200 Hz could elucidate the significant role of entorhinal cortex-dentate pathway in a similar way to the elucidation provided by the peak fEPSP slope. Thus, it will be important in the future to conduct non-invasive local brain

field potential recording and power spectral analysis in conscious animals and humans, including the effect of taurine supplementation on learning and memory.

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Influences of Taurine Pharmacodynamics and Sex on Active Avoidance Learning and Memory

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Keywords

Taurine · Active avoidance test · Aversive learning and memory · Avoidance learning · Sex-dependent differences · GABAergic · Pharmacodynamics

Abbreviations

AAT	Active avoidance test
CS	Conditioned stimulus
EDTA	Ethylenediaminetetraacetic acid
HP	Hippocampus
ITI	Inter-trial-interview
PAT	Passive avoidance test
PND	Postnatal day
Tau	Taurine
UCS	Unconditioned stimulus
VI	Variable interval

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1 Introduction

In review of the taurine literature, many studies have been devoted to examining the effects of taurine supplementation on animal and clinical models of coronary (Bkaily et al. 2020; Qaradakhl et al. 2020; Zulli 2011; Wójcik et al. 2010; Xu et al. 2008), inflammatory (Qaradakhl et al. 2020; Marcinkiewicz and Kontny 2014), bowel (Son et al. 1998), liver (Miyazaki and Matsuzaki 2014; Gentile et al. 2011), and mitochondrial diseases (Fakruddin et al. 2018; Suzuki et al. 2002) or neuropathological conditions (Menzie et al. 2014). The rationale for these approaches were driven by taurine offering neuroprotection and pathological symptom amelioration suggesting it to be an essential amino acid in humans (for review, see Ripps and Shen 2012; Lourenço and Camilo 2002). Given the growing body of literature on the associated observations of clinical and pathological symptom alleviation when supplemented with taurine, the mechanism(s) for its therapeutic effects have been the current focus of the field at this time (for review, see Schaffer

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and Kim 2018) to elucidate its clinical significance as the field moves forward (for review, see Schaffer et al. 2014). Moreover, studies of taurine neuroprotection (Kumari et al. 2013) have been focused on the effects of developmental neurotoxicants (Liu et al. 2020; Neuwirth and Emenike 2021; Neuwirth and El Idrissi, 2021; Piao et al. 2019; Li et al. 2019; Neuwirth et al. 2017, 2018; 2019a, b; Li et al. 2017; Guan et al. 2017; Neuwirth 2014; Liu et al. 2013; Wang et al. 2013; Zhang et al. 2013; Sayed et al. 2012; Ma et al. 2010), early neurodevelopmental disorders (Neuwirth et al. 2013, 2015; El Idrissi et al. 2009a, 2010), and the effects of aging as late neurodevelopmental disorders (Neog et al. 2019; Che et al. 2018; Hou et al. 2018; Gebara et al. 2015; Ito et al. 2015; Kim et al. 2014; Neuwirth et al. 2013; El Idrissi et al. 2009b, 2013a, b; El Idrissi 2008; Louzada et al. 2004; Liljequist and Winblad 1993; Liljequist et al. 1983).

In further review of the literature, studies investigating the influence of taurine supplementation/treatment on active avoidance testing (AAT) of rodent learning and memory are scarce with most occurring more than 30–40 years ago (Wenxiong et al. 1998; Liljequist and Winblad 1993; Liljequist et al. 1983; Valliant 1976; Hruska et al. 1975; Hruska 1975). Moreover, when critically reviewing these original reports, the following findings are glaring: (1) there is a large gap in the literature to bridge the current behavioral and neuropharmacology work on taurine with the AAT reports of the past; (2) these original reports had very small sample sizes of rats limiting their generalization and external validity; (3) there was lack of comparisons of both sexes regardless of species and strains employed; (4) levels of taurine supplementation of administration that would be deemed physiologically relevant by today's standards were not used; and (5) the studies were compounded with other behavioral tests and were not contextualized in the framework of the AAT facilitating excitatory-dependent (i.e., with the exception of Wenxiong et al. 1998) and the passive avoidance test (PAT) facilitating inhibitory-dependent learning and memory processes. Therefore, these studies are to be interpreted with caution, and more work is warranted in the use of taurine supplementation on AAT learning and memory outcomes in neurotypical rats of both sexes, regardless of strain.

Taken together, the range of these emerging and continuing studies on taurine neuroprotection has employed a range of tests using both aversive and non-aversive stimuli that are used to investigate precise brain structure with behavioral functional relationships in these animal models of neuropathological conditions. However, much less is known regarding the effects of taurine supplementation on the cognition of neurotypical animal models and whether or not taurine would have the same pharmacodynamics and pharmacokinetics in proactively preventing the onset of any pathological conditions (for review, see Chen et al. 2019). Furthermore, the use of taurine neuroprotection studies has not evaluated the effects and/or differences between both sexes equitably, and thus the field is either biased towards male sex findings or data on female sex effects are limited. A few reports exist that compare behavioral outcomes as a function of taurine and sex (Brown et al. 2020; Valliant 1976), while other reports have identified that stress increased prefrontal cortical taurine neurotransmitter levels in females when compared to males (Perry et al. 2021). Therefore, the field needs more research that carefully considers the evaluation of both sexes in neurotypical animal models to better understand whether taurine neuroprotection or other potential nootropic effects are possible to proactively prevent against the occurrence of such pathological conditions that are inevitable due to senescence.

The present study sought to examine the effects of sex in neurotypical animals when administered taurine either prior to training or following training on a learning and memory task. The purpose of this methodological approach was to evaluate the effects of taurine on memory priming and consolidation prior to and following training of an AAT. The effects of taurine on these distinct phases of learning (i.e., during training) were then followed up with test-

ing conducted at 24 h, 48 h, and 1 week later to assess memory consolidation and retention. Further, these findings could be also evaluated against datasets that specifically evaluated taurine neuroprotection and symptom amelioration in animal models of neuropathological diseases. Lastly, taurine, a potent $GABA_{AR}$ and partial $NMDA_R$ agonist with established neuroprotective properties, may serve to enhance and recover different neuropathological conditions in unique ways, with sex-specific effects, and may not work in the same ways in neurotypical animal models. Altogether, the present study examined the extent to which acute taurine administration could influence learning and memory behaviors in the AAT as a function of sex and pre- and post-training administration.

2 Methods

2.1 Subjects

In accordance with The SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans Norwegian hooded male (N = 5) and female rats (N = 10) (Taconic, N.J.) were paired for breeding, and their male and female F1 generation offspring were used for the present study. Litters were culled to 8–10 pups in order to control for maternal social influences on neurodevelopmental and behavioral outcomes that were later examined. All rats were fed regularly with Purina rat chow (RHM1000 # 5P07) with regular water 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 is fully matured in rats), male and female rats were randomly selected from the litters and then assigned to the AAT in one of the following treatment groups: (1) Control (i.e., no taurine), (2) Taurine Pre-Training (Taurine Pre-Train), and Taurine Post-Training (Taurine Post-Train). The following sample sizes were used within the AAT: n = 11 Control males, n = 11 Control females, n = 9 Taurine Pre-Train males, n = 10 Taurine Pre-Train females, n = 9 Taurine Post-Train males, and n = 11 Taurine Post-Train females, comprising a sample size of N = 63, respectively.

2.2 Taurine Administration

Rats assigned to either the Taurine Pre-Train or Taurine Post-Train treatment groups were administered 43 mg/kg i.p. Taurine (2-aminoethanesulfonic acid; $C_2H_7NO_3S$) that was dissolved in physiological buffered saline pH 7.4 and sterilized through a 0.4 μm filter and administered 15 min prior to the AAT. Taurine was obtained from Sigma-Aldrich (St. Louis, MO). The Taurine Pre-Train group was used to investigate the effects of taurine on facilitating/enhancing cognition prior to training. In contrast, the Taurine Post-Train group was used to investigate the effects of taurine on improving the memory consolidation from the prior day's training before testing. The influences of taurine on AAT learning and memory in both groups were examined at 24 h, 48 h, and 1 week following training and compared to Control rats as the reference group.

2.3 Active Avoidance Testing

Between PND 60 and 90, rats were subjected to an AAT using the Gemini™ Avoidance Systems with Software Version 1.1.0 from San Diego Instruments (San Diego, CA). Rats were placed in the two-way shuttle box with the guillotine door open during a 60 s acclimation period. This was followed by a 10 s pre-trial interval that occurred with the house light on. The dark and light chambers of the shuttle box served as the conditioned stimuli (CS; i.e., different from the house light), which were randomly initiated on the first trial (i.e., each trial lasted up to 12 s in duration). This was followed by a 0.5 mA foot shock serving as the unconditioned stimulus (UCS) for 2 s in duration. Afterwards, the CS and UCS co-terminated prior to an inter-trial-interval (ITI) that was set on a variable interval (VI) 17 s schedule. This proce-

dure was repeated for another 19 trials, whereby 20 trials defined a single training session. A learning error (i.e., shock) was defined as the rat failing to cross over in the shuttle box (i.e., dark to light chamber) to actively avoid the foot shock. The trial length did not exceed 12 s in duration, and no incorrect crosses were punished. A correct learning response (i.e., avoids/escapes) was defined as the rat crossing over in the shuttle box from the dark to the light chamber either prior to foot shock (i.e., avoids) or during a foot shock prior to the guillotine door closure (i.e., escapes). Rats were trained in 1 day for two sessions with a 1 h break between sessions. This was followed by a series of test conditions at 24 h, 48 h, and 1 week following training.

2.4 Data Analyses

Data were recorded in real time and analyzed using the Gemini™ Avoidance System with Software Version 1.1.0 from San Diego Instruments (San Diego, CA). Data were exported into a Microsoft Excel spreadsheet containing all the parameters specified. The dependent variables of interest were as follows: percent (%) Avoids/Escapes, percent (%) Shocks, and Latency (sec) to cross over in the shuttle box.

2.5 Statistical Analyses

All behavioral data were collated in Microsoft Excel and later analyzed in IBM SPSS V. 24 (IBM, Inc. Armonk, NY). For the AAT behavioral tests, a univariate *ANOVA* was conducted using the *Sex* or *Treatment* as the within-subjects factors and *AAT Test Condition* and *Treatment* as the between-subjects factors for the dependent variables of *Latency* (sec), *% Avoids/Escapes,* and *% Shocks*. The criteria for significance were set at $\alpha = 0.05\%$ with a 95% confidence interval with the data presented as the mean ± SEM. Significant differences were determined by an (un)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 Control Rat's Sex-Dependent Effects on Active Avoidance Behaviors

The ability for rats to make a cognitive decision in the AAT is dependent upon their ability to detect environmental stimuli, make the relevant associations, and respond accordingly and efficiently in this testing context. Therefore, prior to evaluating their learning ability, it is important to evaluate the *Latency (sec)* by which they make such cognitive decisions through the behaviors they exhibit. In the Control males when compared to the Control females, there was a *Sex* effect of *Latency* to cross the shuttle box $F_{(1)} = 32.245$, $p < 0.001^{***}$, $\eta_p^2 = 0.298$, whereby males took longer than females to make a decision to cross (Fig. 1).

With respect to their learning performance on the AAT, there was a *Sex* effect of the *% Avoids/Escapes* $F_{(1)} = 34.239$, $p < 0.01^{**}$, $\eta_p^2 = 0.300$, whereby females had nearly double the learning rate of males and performed the AAT more accurately than males (Fig. 2). Additionally, in the Control males when compared to Control females, there was a *Sex* effect of the *% Shocks* $F_{(1)} = 32.507$, $p < 0.001^{***}$, $\eta_p^2 = 0.289$, whereby males made twice as many learning errors as females (Fig. 3).

3.2 Effects of Taurine Pre- and Post-training on Male's Active Avoidance Behaviors

For males, taurine treatment administered either 15 mins pre- or post-training produced similar response *Latencies* as Control males with the exception of the taurine treatment conditions for the 24 h *Test Condition* ($p < 0.05$*; Fig. 4).

Interestingly, there were no differences in males regarding Treatment for the *% Avoids/Escapes* (Fig. 5). However, there was a *Treatment* effect that was observed for the *% Shocks* $F_{(2)} = 5.525$, $p < 0.01^{**}$, $\eta_p^2 = 0.096$ (Fig. 6). The *multiple pair-wise comparisons* further revealed significant differences between the Control males

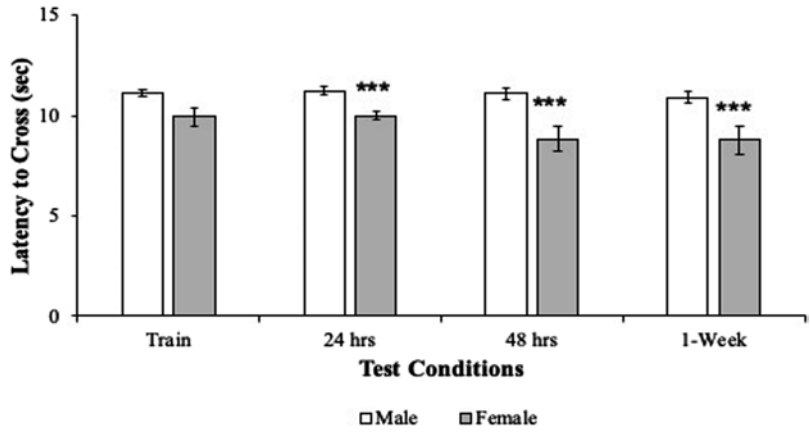

Fig. 1 Illustrates the differences in Control male (solid white bars) and female (solid gray bars) rats' *Latency* to respond to the stimuli in the AAT and cross in the shuttle box. Females showed a reduced latency to cross indicating a faster decision-making process to engage in the AAT at 24 h, 48 h, and 1 week ($p < 0.001$***)

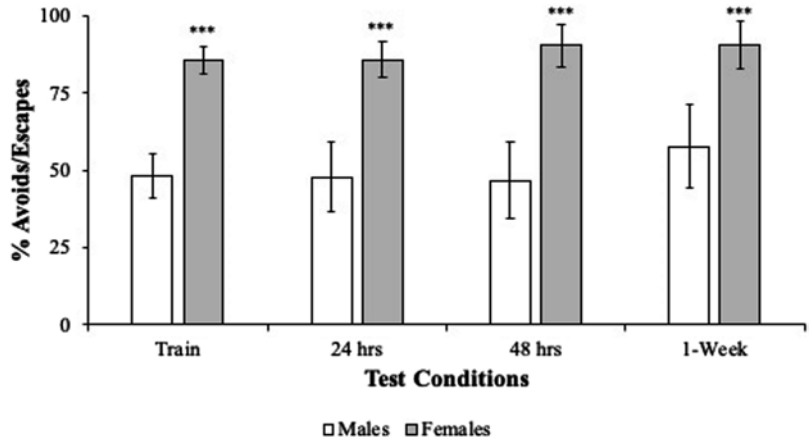

Fig. 2 Illustrates the differences in control male (solid white bars) and female (solid gray bars) rats' percentage in *% Avoids/Escapes* in learning the AAT. Female rats learned nearly two times the rate of males across training and all test conditions thereafter ($p < 0.001$***)

and Taurine Post-Training males ($p < 0.01$**) and the Taurine Pre-Training males and Taurine Post-Training males ($p < 0.001$***; Fig. 6), respectively.

3.3 Effects of Taurine Pre- and Post-training on Female's Active Avoidance Behaviors

For females, there was an effect of *Treatment* $F_{(3)} = 7.729$, $p < 0.001^{***}$, $\eta_p^2 = 0.118$ and an effect of *Test Condition* $F_{(3)} = 5.068$, $p < 0.001^{\#\#\#}$ $\eta_p^2 = 0.116$ for response *Latencies* (Fig. 7). The *multiple pair-wise comparisons* revealed a significant Taurine *Treatment* effect either Pre- or Post-Training when compared to 48 h ($p < 0.05$*) and 1 week ($p < 0.001$*; Fig. 7) for response *Latencies*.

Interestingly, there was also a *Treatment* effect $F_{(2)} = 7.391$, $p < 0.001^{***}$, $\eta_p^2 = 0.113$ and a *Test Condition* effect $F_{(3)} = 4.451$, $p < 0.01^{**}$, $\eta_p^2 = 0.103$ for the *% Avoids/Escapes* (Fig. 8). The *multiple pair-wise comparisons* revealed significant differences between the Control females and the Taurine Post-Training females ($p < 0.01$**) and the Taurine Pre-Training females and the Taurine Post-Training females ($p < 0.001$**; Fig. 8). Moreover, there was a *Treatment* effect $F_{(2)} = 7.891$, $p < 0.001^{***}$, $\eta_p^2 = 0.120$ and a *Test Condition* effect $F_{(3)} = 4.067$, $p < 0.01^{**}$, $\eta_p^2 = 0.095$ that was observed for the *% Shocks* (Fig. 9). Further, the *multiple pair-wise comparisons* revealed significant differences between the Control females and Taurine Post-Training females ($p < 0.001^{***}$) and the Taurine Pre-Training females and Taurine Post-Training females ($p < 0.01$**; Fig. 9), with the greatest effects observed at 48 h ($p < 0.01^{**}$) and 1 week ($p < 0.01^{**}$), respectively.

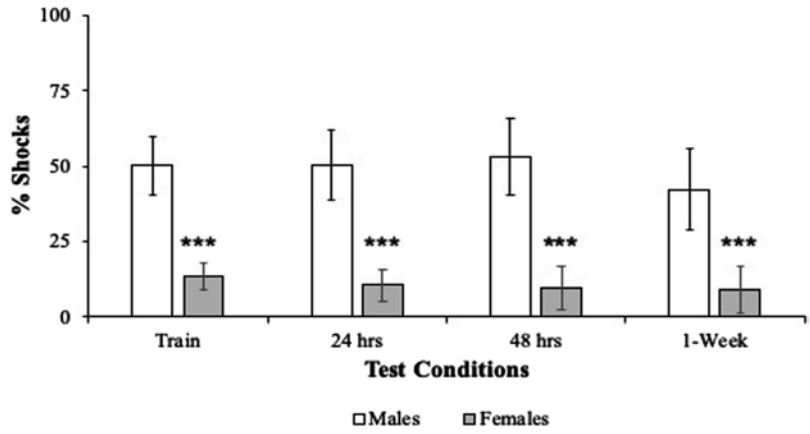

Fig. 3 Illustrates the differences in control male (solid white bars) and female (solid gray bars) rats' AAT learning errors (i.e., *% Shocks*). The Control female rats make far less errors in the AAT across all test conditions ($p < 0.001$***) when compared to Control male rats

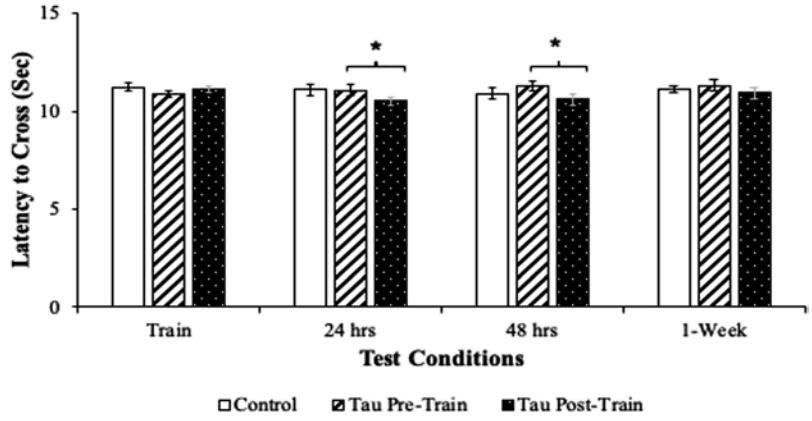

Fig. 4 Illustrates the treatment differences between control (solid white bars), taurine pre-train (striped black bars), and taurine post-train (speckled black bars) male rats' *Latencies* to cross the shuttle box. The taurine post-train male rats had significantly shorter latencies than the taurine pre-train male rats ($p < 0.05$*)

4 Discussion

The present study examined in mature rats whether taurine injected 15 min prior to training or 15 min following training but prior to testing would influence the learning and memory behaviors of the rat in the AAT at 24 h, 48 h, and 1 week. This was done to investigate taurine influences on learning and memory consolidation of the AAT during the Pre-Train and Post-Train phases of experiential learning. In Control male and female rats, female rats showed a decrease in their latency to make a decision within the shuttle box at 24 h, 48 h, and 1 week (Fig. 1). These faster decisions corresponded to increased rates of correct *% Avoids/Escapes* responses (Fig. 2) and a decrease in the rates of incorrect *% Shocks* (i.e., learning errors; Fig. 3). Taken together, these data suggest that females are more sensitive to aversive stimuli (i.e., foot shocks) and as a result in the AAT have increased correct *% Avoids/Escapes* responses when compared to males.

When the rats were separated to examine the sex-dependent effects of Taurine Pre-Train and Taurine Post-Train on their AAT learning and memory, different behavioral traits were observed. In male rats, the Taurine Post-Train rats had reduced *Latencies* to cross over in the shuttle box when compared to the Taurine Pre-Train rats (Fig. 4). Interestingly, there were no influences of taurine in either treatment group for the correct *% Avoids/Escapes*, when compared to Control rats (Fig. 5). Further, the Taurine Post-Train rats reduced latencies to cross over corresponded with decreases in incorrect *% Shocks* in the 24 h and 48 h Test Conditions (Fig. 6). These data suggest that Taurine Post-Train reduced the rates of learning errors when compared to Control and Taurine Pre-Train males, respectively.

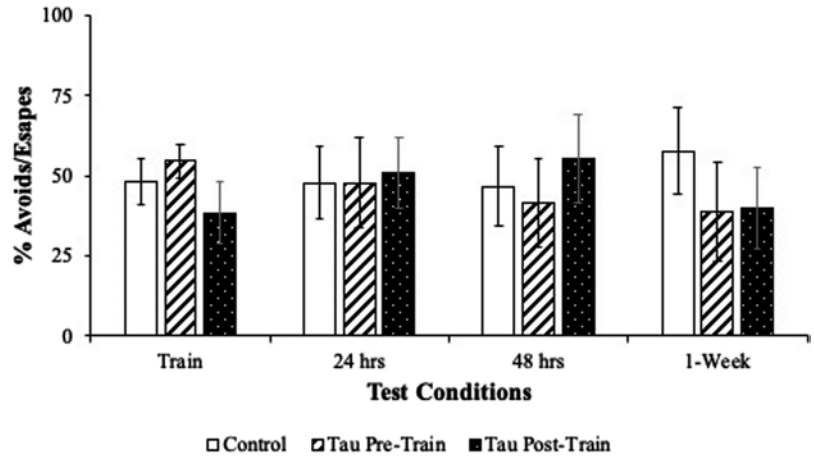

Fig. 5 Illustrates the treatment differences between control (solid white bars), Taurine pre-train (striped black bars), and Taurine post-train (speckled black bars) male rats' *% Avoids/Escapes* in learning the AAT. Neither taurine treatment caused any significant differences in male AAT learning performance

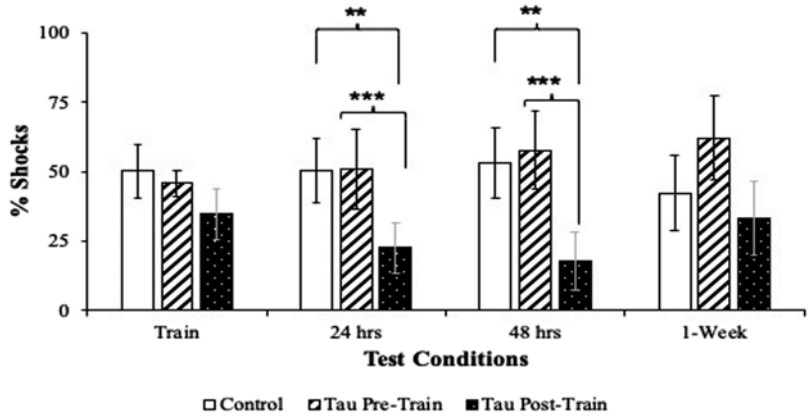

Fig. 6 Illustrates the treatment differences between control solid white bars), Taurine pre-train (striped black bars), and Taurine post-train (speckled black bars) male rats' AAT learning errors (i.e., *% Shocks*). The Taurine Post-Train male rats had significant reductions in their AAT learning errors at 24 h and 48 h when compared to Control male ($p < 0.01$**) and Taurine Pre-Train male ($p < 0.001$***) rats

In contrast, in female rats, the Taurine Post-Train had increased *Latencies* to cross over in the shuttle box during training and at 24 h and 48 h testing when compared to both Control and Taurine Pre-Train rats (Fig. 7). Notably, unlike the male rats, the female rats were influenced by taurine treatment. The Taurine Post-Train female rats had decreased rates of correct *% Avoids/Escapes* responses when compared to Control female rats during Training, 24 h, and 48 h testing, as well as across all Test Conditions when compared to Taurine Pre-Train female rats, respectively (Fig. 8). These data corresponded to increased learning error rates in *% Shocks* in the Taurine Post-Train female rats when compared to Control female rats during training and 24 h testing and across all Test Conditions when compared to Taurine Pre-Train female rats, respectively (Fig. 9).

The findings from the present study raise some important questions in the field of taurine supplementation in studying animal's learning and memory behaviors. Taurine supplementation has been shown to influence motor learning (Santora et al. 2013) and a wide range of learning and memory behaviors in disease models of Fragile X Syndrome (Neuwirth et al. 2013, 2015; El Idrissi et al. 2009a, b, 2010, 2012) and neurodevelopmental lead exposures (Neuwirth et al. 2017, 2019a, b; Neuwirth 2014) as a few well-studied

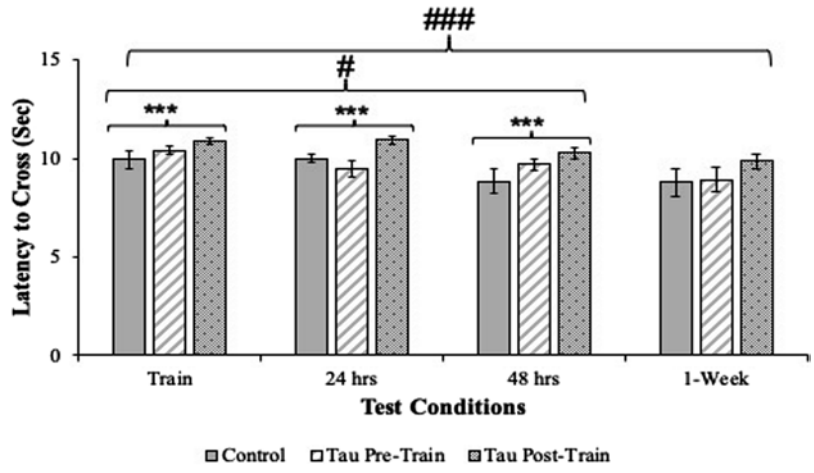

Fig. 7 Illustrates the treatment differences between control (solid gray bars), Taurine pre-train (striped gray bars), and Taurine post-train (speckled gray bars) female rats' *Latencies* to cross the shuttle box. The Taurine post-train female rats had significantly longer latencies than the control female and Taurine pre-train female rats during training, 24 h, and 48 h ($p < 0.001$***). An effect of *Test Condition* was observed between training, 24 h, and 48 h ($p < 0.5^{\#}$) and between training and 1 week ($p < 0.001^{\#\#\#}$)

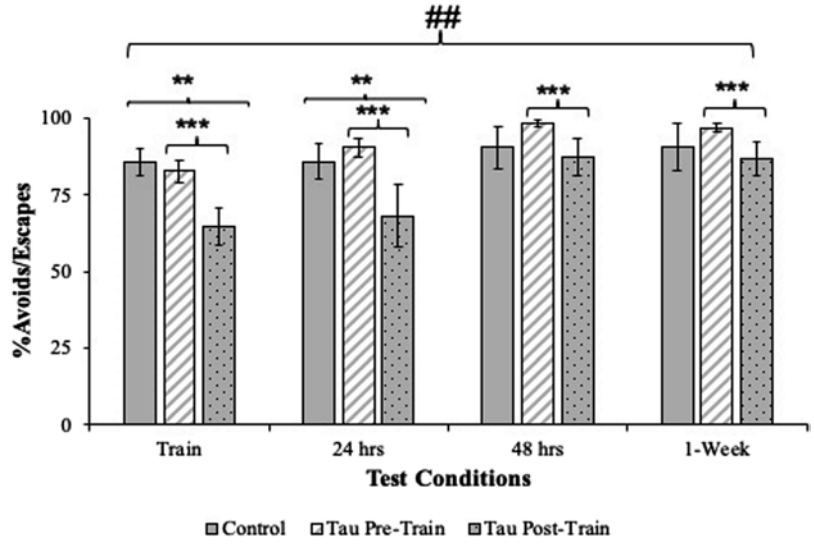

Fig. 8 Illustrates the treatment differences between control (solid gray bars), taurine pre-train (striped gray bars), and taurine post-train (speckled grey bars) female rats' *% Avoids/Escapes* in learning the AAT. The taurine post-train female rats had significantly reduced *% Avoids/Escapes* than the Control female ($p < 0.01$**) and taurine pre-train female rats during training, 24 h, and 48 h ($p < 0.001$***), as well as in the 48 h and 1 week Test Conditions when compared to taurine pre-train rats ($p < 0.001$***). An effect of *Test Condition* was observed across training, 24 h, 48 h, and 1 week ($p < 0.01^{\#\#}$)

examples. However, much less is known regarding taurine's influences on neurotypical rodent models. This is important as reports have shown differences in chronic versus acute taurine administration that can have differential effects on learning and memory-associated outcomes (El

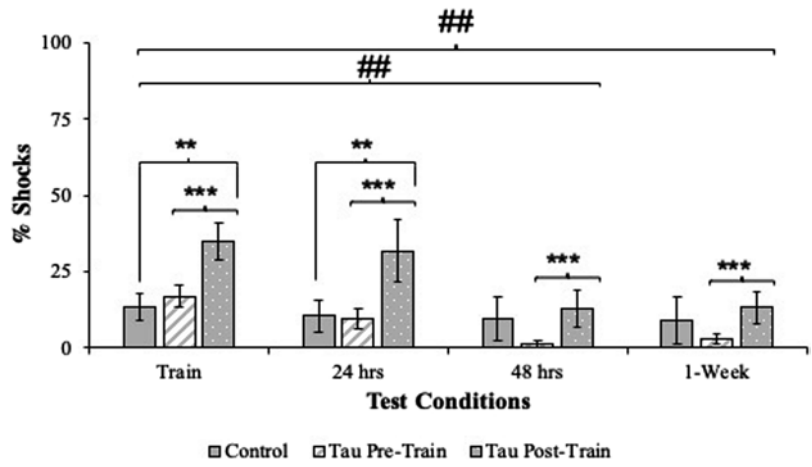

Fig. 9 Illustrates the treatment differences between control (solid gray bars), taurine pre-train (striped gray bars), and taurine post-train (speckled grey bars) female rats' AAT learning errors (i.e., *% Shocks*). The Taurine Post-Train female rats had significantly increased *% Shocks* than the Control female rats during training and 24 h ($p < 0.01$**) and taurine pre-train female rats across all test conditions ($p < 0.001$***). An effect of *Test Condition* was observed between training and 48 h ($p < 0.01^{\#\#}$) as well as training and 1 week ($p < 0.01^{\#\#}$)

Idrissi et al. 2009b, 2010). Additionally, our understanding of the ways in which taurine can modulate the anterolateral or nociceptive system is an underexplored area that now requires considerable attention. It is plausible that differences in sensory receptivity of the anterolateral or nociceptive systems can, in part, contribute to the Pre-Train and Post-Train sex-dependent differences observed herein, and more work in this area would help to clarify the role(s) taurine may facilitate through an integrative systems neuroscience approach.

Further, chronic taurine exposure (i.e., supplemented at 0.05% in the drinking water; equivalent to 43 mg/kg injected i.p. for acute exposures) has been shown to alter the gene expression of the GABA-$_{AR}$s differentially across brain regions (Neuwirth et al. 2018; Shen et al. 2013; Zhang et al. 2009). These observations, along with many others, have been carefully and thoughtfully synthesized to facilitate researcher's understanding of the wide therapeutic implications for how taurine might remedy a number of health ailments and/or psychopathological conditions (for review, see Neuwirth and Emenike 2021; Jakaria et al. 2019; Schaffer and Kim 2018; Chung et al. 2012; Ward et al. 2006; Gupta et al. 2005). However, in the neurotypical model absent of such pathophysiological states, taurine's influences may not affect its target mechanism(s) in the same manner (Corte et al. 2002) when compared to recovering pathological states (Das et al. 2012). Rather, in the neurotypical model, taurine supplementation at times may be used to either validate nootropic mechanisms of action and their associated behaviors or elucidate how it may cause paradoxical mechanisms of action that may disrupt normal cognitive and behavioral functioning.

Using such a deductive framework, researchers that study the effects of environmental stressors on CNS function and pathophysiology (for review, see Arnsten 2009) have shown that taurine supplementation improved learning responses, short-term memory, and motoric/exploratory responses, thereby improving the stress-induced deficits (Ezekiel et al. 2016) and lowering blood pressure (El Idrissi et al. 2013a, b). These reports corroborate with the findings that taurine improves emotional regulation through GABAergic-dependent learning and memory (Neuwirth et al. 2022; Cruz et al. 2022; Vasquez et al. 2022; El Idrissi et al. 2009a, b, 2012) and facilitates inhibitory-dependent learning in brain slices and through behaviors in the PAT (El Idrissi et al. 2010; El Idrissi 2008) and further can mitigate seizure severity (Vasquez

et al. 2022; El Idrissi and L'Amoreaux 2008; El Idrissi et al. 2013a, b; 2003). Other studies have shown taurine to recover fronto-executive functions in response to neurodevelopmental lead poisoning with sex-dependent differences (Neuwirth, 2014; Neuwirth et al. 2019a, c; for review, see Tait et al. 2018; for review, see Neuwirth and Kolb 2020) as well as when challenged by arsenic during neurodevelopment (Piao et al. 2019). Moreover, these cognitive enhancing effects of taurine have been shown to decrease negative emotional traits as well as forestall cognitive decline as a natural consequence of senescence (El Idrissi et al. 2009b, 2013a, b) that are thought to be associated with taurine-dependent changes in the gene expression of GABAergic synapses (Shen et al. 2013) and the pharmacodynamics of the GABA-$_{AR}$s (L'Amoreaux et al. 2010). At this time, the taurine field lacks longitudinal studies on aged rodent models that examine equivocally both sexes and subject them to learning and memory tests using both non-aversive and aversive stimuli. The present study attempted to begin such a discussion within the swiftly growing body of literature within the area of taurine research.

5 Conclusion

Taken together, the present study's data reveal that in mature rats subjected to the AAT, taurine administration is insensitive to positively influence the rate of learning through increasing the rate of correct *% Avoids/Escapes* responses. Interestingly, taurine administration was sensitive to differentially influence the rate of learning errors *% Shocks* as a function of sex, whereby improvements were observed in males and more deficits were observed in females. Further, Taurine Pre-Train did not influence either sex in the AAT learning and memory performance when compared to their sex-matched Control counterparts. Additionally, Taurine Post-Train (i.e., administered 15 min following training but before testing) caused the sex-dependent effects observed in this study. These observations raise some interesting points for future studies to consider: 1) AAT using a large number of trials to train rodents with aversive stimuli that may shift the rodent's cognitive resources from excitatory-dependent learning and memory process (i.e., Glutamatergic) to a strategy that utilizes more of an inhibitory-dependent learning and memory process (i.e., GABAergic). This latter point is critical, as reports on the GABA-shift show that the GABAergic systems precede the functional activation of the glutamatergic system and may ontogenetically help to parse different neurodevelopmental disorders (for review, see Ben-Ari et al. 2012; Ben-Ari 2002). In this way, these rodents may be learning to avoid shock/inhibit penalties rather than learning to follow the light cue within the shuttle box. This would explain why taurine influences were observed in *% Shocks* and not *% Avoids/Escapes*. Future studies that use aversive conditioning protocols may find taurine administration to be more beneficial to reduce and/or ameliorate learning deficits.

Alternatively, since the AAT combines both aversive and non-aversive stimuli during the learning process, it is also possible that by design, cognitive behavioral tests that do not have aversive stimuli would permit the ability to assess taurine influences on cognitive behavioral performances. Thus, researchers studying taurine relationships through cognitive behavioral approaches should be ever mindful of how they design their testing procedures to carefully parse out experimental design constraints that may create false-positive (i.e., effects of taurine when there are no effects) versus false negatives (i.e., no effects of taurine when there are effects). In conclusion, researchers studying aversive and emotional learning and memory behaviors would be predictive of observing taurine influences in reducing cognitive behaviors (i.e., anxiolytic properties), whereas researchers studying solely cognitive behaviors in the absence of aversive emotionally activating stimuli are predictive of determining whether taurine could influence learning and memory (i.e., nootropic properties). Lastly, these examinations will be further complicated by factors such as age, taurine administration, taurine pharmacodynamics, taurine pharmacokinetics, and sex.

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Conflicts of Interest The authors declare no conflicts of interest.

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The Effects of Dietary Taurine-Containing Jelly Supplementation on Cognitive Function and Memory Ability of the Elderly with Subjective Cognitive Decline

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Keywords

Cognitive function · Memory ability · Taurine · Taurine jelly · Elderly

Abbreviations

BMI	Body mass index
DST	Digit span test
K-BNT	Korean version of the Boston naming test
K-SVLT	Korean version-Seoul verbal learning test
MMSE-DS	Mini-mental state examination-dementia screening
SMCQ	Subjective memory complaints questionnaire
TJ	Taurine jelly (dietary taurine-containing jelly)

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1 Introduction

The population aged 65 or older not only in Korea but worldwide is increasing rapidly. With 14.3% of the elderly population, Korea has entered an aged society at a fast pace (Statistics Korea 2019; United Nations 2019). The rapid entry of the aged society has caused a lot of problems. In particular, among the physical and mental function declines that can be experienced as the aging process, degenerative neurological diseases such as mild cognitive impairment and dementia are a heavy socioeconomic burden. The care and treatment of these elderly is a huge problem to the economy and a drain on the overall medical investment of the society (The Kyunghyang Shinmun 2018).

Memory decline can be common in the elderly (Chin et al. 2010) but is often confused with forgetfulness and is sometimes considered part of the aging process. However, memory decline might be a symptom of cognitive impairment and an early sign of dementia, which requires early intervention and prevention (Derouesne et al. 1999). Dementia is a representative disease of cognitive impairment related to aging process and is a mental health problem plaguing people in Korea and elsewhere globally. 50 million people were diagnosed with dementia in 2018, and

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82 million are expected to have the disease by 2030. According to the Alzheimer's Association International (ADI), the population of the elderly with dementia could triple the number of cases in 2050 than in 2018 (National Institute of Dementia 2019). Variables that affect the onset of dementia include age, gender, vascular status, education level, living alone, financial ability, and so forth. Female elderly patients with cardiovascular disease who live alone or are poor showed a higher incidence of dementia (Di et al. 2000; Kim et al. 2019). Nutritional status is also a very important factor in the prevention and treatment of dementia (Lim et al. 2021). Malnutrition and eating disorders normally afflict up to 80% of the elderly with dementia, so it is necessary to prepare countermeasures accordingly (Christina et al. 2010).

There is still no perfect cure for dementia. However, studies have shown a positive effect on delaying the progression of dementia or improving cognitive function (Clare et al. 2003; Woods et al. 2012). Taurine has been shown to improve neuro-communication in many types of the dementia disease and has also been shown to improve memory ability in animal studies (Barthel et al. 2001; Kim et al. 2014). High concentrations of taurine are found in fish, scallops, clams, and other shellfish, as well as octopi, abalone, and red fish. Many kinds of functional beverages and healthy foods also add taurine to play a refreshing effect (Kim et al. 1999; National Institute of Fisheries Science 2021). Taurine enters the human body easily through the brain and blood vessel walls of the body, so it is well supplemented through foods (Yoon et al. 2015). However, the intake of taurine of the elderly is usually insufficient because of the high price and difficulty of cooking seafood.

In Korea, as the prevalence of dementia and the elderly population increases, interest in supplementary food that helps improve cognitive function is high. Also, in recent years, in order to cope with the changes of the aged society, Korea has actively carried out the development of the friendly food for the elderly (Kim and Kim 2016; Kim et al. 2019). However, the development of foods for the elderly with dementia and cognitive impairment is inadequate. This study investigates the effect of cognitive function in the elderly with cognitive decline after taurine supplementation through taurine-containing jelly.

2 Methods

2.1 Design of the Study

This was a single group pretest-posttest examination design study to investigate the effect of dietary taurine-containing jelly supplementation on cognitive function and memory ability of the elderly with subjective cognitive decline.

2.2 Subjects and Taurine-Containing Jelly Supplementation

This study was approved by the Institutional Review Board (IRB) of the Inha University (190225-7AR). The subjects of this study were 26 elderly (8 males and 18 females) with subjective cognitive decline who lived in Incheon, Korea. Subjective cognitive decline was evaluated by self-test results (6 points or more) of the Subjective Memory Complaints Questionnaire (SMCQ). The jelly used in the study contains 3 g of taurine and was supported by Dong-A Pharmaceutical Co. Ltd. It was reprocessed in the soft state like pudding considering the subjects' safe intake and preferences (Fig. 1) and provided once a day for 4 weeks. The researcher monitored the intake and side effects every day directly or over the phone. There was a decrease in the number of subjects to be evaluated due to personal reasons such as indigestion during the study period (2 males and 4 females).

2.3 Data Collection

This study measured the general characteristics (gender, age, education period, and chewing ability), anthropometric data (height, body weight, body mass index (BMI), blood pressure, and blood glucose), and changes in cognitive func-

Fig. 1 Reprocessing of taurine-containing jelly into pudding

tion (Mini-Mental State Examination-Dementia Screening, MMSE-DS) and memory ability (Digit Span Test (DST), Korean version of the Boston Naming Test (K-BNT), Korean version-Seoul Verbal Learning Test (K-SVLT), and subjective memory of the subjects.

2.4 Statistical Analysis

The statistical analysis of all data was conducted using the SPSS 26.0 program. All data were analyzed by dividing the male and female elderly before and 4 weeks after supplementation of dietary taurine-containing jelly (taurine jelly, TJ), respectively. The values were expressed as the percentage or mean ± standard error (SE). The statistical significance in changes of cognitive function and memory ability was determined by a paired t-test. The significant difference of results was found at $p < 0.05$.

3 Results

3.1 General Characteristics of the Subjects

The total average age of the subjects was 72.3 years, and the average education period was 9.3 months (Table 1). Most of them had a high school (46.2%) or elementary school education (34.6%). The average score of chewing ability of the subjects was 3.4 points (out of 5 points), and it was considered that there would be no problem in chewing or swallowing food.

3.2 Anthropometric Data, Blood Pressure, and Blood Glucose Level of the Subjects

The total average height, body weight, and BMI of the subjects were 156.6 cm, 59.5 kg, and 24.3 kg/m^2 before TJ supplementation, respectively (Table 2). The BMI was similar to it (24.1 kg/m^2) of the elderly 65 years and older from the 7th (2016–2018) raw data of the Korea National Health and Nutrition Examination Survey (KNHANES). However, according to the guidelines for the treatment of obesity in 2018 by the Korean Society of Obesity (underweight BMI ≤ 18.4, normal 18.5 ≤ BMI ≤ 22.9, overweight 23.0 ≤ BMI ≤ 24.9, step 1 obesity 25.0 ≤ BMI ≤ 29.9, step 2 obesity 30.0 ≤ BMI ≤ 34.9, and step 3 obesity 35.0 ≤ BMI), it was evaluated as overweight. After 4 weeks supplementation of TJ, the average body weight and BMI were 59.8 kg and 24.4 kg/m^2, respectively, and there were no significant differences from before supplementation of TJ.

The total average blood pressure of the subjects before supplementation of TJ was normal, with systolic and diastolic blood pressures of 133.6 mmHg and 81.2 mmHg, respectively. There were no significant differences from after 4 weeks supplementation of TJ (130.2 mmHg and 78.6 mmHg). The total average fasting blood glucose of the subjects before and after 4 weeks supplementation of TJ were 99.5 mg/dL and 99.6 mg/dL, respectively, and the postprandial glucose were 127.4 mg/dL and 136.8 mg/dL, respectively. There were no significant differences in both blood glucose before and after sup-

Table 1 General characteristics of the subjects

Variables	Total (N = 26)	Male (N = 8)	Female (N = 18)
Age (years)	72.3 ± 1.1	72.3 ± 2.3	72.3 ± 1.3
Education period			
Average (years)	9.3 ± 0.7	9.3 ± 0.7	9.3 ± 0.7
Uneducated	1 (3.8)	0 (0.0)	1 (5.6)
≤ Elementary school	9 (34.6)	1 (12.5)	8 (44.4)
≤ Middle school	4 (15.4)	3 (37.5)	1 (5.6)
≥ High school	12 (46.2)	4 (50.0)	8 (44.4)
Chewing ability	3.4 ± 0.2	3.4 ± 0.5	3.4 ± 0.2

Mean ± SE, N (%) by Student's t-test or chi-squared test

Table 2 Anthropometric data, blood pressure, and blood glucose level of the subjects

Variables		Pre-test	Post-test	p-value [a]
Height (cm)	Total	156.6 ± 1.4	–	–
	Male	163.0 ± 1.2	–	–
	Female	153.8 ± 1.5	–	–
	t-value[b]	–	–	–
Body weight (kg)	Total	59.5 ± 1.5	59.8 ± 1.5	0.390
	Male	61.9 ± 2.7	62.3 ± 2.8	0.142
	Female	58.5 ± 1.7	58.6 ± 1.7	0.705
	t-value	–	–	–
BMI (kg/m^2)	Total	24.3 ± 0.6	24.4 ± 2.2	0.300
	Male	23.3 ± 1.1	23.5 ± 1.1	0.156
	Female	24.8 ± 0.8	24.9 ± 0.8	0.541
	t-value	0.310	0.337	–
Systolic blood pressure (mmHg)	Total	133.6 ± 3.9	130.2 ± 3.9	0.127
	Male	136.4 ± 8.9	132.1 ± 8.9	0.253
	Female	132.3 ± 4.2	129.3 ± 4.3	0.290
	t-value	0.642	0.745	–
Diastolic blood pressure (mmHg)	Total	81.2 ± 1.9	78.6 ± 1.8	0.132
	Male	81.0 ± 3.8	77.6 ± 4.0	0.265
	Female	81.2 ± 2.3	79.1 ± 2.0	0.305
	t-value	0.959	0.723	–
Fasting blood glucose (mg/dL)	Total(n = 8)	99.5 ± 5.2	99.6 ± 4.3	0.965
	Male(n = 3)	104.3 ± 2.6	102.7 ± 1.8	0.713
	Female(n = 5)	96.6 ± 8.2	97.8 ± 7.0	0.775
	t-value	0.511	0.622	–
Postprandial glucose (mg/dL)	Total(n = 18)	126.8 ± 4.4	135.7 ± 7.1	0.147
	Male(n = 5)	135.0 ± 10.6	127.4 ± 11.9	0.572
	Female(n = 13)	123.6 ± 4.5	138.9 ± 8.9	0.025*
	t-value	0.256	0.489	

Total (n = 26), Male (n = 8), Female (n = 18), Mean ± SE
[a]by paired t-test between pre and post, *$p < 0.05$
[b]by Student t-test between male elderly and female elderly

plementation of TJ, and they were at normal levels. However, in the case of the elderly females, there was a significant increase after 4 weeks of supplementation (138.9 mg/dL) compared to before supplementation of TJ (123.6 mg/dL) in the postprandial glucose. It is thought that the manufacturing skill required to reduce the sweetness of jelly as a customized supplementation food for the elderly is needed in the future.

3.3 The Changes in Cognitive Function of the Subjects

The total MMSE-DS score of the subjects increased from 25.9 points before supplementation of TJ to 26.4 points after 4 weeks of supplementation, but there was no significant difference (Table 3). However, in the case of the female elderly, there was a tendency to show some improvement in "visuospatial constructional ability" ($p = 0.083$) (Fig. 2).

3.4 The Changes in Memory Ability of the Subjects

The results of the memory ability tests of the subjects are as shown in Table 4. First, the 'DST-Forward (DST-F)' score (out of 84.0 points) showed a significant increase from 60.6 points before supplementation of TJ to 65.7 points after 4 weeks of supplementation ($p < 0.01$). In particular, in the case of the female elderly, it significantly increased from 57.8 points before supplementation of TJ to 64.3 points after 4 weeks of supplementation ($p < 0.001$). Second, in the 'DST-Backward (DST-B)' score (out of 70.0 points), there was no significant difference from 33.3 points before supplementation of TJ to 34.4 points after 4 weeks of supplementation. Third, the 'K-BNT' score (out of 60.0 points) showed a significant increase from 46.5 points before supplementation of TJ to 51.7 points after 4 weeks of supplementation ($p < 0.001$). In particular, the scores were significantly increased in both male elderly (50.3 points → 55.8 points) and female elderly (44.8 points → 49.9 points) ($p < 0.01$). Fourth, the 'K-SVLT' score (out of 36.0 points) showed a significant increase from 15.2 points before supplementation of TJ to 17.8 points after 4 weeks of supplementation ($p < 0.01$). The scores were significantly increased in both male elderly (13.6 points → 15.6 points) and female elderly (15.8 points → 18.7 points) ($p < 0.05$). Finally, fifth, the 'subjective memory' score (out of 5.0 points) showed a significant increase from 2.6 points before supplementation of TJ to 3.6 points after 4 weeks of supplementation ($p < 0.05$). In particular, in the case of the female elderly, it significantly increased from 2.5 points before supplementation of TJ to 3.5 points after 4 weeks of supplementation ($p < 0.01$).

4 Discussion

In this study, the memory ability of the subjects with subjective cognitive decline showed a positive effect after TJ supplementation. To observe the effect of taurine in cognitive function and memory ability of the elderly with subjective cognitive decline, the taurine was supplemented to the subjects every day for 4 weeks, and the cognitive function and memory ability was tested after TJ (contained 3 g taurine) supplementation. The cognitive function of subjects was tested using the "Mini-Mental State Examination-Dementia Screening" (MMSE-DS) questionnaire, memory ability was tested using three kinds of questionnaires: "digit span test" (DST), "Korean version of the Boston Naming Test" (K-BNT), and "Korean version of the Seoul Verbal Learning Test" (K-SVLT). Additionally, the subjective memory of the subjects was tested at the same time. The total MMSE-DS score of the subjects showed no difference before and after 4 weeks supplementation of TJ. It is thought that this result was likely because the subjects of this study only subjectively felt cognitive decline and had a high probability of having normal cognitive function.

The memory ability test scores by DST, K-BNT, K-SVLT, and subjective memory were significantly increased after 4 weeks supplementation of TJ. According to the previous study

Table 3 The changes in cognitive function (MMSE-DS) of the subjects

Variables		Pre-test	Post-test	p-value[a]
Total average	Total	25.9 ± 0.6	26.4 ± 0.6	0.119
	Male	25.8 ± 1.2	26.1 ± 1.3	0.476
	Female	26.0 ± 0.7	26.6 ± 0.7	0.181
	t-value[b]	0.856	0.754	–
Time orientation	Total	4.5 ± 0.1	4.5 ± 0.1	1.000
	Male	4.4 ± 0.3	4.4 ± 0.3	1.000
	Female	4.6 ± 0.2	4.6 ± 0.1	1.000
	t-value	0.476	0.401	–
Place orientation	Total	4.7 ± 0.1	4.9 ± 0.1	0.185
	Male	4.6 ± 0.3	4.8 ± 0.3	0.351
	Female	4.8 ± 0.1	4.9 ± 0.1	0.331
	t-value	0.562	0.492	–
Memory	Total	5.2 ± 0.2	5.3 ± 0.2	0.212
	Male	5.1 ± 0.3	5.4 ± 0.3	0.170
	Female	5.2 ± 0.2	5.3 ± 0.2	0.495
	t-value	0.918	0.811	–
Attention	Total	3.4 ± 0.3	3.4 ± 0.3	0.882
	Male	3.6 ± 0.5	3.3 ± 0.6	0.402
	Female	3.3 ± 0.3	3.4 ± 0.4	0.734
	t-value	0.567	0.838	–
Language	Total	3.0 ± 0.0	3.0 ± 0.0	–
	Male	3.0 ± 0.0	3.0 ± 0.0	–
	Female	3.0 ± 0.0	3.0 ± 0.0	–
	t-value	–	–	–
Ability to execute	Total	2.6 ± 0.1	2.7 ± 0.1	0.376
	Male	2.4 ± 0.2	2.8 ± 0.2	0.197
	Female	2.7 ± 0.1	2.7 ± 0.2	1.000
	t-value	0.243	0.758	–
Visuospatial constructional ability	Total	0.7 ± 0.1	0.7 ± 0.1	0.327
	Male	0.8 ± 0.2	0.6 ± 0.2	0.351
	Female	0.6 ± 0.1	0.8 ± 0.1	0.083
	t-value	0.512	0.438	–
Judgement and abstract thinking	Total	1.9 ± 0.1	2.0 ± 0.0	0.161
	Male	1.9 ± 0.1	2.0 ± 0.0	0.351
	Female	1.9 ± 0.1	1.9 ± 0.1	0.331
	t-value	0.922	0.516	–

Total (n = 26), Male (n = 8), Female (n = 18), Mean ± SE
[a]by paired t-test between pre and post, *$p < 0.05$
[b]by Student t-test between male elderly and female elderly

(Kim and Kim 2015; Kim et al. 2014) on "the effect of exercise on cognitive function of the elderly with cognitive impairment," compared with the experimental group, the score of "DST-F" in the control group was significantly reduced, while the result of "DST-B" was no difference between the experimental group and the control group, which was similar to the results in this study.

In the previous study (Kim et al. 2004), the K-BNT score of normal female elderly was 47.2 points, which was similar to the results before TJ supplementation in this study. The K-BNT scores of the elderly with mild dementia and severe dementia were 34.5 points and 23.6 points, respectively, which suggested that lower cognitive function was associated with lower K-BNT scores. In another study (Park et al. 2011), the

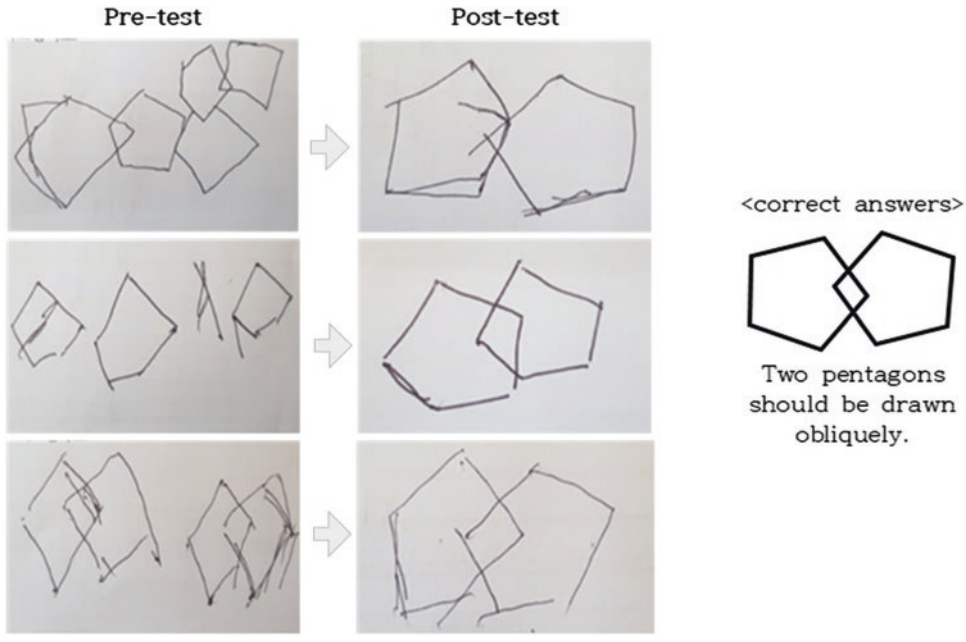

Fig. 2 The results of "visuospatial constructional ability" of some subjects

Table 4 The changes in memory ability test scores of the subjects

Variables		Pre-test	Post-test	p-value[a]
DST-F[c]	Total	60.6 ± 2.8	65.7 ± 2.1	0.001**
	Male	66.8 ± 4.2	68.8 ± 2.9	0.515
	Female	57.8 ± 3.4	64.3 ± 2.7	0.000***
	t-value[b]	0.140	0.325	–
DST-B[d]	Total	33.3 ± 3.1	34.4 ± 2.9	0.567
	Male	34.4 ± 3.2	37.3 ± 4.6	0.401
	Female	32.8 ± 4.3	33.2 ± 3.8	0.880
	t-value	0.816	0.532	–
K-BNT[e]	Total	46.5 ± 1.5	51.7 ± 1.5	0.000***
	Male	50.3 ± 1.4	55.8 ± 1.1	0.001**
	Female	44.8 ± 2.0	49.9 ± 1.9	0.000***
	t-value	0.036*	0.014*	–
K-SVLT[f]	Total	15.2 ± 0.9	17.8 ± 1.0	0.004*
	Male	13.6 ± 1.3	15.6 ± 1.3	0.037*
	Female	15.8 ± 1.1	18.7 ± 1.4	0.023*
	t-value	0.257	0.177	–
Subjective memory	Total	2.6 ± 0.1	3.0 ± 0.2	0.013*
	Male	2.9 ± 0.4	3.1 ± 0.3	0.516
	Female	2.5 ± 0.2	3.0 ± 0.2	0.008**
	t-value	0.249	0.712	–

Total (n = 26), Male (n = 8), Female (n = 18), Mean ± SE

[a]*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ by paired t-test between pre and post

[b]*; $p < 0.05$ by Student t-test between male elderly and female elderly

[c]Digit span test—Forward

[d]Digit span test—Backward

[e]Korean version of the Boston Naming Test

[f]Korean version-Seoul Verbal Learning Test

K-BNT score of the elderly with dementia was 30.4 points, but the elderly with mild cognitive impairment had higher K-BNT scores (37.3 points). In the previous study of reminiscence therapy for the elderly with dementia, the K-BNT scores of the experimental group were increased, similar to the results of this study (Kwon and Kim 2019). In the case of K-SVLT, the scores were significantly increased in both experimental (21.0 points → 24.2 points) and control groups (20.4 points → 22.3 points) in a previous study that identified the effects of individual reminiscence therapy programs on dementia patients, which was similar to the results of this study. However, the subjects in this study have unusually lower K-SVLT scores (15.2 points) than previous studies (21.0 points and 20.4 points), despite having only subjective cognitive decline, not dementia.

The rapid growth of the aging population is a huge social problem in Korea. In the process of aging, both physical and mental health are equally important. The reality is that the overall social welfare and medical care for the elderly is not enough, so it can be expressed as a problem. In particular, the prevalence of mental and chronic diseases in the elderly such as dementia is rapidly increasing, while there is no perfect treatment yet. In the process of aging, cognitive function has a direct impact on the quality of life (Park et al. 2007). Recently, in line with aging society, interest in supplements such as health functional foods, which help the elderly improve their cognitive function or prevent decline, is also increasing in Korea. Many studies have shown that taurine has a very good effect on aging, nerve repair, and the repair of cognitive function and learning ability (Curtis and Watkins 1965; Deleuze et al. 2005; Hussy et al. 2001; Wang et al. 2013). According to a previous study, the higher the frequency of eating octopi, squid, and shellfish with high taurine content as well as more nutrition knowledge related to taurine, the higher the level of cognitive function (Bae et al. 2017, 2019b). In addition, it was confirmed that previous studies showed positive effects on cognitive function and memory improvement through dietary taurine supplementation (Bae et al. 2019a). Therefore, it is considered that there is sufficient potential as a material for customized supplements to improve or prevent cognitive decline of the elderly in the future. According to many animal and human clinical trials, high levels of taurine have been supplemented without any toxicity or adverse reactions, and taurine has the characteristic that it can be easily absorbed by the human body even just through food (Mizushima et al. 1996; Militante and Lombardini 2002; Schaffer et al. 2014; Takahashi et al. 1972). However, this study was a clinical study, so the number of subjects was small. In the future, further studies with a variety of elderly participants in a wider demographic will be needed. It is expected that the results of this study will be used as basic data to improve or prevent cognitive decline in the elderly.

5 Conclusion

The results of this study showed that dietary taurine-containing jelly supplementation had some positive effects on the memory ability of the elderly with subjective cognitive decline. Therefore, it is suggested to supplement taurine in various elderly customized products such as pudding or jelly for improved cognitive function and memory ability of the elderly in the future.

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Effects of Dietary Taurine-Containing Jelly Supplementation on Academic-Related Characteristics and Academic Achievement in Korean College Entrance Examinees: A Pilot Study

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Keywords

Taurine · Supplementation · Academic characteristics · Academic achievement · College entrance exam

Abbreviations

CEES	College entrance examination stress
PG	Placebo group
SAAS-R	School attitude assessment survey-revised
SRL	Self-regulated learning
TSG	Taurine supplementation group

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1 Introduction

Taurine plays an important role in physical and psychological well-being through various functions in the body, such as fatigue recovery, antioxidant effect, protection of visual function, and immunity improvement (Stapleton et al. 1997; Yoon et al. 2015). It was reported that middle-aged women supplemented with 4 g of taurine had decreased uric acid and increased antioxidant effect and serotonin levels compared to the control group (Kang and Lee 2013). This means that supplement intake of taurine had a positive effect on the reduction of fatigue substances in the blood, an increase of antioxidants in the body, and stability of the central nervous system.

Factors that positively affect academic achievement improvement are high levels of academic-related attitudes, self-regulated learning, and self-efficacy (Ablard and Lipschultz 1998; Cho et al. 2012; Kim 2001) and low levels of academic stress and depression (Byun and Kang 2016; Hwang 2008; Kang et al. 2014; Liu and Lu 2011). Adolescents need physical and psychological management so that they can have low fatigue and high stability to improve their academic achievement level. However, students

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have been consuming an energy drink or nutritious tonic which is high in caffeine and taurine to recover from fatigue and prevent drowsiness (Park et al. 2017a). The problem of the side effects of high caffeine intake among adolescents was raised, and a healthy snack to recover from fatigue for students was provided. In 2019, in Korea, taurine-containing jelly products were developed as a snack for fatigue recovery that can be consumed by all age groups. Taurine content in taurine-containing gummy jelly products is 1000–1700 mg per packet, so it contains the same amount of taurine in nutritious tonic and energy drink products on the market. In a previous study on the improvement of dietary taurine intake and academic-related attitudes among Korean high school students, as a result of providing high taurine meal service for 1 week using foods with high taurine content, it was reported that the score of academic-related attitudes in dietary taurine supplementation group was significantly higher compared to the control group (Park et al. 2017b). Intake of taurine-rich food is necessary to relieve the academic stress that students may experience while concentrating on their studies and to recover from physical fatigue.

Therefore, the purpose of this pilot study was to evaluate the effects of dietary taurine-containing jelly supplementation on the academic-related characteristics of Korean college entrance examinees.

2 Methods

2.1 Study Design

When dietary taurine was supplemented at 3 g, it showed a positive effect of reducing oxidative stress (De Carvalho et al. 2017) and improving cognitive function in the elderly with dementia (Bae et al. 2019). When dietary taurine supplementation was taken for 2 weeks, the improvement of diabetic disease (Moloney et al. 2010), lipid metabolism, and liver function is significant (Choi and Lee 2018). Based on these finding, we designed this pilot study (Fig. 1).

This pilot study was conducted as a randomized, double-blind, placebo-controlled study. Taurine-containing jelly and placebo jelly as research prototypes were developed with the same raw material and content ratio as Bacchus flavored jelly by Dong-A Pharmaceutical Co. Ltd. The research prototypes were supported by Dong-A Pharmaceutical Co. Ltd. Taurine-containing jelly contained 3 g of taurine in a packet, and placebo jelly had no taurine. The shape of taurine-containing jelly is solid and oval gummy jelly with a diameter of about 2 cm, and one packet of jelly contains 25–26 gummy jelly to supplement 3 g of taurine; 1 taurine-containing jelly contains 115–120 mg of taurine.

The jelly was provided to the subjects with one packet per day for 2 weeks. During the jelly intake period, the subjects restricted the intake of health functional foods, fatigue recovery drinks, energy drinks, and fish and shellfish with high taurine content as much as possible.

The effect of dietary taurine-containing jelly supplementation was evaluated before and 2 weeks after the intake of jelly by the three academic-related characteristics (Academic-related attitude, College Entrance Examination Stress; CEES, and Self-regulated Learning; SRL) and the academic achievement. The subjects were conducted using a self-administered questionnaire.

2.2 Subjects

The subjects of this pilot study were recruited from 25 college entrance examinees over the age of 19 years who were enrolled at college entrance cram schools located in Incheon Metropolitan City, Korea. These subjects were those who had no food allergy, could consume jelly, understood the purpose of the study, and voluntarily agreed to participate in the study. The subjects were randomly assigned to 13 examinees in the taurine supplementation group (TSG) and 12 examinees in the placebo group (PG). Among them, except for eight examinees who gave up during the jelly intake period, the data of nine examinees in TSG (effective ratio 69.2%) and eight examinees in

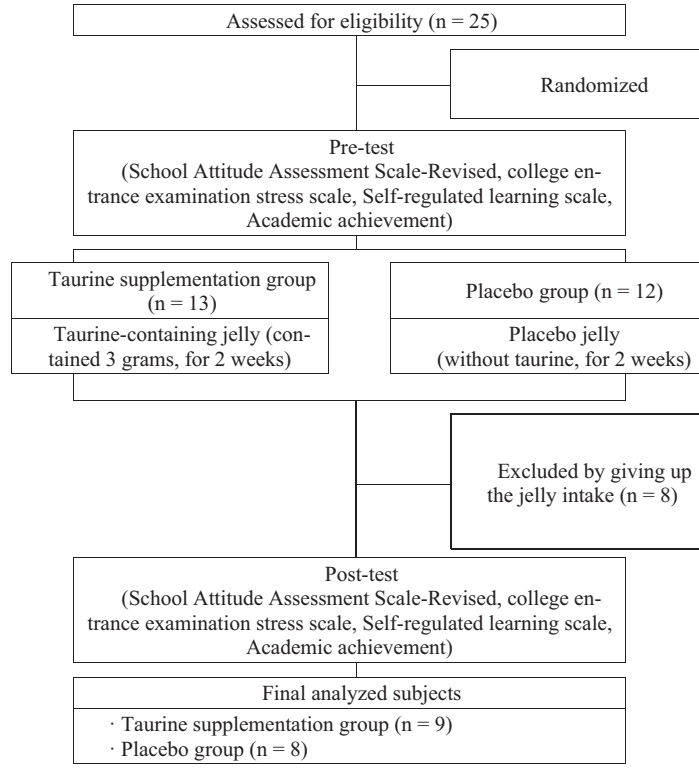

Fig. 1 Process flowchart of the study

PG (effective ratio 66.7%) were used in the final analysis. The study was conducted from September to November 2019 and was approved by the institutional review board of Inha University, Korea (No. 190225-4AR).

2.3 Academic-Related Characteristics

The academic-related attitude was evaluated using SAAS-R (School Attitude Assessment Scale-Revised) (Ahn and Kim 2009), CEES (Lee and Chung 1997), and SRL (Kim 2005) scales, and all items were measured using a Likert five-point scale. It was ranging from 5 points of "strongly agree" to 1 point of "strongly disagree," and the higher the score, the higher the level of the SAAS-R, CEES, and SRL.

The academic-related attitude was assessed using adolescent's SAAS-R developed by Ahn and Kim (2009). SAAS-R consists of 29 items for 5 sub-factors, and the number of items for each sub-factor consists of 7 items of academic self-perceptions, 7 items of attitudes toward teachers, 5 items of attitude toward cram school, 4 items of goal valuation, and 6 items of motivation/self-regulation. In this study, the reliability coefficient Cronbach's α of the sub-factor scale of academic-related attitudes was 0.738 academic self-perceptions, 0.858 attitudes toward teachers, 0.847 attitude toward cram school, 0.735 goal valuation, and 0.799 motivation/self-regulation.

CEES was the stress experienced in the process of preparing for college entrance examination and assessed using the CEES scale developed by Lee and Chung (1997). CEES scale consists of 24 items for 4 sub-factors, and the number of items for each sub-factor consists of 9 items of parental pressure, 6 items of test and grade anxiety, 6 items of lack of leisure, and 3 items of uncertainty of the future. In this study, the reliability coefficient Cronbach's α for the sub-factor scale of CEES was 0.928 parental pressure, 0.791

test and grade anxiety, 0.640 lack of leisure, and 0.775 uncertainty of the future.

SRL refers to the learner's self-directed learning, planning, and cognitive control, and in this study, on the basis of the SLS scale developed by Yang (2000), a scale reconstructed in a shortened form in Kim's study (2005) was used. SRL scale consisted of 41 items for three sub-factors, and the number of items for each sub-factor consisted of 12 items of cognitive control, 16 items of motivation control, and 13 items of action control. In this study, the reliability coefficient Cronbach's α of the sub-factor scale of SEL was 0.681 for cognitive control, 0.886 for motive control, and 0.660 for action control.

2.4 Academic Achievement

Academic achievement refers to the degree to which the subjects have achieved academically and measured on three scales. Scale items were the satisfaction of subjects' mock examination score and levels of effort and willingness to improve the subjects' mock examination score and measured using a Likert five-point scale. The score was given 5 points "strongly agree," 3 points "so-so," or 1 point "strongly disagree," and academic achievement of subjects was measured by calculating the average of scores on the three scales.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS 25.0 version program (SPSS Inc., Chicago, IL, USA). The difference between the two groups before and after jelly intake was analyzed using the chi-square test and Student *t*-test, and the difference between before and after jelly intake within each group was analyzed using paired t-test. However, scale factors that showed a significant difference in the homogeneity test before supplementation with dietary taurine were analyzed for differences by using ANCOVA (analysis of covariance). Correlations between academic-related attitude, CEES, and SRL on academic achievement were determined Pearson's correlation coefficient, and explanatory power of variables showing correlations with academic achievement was analyzed through simple regression analysis. Statistical significance was set at $P < 0.05$.

3 Results

3.1 General Characteristics of Subjects

As for the general characteristics of the subjects by dietary taurine supplementation, as shown in Table 1, there were no significant differences between the two groups in gender, repeating a college entrance examination, and major field.

3.2 Homogeneity Test Between the TSG and PG Before Intake of Jelly

Before dietary taurine supplementation, homogeneity of academic-related characteristics and academic achievement was verified between the TSG and the PG (Table 2). However, there was a significant difference only in the average score of academic self-perceptions among the sub-factors of academic-related attitudes. When analyzing the difference between the groups after dietary taurine supplementation, academic self-perception factor of academic-related attitudes before dietary taurine supplementation was taken as a covariate.

3.3 Difference Between the TSG and PG After Intake of Jelly

As shown in Table 3, there were no significant differences between the groups according to dietary taurine supplementation. However, the total score ($p = 0.069$) and motivation/self-regulation score ($p = 0.060$) in SAAS-R tended to be

Table 1 General characteristics of the subjects

Variables	Taurine supplementation group (n = 9)	Placebo group (n = 8)	Total (n = 17)	χ^2 (p)[b]
Gender				
Males	6 (66.7)[a]	4 (50.0)	10 (58.8)	0.486 (0.486)
Females	3 (33.3)	4 (50.0)	7 (41.2)	
Repeating a college entrance examination				
Yes	2 (22.2)	1 (12.5)	3 (17.6)	0.275 (0.600)
No	7 (77.8)	7 (87.5)	14 (82.4)	
Major field				
Liberal arts	7 (77.8)	4 (50.0)	11 (64.7)	1.431 (0.232)
Natural science	2 (22.2)	4 (50.0)	6 (35.3)	

[a]*n* (%)
[b]*p* values were analyzed by chi-square test

Table 2 Homogeneity test between the TSG and PG before intake of jelly

Variables	Taurine supplementation group (n = 9)	Placebo group (n = 8)	t (p)[b]
School attitude assessment			
Academic self-perceptions	3.4 ± 0.5[a]	2.9 ± 0.4	2.413 (0.029)*
Attitudes toward teachers	3.4 ± 0.6	3.4 ± 0.4	0.128 (0.900)
Attitudes toward school	3.2 ± 0.8	3.3 ± 0.6	−0.376 (0.712)
Goal valuation	3.5 ± 0.5	4.0 ± 0.4	−1.993 (0.065)
Motivation/self-regulation	3.5 ± 0.7	3.3 ± 0.4	0.756 (0.461)
Total score	3.4 ± 0.4	3.3 ± 0.3	0.500 (0.625)
College entrance examination stress			
Parental pressure	2.4 ± 1.1	2.5 ± 0.6	−0.129 (0.899)
Test and grade anxiety	3.4 ± 0.6	3.8 ± 0.5	−1.643 (0.121)
Lack of leisure	2.9 ± 0.7	3.0 ± 0.5	−0.325 (0.750)
Uncertainty of the future	3.0 ± 0.6	3.4 ± 0.8	−1.250 (0.230)
Total score	2.9 ± 0.7	3.1 ± 0.4	−0.771 (0.454)
Self-regulated learning			
Cognitive control	3.4 ± 0.5	3.4 ± 0.3	−0.144 (0.887)
Motivation control	3.3 ± 0.7	3.1 ± 0.3	0.989 (0.343)
Action control	3.2 ± 0.7	3.0 ± 0.3	0.643 (0.530)
Total score	3.3 ± 0.5	3.2 ± 0.2	0.720 (0.482)
Academic achievement	3.2 ± 0.5	2.9 ± 0.3	1.159 (0.265)

[a]mean ± SD
[b]*p* values were analyzed by Student *t*-test. *$p < 0.05$

significantly higher in the TSG after 2 weeks of supplementation compared to before supplementation.

3.4 Differences in Changes Before and After Intake of Taurine-Containing Jelly in TSG

As shown in Table 4, there was no significant difference in the change in academic-related characteristics and academic achievement before and after intake of jelly in the TSG. Academic self-perceptions, a sub-factor of academic-related attitude, showed a tendency to improve from 3.4 points before intake of taurine-containing jelly to 3.9 points after intake of them (p = 0.086), and action control, a sub-factor of self-regulated learning ability, also showed a tendency to improve from 3.2 points before intake of taurine-containing jelly to 3.5 points after intake of them (p = 0.098).

However, in the case of the PG, a score of parental pressure, which is a sub-factor of the

Table 3 Differences between the TSG and PG after intake of jelly

Variables	Taurine supplementation group (n = 9)	Placebo group (n = 8)	F/t (p)
School attitude assessment			
Academic self-perceptions	3.9 ± 0.9[a]	2.8 ± 0.6	2.859 (0.113)[b]
Attitudes toward teachers	3.6 ± 0.6	3.1 ± 0.5	1.594 (0.132)[c]
Attitudes toward school	3.2 ± 0.7	3.3 ± 0.5	−0.421 (0.680)[c]
Goal valuation	3.7 ± 0.7	3.9 ± 0.6	−0.685 (0.504)[c]
Motivation/self-regulation	3.7 ± 0.5	3.3 ± 0.4	2.032 (0.060)[c]
Total score	3.7 ± 0.5	3.2 ± 0.3	3.880 (0.069)[b]
College entrance examination stress			
Parental pressure	2.3 ± 1.3	2.8 ± 0.6	−1.134 (0.293)[c]
Test and grade anxiety	3.5 ± 0.4	3.8 ± 0.6	−0.998 (0.334)[c]
Lack of leisure	3.4 ± 0.8	3.1 ± 0.5	0.838 (0.415)[c]
Uncertainty of the future	3.0 ± 0.6	3.3 ± 1.0	−0.950 (0.357)[c]
Total score	3.0 ± 0.6	3.2 ± 0.5	−0.877 (0.395)[c]
Self-regulated learning			
Cognitive control	3.7 ± 0.5	3.7 ± 0.5	−0.257 (0.801)[c]
Motivation control	3.5 ± 0.5	3.1 ± 0.4	1.365 (0.192)[c]
Action control	3.5 ± 0.6	3.1 ± 0.4	1.518 (0.150)[c]
Total score	3.5 ± 0.4	3.3 ± 0.3	1.274 (0.222)[c]
Academic achievement	3.3 ± 0.7	2.8 ± 0.6	1.468 (0.163)[c]

[a]mean ± SD
[b]p values were analyzed by ANCOVA
[c]p values were analyzed by Student t-test

Table 4 Differences in scores of academic-related characteristics and academic achievement at the before and after intake of taurine-containing jelly in TSG

Variables	Pre	Post	△Post − Pre	t (p)[b]
School attitude assessment				
Academic self-perceptions	3.4 ± 0.5[a]	3.9 ± 0.9	0.5 ± 0.8	−1.960 (0.086)
Attitudes toward teachers	3.4 ± 0.6	3.6 ± 0.6	0.1 ± 0.4	−0.973 (0.359)
Attitudes toward school	3.2 ± 0.8	3.2 ± 0.7	0.0 ± 0.6	0.000 (1.000)
Goal valuation	3.5 ± 0.5	3.7 ± 0.7	0.2 ± 0.7	−0.855 (0.417)
Motivation/self-regulation	3.5 ± 0.7	3.7 ± 0.5	0.2 ± 0.8	−0.918 (0.385)
Total score	3.4 ± 0.4	3.7 ± 0.5	0.2 ± 0.5	−1.458 (0.183)
College entrance examination stress				
Parental pressure	2.4 ± 1.1	2.3 ± 1.3	−0.1 ± 0.7	0.552 (0.596)
Test and grade anxiety	3.4 ± 0.6	3.5 ± 0.4	0.2 ± 0.6	−0.912 (0.388)
Lack of leisure	2.9 ± 0.7	3.4 ± 0.8	0.5 ± 1.0	−1.400 (0.199)
Uncertainty of the future	3.0 ± 0.6	3.0 ± 0.6	−0.0 ± 0.6	0.189 (0.855)
Total score	2.9 ± 0.7	3.0 ± 0.6	0.1 ± 0.4	−0.907 (0.391)
Self-regulated learning				
Cognitive control	3.4 ± 0.5	3.7 ± 0.5	0.2 ± 0.6	−1.273 (0.239)
Motive control	3.3 ± 0.7	3.5 ± 0.5	0.1 ± 0.6	−0.679 (0.517)
Action control	3.2 ± 0.7	3.5 ± 0.6	0.3 ± 0.4	−1.873 (0.098)
Total score	3.3 ± 0.5	3.5 ± 0.4	0.2 ± 0.5	−1.283 (0.235)
Academic achievement	3.2 ± 0.5	3.3 ± 0.7	0.2 ± 0.8	−0.543 (0.602)

[a]mean ± SD
[b]p values were analyzed by paired t-test

entrance examination stress, increased significantly from 2.5 points before intake of placebo jelly to 2.8 points after intake of them ($p < 0.001$, data not shown).

3.5 Correlation Between Academic-Related Characteristics and Academic Achievement for Differences in Post- and Pre-scores

In the TSG, as the degree of difference in the score of self-regulated learning increased, the degree of difference in academic achievement also increased positively, showing a positive correlation. However, no correlation was found in the placebo group (Table 5).

3.6 Effect of Self-Regulated Learning Score on Academic Achievement in the TSG

In the TSG, a regression analysis was performed between the two variables as there was a correlation between the degree of change in academic achievement and the degree of change in self-regulated learning before and after intake of taurine-containing jelly. As for Table 6, it was found that the degree of change in self-regulated learning affects the degree of change in academic achievement ($p = 0.033$). The explanatory power of the degree of change in academic achievement to explain the degree of change in self-regulated learning ability was 42.9%.

4 Discussion

The present pilot study may support the hypothesis that college entrance examinees need taurine supplementation. In TSG of this pilot study, the motivation/self-regulation score ($p = 0.060$) in SAAS-R tended to be significantly higher after 2 weeks of supplementation when compared to the score before supplementation. The higher the academic achievement level, the higher the SAAS-R score showed; in motivation/self-regulation factors, the average score of the high score group (3.7 points) was shown significantly higher than that of the middle score group (3.1 points) (Ahn and Kim 2009). The motivation/self-regulation factor refers to psychological stability regarding concentration and voluntary actions on studies, and taurine plays the function of psychological stability and stress relief (Neuwirth et al. 2013). Dietary taurine supplementation may have effects of the difference in the significant trends on the score of motivation/self-regulation factors between the TSG and PG.

It was reported that traits such as attitude toward teachers, goal valuation, motivation/self-regulation, and total score of SAAS-R in Korean high school students were significantly improved after supplementing an average of 500 mg of taurine-rich food in school meals for 5 days (Park et al. 2017b). In this pilot study, there was no significant difference in academic-related attitudes, but there was a trend of significant differences in academic self-perception. It may be seen that taurine intake through taurine-containing jelly supplementation has the potential to be a factor that may bring about positive changes in academic-related attitudes.

Table 5 Correlation between academic-related characteristics and academic achievement for differences in post and pre-scores

Variables	△ Post-pre score at academic achievement		
	△ Post-pre score at total score		
	School attitude assessment	College entrance examination stress	Self-regulated learning
Taurine supplementation group	0.425 (0.254)	−0.645 (0.060)	0.707 (0.033)*[a]
Placebo group	−0.525 (0.182)	0.013 (0.975)	−0.348 (0.398)

[a]Values were Pearson's correlation coefficient. *$p < 0.05$

Table 6 Effect of self-regulated learning score on academic achievement in the taurine supplementation group

Dependent variable	Independent variable	*B*	S.E.	*β*	*t*	*p*
△ post-pre score at academic achievement	(Constant)	−0.099	0.226		−0.439	0.674
	△ post-pre score at self-regulated learning	1.218	0.460	0.707	2.646	0.033*[a]
$F = 7.002$ ($p = 0.033$), $R^2 = 0.500$, adj $R^2 = 0.429$, Durbin-Watson = 3.099						

[a]*p* value was analyzed by simple regression analysis. *$p < 0.05$

It has been reported that adolescents' self-regulated learning was higher when psychological instability was lower (Son et al. 2006), and students with higher self-regulated learning were higher academic achievers (Castejón et al. 2016). In this pilot study, it is also considered that taurine supplementation had a positive effect on the degree of change in self-regulated learning and the degree of change in academic achievement due to psychological stability.

5 Conclusion

This pilot study resulted in the possibility that taurine intake through taurine-containing jelly supplementation has a positive effect on academic-related characteristics in Korean college entrance examinees. It is considered that taurine supplementation seems to be beneficial in improving academic self-perceptions, motivation/self-regulation, and self-regulated learning for college entrance examinees' academic work.

Although this pilot study can be utilized as base data on the need for taurine-containing jelly supplementation for students who study, further study is needed to clarify the effect of intake of taurine-containing jelly. Therefore, subsequent evaluations of the effectiveness of taurine-containing jelly should enlarge the sample size for college entrance examinees. In addition, further studies are required to substantiate the range and content of taurine intake.

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Improvement in Willingness to Purchase Taurine-Containing Jelly Needs High Degrees of Interest and Preference: Focused on Korean College Students

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Keywords

Jelly · Willingness · Purchase · Korean college students · Dietary taurine

1 Introduction

Taurine is a β-amino acid that exerts multiple physiological actions; many of taurine plays a role in recovering from physical and psychological fatigue, relieving anxiety and stress, and improving cognitive function (Yoon et al. 2015). It protects brain nerve cells by inhibiting their death and can cross the blood-brain barrier, blocking neurotransmitters and regulating the functions of the central nervous system, such as enhancing the function of the hippocampus (Kendler 1989; Yoon et al. 2015). Taurine, along with caffeine, is used as the main ingredients of energy drinks and nutritious tonics that students consume to recover from fatigue. Since high caffeine intake causes side effects such as arousal and insomnia, problems with energy drink intake by students have been raised (Park et al. 2017). However, taurine is called a natural anti-fatigue agent and is known as a nutrient with few side effects because it is excreted through the urine or bile even when consumed in excess of required amounts (Cha et al. 1999).

Recently, in Korea, taurine-containing jelly was developed that has the same taste and smell as energy drinks or nutritious tonics but does not contain caffeine. There are two types of taurine-containing jellies on the market in Korea, gummy and konjac, and one packet contains 500–1700 mg of taurine. So, commercially available taurine-containing jellies provide a convenient way for students to supplement their dietary taurine. If students consume taurine-containing jelly as a snack while studying, the positive effects of taurine may be felt.

Therefore, the purpose of this study was to investigate recognition, consumer preference, and improvement points to increase willingness to purchase taurine-containing jelly as a snack among Korean college students.

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2 Methods

2.1 Subjects

The subjects of this study were 600 college students in Incheon who understood the purpose and process of the research and voluntarily agreed to participate in this study. The survey and preference evaluation were conducted from June to November 2019.

The survey was conducted using an anonymous self-administered questionnaire, with 561 copies (recovery rate of 93.5%) used for the final analysis data. This study was approved by the institutional review board of Inha University, Korea (No. 190225-5AR).

2.2 General Characteristics and Interest in Taurine-Containing Jelly

General characteristics of the subjects included gender, age, grade, major field, residence type, average monthly pocket money, and average daily snack money.

The degree of interest in taurine-containing jelly was evaluated using a five-point Likert scale (1 point: not very interested, 3 points: moderate interest, 5 points: very interested). Subjects ranging from 3 to 5 points were classified as the taurine-containing jelly interest group, and subjects ranging from 1 to 2 points became the taurine-containing jelly indifference group.

2.3 Consideration in Buying Taurine-Containing Jelly

Those considering the purchase of taurine-containing jelly were surveyed for taste, price, efficacy (functionality), brand, nutrient, promotion, weight, and packaging type by referring to a previous study (Choi and Lee 2018) and were measured using a five-point Likert scale.

2.4 Recognition of Taurine-Containing B Jelly

The recognition of commercially available taurine-containing jellies, including taurine-containing Bacchus flavored jelly (B jelly), was examined. The survey items were constructed by referring to previous studies (Lee 2016; Park and Kim 2018; Yang et al. 2018). Recognition of taurine-containing jelly B was examined for knowledge of taurine, image familiarity (five-point Likert scale), type of food, experience to purchase and intention to repurchase, purpose of purchase, and subjective fatigue recovery effect after intake. The purpose of purchasing the product and subjective fatigue recovery after intake of taurine-containing B jelly were investigated for 85 subjects with experience past purchases and intention to repurchase.

2.5 Evaluation of Consumer Preference for Taurine-Containing B Jelly

To evaluate consumer preference, taurine-containing B jelly was supported by Dong-A Pharmaceutical Co. Ltd. It was put three each in small white paper cups and provided to the subjects to taste. Referring to previous studies (Yang et al. 2018), the appearance, color, aroma, taste, texture, and overall preference were evaluated using a nine-point hedonic scale (1 point "not at all," 9 points "strongly agree").

2.6 Acceptability to Purchase Product After Tasting Taurine-Containing B Jelly

Willingness to purchase and intention to recommend taurine-containing B jelly were assessed using a five-point Likert scale. Improvement points of taurine-containing B jelly were examined with multiple responses.

2.7 Statistical Analysis

For statistical analysis of this study data, SPSS version 25.0 program (SPSS Inc., Chicago, IL, USA) was used. According to gender and interest in taurine-containing jelly, variables were analyzed using the chi-square test and Student t-test. For each group (males and females) by gender and each group (interest and indifferent groups) by interest, significant differences in consideration of buying and evaluation of consumer preference were analyzed using one-way ANOVA and Scheffe test. Effects of recognition and consumer preference for taurine-containing B jelly on willingness to purchase were analyzed using multiple regression analysis. All significance tests were performed at the $p < 0.05$ level.

3 Results

3.1 General Characteristics and Interest in Taurine-Containing Jelly of the Subjects

General characteristics of the subjects of this study are shown in Table 1. The proportion of male and female college students with an average age of 23.3 years were 46.3% and 53.7%, respectively. The average monthly pocket money and daily snack purchase cost of 45.6% and 49.4% of the subjects were over 400,000 won and 1000 won to 5000 won, respectively.

As for the degree of interest in taurine-containing jelly, the proportion of the interest group (very to moderately interested) and the indifferent group (indifferent to very indifferent) were 49.2% and 50.8%, respectively.

Table 1 General characteristics and interest in taurine-containing jelly of the subjects

Variables		Total ($n = 561$)
Gender	Male	260 (46.3)[1]
	Female	301 (53.7)
Age (years)		23.3 ± 3.1[2]
Grade	Freshman	128 (22.8)
	Sophomore	145 (25.8)
	Junior	134 (23.9)
	Senior	154 (27.5)
Major field	Engineering	146 (26.0)
	Natural science	145 (25.8)
	Liberal arts	194 (34.6)
	Arts and physical education	63 (11.2)
	Others	13 (2.3)
Residence type	Home	327 (58.3)
	Dormitory	42 (7.5)
	Boarding	182 (32.4)
	Others (relative house, etc.)	10 (1.8)
Average monthly pocket money (10,000 won)	< 10	54 (9.6)
	10 to < 19	29 (5.2)
	20 to < 29	91 (16.2)
	30 to < 39	131 (23.4)
	≥40	256 (45.6)
Average daily snack purchase cost (1000 won)	<1	79 (14.1)
	1 to < 5	277 (49.4)
	5 to < 10	114 (20.3)
	10 to < 20	39 (7.0)
	≥20	52 (9.3)
Degree of interest in taurine-containing jelly	Interest	276 (49.2)
	Indifferent	285 (50.8)

[1] n (%)
[2] mean ± SD

3.2 Considering the Purchase of Taurine-Containing Jelly

Comparing those who considered buying (or trying to buy) taurine-containing jelly differed according to gender (Table 2), taste ($p < 0.05$), and brand ($p < 0.01$), with female college students scoring significantly higher compared to male college students, but scores of price ($p < 0.05$) and efficacy ($p < 0.05$) were significantly higher in male college students compared to female college students. Among male students, taste and price were considered the most important when purchasing taurine-containing jelly, while weight and packaging were among the least important factors ($p < 0.001$). Among female students, taste was considered the most important factor when purchasing taurine-containing jelly, while brand and packaging were the least important factors ($p < 0.001$).

Table 2 Consideration in buying (or trying to buy) taurine-containing jelly according to gender

Variables	Males (n = 260)	Females (n = 301)	Total (n = 561)	t (p)[1]
Taste	4.4[a] ± 0.8[2]	4.5[a] ± 0.7	4.5 ± 0.7	−2.505*(0.013)
Price	4.3[a] ± 0.7	4.2[b] ± 0.7	4.2 ± 0.7	2.076* (0.038)
Efficacy	3.8[b] ± 1.0	3.7[c] ± 1.0	3.8 ± 1.0	2.018* (0.044)
Brand	2.9[d] ± 1.1	3.1[d] ± 1.0	3.0 ± 1.1	−3.046** (0.002)
Nutrient	3.6[b,c] ± 1.0	3.6[c] ± 1.0	3.6 ± 1.0	−0.237 (0.813)
Promotion	3.5[c] ± 1.1	3.6[c] ± 0.9	3.6 ± 1.0	−1.898 (0.058)
Weight	3.8[b] ± 0.9	3.8[c] ± 0.9	3.8 ± 0.9	0.873 (0.383)
Packaging type	3.0[d] ± 1.1	3.1[d] ± 1.1	3.0 ± 1.1	−0.626 (0.532)
F (p)[3]	78.553*** (0.000)	72.282*** (0.000)		

[1]p values were analyzed by Student t-test. *$p < 0.05$
[2]mean ± SD
[3]p values were analyzed by one-way ANOVA and Scheffe test. ***$p < 0.001$. Means in a hole column with different letters (a, b, c, d) are significantly different

Table 3 Consideration in purchasing of (or trying to buy) taurine-containing jelly according to degree of interest

Variables	Interest group (n = 276)	Indifferent group (n = 285)	Total (n = 561)	t (p)[1]
Taste	4.5[a] ± 0.7[2]	4.5[a] ± 0.8	4.5 ± 0.7	0.595 (0.552)
Price	4.2[a] ± 0.7	4.3[a] ± 0.8	4.2 ± 0.7	−0.683 (0.495)
Efficacy	3.8[b,c] ± 1.0	3.7[b] ± 1.1	3.8 ± 1.0	1.594 (0.111)
Brand	3.1[d] ± 1.0	2.9[c] ± 1.1	3.0 ± 1.1	1.770 (0.077)
Nutrient	3.6[b,c] ± 0.9	3.5[b] ± 1.1	3.6 ± 1.0	2.015* (0.044)
Promotion	3.6[c] ± 0.9	3.5[b] ± 1.0	3.6 ± 1.0	0.607 (0.544)
Weight	3.9[b] ± 0.8	3.7[b] ± 1.0	3.8 ± 0.9	2.026* (0.043)
Packaging type	3.1[d] ± 1.0	3.0[c] ± 1.1	3.0 ± 1.1	1.896 (0.058)
F (p)[3]	64.873*** (0.000)	83.537*** (0.000)		

[1]p values were analyzed by Student t-test. *$p < 0.05$
[2]mean ± SD
[3]p values were analyzed by one-way ANOVA and Scheffe test. ***$p < 0.001$. Means in a hole column with different letters (a, b, c, d) are significantly different

According to interest in taurine-containing jelly (Table 3), scores of nutrient value and weight in the interest group were significantly higher compared to those of the indifferent group ($p < 0.05$). In the interest group, taste and price were considered the most important factors when purchasing taurine-containing jelly, while brand and packaging were considered least important ($p < 0.001$). Among the indifferent group, taste and price were considered the most important factors when purchasing taurine-containing jelly, while brand and packaging were among the least important factors ($p < 0.001$).

When buying taurine-containing jelly, the highest consideration was taste 4.5 points, price 4.2 points, and efficacy 3.8 points in order, and the lowest consideration factors were packaging type and brand 3.0 points.

3.3 Recognition of Taurine-Containing B Jelly

As shown Table 4, male college students were significantly more knowledgeable about taurine than female college students ($p < 0.001$). As for the food type, the subjects associated with taurine-containing B jelly, candy, and health functional food were rated 49.2% and 64.8% among male college students and 38.8% and 31.9% among female college students, respectively ($p < 0.001$). Of the 85 subjects who have already purchased the product or intended to repurchase taurine-containing B jelly, the purpose of purchasing taurine-containing B jelly was fatigue recovery by 62.2% and as a snack and for good taste by 13.5% among male college students but was chosen because it was a snack

Table 4 Recognition of taurine-containing B jelly according to gender

Variables	Males (n = 260)	Females (n = 301)	Total (n = 561)	χ^2/t (p)[1]
Knowledge of taurine				
Yes	220 (84.6)	231 (70.8)	433 (77.2)	15.198*** (0.000)
No	40 (15.4)	88 (29.2)	128 (22.8)	
Image familiarity	3.8 ± 1.0[2]	3.8 ± 1.0	3.8 ± 1.0	−0.653 (0.514)
Type of food				
Medicine	15 (5.8)	4 (1.3)	19 (3.4)	22.718*** (0.000)
Health functional food	101 (38.8)	96 (31.9)	197 (35.1)	
Candy	128 (49.2)	195 (64.8)	323 (57.6)	
Not know	14 (5.4)	6 (2.0)	20 (3.6)	
Others	2 (0.8)	0 (0.0)	2 (0.4)	
Experience to purchase and intention to repurchase				
Yes	37 (14.2)	48 (15.9)	85 (15.9)	0.324 (0.850)
Only purchasing experience	34 (13.1)	38 (12.6)	72 (12.6)	
No	189 (72.7)	215 (71.4)	404 (72.0)	
Purpose of purchases (n = 85)				
Fatigue recovery	23 (62.2)[3]	10 (20.8)	33 (38.8)	17.075** (0.004)
Snack	5 (13.5)	17 (35.4)	22 (25.9)	
Taste	5 (13.5)	17 (35.4)	22 (25.9)	
Caffeine free	2 (5.4)	2 (4.2)	4 (4.7)	
Nutritional supplement	1 (2.7)	1 (2.1)	2 (2.4)	
Improvement of concentration	1 (2.7)	1 (2.1)	2 (2.4)	
Subjective fatigue recovery effect after intake (n = 85)				
Yes	17 (45.9)	11 (23.4)	28 (33.3)	4.830 (0.089)
So-so	12 (32.4)	20 (42.6)	32 (38.1)	
No	8 (21.6)	16 (34.0)	24 (28.6)	

[1]p values were analyzed by chi-square test or Student t-test. ***$p < 0.001$, **$p < 0.01$
[2]Average value was calculated on five-point Likert scales. Mean ± SD
[3]n (%)

and had good taste by 35.4% each and fatigue recovery by 20.8% among female college students, respectively ($p < 0.01$).

According to the interest in taurine-containing jelly (Table 5), the interest group was significantly more knowledgeable about taurine than the indifferent group ($p < 0.001$). Image familiarity of taurine-containing B jelly in the interest group (4.0 points) was significantly higher compared to that of the indifferent group (3.6 points) ($p < 0.001$). There was a significant difference between the two groups, with 42.4% of the interested group and 28.1% of the indifferent group who recognized the food type of taurine-containing B jelly as a health functional food ($p < 0.01$). Subjects who have the experience of purchasing the product or intend to repurchase it were 23.9% among the interest group and 6.7% among the indifferent group ($p < 0.001$).

3.4 Evaluation of Consumer Preference for Taurine-Containing B Jelly

Consumer preference for taurine-containing B jelly according to gender (Table 6), aroma ($p < 0.01$), and taste ($p < 0.05$) was significantly higher among male college students than female college students. There was no significant difference in the evaluation of consumer preference among male students, but among female students, color was one of the important preference variables, while aroma was less important ($p < 0.01$).

According to the degree of interest in taurine-containing jelly (Table 7), scores of all items in the interest group were significantly higher compared to those of the indifferent group ($p < 0.01$). There was no significant difference in the evaluation of

Table 5 Recognition of taurine-containing B jelly according to degree of interest

Variables	Interest group (n = 276)	Indifferent group (n = 285)	Total (n = 561)	χ^2/t (p)[1]
Knowledge of taurine				
Yes	240 (87.0)	193 (67.7)	433 (77.2)	29.465*** (0.000)
No	36 (13.0)	92 (32.3)	128 (22.8)	
Image familiarity	4.0 ± 0.8[2]	3.6 ± 1.1	3.8 ± 1.0	5.035*** (0.000)
Type of food				
Medicine	7 (2.5)	12 (4.2)	19 (3.4)	16.100** (0.003)
Health functional food	117 (42.4)	80 (28.1)	197 (35.1)	
Candy	146 (52.9)	177 (62.1)	323 (57.6)	
Not know	5 (1.8)	15 (5.3)	20 (3.6)	
Others	1 (0.4)	1 (0.4)	2 (0.4)	
Experience to purchase and intention to repurchase				
Yes	66 (23.9)	19 (6.7)	85 (15.2)	33.126*** (0.000)
Only purchasing experience	35 (12.7)	37 (13.0)	72 (12.8)	
No	175 (63.4)	229 (80.4)	404 (72.0)	
Purpose of purchases (n = 85)				
Fatigue recovery	29 (43.9)[1]	4 (21.1)	33 (38.8)	7.917 (0.161)
Snack	15 (22.7)	7 (36.8)	22 (25.9)	
Taste	14 (21.2)	8 (42.1)	22 (25.9)	
Caffeine free	4 (6.1)	0 (0.0)	4 (4.7)	
Nutritional supplement	2 (3.0)	0 (0.0)	2 (2.4)	
Improvement of concentration	2 (3.0)	0 (0.0)	2 (2.4)	
Subjective fatigue recovery effect after intake (n = 85)				
Yes	25 (38.5)	3 (15.8)	28 (33.3)	3.945 (0.139)
So-so	24 (36.9)	8 (42.1)	32 (38.1)	
No	16 (24.6)	8 (42.1)	24 (28.6)	

[1]p values were analyzed by chi-square test or Student t-test. ***$p < 0.001$, **$p < 0.01$
[2]Average value was calculated on five-point Likert scales. Mean ± SD
[3]n (%)

Table 6 Evaluation of consumer preference for taurine-containing B jelly according to gender

Variables	Males (n = 260)	Females (n = 301)	Total (n = 561)	t (p)[1]
Appearance	6.9 ± 1.3[2]	6.8 ± 1.4	6.8 ± 1.3	0.631 (0.528)
Color	7.0 ± 1.3	7.0[a] ± 1.3	7.0 ± 1.3	−0.044 (0.965)
Aroma	6.8 ± 1.5	6.4[b] ± 1.8	6.6 ± 1.7	2.809** (0.005)
Taste	7.1 ± 1.4	6.8 ± 1.7	6.9 ± 1.6	2.456* (0.014)
Texture	7.1 ± 1.4	6.8 ± 1.6	6.9 ± 1.5	2.784** (0.006)
Overall preference	6.9 ± 1.4	6.7 ± 1.5	6.8 ± 1.5	1.666 (0.096)
F (p)[3]	3.733 (0.080)	9.759** (0.001)		

[1]p values were analyzed by Student t-test. **$p < 0.01$, *$p < 0.05$
[2]Average value was calculated on nine-point hedonic scales. Mean ± SD
[3]p values were analyzed by one-way ANOVA and Scheffe test. **$p < 0.01$. Means in a hole column with different letters (a, b) are significantly different

Table 7 Evaluation of consumer preference for taurine-containing B jelly according to degree of interest

Variables	Interest group (n = 276)	Indifferent group (n = 285)	Total (n = 561)	t (p)[1]
Appearance	7.0 ± 1.3[2]	6.6 ± 1.4	6.8 ± 1.3	3.612*** (0.000)
Color	7.2 ± 1.2	6.8[a] ± 1.4	7.0 ± 1.3	3.263** (0.001)
Aroma	7.0 ± 1.6	6.3[b] ± 1.7	6.6 ± 1.7	4.862*** (0.000)
Taste	7.2 ± 1.4	6.6 ± 1.7	6.9 ± 1.6	4.953*** (0.000)
Texture	7.2 ± 1.3	6.7 ± 1.7	6.9 ± 1.5	4.305*** (0.000)
Overall preference	7.1 ± 1.2	6.5 ± 1.6	6.8 ± 1.5	5.258*** (0.000)
F (p)[3]	3.075 (0.120)	9.114**(0.003)		

[1]p values were analyzed by Student t-test. ***$p < 0.001$, **$p < 0.01$
[2]Average value was calculated on nine-point hedonic scales. Mean ± SD
[3]p values were analyzed by one-way ANOVA and Scheffe test. **$p < 0.01$. Means in a hole column with different letters (a, b) are significantly different

Table 8 Acceptability to purchase taurine-containing B jelly after tasting according to gender

Variables	Males (n = 260)	Females (n = 301)	Total (n = 561)	χ^2/t (p)[1]
Willingness to purchase	3.1 ± 1.0[2]	3.1 ± 1.1	3.1 ± 1.0	−0.025 (0.980)
Recommendation intention	3.1 ± 0.8	3.2 ± 0.9	3.1 ± 0.8	−1.293 (0.197)
Improvement of taurine-containing B jelly (multiple responses)				–
Functionality	43 (16.5)[3]	46 (15.3)	89 (13.0)	
Taste	42 (16.2)	50 (16.7)	92 (13.5)	
Chewiness	33 (12.7)	54 (18.0)	87 (12.7)	
Aroma	30 (11.5)	55 (18.3)	85 (12.4)	
Nutrient	24 (9.2)	23 (7.7)	47 (6.9)	
Packaging type	8 (3.1)	8 (2.7)	16 (2.3)	
Affordable price	47 (18.1)	43 (14.3)	90 (13.2)	
Not know	68 (26.2)	55 (18.3)	123 (18.0)	
Others	23 (8.8)	32 (10.7)	55 (8.0)	

[1]p values were analyzed by Student t-test
[2]Average value was calculated on five-point Likert scales. Mean ± SD
[3]n (%)

consumer preference among the interest group, but among the indifferent group, color was a more important preference variables than aroma ($p < 0.01$).

3.5 Acceptability to Purchase Taurine-Containing B Jelly After Tasting

As shown in Table 8, willingness to purchase the taurine-containing B jelly was found to be 3.1 points for both male and female students after tasting it.

According to the degree of interest in taurine-containing jelly (Table 9), scores of willingness to purchase ($p < 0.001$) and intention to recommend ($p < 0.001$) among the interest group (3.4 and 3.4 points) were significantly higher compared to that of the indifferent group (2.8 and 2.9 points).

Improvement in purchasing taurine-containing B jelly in all subjects depended upon taste for 13.5%, affordable price 13.2%, and functionality for 13.0% in that order.

Table 9 Acceptability to purchase taurine-containing B jelly after tasting according to degree of interest

Variables	Interest group (n = 276)	Indifferent group (n = 285)	Total (n = 561)	χ^2/t (p)[3]
Willingness to purchase	3.4 ± 0.9[1]	2.8 ± 1.0	3.1 ± 1.0	7.315*** (0.000)
Recommendation intention	3.4 ± 0.8	2.9 ± 0.8	3.1 ± 0.8	8.745*** (0.000)
Improvement of taurine-containing B jelly (multiple responses)				–
Functionality	50 (18.1)[2]	39 (13.7)	89 (13.0)	
Taste	38 (13.8)	54 (19.0)	92 (13.5)	
Chewiness	38 (13.8)	49 (17.3)	87 (12.7)	
Aroma	37 (13.4)	48 (16.9)	85 (12.4)	
Nutrient	29 (10.5)	18 (6.3)	47 (6.9)	
Packaging type	9 (3.3)	7 (2.5)	16 (2.3)	
Affordable price	42 (15.2)	48 (16.9)	90 (13.2)	
Not know	54 (19.6)	69 (24.3)	123 (18.0)	
Others	32 (11.6)	23 (8.1)	55 (8.0)	

[1]Average value was calculated on five-point Likert scales. Mean ± SD
[2]n (%)
[3]p values were analyzed by Student t-test

Table 10 Effects on willingness to purchase taurine-containing B jelly

Dependent variable	Independent variables	B	S.E.	β	t	p[1]	VIF
Willingness to purchase	(Constant)	−0.236	0.182		−1.297	0.195	
	Degree of interest	0.119	0.034	0.122	3.470	0.001**	1.267
	Image familiarity	0.022	0.033	0.022	0.660	0.510	1.118
	Overall preference	0.203	0.025	0.288	7.979	0.000***	1.336
	Recommendation intention	0.511	0.047	0.417	10.848	0.000***	0.658
F = 117.733 (p = 0.000), R^2 = 0.459, adj R^2 = 0.455, Durbin-Watson = 1.062							

[1]p values were analyzed by multiple regression analysis. ***$p < 0.001$

3.6 Effects on Willingness to Purchase Taurine-Containing B Jelly

Table 10 presents the result of multiple regression analysis. Variables that were positively correlated with effects on willingness to purchase taurine-containing B jelly were interested in taurine-containing jelly (t = 3.470, $p < 0.01$), overall preference (t = 7.979, $p < 0.001$), and recommendation intention (t = 10.848, $p < 0.001$). In addition, in the order of recommendation intention (β = 0.417), overall preference (β = 0.288), and degree of interest (β = 0.122), the association of the effect on willingness to purchase the taurine-containing B jelly was higher.

4 Discussion

The present study suggested directions to increase willingness to purchase taurine-containing jelly among Korean college students. Consideration in buying taurine-containing jelly depended on taste, price, capacity, and efficacy in that order. In surveys on the selection criteria for purchasing snacks by college students (Lee et al. 2010) and consideration for selecting processed foods by adolescents (Song and Choi 2013), it was found that taste was a top priority when purchasing products. As a consideration in purchasing a functional bakery product by adults, taste, functionality, and price were considered most important, giving priority to taste over functionality

(Kim and Lee 2004). The addition of taurine to jellies is a great value factor for these products, but the taste of the product is a major factor in product selection. Therefore, the development of taurine-containing jelly should be done with an emphasis on taste.

The variables that positively affected the willingness to purchase the taurine-containing B jelly were interested in taurine-containing jelly, overall preference, and recommendation intention. Also, knowledge of taurine was related to interest in taurine-containing jelly. In this study, just as the degree of interest in taurine-containing jelly affects willingness to purchase, consumers who are more interested in the national certification mark when purchasing products have an effect on willingness to purchase (Kim and Kwon 2015), and in the case of health functional food, it was reported that the higher the interest in functionality and health, the higher the willingness to purchase (Yu and Kim 2016). In other words, in order to increase the willingness to purchase taurine-supplemented jelly among college students, it is necessary to focus on marketing to increase the recommendation intention and interest in the taurine-containing jelly and to improve overall preferences. The higher the satisfaction with taste and quality, the higher the purchase intention in college students' selection of home convenience food-related products (Kim 2018). It was reported that the higher the recommendation intention, the higher the purchase intention not only in food but also in fashion and cosmetics (Choi et al. 2018; Kim et al. 2016; Park 2017). Therefore, it is necessary to increase the degree of interest and to improve knowledge about taurine in order to improve willingness to purchase taurine-containing jelly in Korean college students.

5 Conclusion

Taurine is a nutrient that college students require because it plays a role in recovering from fatigue, protecting eye-sight, and stabilizing nerves. In order to improve the willingness to purchase taurine-containing jelly by college students, price competitiveness is important, but above all, it is necessary to increase the degree of interest, preference, and recommendation intention based on taurine-containing jelly with improved taste and efficacy. In particular, since the subjects who have some knowledge of taurine were more interested in taurine-containing jelly, it is advisable to promote nutritional education on taurine, in order to stimulate interest in taurine-containing jelly.

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Positive Eusocial Impacts on Ants by Taurine Derivatives

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Keywords

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

Abbreviations

CDI	Cooperative defense index
GT	Galactose-taurine
XT	Xylose taurine

1 Introduction

Eusociality is one of the highly advanced social systems in the Kingdom Animalia. The eusocial characteristics evolved over 3 million years ago after continuous modifications of social behaviors (Gadau et al. 2012). Although it is rare in Hymenoptera, such as bees and wasps, the vast majority of ant species show a form of eusociality (Wilson and Holldobler 2005). One of the distinctive features for eusociality is a division of labor between reproductive and non-reproductive castes (Hunt 2012). A eusocial colony has a unique ranking system which is maintained under a strict social order. Queens and reproductive males are the only breeding members, whereas most offspring assume the roles of workers or soldiers who amass food, raise the progenies, or protect the colony (Simola et al. 2013). Eusocial ants have a high level of interaction among peers and are highly collaborative with other members to ensure the success of their community (Thorne and Traniello 2003; Wilson and Nowak 2014). Eusocial ants defend their colonies by subduing invaders and alert nest mates to rich food sources (Ferguson et al. 2021). Soldiers and workers are interdependent on the overall growth, solidarity, and proliferation of the colony for their own survival (Linksvayer and Wade 2005; Norman and Hughes 2016).

Taurine or 2-aminoethanesulfonic acid harbors sulfonic acid and amino functions and is naturally derived from cysteine. Taurine has many essential biological roles in antioxidation, osmoregulation, membrane stabilization, and modulation of calcium signaling (Schaffer and Kim 2018). Taurine is an essential nutritional component, particularly at the stages of development, which plays an important role in muscle maturation. Taurine is widely found in animal organs and accounts for 0.1% of total body weight of humans (Wen et al. 2019). It protects

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the brain from various impairments, alleviates withdrawal syndromes, and enhances visual function. Taurine is essential for maintaining cardiovascular, skeletal muscle, and central nervous systems as well as development and function of the retina.

Ants have an unusual amount of taurine compared to other insects (Kim and Lee 2018). Ants are typically eusocial insects and referred to as true social insects with complex and diverse biological traits. The taurine concentration in ants is comparable to those found in the heart of cows (*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 been fully elucidated; however, taurine or antioxidants may play a major functional role in reducing stress in the ant community (Schneider et al. 2016; Kamhi and Traniello 2013).

Recent studies show that taurine derivatives are highly effective in stabilizing biomembranes, scavenging reactive oxygen species (ROS), and reducing the peroxidation of unsaturated membrane lipids compared to taurine (Kim et al. 2021, 2017; Jo et al. 2019). Xylose-taurine (XT) exerts more cytoprotective effect against oxidative stress in cultured hepatocytes, thus improving cell viability and reducing ROS production (Jo et al. 2019). XT may be a bio-agent for the therapy of hepatic diseases. XT regulates the level of expression for Bcl-2/Bax and caspase-3, allowing exposed cells to cope against apoptosis.

Galactose-taurine (GT), another taurine derivative, is considered a very effective antioxidant with special anti-inflammatory activity. GT effectively reduces the productions of nitric oxide, reactive oxygen species, and cell death in zebrafish embryos (Kim et al. 2017). GT diminishes the degree of inflammation and improves the survival rate of LPS-treated zebrafish embryos. GT modulates the expression of proinflammatory factors, such as TNF-α, IL-6, inducible NOS, and cyclooxygenase 2.

The purpose of the present study is to characterize the effect of taurine and a pair of its prime derivatives with antioxidant properties, XT and GT, on eusocial ants. This study employed Japanese carpenter ants (*Camponotus japonicas*) to investigate the potential effect of taurine, GT, and XT on eusociality. Ants were fed with taurine-supplemented diets whose effects were tested according to eusocial indexes. The effect of taurine, XT, and GT as potential eusocial enhancers was analyzed using four classes of criteria: number of chamber unit, survivorship under exposure to excessive light and high temperature, cooperative defense index, and population size.

2 Methods

2.1 Taurine Content Measurement

Taurine and its derivatives (GT and XT) were provided by Dong-A Pharmaceutical Company and Konkuk University (Chemistry Department), respectively. After culture with taurine, XT, or GT for 3 days, three ants were homogenized and sonicated in saline to prepare samples. Taurine content was determined using Taurine Assay Kit (Abcam, Cambridge, UK) which utilizes the ability of an enzyme (taurine dioxygenase) to split taurine into sulfite and aminoacetaldehyde. The resulting sulfite was quantitated by measuring optical density of a colorimetric probe at 415 nm using a microplate reader (R&D Systems Inc.).

2.2 Maintenance of Ant Farms

Ant stocks (*Camponotus japonicas*) were purchased from Biobiba Ants (Daegu, Korea). They were maintained in transparent PET bottles containing autoclaved soil. Ant farms were assembled in a 2 l PET bottle where an empty 500 ml PET bottle was positioned upside down. To accommodate the 500 ml bottle, the larger bottle was cut open horizontally then sealed using transparent tape after the smaller bottle was inserted. The gap

between the two bottles was packed with autoclaved soil; ants were introduced afterward.

Openings were made for ventilation by chopping the outer covering with a scalpel. Soil was moisturized through the ventilation slits twice a week. Ants were raised primarily with 1 mM sucrose solution. The ant farms were normally kept in the dark at 15 °C unless ants were subjected to stress conditions for light exposure or high temperature. Separate groups of ants were fed a non-taurine or taurine diet. The effects of taurine and its derivatives were analyzed according to the ant eusocial indexes. Taurine diets were created using 0.1 mM taurine in sucrose solution. Four indexes of eusocial strength were used to investigate the impact of taurine on group activity: number of nest chamber unit, survival at stressful conditions (light exposure or high temperature), cooperative defense efforts, and population size (or ant number and composition).

2.3 Monitoring Newly Formed Chamber Units

One queen and 20 workers were introduced into the ant farms to initiate one unit of colonies. The colonies were maintained for 30 days and fed with a mixture of taurine and sucrose at the concentrations above. The chambers were monitored daily, and newly formed unit of chamber was marked with a color pen. The cumulative number of chamber unit was recorded every 2 days.

2.4 Growing Under Stressful Conditions

Ants were grown under two stressful conditions: long exposure to light and high temperature at 35 °C. Different from normal conditions, ants were subjected to a 14/10-h light/dark cycle. Additional group of ants were raised in a plant growth chamber whose temperature was set at 35 °C to evaluate resistance at high temperature. Survivorship was evaluated every other day for each experimental plan.

2.5 Evaluation of Cooperative Defense Efforts

The extent of cooperative defense effort was evaluated based upon the time leading to establish a defense formation to cope with a foreign queen. A queen was carefully picked up from another group and added to an established farm using a pair of forceps to prompt a defensive response. Soldiers or workers were then allowed to respond to the incoming queen. We measured the consumption of time which was required for several workers to tug each leg of the queen. The time taken in hour [T_H] was transformed into reciprocals to achieve the cooperative defense index (CDI). The CDI was obtained using the formula of CDI = $1/[T_H]$.

2.6 Comparison of Population Mass

The number of ants and eggs in each group was counted along with the composition of the group at the termination of the experiment. Thirty days after implanting ants into the farm, the whole ant farm was discharged into a wide tray, and the soil was carefully removed with a pair of tweezers to count the ants in each class. The number of ants was evaluated between taurine-free and taurine-supplemented groups.

3 Results

To understand a potential effect of taurine or its derivative on the eusociality of ant, four eusocial strength indices were measured in the study: measurement of formicaries formed, survivorship at extreme conditions, CDI, and group size and composition.

3.1 Taurine Content Measurement

After ants were fed with taurine or its derivatives, taurine content in ants was determined using taurine assay kit. Figure 1 shows internal taurine

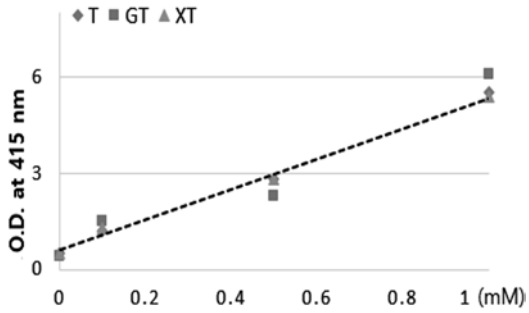

Fig. 1 Measurement of internal taurine content after feeding taurine and derivatives. After ants were fed with taurine or taurine derivatives, internal taurine content was measured as described in Sect. 2. Taurine content was determined by measuring the optical density (O.D.) at 415 nm. The optical density data concurred with the amount of dietary taurine or derivatives, XT or GT

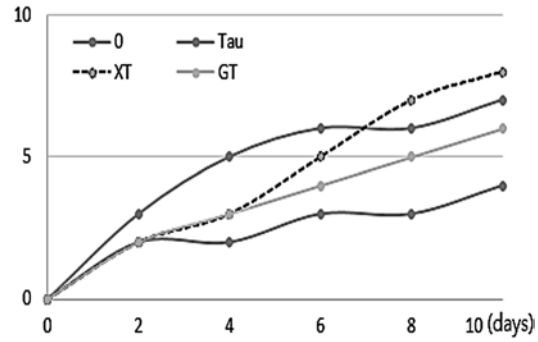

Fig. 2 Comparison of chamber units formed. The formicary chamber construction was monitored, while ants were maintained on different taurine diets. The number of chambers was counted every 2 days during a 10-day period from the placement into the ant farm. The numbers refer to the total count of chambers which were observed on the day of counting. The *x*-axis refers to days after transplantation and *y*-axis, # of chambers. Error bars were omitted for clarification ($n = 3$)

content after nurturing with taurine diet. Ants shows an internal taurine content which concurred with the external supply of taurine or GT or XT.

3.2 Number of Chamber Units Formed

Ants began to assemble formicaries as early as 2 days after 1 queen and 20 ants were introduced into the ant farms. The number of formicary units in the different taurine concentrations was counted every 2 days. Figure 2 shows the cumulative number of chamber units up to 10 days after introduction into the ant farms. The number of chamber units increased after taurine was added in the diet. This indicated that taurine or its derivative might stimulate ants to build more chambers and/or that more dynamic activity, stimulated by taurine or its derivative, might result in a higher number of chamber units.

3.3 Survival at Severe Conditions

Ants were subjected to excessive light or high-temperature condition. Figure 3a shows survivorship under exposure to light. When exposed to light conditions daily for 14 h, ants appeared to respond negatively. Increasing cases of death were evident. The extent of fatality increased as ants were subjected longer to the light conditions. However, the taurine-supplemented group showed a higher survivorship in the presence of excessive light.

Ants were also grown at 35 °C. As in the light exposure experiments, ants responded negatively to high temperature. Fatality increased when ants were grown at the applied temperature. Addition of taurine or its derivative in the diet, however, appeared to exert a positive outcome. Taurine supplementation evidently decreased mortality under the high-temperature condition. Figure 3b shows that taurine enabled ants to survive the high temperature. Particularly with XT, ants survived better under the high-temperature condition.

3.4 Cooperative Defense Index (CDI)

After a foreign queen was introduced into the ant colony, the effect of different taurine diets on the effort by workers to protect the colony was monitored. The defensive response began as early as 5 min after the introduction of a foreign queen. The cooperative defense index (CDI) was

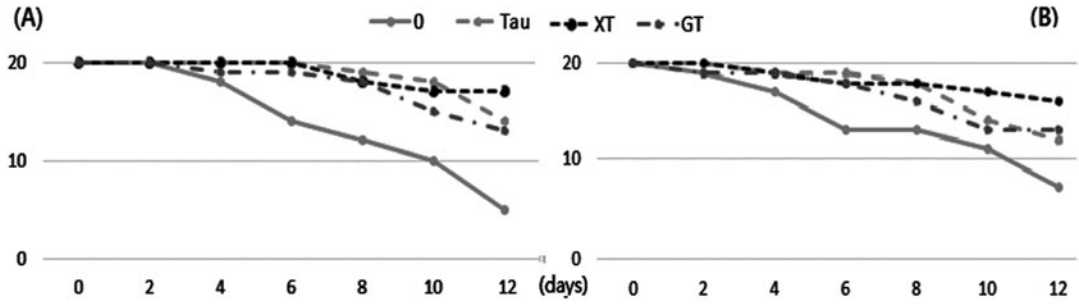

Fig. 3 Survivorship under stressful conditions. Ants were subjected to two different stressful conditions for 12 days. Their survival was monitored every other day. (**a**) Ants were grown under exposure to strong light intensity, a 14/10 h light/dark cycle. (**b**) Ants were grown at 35 °C, and their survivorship was monitored every other day. Error bars were omitted for clarification ($n = 4$)

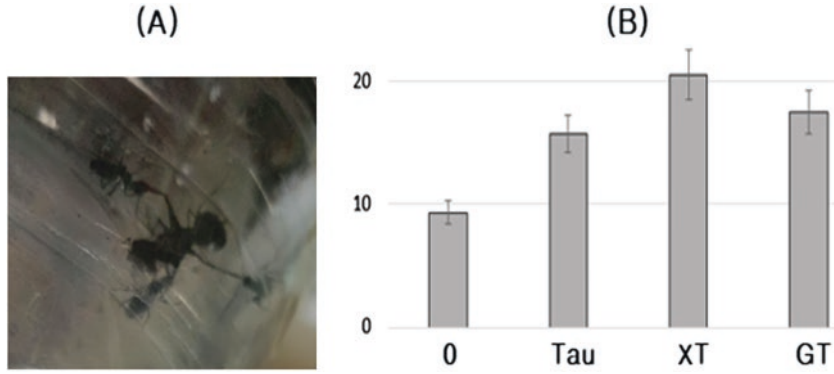

Fig. 4 Cooperative defense (CD) effort. Defensive response was observed immediately after introduction of a foreign queen. The level of cooperative defense effort was quantitated based on the level of readiness against a newly planted foreign queen ant. (**a**) Typical image of CD. (**b**) Cooperative defense index (CDI) of groups treated with taurine or taurine derivatives. CDI = $1/[T_H]$ (H T_H: hours taken for completion of the defensive form against a foreign queen). Error bars represent standard deviation ($n = 3$)

obtained by calculating the reciprocal of the time leading to conclusion of the defensive response, which means at least three ants holding each leg of the foreign queen. We compared the time (in hours) required to observe a defensive formation between the taurine-free and taurine-supplemented groups. Figure 4 shows that a substantial difference was evident in the time to complete the defensive formation among the groups. Taurine-supplemented groups displayed a quicker response than the control. The taurine groups subsequently had a higher CDI. That is, the taurine-supplemented groups showed a higher defensive willingness against the imbedded foreign queen. The highest values were found in the group which was on the XT-supplemented diet. Since successful defensive efforts are essential to sustain a community, taurine and its derivatives may play a significant role in guarding their own eusocial community.

3.5 Population Size and Composition

Ant colonies were initiated using 1 queen and 20 workers and maintained for 30 days. The population size and composition were compared among the ant groups at the end of the experiment. There was a substantial difference among the groups.

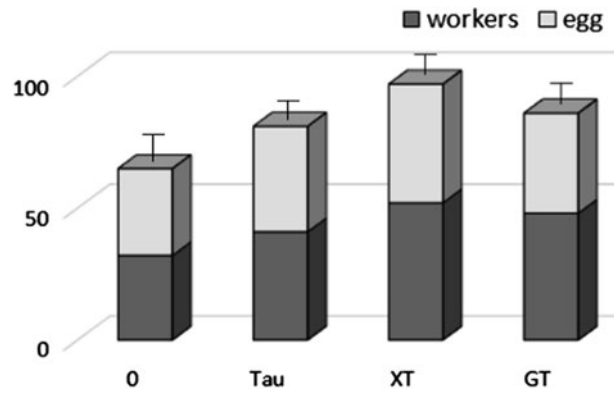

Fig. 5 Population size and composition. Ant farms were maintained on taurine-free or taurine-supplemented diet for 30 days. At the termination of the experiment, the ant groups were evaluated according to the total number and colony composition of the workers and eggs, except for the queen. Error bars represent standard deviation of the mean ($n = 4$)

Figure 5 shows the population size and composition of each group at the termination of the experiment. The taurine-supplemented groups contained more workers and eggs than did the non-taurine control group. A substantial difference was apparent between the non-taurine and taurine groups. There was an obvious difference in the number of workers between the taurine, XT, and GT groups, suggesting the effect was also dependent on the derivative of taurine. A significant difference was also evident in the number of eggs between the taurine-free and taurine-supplemented groups.

4 Discussion

In the present study, taurine and its derivatives steadily strengthened ant workers in boosting their eusocial performance. In the taurine-supplemented groups, ants built more resident chamber units following treatments with taurine or its derivatives. They also enabled ants to react rapidly to an alien queen by reducing the time taken to accommodate cooperative defense establishments. Taurine and its derivatives increased the fecundity of the queen ant and the contribution of workers to the care of the young. Undeniably, the positive effects of taurine on all four indices of group activity are significant in supporting the eusocial community.

Eusociality is a highly advanced system and a relatively recent progress in this group of insects during the course of evolution (Nowak et al. 2010; Ratnieks and Helantera 2009). The evolution of eusociality requires an escalation in complexity. The continual interaction between biological organization and natural selection results in a unique system requiring compulsory collaboration between individuals (Opachaloemphan et al. 2018). The eusocial system is very well-organized for the survival of a group; however, it can be very demanding for eusocial members who must concede their opportunity for reproduction and conform to the mission of the colony (Rehan and Toth 2015; Purcell et al. 2014). Most members of an ant colony must provide continual labor without any immediate benefit. Under circumstances, the unusual amount of taurine in Formicidae may contribute to relieve the stress among members (Nishikawa et al. 2012).

Xylose-taurine significantly induced metabolic factors and increased the activities of hepatic antioxidant enzymes, catalase, glutathione peroxidase, and superoxide dismutase in mammals. Considering XT exhibits powerful reducing power and free radical scavenging

activities, the higher performance by XT-fed ants shows it also performed a similar effect in insects. Especially, its antioxidant effect greatly enables ants to overcome the harsh conditions as eusocial members.

Our results for population size and composition were surprising. The augmented population of ants is a strong indicator of the fecundity by queens. Since queens are the only reproductive members in the ant community, her fertility and capability to care for the progenies reveal the inclusive sustainability of the community. Taurine may have applied constructive effects equally on the queen and workers in producing and caring for offspring in spite of restricted means in the farms. The increased fecundity, however, may indicate that a stress level went up due to taurine treatment. The enhanced level of CDI and survivorship under harsh conditions strongly support a role for taurine and its derivatives in eusocial performance.

Despite extensive reports concerning mammals, no report is available on the effects of taurine in insects. Taurine is abundant in ants (Hymenoptera: Formicidae) and can account for up to 0.65% of dry matter (Spitze et al. 2003). This amount in ants even surpasses that of mollusks and many oceanic fishes. This is unexpected, since taurine content does not exceed 0.1% in most insects. Despite its extremely high content in ants, the actual function of taurine in ants is still unknown. However, individual members within a eusocial ant community may encounter a high level of pressure and stress, which taurine may help them overcome, thereby ensuring their membership.

Taurine might have reinforced relevant behavior during the course of evolution leading to eusociality in ants. For instance, taurine may lessen stress from mental and physical efforts to conform with a structure dominated by a queen. In addition, ants, especially workers, perform their allocated roles ceaselessly. This would be unbearable without taurine, which is a powerful antioxidant. Our results suggest that ants may utilize taurine to diminish stress related to membership in a eusocial community. Ants were highly reactive to taurine reinforcement under eased stress or pressure situations. Ants receiving a taurine-supplemented diet built more residence chamber units. Also, ant fecundity and population size are affected in response to different taurine derivatives. Workers answered in a shorter time to the infiltration of a foreign queen when their food was reinforced with taurine or its derivatives.

Using quantitative indices of eusociality, the potential effect of taurine and its derivatives on eusocial organisms was characterized in the present study. Antioxidants may be indispensable biomolecules which help overcome social stress and in handling aggressive interactions as eusocial members, which include insects and higher organisms (Schneider et al. 2016). The results in the present study strongly point out that taurine and its derivatives play an important role in elevating antioxidant level in individual ants and eventually in maintaining ant communities as a eusocial superorganism. There is a difference in terms of eusocial effect among taurine and its derivatives, with XT being the most effective. Future study is needed to define whether taurine effectively helps social organisms increase their eusocial strength.

5 Conclusion

The eusociality apparently leveled up in ant groups when fed with diets containing taurine or its derivative. Ants built more formicary units in response to taurine supplementation, especially to that of XT. Taurine apparently prompted ants to participate readily in a cooperative defense formation. XT helped ants to endure a pair of stress conditions: excessive light and temperature. Among taurine derivatives, XT augmented the fecundity of queen ants and the care of young by workers. This study may expand the scope of understanding in the eusociality and the impact of taurine and its derivatives on maintaining the level of eusociality.

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Part VIII

Effect of Taurine Against Toxic Agents

Taurine Prevents AFB1-Induced Renal Injury by Inhibiting Oxidative Stress and Apoptosis

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Keywords

Taurine · Aflatoxin B1 · Renal injury · Antioxidant ability · Apoptosis

Abbreviations

AFB1	Aflatoxin B1
BUN	Blood urea nitrogen
CAT	Catalase
CRE	Blood creatinine
cys-c	Cystatin
GCLC	Glutamate cysteine ligase catalytic
HO-1	Heme oxygenase-1
NQO1	Nicotinamide adenine dinucleotide phosphate-quinine oxidoreductase 1
SOD	Superoxide dismutase
T-AOC	Total antioxidant capacity
UA	Blood uric acid
γ-GCS	γ-glutamylcysteine synthetase

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1 Introduction

The kidney is an important excretion organ of the body. Many prototypes or metabolites of drugs and poisons are excreted from the body through the urine (Radi 2019). Aflatoxin is the most toxic mycotoxin to date, with aflatoxin B1 (AFB1) being the most toxic aflatoxin. It is very easy to contaminate and pose a threat to the sanitation of animal feed (Xue et al. 2019). The kidney participates in the metabolism of AFB1, which remains in the kidney, causing injury (Rasooly et al. 2013). AFB1 mediates apoptosis of hepatocytes by generating ROS and activating the oxidative stress pathway, leading to excessive accumulation of ROS, an imbalance in the cellular antioxidant defensive system, and cell apoptosis (Chen et al. 2019). Other scholars have found that AFB1 induces exogenous and endogenous apoptosis. AFB1 expresses high levels of hepatocyte death receptors through the exogenous pathway, leading to several liver diseases, including liver cirrhosis, cholestasis, viral hepatitis, and hepatocellular carcinoma (Wang 2014).

Taurine, which is a β-sulfur-containing amino acid with many important functions, is the most abundant amino acid in most tissues and cells of the body. It is widely distributed in various animals, but the synthesis of taurine in the body is limited to the liver, although in humans the main source of taurine is from food intake (Huxtable 1992). A large number of studies have confirmed

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that taurine has a variety of physiological functions, including diminished generation of oxygen free radicals, regulation of osmotic pressure, maintenance of cell calcium homeostasis, reduction in peroxidative damage, and maintenance of membrane stability and regulation of energy metabolism (Das et al. 2009, 2011; Lou et al. 2018). Taurine has preventive and protective effects on STZ-induced diabetic nephropathy (Zhang et al. 2020) and diminishes renal ischemia-reperfusion injury (Cavdar et al. 2017).

2 Methods

2.1 Animal Treatment

The temperature in the feeding room of SPF rats was controlled at 22 °C ± 2 °C, drinking and eating freely. After a week of adaptive feeding, rats were randomly divided into control group (C), taurine control group (T), DMSO control group (DMSO), model group (M), 1% taurine treatment group (M + T1), 2% taurine treatment group (M + T2), and 4% taurine treatment group (M + T3) and were raised in separate cages. AFB1 was dissolved in DMSO to prepare the required concentration. Urine, serum, and kidney tissue were collected on the 40 d.

2.2 Measurement of Renal Index and Histological Observation

The kidney was weighed by analytical balance, and the kidney index was calculated according to the formula (kidney weight/body weight). The ipsilateral kidney was fixed in formalin solution for 24 h. After treatment with gradient ethanol and xylene, it was immersed in paraffin for routine paraffin embedding and sectioning and finally stained with hematoxylin and eosin. The histological changes of kidney were observed by microscope.

2.3 Detection of Renal Injury-Related Indicators

The commercial kit purchased from Nanjing Jiancheng Institute of Biological Engineering is used to determine the blood urea nitrogen (BUN), blood creatinine (CRE), blood uric acid (UA), and cystatin (Cys-c) in serum. The specific steps were according to the protocols.

2.4 Antioxidant Indexes Analysis

Superoxide dismutase (SOD), total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), succinate dehydrogenase (SDH), and malonaldehyde (MDA) were assayed with commercial kits (Beyotime, Nantong, China) according to the protocols.

2.5 Total RNA Extraction and Quantitative RT-PCR

The extraction of total RNA from kidney tissue was done by using TRIzol reagent (TaKaRa, Dalian, Japan). Then, a RT-PCR kit (TaKaRa, Dalian, China) was used to synthesize cDNA from the extracted total RNA (1 μg). For standardization, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the housekeeping gene. The reaction system consisted of 400 nM primers, 2 μl cDNA template, and 10 μl TB Green Premix Ex Taq (TaKaRa, Dalian, China). Use the IQ5 real-time quantitative PCR (qRT-PCR) machine (BioRad, Hercules, USA) to perform the reaction as follows: preheat at 95 °C for 1 min, and then perform 40 amplification cycles (30 s at 95 °C, 50 °C for 1 min), then at 72 °C for 30 s, and then at 72 °C for 5 min. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of the primers were as follows:

Nrf2
F:TAGATCTTGGGGTAAGTCGAGA
R:CTCTTGTCTCTCCTTTTCGAGT

SOD1
F: ATTCCATCATTGGCCGTACTAT
R: CTCAGACCACATAGGGAATGT

NQO1
F: GTTGAGTCATCTCTGGCGTATA
R: TATTCTGGAAAGGACCCTTGTC

HO-1
F: GAAGAGGAGATAGAGCGAAACA
R: CAATCTTCTTCAGGACCTGACC

GCLC
F: CTGAACTCTGCCAGCTATTAGA
R: ATTTGACAGAGCTCTTGGTGTA

Bcl-2
F: GGGATGCCTTTGTGGAACTATA
R: CTTTTGCATATTTGTTTGGGGC

Bax
F: TTTTTGCTACAGGGTTTCATCC
R: CCAGTTCATCGCCAATTCG

Bak1
F: TCTATGGTCACAAAGTCTCGAG
R: CAGTTTGAAGACAAAGATCCGG

Cyt-c
F: CATGGTCTGTTTGGGCGGAA
R: GGGTATCCTCTCCCCAGGTG

Apaf1
F: TCTTTAGTTGGTGCGCTTTTAC
R: ATGGAGAGGTCTGTGTAGTAGT

Caspase-3
F: GATCCCGTGTATTGTGTCAATG
R: CTGACAGTTTTCTCATTTGGCA

Caspase-9
F: TGCCTCATTTTTCTCCCAAAAG
R: CAGTTCGAAATCCTTGCTTCAC

β-actin
F: ATCCTGACCGAGCGTGGCTAC
R: AGGAAGAGGATGCGGCAGTGG

2.6 Cell Apoptosis Analysis

The paraffin-embedded tissue blocks were sectioned and treated with xylene and ethanol gradient. Perform the TUNEL test according to the manufacturer's kit (Servicebio, Wuhan, China) instructions to detect apoptosis.

2.7 Statistical Analysis

All data from the assays were analyzed by ANOVA in SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and are expressed as the mean ± SEM. A *P*-value of <0.05 was considered significant.

3 Results

3.1 Taurine Inhibits AFB1-Induced Renal Injury in Rats

The results of renal index of rats in different groups are shown in Fig. 1. Compared with group C, the renal index of group M was sig-

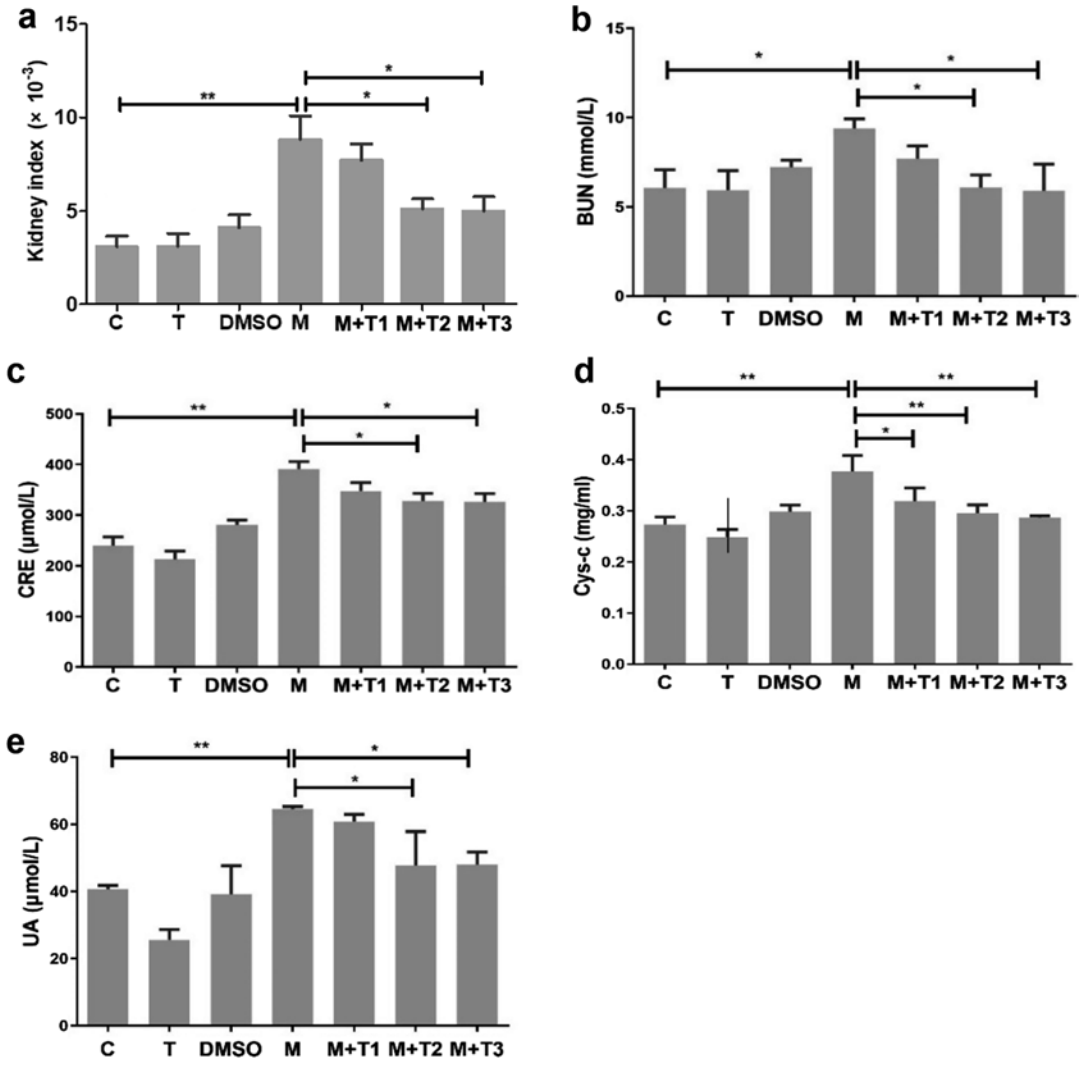

Fig. 1 Effect of taurine feeding on the renal injury in rats. (**a**) Kidney index. (**b**) BUN content. (**c**) CRE content. (**d**) Cys-c content. (**e**) UA content. The results are presented as means ± SEM ($n = 5$). *$P < 0.05$: the difference between groups is significant. **$P < 0.01$: the difference between groups is extremely significant

nificantly increased ($P < 0.01$). The renal index of M + T2 and M + T3 groups compared with group M was significantly decreased ($P < 0.05$). The levels of Bun, CRE, UA, and Cys-c were significantly higher in group M than in group C ($P < 0.05$). In contrast to group M, the content of Bun, CRE, UA, and Cys-c was lower in the M + T2 and M + T3 groups ($P < 0.05$). As shown in Fig. 2, taurine attenuates the damage of AFB-1 to the structural integrity of renal cells. The experimental results suggest that taurine prevents renal injury induced by AFB1 in rats.

3.2 Taurine Improves the Antioxidant Capacity of Renal Cells

In order to explore the antioxidant capacity of taurine, SOD, T-AOC, CAT, GSH, GSH-Px, SDH, and MDA content of renal cells were

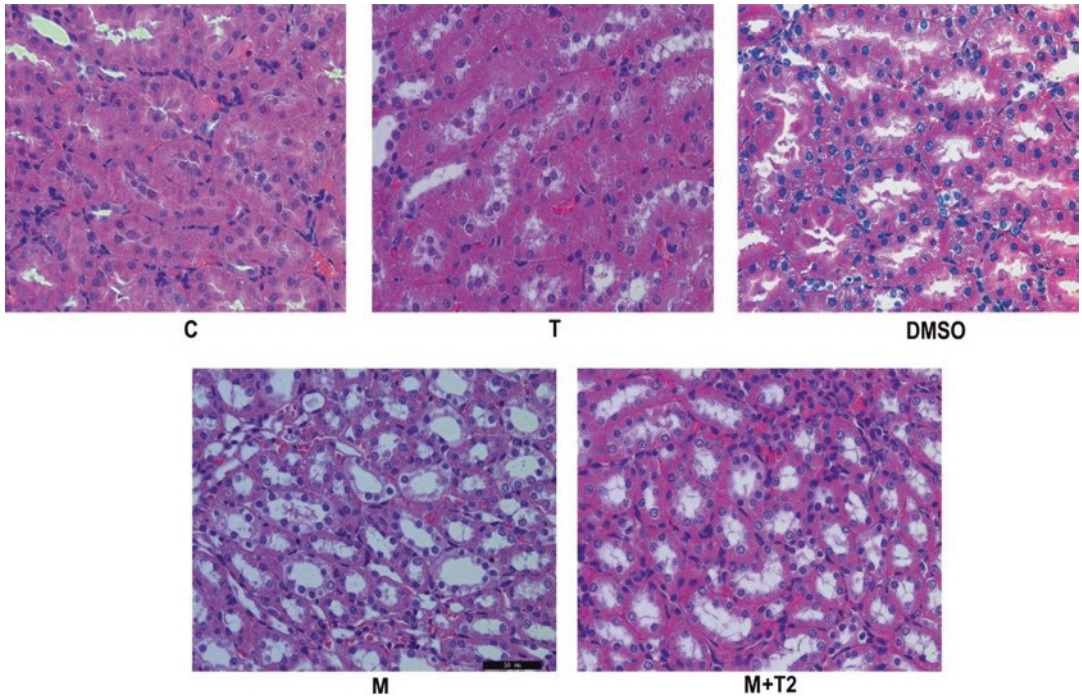

Fig. 2 Effect of taurine feeding on the structural integrity of renal cells

examined. As shown in Fig. 3, the content of SOD, T-AOC, CAT, GSH, GSH-Px, and SDH was significantly reduced in group M compared with group C ($P < 0.01$). Compared with group M, SOD, T-AOC, CAT, GSH, GSH-Px, and SDH content in the M + T2 and M + T3 groups was significantly increased ($P < 0.05$). By contrast, the MDA content of group M was significantly elevated relative to that of the M + T2 and M + T3 groups ($P < 0.05$). Secondly, as shown in Fig. 4, the mRNA levels of oxidative stress regulatory genes Nrf2, SOD, NQO1, HO-1, and GCLC in renal cells were elevated in group M compared with those of group C ($P < 0.05$). Also, compared with the mRNA levels of Nrf2, SOD1, NQO1, HO-1, and GCLC in group M, the levels in group M + T2 were significantly elevated ($P < 0.05$). These results indicate that taurine elevates the antioxidant capacity of renal cells.

3.3 Taurine Inhibits Renal Cell Apoptosis Induced by AFB1

In order to explore the inhibitory effect of taurine on renal cell apoptosis, we used the TUNEL method to detect the apoptotic rate, and the qPCR method was used to detect the mRNA expression of the relevant apoptotic factors. As shown in Fig. 5, the results of the apoptotic rates showed that it was significantly higher in group M than in group C. In contrast to group M, taurine decreased the apoptotic rates significantly in group M + T2 ($P < 0.01$) (Fig. 5).

As shown in Fig. 6, compared with group C, the mRNA expression of Bax, Bak-1, Apaf-1, caspase-9, and caspase-3 in group M was significantly increased ($P < 0.05$). The mRNA expression of Bcl-2 in group M was significantly decreased than that of group C ($P < 0.05$). In contrast to group M, taurine reversed the apoptotic trends of these apoptotic factors significantly in group M + T2 ($P < 0.01$) (Fig. 6) These data suggest that taurine is involved in the regulation of renal cell apoptosis.

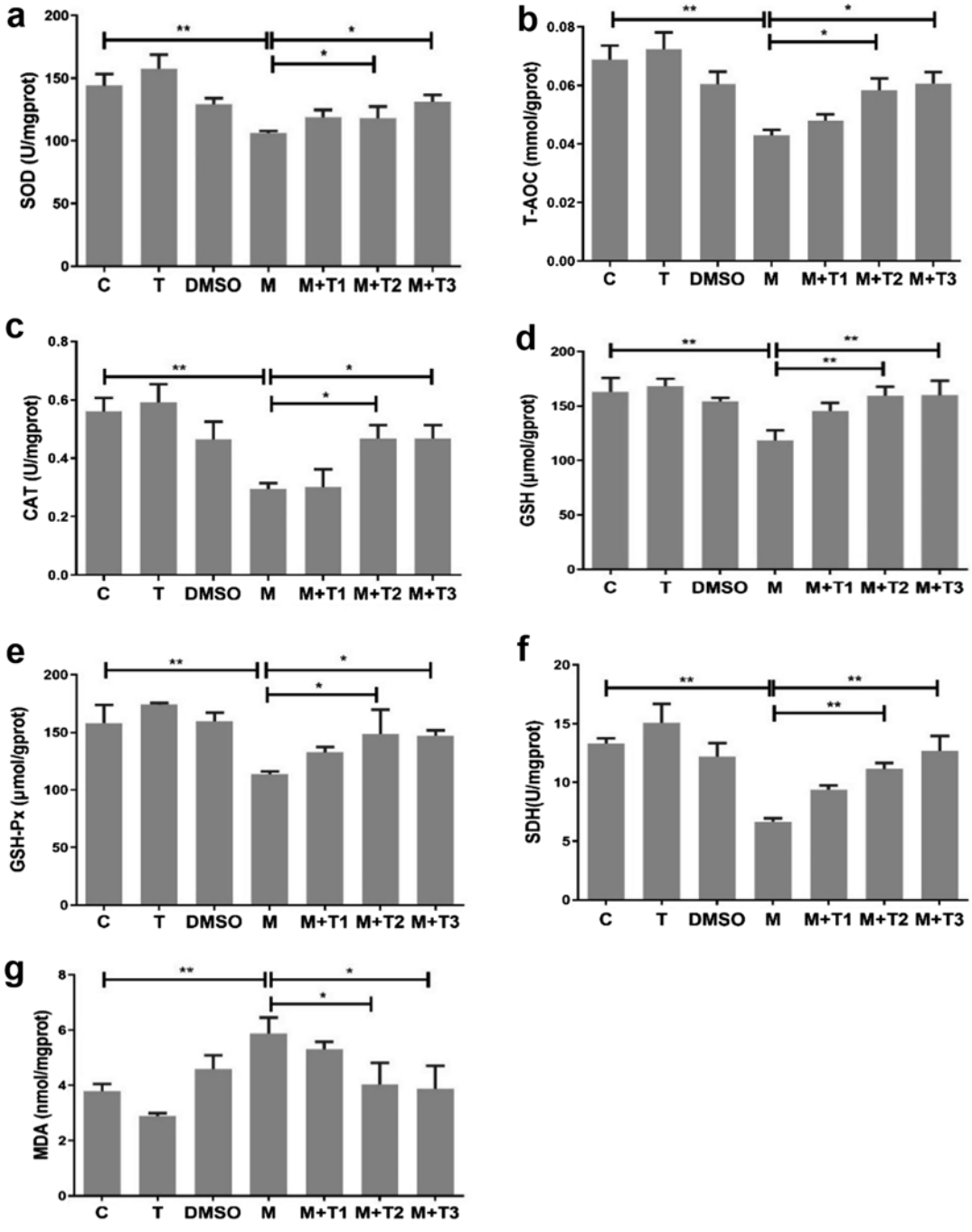

Fig. 3 Effect of taurine feeding on the antioxidant ability of rat liver. (**a**) SOD. (**b**) T-AOC. (**c**) CAT. (**d**) GSH. (**e**) GSH-Px. (**f**) SDH. (**g**) MDA. The results are presented as means ± SEM ($n = 5$). *$P < 0.05$: the difference between groups is significant. **$P < 0.01$: the difference between groups is extremely significant

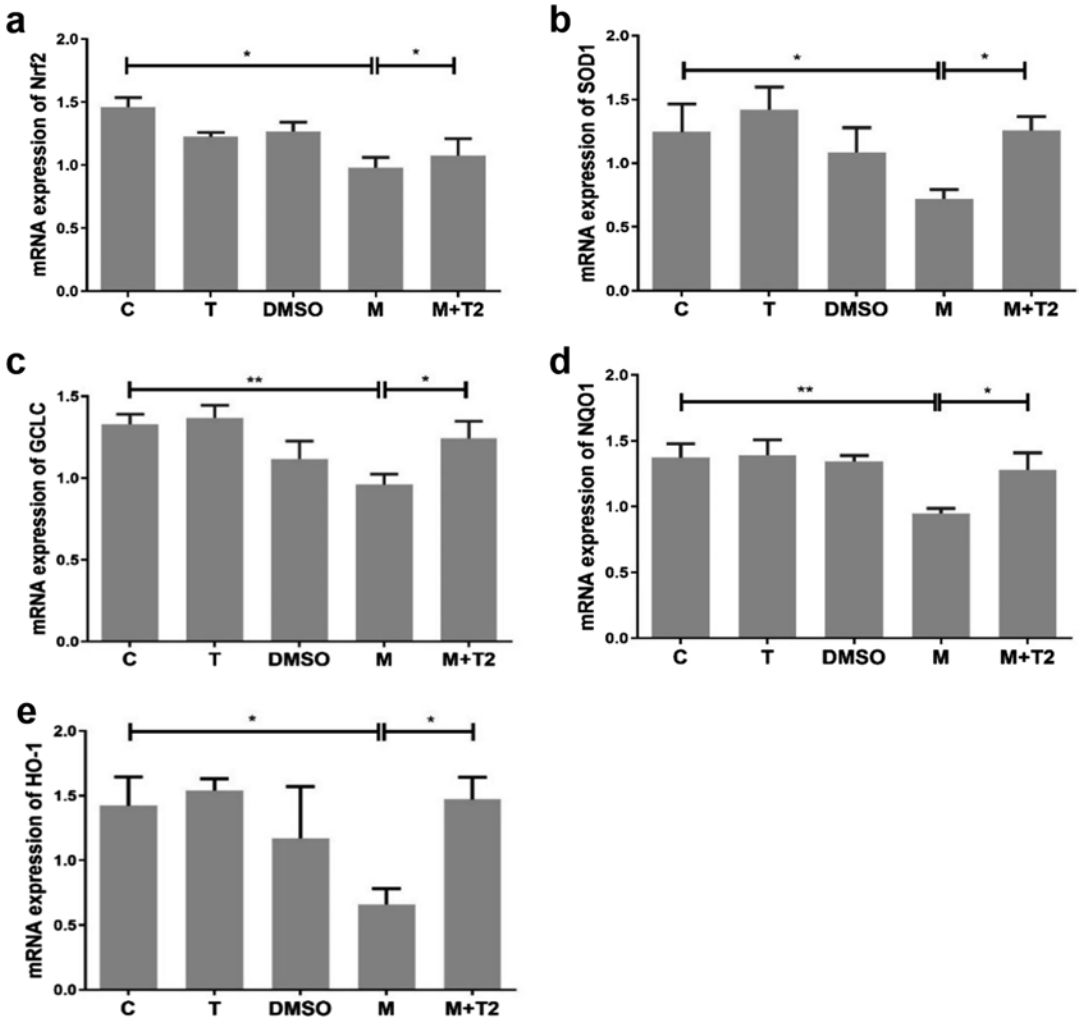

Fig. 4 Effect of taurine feeding on the mRNA levels of oxidative factors in rat liver. (**a**) Nrf2. (**b**) SOD1. (**c**) GCLC. (**d**) NQO1. (**e**) HO-1. The results are presented as means ± SEM ($n = 5$). *$P < 0.05$: the difference between groups is significant. **$P < 0.01$: the difference between groups is extremely significant

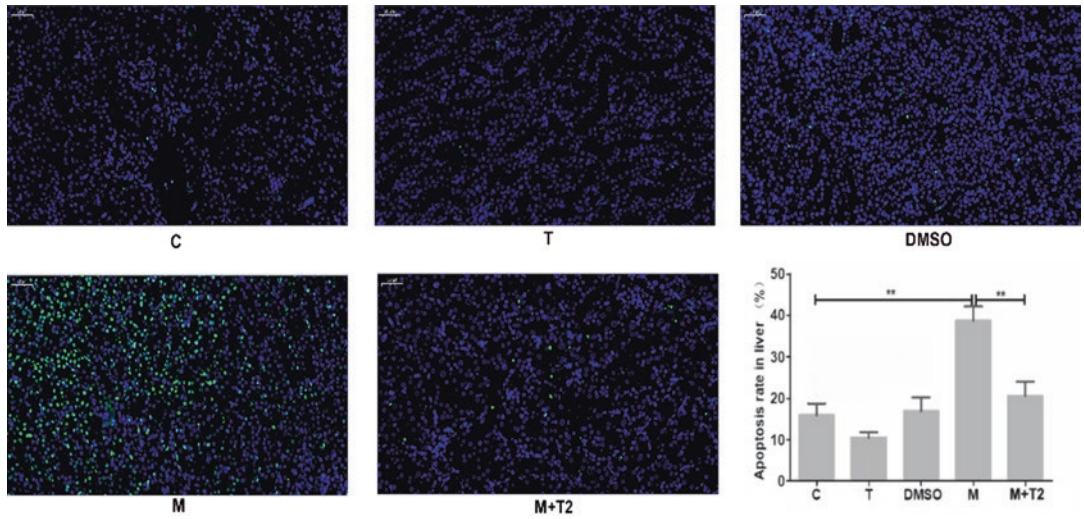

Fig. 5 Effect of taurine feeding on apoptosis of renal cells. The results are presented as means ± SEM ($n = 5$). *$P < 0.05$: the difference between groups is significant. **$P < 0.01$: the difference between groups is extremely significant

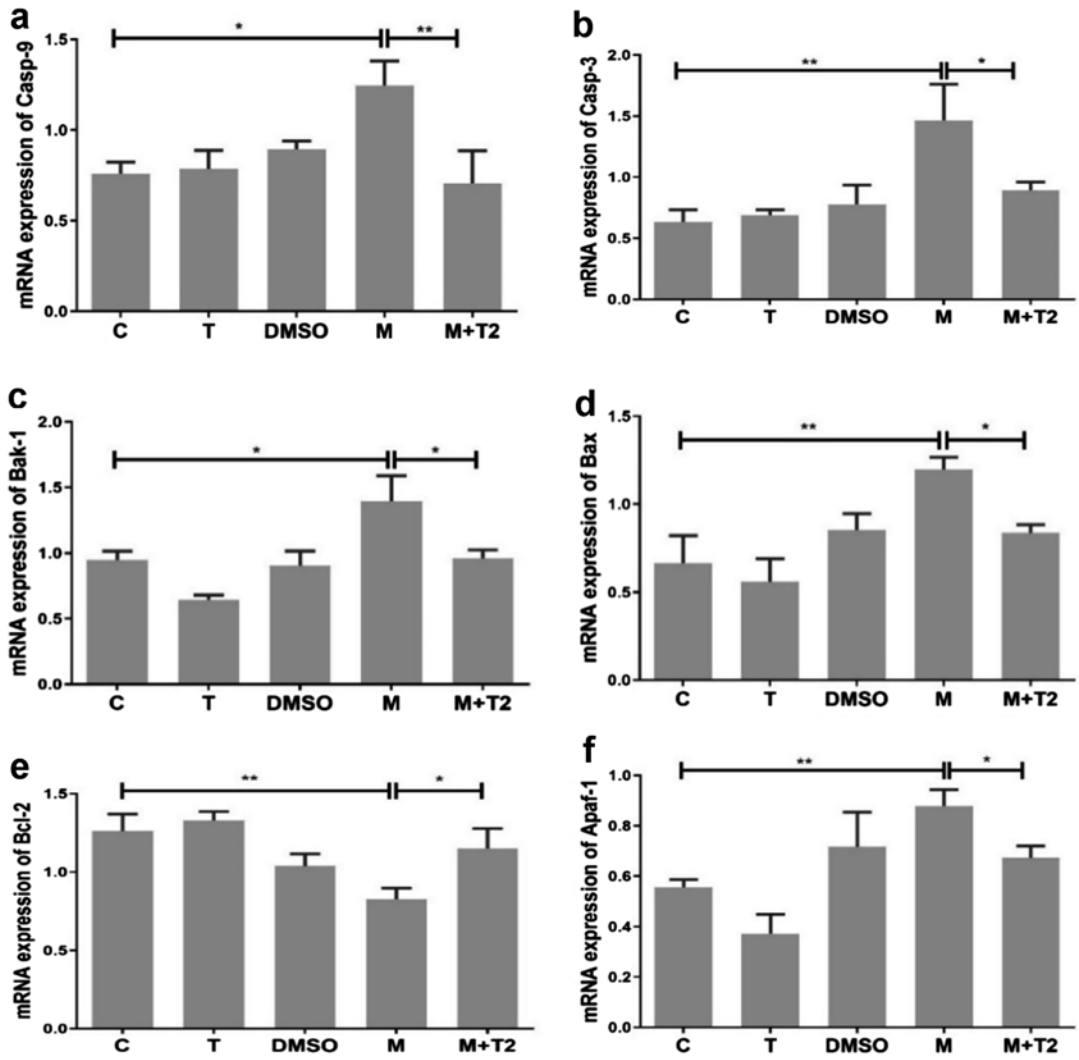

Fig. 6 Effect of taurine feeding on apoptotic factors of renal cells. (**a**) Caspase-9 (Casp-9). (**b**) Caspase-3 (Casp-3). (**c**) Bak-1. (**d**) Bax. (**e**) Bcl-2. (**f**) Apaf-1. The results are presented as means ± SEM (n = 5). *P < 0.05: the difference between groups is significant. **P < 0.01: the difference between groups is extremely significant

4 Discussion

Aflatoxin B1 (AFB1) is the most toxic mycotoxin among the aflatoxins (Murcia and Diaz 2020). The bifuran ring and the coumarin-like moiety of its structure are closely related in toxicity and carcinogenicity (Marin et al. 2013). In both animal models and humans, AFB1 can cause kidney injury (Karabacak et al. 2015; Díaz de León-Martínez et al. 2019). One study reported that AFB1 can cause kidney injury that is detected by the renal index, biochemical indicators, and pathological staining (Li et al. 2019). In the present study, we found that taurine attenuates AFB1-induced kidney injury, presumably by decreasing Bun, CRE, UA, and Cys-c content. According to Adedara et al. (2019) taurine reduces cisplatin-induced renal injury in rats (Kato et al. 2017). Taurine can also reduce kidney damage in hypertensive rats mediated by L-NAME (Adedara et al. 2019).

Many studies have shown that AFB1 can induce oxidative stress in multiple tissues and organs

(Zhou et al. 2019; Huang et al. 2019b). After treatment with aflatoxin B, the antioxidant activity of liver and kidney cells in rats was reduced (Abdel-Wahhab et al. 2020). AFB1 also induces kidney and heart damage while increasing oxidative stress in rats (Yilmaz et al. 2018). In addition, aflatoxin B1 can induce nephrotoxicity and oxidative stress in rat kidney cells (Abdel-Hamid and Firgany 2015). The present results show that taurine significantly reduces oxidative stress induced by AFB1 in kidney cells, an effect associated with increased mRNA expression of SOD, NQO1, HO-1, and GCLC. A bottleneck in mitochondrial electron transport can result in the transfer of electrons from the respiratory chain to oxygen, forming superoxide, which leads to a decrease in respiratory chain activity and an increase in oxidative stress. Taurine can regulate mitochondria by improving this process and exert its antioxidant effect (Jong et al. 2012). Taurine supplements also protect kidney damage triggered by carbon tetrachloride, which is associated with reduced oxidative stress and regulation of mitochondrial function (Heidari et al. 2019). Supplementing taurine in the regular diet also diminishes alloxan-induced diabetic kidney damage in rats, perhaps by altering signal pathways related to oxidative stress and apoptosis. Taurine can reduce kidney oxidative damage and abnormal thyroid function in rats exposed to fluoride for a prolonged period of time (Adedara et al. 2017).

For healthy living bodies, many studies have found that the intake of AFB1 is closely related to the occurrence of apoptosis (Wang et al. 2017; Huang et al. 2019a). AFB1 in the diet of broiler chickens can cause kidney cell apoptosis. In vitro experiments using porcine kidney 15 cells (PK-15), AFB1 induces ROS generation and apoptosis (Lei et al. 2013). Our results indicate that taurine inhibits kidney cell apoptosis caused by AFB1. Previous studies have shown that taurine can reduce OTA-induced nephrotoxicity and renal cell apoptosis (Liu et al. 2020). Taurine treatment of mice significantly reduces apoptotic-related proteins (p53, phosphorylated p53, caspase 9, and caspase 3) in an acute kidney injury model induced by Adriamycin (Kim et al. 2017). Taurine exerts its potential therapeutic activity by preventing oxidative stress and apoptosis of renal tubular epithelial cells induced by oxidized LDL (Chang et al. 2014).

5 Conclusion

In conclusion, our results suggest that taurine can prevent AFB1-induced renal injury by acting as an antioxidant and anti-apoptotic agent.

Acknowledgments In conclusion, our results suggest that taurine can prevent AFB1-induced renal injury by acting as an antioxidant and anti-apoptotic agent.

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Taurine-Derived Compounds Produce Anxiolytic Effects in Rats Following Developmental Lead Exposure

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Keywords

Developmental lead (Pb2+) exposure · Lead poisoning · Taurine derivatives · Anxiety-like behaviors · Anxiolytic drugs · GABAergic system

Abbreviations

Pb^{2+}	lead
TD	taurine derivatives
E/I	excitation-to-inhibition
BLL	blood lead levels
GAD	glutamic acid decarboxylase
PND	postnatal day
EDTA	ethylenediaminetetraacetic acid
ASV	anodic stripping voltammetry
OF	open field
EPM	elevated plus maze
OTC	open-to-closed ratio

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1 Introduction

Environmental lead (Pb^{2+}) poisoning since antiquity (Hernberg 2000; Taylor et al. 2011) has remained an elusive public health problem in many developed and underdeveloped countries (Shinkuma and Huong 2009; Chen et al. 2011; Eneh and Agunwamba 2011; Laidlaw and Taylor 2011; Yang et al. 2013). Further, Pb^{2+} is a well-established neurotoxicant that causes significant damage to the developing brain with consequences that persist across the lifespan and may further serve as an epigenetic modifier of senescence (Eid and Zawia 2016). Globally, children and young adolescents who reside or work near toxic exposure sites are at elevated risk for developmental neuropathologies (Tandon et al. 2001; Hu et al. 2007; Huo et al. 2007; Meyer et al. 2008; Zheng et al. 2008; Leung et al. 2008; Guo et al. 2010; Chen et al. 2011; Mehrpour et al. 2012; Yan et al. 2013; Caravanos et al. 2016; Dowling et al. 2016; Johnston and Hricko 2017; O'Connor et al. 2020; Zhang et al. 2020). Clinical reports have cogently and carefully established the relationship between Pb^{2+} serving as both an environmental contaminant at high- and low-level exposures and a central nervous system (CNS) neurotoxicant, specifically at low-level exposures (Bellinger 2008; Lanphear et al. 2000, 2005). Lower levels of Pb^{2+} exposures are desirable for both the environment and humans, but what remains concerning is that low-level Pb^{2+} exposures can cause a wide range of neuropathologies in the developing brains of children. Thus, low-level Pb^{2+} exposures in the environment can significantly impact children's intellectual and behavioral functioning at multiple stages of neurodevelopment (i.e., *in utero*, during critical periods from birth through the first few years of postnatal life, and have persistent effects across the lifespan). Unfortunately, waiting until 2 years of age to conduct blood lead level (BLL) screening is too late and not done consistently to be useful in the early detection of a child with Pb^{2+} poisoning (Neuwirth 2018). Unfortunately, this situation increases the likelihood that many children will not undergo BLL screenings, and if Pb^{2+} poisoned, they may not ever be evaluated or diagnosed.

Moreover, these Pb^{2+}-induced neurodevelopmental insults have been associated with reduced cognitive abilities and the potential for academic achievement (Bellinger et al. 1992; Bellinger 2008; Lanphear et al. 2000, 2005; Lidsky and Schneider 2003; Zhang et al. 2013; Sorensen et al. 2018) suggesting that primary prevention is the only solution (Rosen and Mushak 2001). Further, the populations most identified to reside near toxic waste sites and face lower academic achievement rates are often underrepresented minority or low socially and economically disadvantaged populations (Rau et al. 2015; Neuwirth 2018). These disparities as they relate to at-risk populations have been further exacerbated by COVID-19 (Dang et al. 2021). These persistent public health and economic problems caused by Pb^{2+} poisoning that have deleterious effects on children have brought to light the need for critical evaluation of fronto-executive functions. A growing number of reports in the field suggest that Pb^{2+} poisoning should be classified in the *Diagnostic and Statistical Manual for Mental Disorders 5th Ed.* (*DSM-5*) as a neurodevelopmental disorder of fronto-executive function (for review, see Neuwirth et al. 2020).

Consistent with the scientific evidence of neurotoxicant Pb^{2+}-exposures, in the USA, the rationale for lowering environmental Pb^{2+} exposure action levels (i.e., from 5 μg/dL to 2 μg/dL) remains a contentious debate (Gilbert and Weiss 2006). However, there is consensus for lowering these Pb^{2+} exposures, but ensuring that the sources for such exposures can be reduced or will not grow larger over time remains the conundrum for the federal and state governments to resolve. The agreement is that no environmental Pb^{2+} level should be deemed "safe" for children (Bellinger and Dietrich 1994; Barbosa Jr et al. 2005; Lidsky and Schneider 2003, 2006). In the absence of a clear environmental resolution to this antiquated problem (Hernberg 2000; Taylor et al. 2011), low-level Pb^{2+} exposure remains a modern challenge to reduce and/or eliminate neurodevelopmental Pb^{2+} exposures in children (Grandjean and Hertz 2015). Consistent with this framework for lowering environmental Pb^{2+} exposures, neurotoxicology studies have established a curvilinear relationship between Pb^{2+}

exposures and the resultant brain damage (Needleman and Gatsonis 1990; Schwartz 1994; Bellinger and Needleman 2003; Canfield et al. 2003; Lidsky and Schneider 2006). High-levels of Pb^{2+} exposures cause a range of neuropathological and neuropsychological syndromes with varied behavioral and cognitive symptoms (Wong et al. 1991; Bellinger and Dietrich 1994; Schütz et al. 1996; Bleecker et al. 1997; Kreig Jr et al. 2005; Lidsky and Schneider 2006; Bergdhal and Skerfving 2008; Rentschler et al. 2012). Notably, both low- and high-level Pb^{2+} exposures are equally detrimental to the child's neurodevelopment (Needleman 2004). Moreover, children that absorb Pb^{2+} from their environment exhibit an inverse relationship between elevated BLLs and reduced intelligence quotient (IQ) (Needleman and Gatsonis 1990; Schwartz 1994; Bellinger and Needleman 2003; Canfield et al. 2003; Lidsky and Schneider 2003, 2006). Thus, when children's circulating BLLs continue to elevate or remain elevated from source exposures, their intellectual and behavioral functions decrease. Chelation therapy remains an effective treatment for children with high-level Pb^{2+} exposures (*i.e.,* > 39 μg/dL), yet it is an inappropriate therapy for lower levels of Pb^{2+} poisoning (Rogan et al. 2001; Dietrich et al. 2004). Therefore, new therapeutic approaches are warranted for children that continue to face low-level Pb^{2+} exposures in the 21st century.

The present study sought to build upon prior reports (Neuwirth et al. 2019a) that evaluated the effects of acute taurine and taurine derivatives as a neuropsychopharmacotherapeutic treatment (43 mg/Kg i.p. 15 minutes prior to behavioral testing) in ameliorating anxiety-like behaviors induced by neurodevelopmental Pb^{2+} exposure. The rationale for this work followed the research showing that Pb^{2+} exposures can induce excitation/inhibition (E/I) imbalances resulting in learning and memory deficits that were recovered by acute taurine treatment through the $GABA\text{-}_{AR}$ system (Neuwirth 2014, 2018; Neuwirth et al. 2017, 2019a, b). When the brain's E/I balancing between the glutamatergic (*i.e.,* excitatory) and GABAergic (*i.e.,* inhibitory) systems is perturbed in early neurodevelopment, it has been identified as a neurodevelopmental risk factor for seizure and other neuropathologies (Ben-Ari 2002; Ben-Ari et al. 2012). Taurine has been shown to protect against hippocampal E/I imbalances in animal models of epilepsy (El Idrissi et al. 2003; El Idrissi & L'Amoreaux, 2008) through upregulating *glutamic acid decarboxylase* (GAD) as well as through interactions with the $GABA\text{-}_{AR}$ β2/β3 subunits (L'Amoreaux et al. 2010). Moreover, taurine provides neuroprotection by maintaining GABAergic tone during senescence when the E/I balance begins to weaken in an age-dependent manner (El Idrissi et al. 2013). This is consistent with taurine's ability to maintain, support, and/or facilitate improvement in learning (El Idrissi 2008; Neuwirth et al. 2013) and motor abilities (Santora et al. 2013) of aged animals. Therefore, the studies investigating the expression of cognitive traits in Pb^{2+}-exposed rodent models through behaviors regulated by the GABAergic system may prove useful for developing novel pharmacotherapeutics in treating Pb^{2+} poisoning (Neuwirth and Emenike 2021).

Using that specific framework and to further expand upon the report of Neuwirth et al. (2019a), the present study evaluated the effects of developmental Pb^{2+} exposure on locomotor activity and anxiety-like behaviors that are partially regulated by the GABAergic system. Consistent with previous reports (Neuwirth 2014; Neuwirth et al. 2017, 2019a, b), the present study evaluated whether the acute administration of taurine and taurine derivatives would recover the sex-dependent Pb^{2+}-induced neurobehavioral aberrations in the rat model to provide the field with new information on developing novel pharmacotherapeutics with the goal of addressing Pb^{2+} poisoning in children (Neuwirth and Emenike 2021).

2 Methods

2.1 Subjects

In accordance with the SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans Norwegian hooded male (N = 40) and female rats (N = 80) (Taconic, N.J.) were paired for breeding, and their male F1 generation was used for future experimentation. This breeding

paradigm was conducted over a 5-year time period for this study. Whenever possible, rat litters were culled to 8–10 pups to control for maternal socialization that might influence neurodevelopment and its associated behavioral outcomes. Rats were fed *ad libitum* with Purina rat chow (RHM1000 # 5P07). Notably, Control rats were provided regular water, while the Pb^{2+}-exposed rats were provided 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 Pb^{2+} developmental exposure model). At PND 22, Pb^{2+} exposures ceased, and all rats returned to a regular water regimen. Rats that were assigned to the Peri-22 150 ppm group (drank a lead acetate water of [363.83 μM]) and the Peri-22 1,000 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 the open field test and 24 h later the elevated plus maze test.

2.2 Blood Pb^{2+}-Level Analyses

Blood samples were collected and analyzed using the same methods as prior reports (Neuwirth 2014; Neuwirth et al. 2017, 2018, 2019a, b, c; Neuwirth and El Idrissi 2021). Briefly, a separate cohort of (n = 4) male and (n = 4) female pups at PND 22 were randomly selected and sacrificed from the Peri-22 150 ppm and the Peri-22 1,000 ppm treatment groups. The blood samples were collected within 2 mL anticoagulant *ethylenediaminetetraacetic acid* (EDTA) coated syringes (Sardstedt, Germany), mixed to prevent coagulation and temporarily stored at -80 °C. Blood samples were analyzed using a commercial ESA LeadCare II Blood Lead Analyzer system (Magellan Diagnostics, North Billerica, MA) through electrochemical anodic stripping voltammetry (ASV). 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 inserting 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 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 The Open Field Test

Behaviorally naivë rats were tested between PND days 36–45 from the F1 generation offspring (N = 432) comprised of both males (n = 216) and females (n = 216) were subjected to an open field test (OF). The treatment groups were as follows: Control males (n = 72), Peri-22 150 ppm Pb^{2+} males (n = 72), and Peri-22 1,000 ppm Pb^{2+} males (n = 72) and Control females (n = 72), Peri-22 150 ppm Pb^{2+} females (n = 72), and Peri-22 1,000 ppm Pb^{2+} females (n = 72), 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 differences as a consequence of sex and disruptions as a function 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 of locomotor activity, rats were assigned to an elevated plus maze (EPM) to assess anxiety-like behaviors. The (n = 72) male and (n = 72) female rats from each *Treatment* group 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),

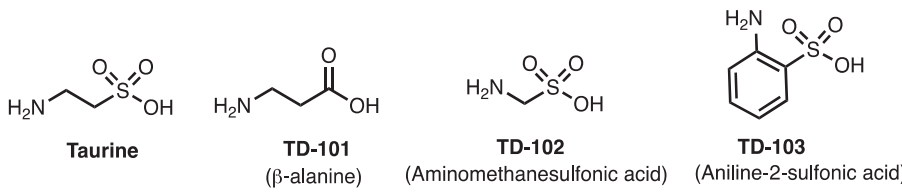

Fig. 1 Comparisons of the chemical structure for taurine and taurine derivatives (TD), TD-101, TD-102, and TD-103. (Neuwirth et al. 2019a)

or TD-103 ($C_6H_7NO_3S$ 173.19 g/mol), consistent with prior studies (Neuwirth et al., 2019a; Fig. 1). This resulted in a (n = 12) sample size for each *Drug Treatment* condition for each *Sex* per *Treatment* group. All taurine and TD compounds were dissolved in physiological buffered saline (PBS) with a pH of 7.4. This resulted in a final systemic taurine and TD concentration of [10 mM]. Prior to i.p. administration, the saline, taurine, and TDs were sterilized by syringe filtration (0.2 μm).

Rats were administered their randomly assigned *Drug Treatment* as a triple-blind procedure via i.p. injection 15 min prior to EPM testing. All drugs were administered as equivalent 43 mg/kg drug injections (*i.e.,* to standardize against Taurine as a reference) across all treatments to draw appropriate comparative outcomes consistent with prior reports (Neuwirth et al. 2019a). Following the 15 min post-injection period, the rats were examined during 10 min of anxiety-like behavioral assessments in the EPM. The EPM apparatus (external dimensions: 800.1 mm H × 1,104.9 mm W × 1,104.9 mm L; closed arm dimensions: 101.6 mm W × 1,104.9 mm L × 304.8 mm H walls; open arm dimensions: 101.6 mm W × 1,104.9 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 EPM was used to evaluate anxiety-like behaviors as a function of *Sex*, *Treatment*, and *Drug Treatment* following neurodevelopmental Pb^{2+} exposure. The anxiety-like behavioral variables included the *Open-to-Closed (OTC) Ratio* and the *Open Arm Time Mobile (sec)*.

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 1,280 × 720 pixels and 5MP 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 1,600 MHZ (4 GB × 2), 1 TB 7,200 PRM Hard Drive, and a 4th Generation Intel® Core™ i3-4170 Processor (3 M Cache, 3.70 GHz). The video was then displayed through a Dell 20" E2016H monitor with an optimal resolution of 1,600 × 900 pixels at 60 Hz. Data were recorded as digital videos that were post-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 (i.e., 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 t-test was used to compare *Sex* differences in the Control rats for the *Total Distance Traveled (m)*

and *Overall Average Speed (sec)*. Next, an *ANOVA* was used to assess *Treatment* effects for both male and female rats for the *Traveled (m)* and *Overall Average Speed (sec)* in the OF. For the EPM tests, a *t*-test was used to compare *Sex* differences in the Control rats for the *OTC* and the *Open Arm Time Mobile (sec)*. Next, an *ANOVA* was used to assess *Treatment* and *Drug Treatment* effects for both male and female rats for the *OTC* and the *Open Arm Time Mobile (sec)* in the EPM. The criteria for significance were set at $\alpha = 0.05\%$ with a 95% confidence interval ± 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 *effect sizes* where applicable.

3 Results

3.1 BLLs as a Function of Sex and Pb^{2+} Exposure Prior to Behavioral Testing

The separate cohort of rats that were used to determine the BLLs for the Peri-22 150 ppm and the Peri-22 1,000 ppm treatment groups revealed no sex-dependent differences and was less than the 39 µg/dL chelation therapy limit. Each Pb^{2+} treatment at the time of sample collection resulted in BLLs ranging from 4.5 to 12.6 µg/dL (SD ± 1.93) for Peri-22 150 ppm rats ($p < 0.001$***) and from 10.2 to 20.4 µg/dL (SD ± 2.97) for Peri-22 1,000 ppm ($p < 0.001$***), respectively. The Control rats were Pb^{2+} negative. Thus, the BLL samples obtained in this study were less than 3.33 ug/dL. The BLL samples from the behaviorally tested rats were also drawn at PND 55 following the conclusion of the study. However, their BLLs were below the 3.3 µg/dL detection limit consistent with reports from the US Agency for Toxic Substances and Disease Registry (U.S. Department of Health and Human Services 2007) that Pb^{2+} is not uniformly distributed in the bone, blood, and soft mineralizing tissues.

3.2 Developmental Pb^{2+} Exposure-Induced Sex-Based Differences in Locomotor Activity

The OF was used to assess any differences in locomotor activity as a function of *Sex* and *Treatment* (i.e., Pb^{2+}-exposure) to understand how it might influence carryover or order effects within the subsequent EPM test that followed. The data revealed that there were sex-dependent differences between the Control male and female rats for the *Total Distance Travelled (m)* $t_{(142)} = -3.882$, $p < 0.001$*** (Fig. 2a) as well as the *Overall Average Speed (m/sec)* $t_{(142)} = -4.482$, $p < 0.001$*** (Fig. 2b).

Following the sex-dependent 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 1,000 ppm Pb^{2+} exposures. For the OF, the male locomotor activity data revealed that there were sex-dependent differences between *Treatment* for the *Total Distance Traveled (m)* $F_{(2)} = 6.020$, $p < 0.01$**, $\eta_p^2 = 0.165$ (Fig. 3a) as well as the *Overall Average Speed (m/sec)* $F_{(2)} = 11.506$, $p < 0.001$***, $\eta_p^2 = 0.277$ (Fig. 3b).

In contrast, the female OF locomotor activity data revealed that there were sex-dependent differences between *Treatment* for the *Total Distance Traveled (m)* $F_{(2)} = 6.056$, $p < 0.05$*, $\eta_p^2 = 0.121$ (Fig. 4a) and the *Overall Average Speed (m/sec)* $F_{(2)} = 6.091$, $p < 0.001$***, $\eta_p^2 = 0.121$ (Fig. 4b).

3.3 Developmental Pb^{2+} Exposure-Induced Sex-Based Differences in Anxiogenic Behaviors that Were Recovered by Taurine and Taurine Derivative Anxiolytic Drug Treatments

Twenty-four hours following the OF, rats were subjected to the EPM to compare the within-sex differences in response to both developmental

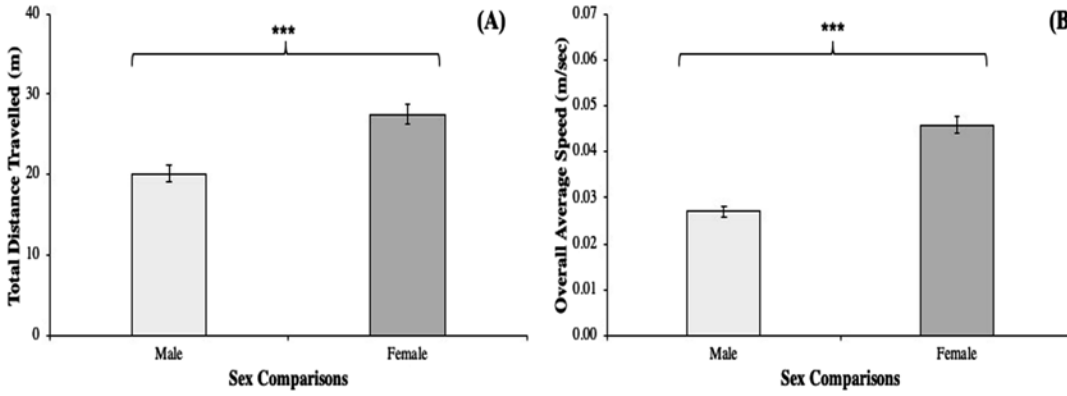

Fig. 2 Assessment of rat's locomotor activity in the OF as an effect of *Sex* (males = white bars; females = gray bars). Data for both rat's *Total Distance Traveled (m; $p < 0.001$***;* (**a**) and *Overall Average Speed (m/s; $p < 0.001$***;* (**b**) were significantly different in OF locomotor activity as a function of *Sex*. Data are presented as the mean ± SEM

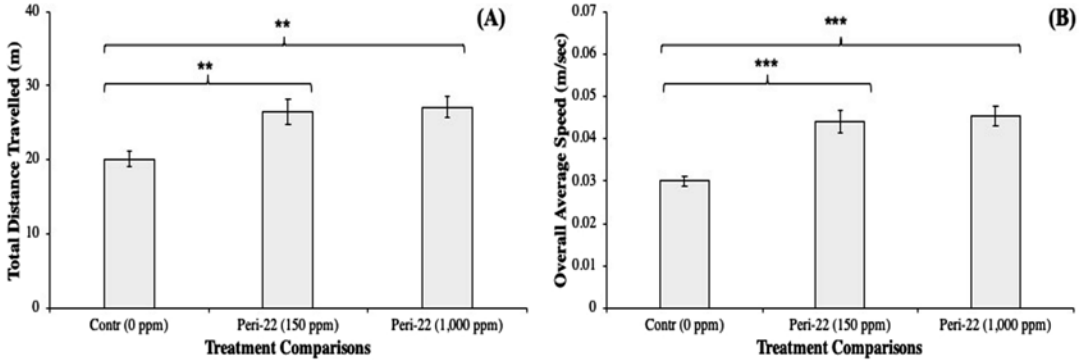

Fig. 3 Assessment of rat's locomotor activity in the OF as an effect of *Treatment* (0 ppm, 150 ppm, and 1,000 ppm). Data for both the male rat's *Total Distance Traveled (m; $p < 0.01$**;* (**a**) and *Overall Average Speed (m/s; $p < 0.001$***;* (**b**) were significantly different in OF locomotor activity as a function of *Treatment*. Data are presented as the mean ± SEM

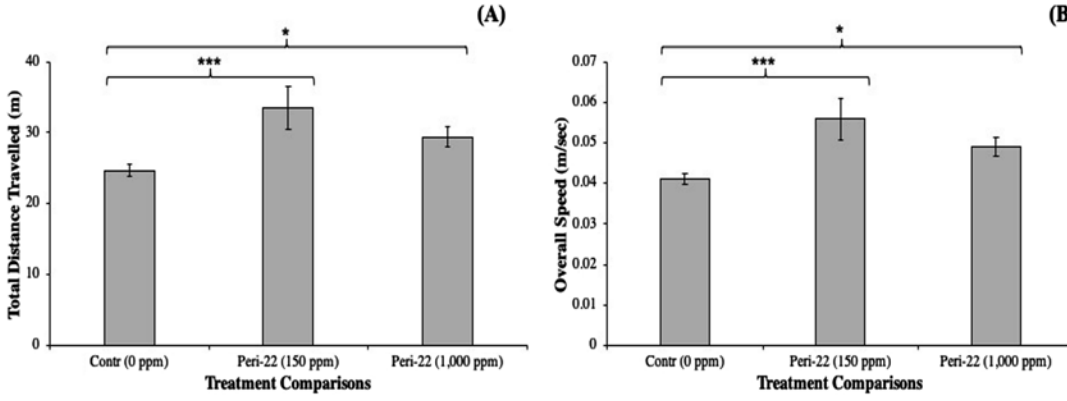

Fig. 4 Assessment of rat's locomotor activity in the OF as an effect of *Treatment* (0 ppm, 150 ppm, and 1,000 ppm). Data for both the female rat's *Total Distance Traveled (m; $p < 0.05$*;* (**a**) and *Overall Average Speed (m/s; $p < 0.001$***;* (**b**) were significantly different in OF locomotor activity as a function of *Treatment*. Data are presented as the mean ± SEM

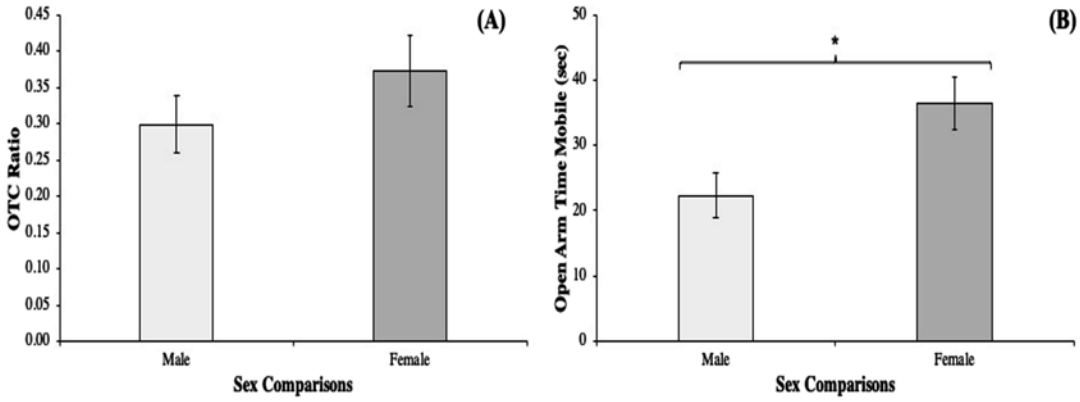

Fig. 5 Assessment of rat's anxiety-like behavioral activity in the EPM as an effect of *Sex*. Data for both the rat's *Total Distance Traveled* revealed no sex-dependent differences (**a**). Alternatively, the *Overall Average Speed (m/s; $p < 0.05$**; (**b**) was significantly different in OF locomotor activity as a function of *Sex*. Data are presented as the mean ± SEM

Pb^{2+} exposure as a function of *Treatment* and *Drug Treatment Condition* effects on the *OTC ratio* and the *Open Arm Time Mobile (sec)*. In comparing the Control male and female rats, the data revealed no significant effect of *Sex* for the *OTC ratio* $t_{(22)} = -1.050$, $p = 0.299$ (Fig. 5a), but there was a significant *Sex* effect of *Open Arm Time Mobile (sec)* $t_{(22)} = -2.182$, $p < 0.05$* (Fig. 5b).

In response to Pb^{2+} exposure, the male rat's *OTC ratio* data revealed that there were no significant effects of *Treatment* $F_{(2)} = 0.757$, $p = 0.471$ nor a significant *Treatment X Drug* interaction $F_{(2,5)} = 1.014$, $p = 0.436$ (Fig. 6a). However, for the male rat's *OTC ratio*, there was a significant effect of *Drug* $F_{(5)} = 2.498$, $p < 0.05$*, $\eta_p^2 = 0.092$ (Fig. 6a). Additionally, for the male rat's *Open Arm Time Mobile (sec)* data, there were no significant effects of *Treatment* $F_{(2)} = 1.263$, $p = 0.287$, nor *Drug* effect $F_{(5)} = 0.530$, $p = 0.753$, and no *Treatment X Drug* interaction $F_{(2,5)} = 1.377$, $p = 0.201$ (Fig. 6b).

In contrast, in response to Pb^{2+} exposure, the female rat's *OTC ratio* data revealed no effect of *Treatment* $F_{(2)} = 0.833$, $p = 0.438$, but there was a significant effect of *Drug* $F_{(5)} = 3.976$, $p < 0.001$***, $\eta_p^2 = 0.155$ and the *Treatment X Drug* interaction approached a trend $F_{(2,5)} = 1.804$, $p = 0.68$, $\eta_p^2 = 0.143$ (Fig. 7a). Additionally, the female rat's *Open Arm Time Mobile (sec)* data revealed no effect of *Treatment* $F_{(2)} = 1.851$, $p = 0.162$, but a significant effect of *Drug* $F_{(5)} = 3.224$, $p < 0.001$***, $\eta_p^2 = 0.134$, and a significant *Treatment X Drug* interaction $F_{(2,5)} = 4.292$, $p < 0.001$***, $\eta_p^2 = 0.292$ (Fig. 7b).

4 Discussion

The present study served to expand upon the previous reports of Neuwirth et al. (2019a) in assessing the effects of neurodevelopmental Pb^{2+} exposure on locomotor activity within the OF and anxiogenic behaviors within the EPM. First, the BLLs for both the Peri-22 150 ppm rats and the Peri-22 1,000 ppm rats did not differ by sex, but were in the clinically relevant range observed in children at low-levels of Pb^{2+} exposure. Thus, the animal model used herein has good construct and external validity. Next, the effects of Pb^{2+} exposure (i.e., PPM) and drug (i.e., saline, taurine, or taurine derivatives) treatments were evaluated as a function of sex. In the OF (i.e., under calming low-light conditions that served to promote locomotor activity), the Control female rats exhibited increased locomotor activity levels for both *Total Distance Traveled* and *Overall Average Speed* when compared to Control male rats. When Control male rats were compared to Peri-22 150 ppm and Peri-22 1,000 ppm male rats in the OF, the Pb^{2+}-exposed rats, irrespective of dose, were more hyperactive than the Control

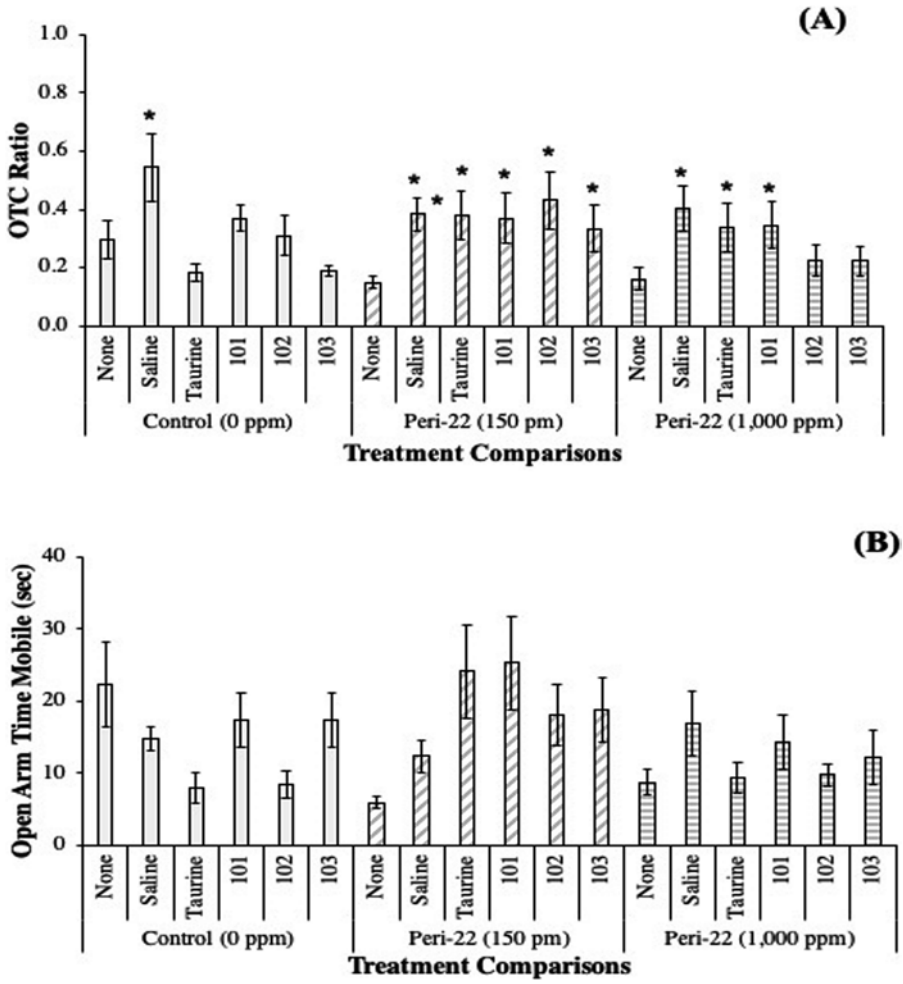

Fig. 6 Assessment of male rat's anxiety-like behavioral activity in the EPM as an effect of *Treatment* and *Drug* condition. Data for the male rat's *OTC ratio* revealed a significant effect of *Drug* ($p < 0.05$*; (**a**). Alternatively, the male rat's *Open Arm Time Mobile (sec)* data revealed no significant effects of *Treatment, Drug,* nor interactions (**b**). Data are presented as the mean ± SEM

male rats for both *Total Distance Traveled* and *Overall Average Speed*. Similarly, the Peri-22 150 ppm female rats and the Peri-22 1,000 ppm rats were more hyperactive than the Control female rats for both *Total Distance Traveled* and *Overall Average Speed*. These results indicate that neurodevelopmental Pb^{2+} exposure produces a hyperactive locomotor profile in the OF that may carry over into the EPM.

In the EPM (i.e., under anxiety-provoking light conditions), the Control female rats remained more active in the *Open Arms Time Mobile* when compared to the Control males. However, they had similar *OTC ratios*. Typically, rodents in the EPM will not venture into the open arms, and if they do, they often freeze constituting a hypoactive anxiety-like behavioral response. Alternatively, if the rodents were to enter the open arms and frequent it multiple times, over greater duration of time, this would constitute a hyperactive anxiety-like response (for review, see Walf and Frye 2007). Thus, the Control female rats exhibited a hyperactive anxiety-like behavioral phenotype in the EPM only for the *Open*

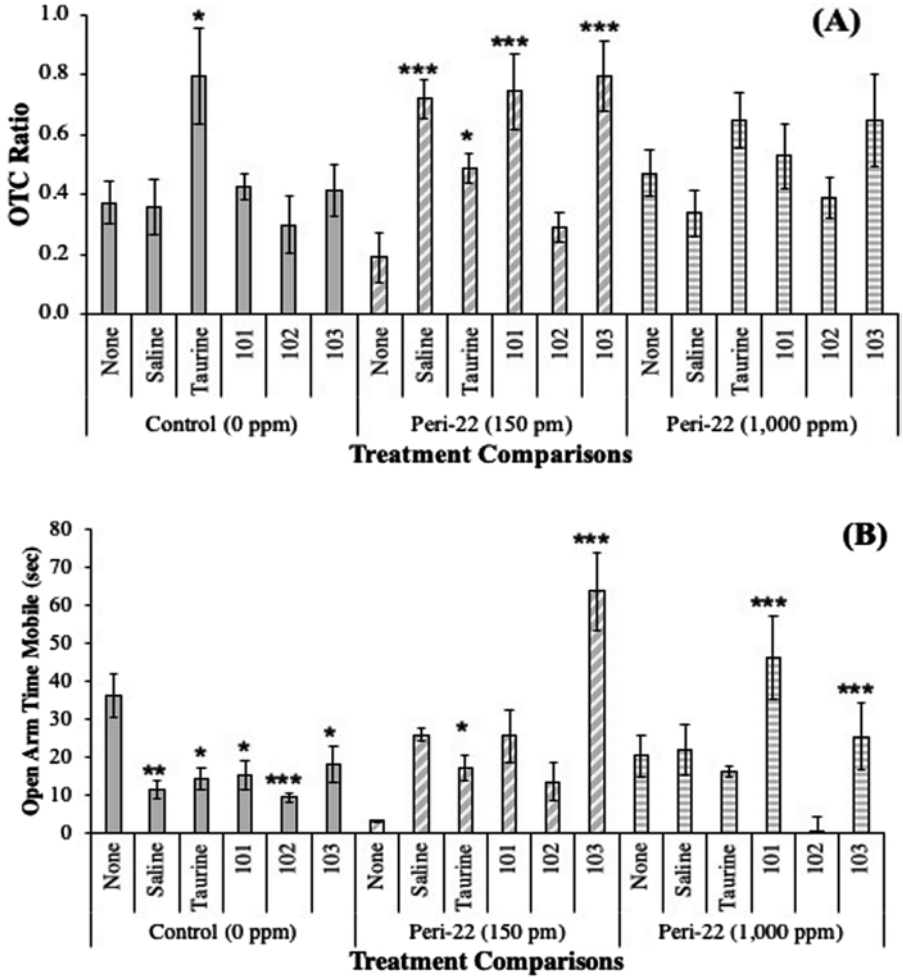

Fig. 7 Assessment of female rat's anxiety-like behavioral activity in the EPM as an effect of *Treatment* and *Drug* condition. Data for the female rat's *OTC ratio* revealed a significant effect of *Drug* ($p < 0.001$***; (**a**). Alternatively, the female rat's *Open Arm Time Mobile (sec)* data revealed a significant effect of *Drug* ($p < 0.01$*) *and a Treatment X Drug interaction* ($p < 0.001$***; (**b**). Data are presented as the mean ± SEM

Arm Time Mobile. For the male rats, the Peri-22 150 ppm and Peri-22 1,000 ppm male rats exhibited a hypoactive anxiety-like behavioral phenotype when compared to the Control male rats in the EPM for both *OTC ratio* and *Open Arm Time Mobile.* Similarly, the Peri-22 150 ppm and Peri-22 1,000 ppm female rats exhibited a hypoactive anxiety-like behavioral phenotype when compared to the Control female rats in the EPM for both *Total Distance Traveled* and *Overall Average Speed.* These data suggest that Control male and female rats are more active in the EPM than their Pb^{2+}-exposed counterparts which is the opposite phenomena observed from the OF with respect to locomotor activity. This suggests that the anxiogenic effects of the EPM had substantial internal validity in provoking anxiety-like behaviors, yet interestingly, the Pb^{2+}-exposed rats, regardless of sex, exhibited a far greater level of inhibition of their locomotor activity in the EPM. Thus, if agonistic pharmacological properties of taurine or the taurine derivatives understudy were to influence the GABAergic system in these neurodevelopmentally Pb^{2+}-exposed rats, then one would presume that the calming effects of increased chloride conductance through the $GABA\text{-}_{AR}$

would promote more activity to enter the Open Arms and to increase the time that they would be mobile within the Open Arm.

When the drug treatment conditions were employed in the EPM, the Control male rats that were exposed to taurine and its derivatives had no influences on the EPM behaviors, but across both the *OTC ratio* and the *Open Arm Time Mobile* TD-101 increased locomotor activity when compared to taurine. Interestingly, for the Peri-22 150 ppm male rats, TD-101, TD-102, and TD-103 proved to be equally as effective as taurine for both the *OTC ratio* and the *Open Arm Time Mobile*. Moreover, for the Peri-22 1,000 ppm male rats, there were no differences between taurine and the taurine derivatives, but TD -101 had trends of being equal to or potentially more effective than taurine in the *OTC ratio*, whereas TD-101 and TD-103 showed the same trend for the *Open Arm Time Mobile*.

For Control female rats, taurine was the most effective drug for the *OTC ratio*, whereas TD-101 and TD-103 were equally as effective as taurine for the *Open Arm Time Mobile*. In contrast, the Peri-22 150 ppm female rats TD-101 and TD-103 were more effective than taurine for the OTC ratio, and in the Open Arm Time Mobile, TD-101 had a trend of being more effective than taurine, but TD-103 was far better than taurine for this behavioral measure. For the Peri-22 1,000 ppm female rats, TD-102 was the least effective drug, and TD-101 and TD-103 were as equally effective as taurine for the *OTC ratio*. Moreover, for the *Open Arm Time Mobile*, TD-101 and TD-103 were more effective than taurine, and TD-102 was the least effective drug.

Taken together, as a function of dose (i.e., 150 ppm and 1,000 ppm) in Pb^{2+}-exposed male rats, taurine or TD-101 could be used as a potential pharmacotherapeutic compound to recover Pb^{2+}-induced behavioral deficits. In contrast, as a function of dose (i.e., 150 ppm and 1,000 ppm) in Pb^{2+}-exposed female rats, TD-101 or TD-103 could be used as a potential pharmacotherapeutic compound in addition to taurine to recover Pb^{2+}-induced behavioral deficits. These findings suggest that perhaps the neurodevelopmental effects of Pb^{2+} exposure caused sex-specific effects that persisted across the lifespan and altered the pharmacodynamics of the GABA-$_{AR}$ system. As such, this could explain the differences in drug responsivity in the EPM and their associated behavioral changes that are directed from the CNS's ability to regulate GABAergic emotionality. Similar work has been done in the characterization of the fragile X syndrome mouse model (El Idrissi et al. 2009, 2010, 2012; Neuwirth et al. 2013, 2015; Santora et al. 2013; Shen et al. 2013) including effects on peripheral blood flow and hypertension (El Idrissi et al. 2013), but has been limited to only acute and chronic taurine treatment conditions (El Idrissi et al. 2009, 2012) which may have sex-specific influences in learning and memory as shown in rats (Zhu et al. 2022). It remains to be elucidated how concurrent taurine or taurine derivative exposure would augment the same behaviors understudy herein, or whether prolonged/chronic taurine or taurine derivative exposures would be beneficial, or would acute treatment regimens be most effective. This study paves the way for more work to continue in this area to find a novel pharmacotherapeutic treatment intervention for treating Pb^{2+} poisoning.

What has been brought to light from this study is that 2-aminoethanesulfonic acid (i.e., taurine), ß-alanine (i.e., TD-101), and analine-2-sulfonic-acid (i.e., TD-103) appear to be prospective drug candidate worth further pursuing, whereas aminomethanesulfonic acid (i.e., TD-102) does not appear to be worthwhile as treatments for Pb^{2+} poisoning. Further, Pb^{2+}-exposed male and female rats are responsive to ß-alanine (i.e., TD-101) irrespective of sex, whereas Pb^{2+}-exposed female rats are selectively more responsive to analine-2-sulfonic-acid (i.e., TD-103), and it outperforms taurine by far in the EPM for recovering anxiogenic behaviors. More studies are required to obtain a full composite of the effects of TD-101 and TD-103 on other cognitive learning and memory as well as social-emotional behaviors. However, the present study has advanced the direction of the field in this particular area.

5 Conclusion

In summary, this study shows that neurodevelopmental Pb^{2+} exposure can have persistent effects on GABAergic regulated locomotor and anxiety-like behaviors that can be evaluated through the OF and EPM. Further, novel drug candidates can be easily screened for preclinical assessment of a drug to treat Pb^{2+} poisoning. The current study builds upon prior studies that sought to evaluate the effects of taurine in ameliorating neurodevelopmental Pb^{2+} exposure (Jiang et al. 1956; Zhu et al. 2005; Li et al. 2006; Yu et al. 2007; Neuwirth 2014; Neuwirth et al. 2017, 2019a, b, c; Cruz et al. 2022; Vasquez et al. 2022) with emphasis on fronto-executive functions (Neuwirth 2014; for review, see Tait et al. 2018; for review see Neuwirth and Kolb 2020). However, only recently have studies focused on low-level Pb^{2+} exposure from these past reports, and one should be cautious as Pb^{2+} exposure can, perhaps, influence the arrangement of the $GABA\text{-}_{AR}$ in ways that alter its pharmacodynamic responsivity to $GABA\text{-}_{AR}$ agonists. This preclinical experimental approach would be consistent with reports of Pb^{2+} exposure altering the inhibition of cultured hippocampal neuron currents through $NMDA_R$s (Ujihara and Albuquerque 1992). Further, earlier and more current reports have worked towards synthesizing the literature and/or theorizing how taurine derivatives might be developed and tested with the goal of treating a range of CNS disorders (Messiha 1979; Madura et al. 1997; Della Corte et al. 2002; Gupta and Kim 2003; Frosini et al. 2003, 2006; Gupta et al. 2005; Gupta, 2006; Bianchi et al. 2006; Ward et al. 2006; Palmi et al. 2006; Chung et al. 2012). However, limited studies have been conducted using these compounds in animal models to assess its therapeutic value in recovering CNS disorders, thus warranting further investigation similar to the present study employing TD-101, TD-102, and TD-103. Notably, the chemical structure of the taurine derivatives (i.e., TD-101 and TD-103) provides new insights into examining specific drug treatments addressing dose-dependent effects (i.e., TD-101) and sex-dependent effects (i.e., TD-103) that might further customize the development of novel pharmacotherapeutics for addressing the longstanding issues of neurodevelopmental Pb^{2+} exposure. It is therefore imperative that future research on neurodevelopmental Pb^{2+} exposure should make a concerted effort to provide children with psychopharmacotherapies to improve their quality of life across their lifespan (i.e., either by protecting against Pb^{2+} exposure or reducing its insults to the brain). This would be considered a secondary level of prevention, when the primary level of intervention should be to remove and eliminate all sources of Pb^{2+} exposure from the environment.

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Developmental Lead Exposure in Rats Causes Sex-Dependent Changes in Neurobiological and Anxiety-Like Behaviors that Are Improved by Taurine Co-treatment

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Developmental lead exposure · Anxiety-like behaviors · Hyperactivity · Neuronal cell counts · GABAergic systems · Neurotoxicology · Developmental behavioral neurotoxicology · Prefrontal cortex · Hippocampus

Abbreviations

Pb^{2+}	lead
MRI	magnetic resonance imaging
fMRI	functional magnetic resonance imaging
MRS	magnetic resonance spectroscopy
Tau	taurine
PFC	prefrontal cortex
HP	hippocampus
DE	diencephalon
NAA	N-acetyl aspartate
ASST	attention set-shift test
E/I	excitation-to-inhibition
BLL	blood lead levels
GAD	glutamic acid decarboxylase
EQ	encephalization quotient
ITF	isotropic fraction
PND	postnatal day
EDTA	ethylenediaminetetraacetic acid
ASV	anodic stripping voltammetry
OF	open field
EPM	elevated plus maze
IRR	inter-rater-reliability
US	United States

1 Introduction

Childhood lead (Pb^{2+}) poisoning remains a persistent global public health and environmental problem for many developed and underdeveloped countries, whereby the latter experience greater deleterious outcomes (Yuan et al. 2007; Shinkuma and Huong 2009; Chen et al. 2011; Eneh and Agunwamba 2011; Laidlaw and Taylor 2011; Yang et al. 2013a). Despite the advancements through the removal of leaded paints and leaded gasoline (Brody et al. 1994), modern sources of Pb^{2+} exposure occur in the form of contaminated water supplies (Edwards et al. 2009) and electronic waste sites (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; Yang et al. 2013a, b). In adults (Rao et al. 2014), occupational elevated Pb^{2+} exposure (i.e., $\geq$ 10 μg/dL) is considered rare by today's health and safety standards, but when it does occur, it can significantly reduce the affected individual's brain volume across various cortical and subcortical regions, as well as decrease the myelination of fine fibers (Andrzej et al. 2019). However, it is important to note that when adults who were exposed to Pb^{2+} poisoning as children were examined by magnetic resonance imaging (MRI), evidence of neurodegeneration was observed that emanated from the early neurodevelopmental spectrum of their lifespan (Stewart and Schwartz 2007; Stewart et al. 2006). Considering this information, children who reside in or in close proximity to toxic exposure sites (e.g., superfund sites) are at increased risk for developmental neuropathologies (Leung et al. 2008; Chen et al. 2011; Yang et al. 2013a, b). As such, it has become increasingly apparent that Pb^{2+} is a neurotoxicant (Grandjean and Hertz 2015; Bellinger 2008a; Lanphear et al. 2000) that causes a range of neurobehavioral problems that persist across the lifespan (Schwartz 1994; Bellinger 2008b).

In the United States (US), there is continued debate to advocate for lowering the environmental Pb^{2+} exposure action levels from the current 5 μg/dL to 2 μg/dL (Gilbert and Weiss 2006); however, the consensus is that no blood lead level (BLL) should be deemed "safe" for children (Bellinger and Dietrich 1994; Barbosa Jr et al. 2005; Lidsky and Schneider 2006). Moreover, in the US, children are not mandated to undergo BLL screenings, and as a consequence, children are either missed or inconsistently/irregularly screened for a BLL at 1 and 2 years of age. Thus, suggesting that more consistent and proactive BLL monitoring programs during pregnancy may help circumvent this issue (for review, see Neuwirth 2018). Assessing a mother for a BLL

prior to and during each trimester of pregnancy would determine if the developing fetus is at risk for Pb^{2+} exposure in time to devise a trimester-dependent early medical treatment plan if a positive test for Pb^{2+} exposure is identified. This type of proactive BLL screening intervention program would eliminate having to wait until the child reaches 1 to 2 years of age to be tested for Pb^{2+} exposure. Reports suggest that during 31–42 postmenstrual weeks in pregnancy that the functional connectome network segregation occurs in the third trimester and provides the early architectural plans for the brain's topological development thereafter (Cao et al. 2017). Although waiting until 1 to 2 years of age to test a child for Pb^{2+} exposure is a typical practice for pediatricians, the reality is that at the neuronal level, this delay in testing presents a challenge for utilizing timely and effective interventions for prenatal Pb^{2+} exposures (Thomason et al. 2019; Forsyth et al. 2018; Shannon 2003), since much of the brain and many organizational processes have already occurred during the first 24 months of postnatal life (Gao et al. 2015; Qiu et al. 2015; Fan et al. 2011; Gao et al. 2011; Knickmeyer et al. 2008; Johnson 2001; Paus et al. 2001; Casey et al. 2000).

Neurotoxicology studies show that low- and high-levels of Pb^{2+} exposures are associated with a range of neuropathological and neuropsychological syndromes with varied behavioral and cognitive symptoms (Wong et al. 1991; Bellinger and Dietrich 1994; Schütz et al. 1996; Bleecker et al. 1997; Bellinger and Needleman 2003; Canfield et al. 2003; Kreig Jr. et al. 2005; Lidsky and Schneider 2006; Bellinger 2008b; Bergdhal and Skerfving 2008; Rentschler et al. 2012). These reports coupled with the developmental mapping of age-dependent structure-function relationships of children's brains (Huang et al. 2015; Paus et al. 2001; Lange et al. 1997; Caviness et al. 1996; Giedd et al. 1996) provide useful reference datasets for neurodevelopmental disorders. Consistent with this framework, Yuan et al. (2006) used a verb generation task during functional magnetic resonance imaging (fMRI) and reported persistent aberrant gray matter reorganization in the left prefrontal cortex (PFC) and reduced activation in the left middle temporal gyrus, and the right PFC had increased activation in adults with elevated BLLs as children.

From longitudinal studies of adults that were Pb^{2+}-poisoned as children, the field has gained substantial insight regarding its persistent effects. For example, using MRI measures, Cecil et al. (2008) found greater loss of brain volume in males when compared to females, with the greatest gray matter loss occurring in the PFC and the anterior cingulate cortex. Additionally, adults that had elevated BLLs as children showed persistent altered white matter microstructure of the corona radiata as well as the genu and splenium of the corpus callosum (Brubaker et al. 2009). Moreover, Brubaker et al. (2010) showed that a relationship was observed between BLLs acquired during later childhood that were associated with greater loss in gray matter volume in the PFC that were more pronounced in males than females. Taken together, these findings further suggest that sex, in addition to the age during neurodevelopment when a child might be Pb^{2+}-poisoned, can have a significant negative influence/interaction on the number of neuronal and glial cell populations that may have persistent structure-function consequences across a number of brain regions and associated cognitive and behavioral systems.

Interestingly, Trope et al. (2001) used magnetic resonance spectroscopy (MRS) to study the effects of childhood Pb^{2+} exposure on adult's gray matter metabolites *in vivo* and found in the PFC that *N-acetyl aspartate* (NAA) and phosphocreatine ratios were altered when compared to age-matched controls. Given the unique value of MRS to detect abnormalities in the brains of affected patients *in vivo*, this method has been expanded to investigate a wide range of neurodevelopmental and metabolic disorders (Cecil 2006), including Pb^{2+} poisoning. In one study, a short-echo proton magnetic resonance spectroscopy (pMRS) was used to evaluate *in vivo* brain metabolites of adults that were Pb^{2+}-poisoned as children, and the results indicated that gray mater NAA and creatine were decreased in the basal ganglia (Cecil et al. 2011). Additionally, a decrease in NAA and choline in the cerebral cor-

tex (particularly choline reductions in the PFC) and a decrease in glutamate/glutamine in the parietal cortical white matter were observed (Cecil et al. 2011).

Along these lines of investigation, a case report observed that a 7-month-old infant with Pb^{2+}-induced encephalopathy that exhibited seizures, regression of developmental milestones, and altered sensory functioning underwent an MRI that was remarkable for reduced myelination in the periventricular and subcortical white matter in MRI scans with a neurological profile consistent with a neurometabolic disorder (Sahu et al. 2010). These data further provided substantial evidence that not only does childhood Pb^{2+}-exposure cause volumetric loss to the CNS gray and white matter but also reduces critical brain metabolites that persist across the lifespan as evidence of Pb^{2+}-induced neurotoxicity and subsequent early spectrum neurodegeneration clinical profiles. The aforementioned clinical data corroborate with previous findings (Neuwirth et al. 2019a, b) of persistent sex-dependent changes in neurotransmitter profiles within the PFC and hippocampus (HP) of perinatal exposed rats (i.e., producing BLLs of 3–10.7 μg/dL for Peri-22 150 ppm rats and from 9.0 to 17.8 μg/dL for Peri-22 1,000 ppm, respectively) with associated deficits in the attention set-shift test (ASST; for review, see Tait et al. 2018; Neuwirth et al. 2019c). These preclinical findings suggest that animal models of childhood Pb^{2+} poisoning can provide useful construct, face, and externally valid models for investigating the basic mechanisms underlying such clinical observations of Pb^{2+} poisoning consistent with the suggested approaches of Neuwirth and Kolb (2020).

At present, chelation therapy is an effective treatment for most children who experience metal toxicity at high-level Pb^{2+} exposures (*i.e.,* ≥ 39 μg/dL). Alternatively, chelation therapy is an ineffective treatment and may be clinically inappropriate for children with BLLs ≤ 10 μg/dL (Rogan et al. 2001; Dietrich et al. 2004). Therefore, in order to address this issue of treating low-level Pb^{2+} exposures to prevent lifelong neurotoxicant impacts and early-stage neurodegeneration from childhood Pb^{2+} poisoning, new therapeutic approaches are warranted. In the absence of such interventions, the brain is vulnerable to low-level Pb^{2+} exposures altering the GABA-shift (Neuwirth 2014, 2018; Neuwirth et al. 2017; Neuwirth and El Idrissi 2021) that is critical for the developmentally regulated excitation-to-inhibition (E/I) balancing (Ben-Ari 2002; Ben-Ari et al. 2012). Moreover, studies have shown that taurine co-treatment (i.e., 0.05% administered through the drinking water) during neurodevelopmental Pb^{2+} exposure (Neuwirth et al. 2019b; Neuwirth and Emenike 2021) or taurine acute treatment (i.e., 43 mg/kg injected i.p. prior to behavioral assays) in mature animals following neurodevelopmental Pb^{2+} exposures (Neuwirth 2014; Neuwirth et al. 2017, 2019a, b, 2022; Vasquez et al. 2022; Neuwirth and Emenike 2021) can ameliorate the neurotoxicant effects of Pb^{2+} poisoning. When the GABA-shift is disrupted, it has been identified as an ontogenetic mechanism contributing to the risk for seizure and other closely related neurodevelopmental disorders (Ben-Ari 2002; Ben-Ari et al. 2012). Given the reported neurotoxicant effects of Pb^{2+} poisoning disrupting the GABA-shift and producing lifelong fronto-executive deficits identified through behavioral and cognitive systems, efforts have begun to advocate for Pb^{2+} poisoning to be formally recognized as a neurodevelopmental disorder in future editions of the *Diagnostic and Statistical Manual of Mental Disorders 5th Edition* (Neuwirth et al. 2020).

Taurine has been shown to provide neuroprotection against brain E/I imbalances in animal models of epilepsy (El Idrissi et al. 2003; El Idrissi & L'Amoreaux, 2008; Vasquez et al. 2021) through upregulation of *glutamic acid decarboxylase* (GAD) and has also been shown to interact with β2/β3 subunits of the $GABA\text{-}_{AR}$ (L'Amoreaux et al. 2010) and modify gene expression of $GABA\text{-}_{AR}$s (Shen et al. 2013). Moreover, reports suggest that taurine protects neurons against the loss of GABAergic tone/activity as a function of senescence whereby, on the later neurodevelopmental continuum, the E/I balance begins to weaken with age (El Idrissi et al. 2013). These reports are consistent with evidence supporting taurine treatment improves

learning in fragile X syndrome mouse models (El Idrissi et al. 2009; El Idrissi 2008; El Idrissi et al. 2010, 2012; Neuwirth et al. 2013, 2015) and motor abilities (Santora et al. 2013) of aged animals as well as regulating vasoactivity that could be related to vascular dementias (El Idrissi et al. 2013). However, in non-diseased animal models, taurine pharmacodynamics may be different as a function of sex and the type of learning paradigm employed (for review, see Zhu et al. 2022).

In addition to investigating sex-dependent behavioral differences, the present study sought to examine the relationships between neurotoxicant effects of neurodevelopmental Pb^{2+} exposure and its associated disruptions in anxiety-like rat behaviors through the open field and elevated plus maze tests. Moreover, the present study evaluated whether developmental Pb^{2+} exposure altered rat's encephalization quotient (EQ; an equation used to measure an organism's brain mass relative to their body weight across species to compare their intellectual ability across evolution). Further, the EQ was compared against the total number of estimated cell counts, as well as neuronal and glial cell population estimates using isotropic fractionation (ITF) to assess the potential for Pb^{2+}-induced early neurodegeneration and whether taurine could protect against these Pb^{2+} insults consistent with previous reports (Vasquez et al. 2022; Neuwirth 2014; Neuwirth et al. 2017, 2019a, b, 2022).

2 Methods

2.1 Subjects

In accordance with the SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans hooded male ($N = 15$) and female rats ($N = 30$) (Taconic, N.J.) were paired for breeding, and their male F1 generation were used for future 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*. Although all rats received water *ad libitum*, the experimental rats were fed water containing lead acetate (Sigma Aldrich, St. Louis, MO). Consequently, lead acetate water was provided from breeding throughout gestation and continued through weaning at postnatal day (PND) 22 (*i.e.,* constituting a Perinatal Pb^{2+} developmental exposure model). At PND 22, 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 1,000 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 32_48, rats were assigned to the open field test and 24 h later the elevated plus maze test. An additional cohort of rats was bred in a similar manner as the Peri-22 150 ppm and Peri-22 1,000 ppm rats, but was co-treated with 0.05% taurine (2-aminoethansulfonic acid [Sigma Aldrich, St. Louis, MO]) supplemented in their drinking water for the duration of their neurodevelopmental Pb^{2+} exposure to evaluate its neuroprotective effects as an add-on experiment for the ITF study (see below).

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 1,000 ppm treatment group), and their blood samples were collected and analyzed consistent with previous reports (Neuwirth 2014; Neuwirth et al. 2017, 2018, 2019a, b, 2022; Neuwirth and El Idrissi 2021; Vasquez et al. 2022). Briefly, blood samples were collected within 2 mL anti-coagulant *ethylenediaminetetraacetic acid* (EDTA)-coated syringes (Sardstedt, Germany), followed by electrochemical anodic stripping voltammetry (ASV) used by taking 50 μL of whole blood mixed with 250 μL of hydrochloric acid solution (0.34 M) in a commercial ESA LeadCare II Blood Lead Analyzer system (Magellan Diagnostics, North

Billerica, MA) to eliminate any potential for experimenter bias. 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 The Open Field Test

Between PND 32–48, behaviorally naivë rats from the F1 generation offspring ($N = 72$) were subjected to an open field (OF) test. The treatment groups were as follows: Control males ($n = 12$), Peri-22 150 ppm Pb^{2+} males ($n = 12$), and Peri-22 1,000 ppm Pb^{2+} males ($n = 12$) and Control females ($n = 12$), Peri-22 150 ppm Pb^{2+} females ($n = 12$), and Peri-22 1,000 ppm Pb^{2+} females ($n = 12$), respectively. All rats were examined for 10 min to assess baseline locomotor activity in the OF apparatus (376 mm H x 914 mm W x 615 mm L) in a dark room illuminated with red lighting (30 Lux). These testing conditions promoted the evaluation of the rat's locomotor activity for any sex-dependent differences and any deficits that could be observed 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)* that were analyzed through Anymaze® Stoelting Co. (Wood Dale, IL).

2.4 The Elevated Plus Maze Test

The next day following the OF assessment, the male and female rats were randomly assigned to the EPM and examined for 10 min to assess anxiety-like behaviors. The EPM apparatus (external dimensions: 800.1 mm H × 1,104.9 mm W × 1,104.9 mm L; closed arm dimensions: 101.6 mm W × 1,104.9 mm L × 304.8 mm H walls; open arm dimensions: 101.6 mm W × 1,104.9 mm L; the platform was elevated off the floor by 495.3 mm H) was within a brightly illuminated room (300 Lux) thereby inducing an anxiogenic response as the motivational factor for the test. The effects of Pb^{2+} exposure in evoking anxiety-like behaviors were assessed. Anxiety-like behavioral variables included the *Open Arms Time Mobile (sec)* and *Closed Arms Time Mobile (sec)* to assess activity across the 10 min of the EPM that were analyzed through Anymaze® Stoelting Co. (Wood Dale, IL).

2.5 The Encephalization Quotient

The encephalization quotient (EQ) is a numerical measure that permits within and between specie comparisons of the relative brain weight to body weight ratio by using the formula (Saganuwan 2021; Jerison 1955) described in Fig. 1. In the present study, it was used to determine if Pb^{2+} exposure reduced brain mass relative to body weight as a function of *Sex* and *Treatment*.

2.6 Isotropic Fractionation

Immediately following testing, rats were sacrificed and their brains were fresh-frozen extracted and stored at -80 °C. For each treatment group and sex ($n = 6$) rat, brains were dissected and subjected to isotropic fractionation (ITF), including the prefrontal cortex (PFC), hippocampus (HP), and the diencephalon (DE). The procedures for ITF were conducted based on the methods of Herculano-Houzel and Lent (2005) to evaluate

$$\text{Encephalization Quotient } (EQ) = \frac{\text{Brain weight}}{0.12^{*}(\text{Body weight})\ 2/3}$$

Fig. 1 The encephalization quotient formula

the total cell counts as well as neuron and non-neuronal cells within the brain regions examined. Individual dissected regions were weighed prior to being processed in a hand-held glass homogenizer containing a mild detergent-based dissociation solution (40 mM sodium citrate and 1% Triton-X100; Sigma Aldrich, St. Louis, MO). Tissue samples were homogenized until no remaining pieces were visible, and the contents were poured into a graduated cylinder for measuring. The glass homogenizer was rinsed once with additional dissociation solution to capture any remaining cells. A 10 mg/mL DAPI solution (Sigma Aldrich, St. Louis, MO) was then added to the homogenates, and the final volume was recorded. Two 1 mL aliquots were removed for sampling. The first aliquot was used to assess total cell counts, and 10 µL of the homogenates were placed onto a hemocytometer and observed under a microscope at 40X. The center 1×1 mm^2 square (i.e., which contained 25 smaller squares) was brought into focus, and cell counts were obtained from 10 of the 25 squares and were visualized in the DAPI fluorescent channel. This process was repeated at least three more times, with a minimum of 40 total squares counted per sample. The coefficient of variation across the four counting trials was constrained to 15%; otherwise additional counts were taken to ensure cell estimates were accurate within this preset tolerance range. Inter-rater reliability (IRR) was assessed when more than one observer contributed to data collection. Counts from the same sample were required to fall within 10% of each other in order for both observers to proceed with data collection and analysis (i.e., IRR = 90%). Data from these trials were then entered into the following equation to extrapolate total regional cell densities for each rat:

$$\text{Average nuclei count} \times \left(\frac{1{,}000{,}000 / \text{volume}(\text{nL})}{\text{counted}} \right) \times \text{total sample volume}\,(\text{mL})$$

The second 1 mL aliquot was utilized for determining neuronal percentages within each sample by co-staining with anti-NeuN (ABN78C3 [1:300] (Millipore Sigma, Burlington, MA), a well-established protein target marker due to its exclusive expression in neurons. Homogenates were collected into a pellet following centrifugation (5 min, 1500 x *g*) and subjected to an antigen retrieval step (i.e., 1 h at 75 °C in 0.2 M boric acid with pH 9) prior to staining. Samples were cooled to room temperature and centrifuged again (5 min, 1500 × *g*), followed by resuspension of the pellet in PBS-BSA (5%) buffer containing the NeuN antibody, and allowed to incubate for 24–48 h at 4 °C. Samples were then pelleted by centrifugation (5 min, 1500 × *g*) and resuspended in fresh PBS for counting. A sample of 10 µL was placed on a hemocytometer and 100 nL volumes were counted, utilizing the same 1×1 mm^2 center square described earlier. Photomicrographs of cell nuclei were captured under each of the DAPI and TRITC filters to assess those nuclei that exhibited staining with DAPI only or DAPI and NeuN staining. A minimum count of 500 cells had to be reached under the DAPI channel, prior to calculating NeuN+ cell percentages. Neuron percentages for each sample were determined by dividing the number of co-stained nuclei by the total number of DAPI-stained nuclei and multiplied by 100.

2.7 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 and EPM tests as well as the EQ and ITF experiments, a *t*-test was used to evaluate the *Sex* differences between Control male and female rats, and an *ANOVA* was used to evaluate the effects of *Treatment* as the between-subjects factors. The criteria for significance was set at $\alpha = 0.05\%$ with a 95% ± SEM. Significant differences were determined by an (un)equal *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 Pb^{2+}-Exposure Cessation Prior to Behavioral Testing

The results showed no differences in BLL as a function of gender. Each Pb^{2+} treatment at the time of sample collection resulted in BLLs ranging from 3.1 to 11.9 μg/dL (SD ± 1.43) for Peri-22 150 ppm rats ($p < 0.001$***) and from 10.1 to 19.8 μg/dL (SD ± 2.94) for Peri-22 1,000 ppm ($p < 0.001$***), when compared to Control rats. The control rats were Pb^{2+} negative (i.e., below the 3.3 μg/dL detection limit). The BLL samples from the behaviorally tested rats were also drawn at PND 55 following the conclusion of the study, and their BLLs were below the 3.3 μg/dL detection limit. This reduction in BLL is consistent with reports from the USA Agency for Toxic Substances and Disease Registry (U.S. Department of Health and Human Services 2007) that Pb^{2+} is not uniformly distributed in the bone, blood, and soft mineralizing tissues.

3.2 Neurodevelopmental Pb^{2+} Exposure Induced Sex-Dependent Differences in Anxiogenic Behaviors

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. When evaluating the Control male and female rats, there was an effect of *Sex* $t_{(22)}$ = -2.923, $p < 0.05$* for the *Total Distance Traveled* (*m*) (Fig. 2a) and an effect of *Sex* $t_{(22)}$ = -2.917, $p < 0.05$* for the *Mean Speed* (*m/s*) (Fig. 2b) in the OF. Control female rats were more active, as they covered more ground, and moved much faster than the Control males.

Next, the effects of neurodevelopmental Pb^{2+} exposure were evaluated in males to assess any differences in locomotor activity. For the males, an effect of *Treatment* $F_{(2)} = 8.548$, $p < 0.001$***, $\eta_p^2 = 0.341$ for *Total Distance Traveled* (*m*) (Fig. 3a) and an effect of *Treatment* $F_{(2)} = 8.830$, $p < 0.001$***, $\eta_p^2 = 0.349$ for *Mean Speed* (*m/s*) (Fig. 3b) were observed. Interesting, the results showed that the Peri-22 150 ppm male rats covered more ground ($p < 0.001$***; Fig. 3a) and moved faster ($p < 0.001$***; Fig. 3b) than the Control male rats. The Peri-22 1,000 ppm male rats covered more ground ($p < 0.05$*; Fig. 3a) but moved slower ($p < 0.05$*; Fig. 3b) than the Peri-22 150 ppm male rats in the OF. This suggests differential effects of Pb^{2+} exposure on locomotor activity are observed dependent upon the dose of exposure.

Additionally, the effects of neurodevelopmental Pb^{2+} exposure were evaluated in females to assess any differences in locomotor activity. Unlike the male rats, neurodevelopmental Pb^{2+} exposure did not have a significant effect on locomotor activity for *Total Distance Traveled* (*m*)

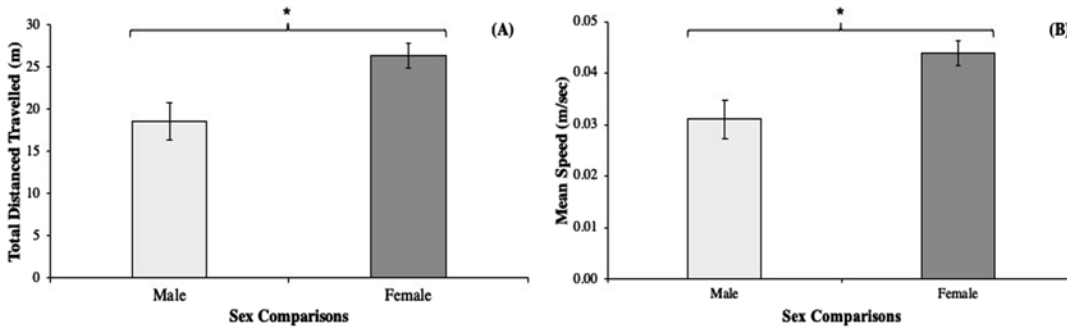

Fig. 2 Preliminary assessment of rat locomotor activity in the OF as an effect of *sex* (males = white bars; females = gray bars). Data shown for both *Total Distance Traveled* (*m*) (**a**) and *Mean Speed* (*m/s*) (**b**) and the Control female rats covered more ground ($p < 0.05$*) and moved faster ($p < 0.05$*) when compared to Control male rats. Data are presented as the mean ± SEM

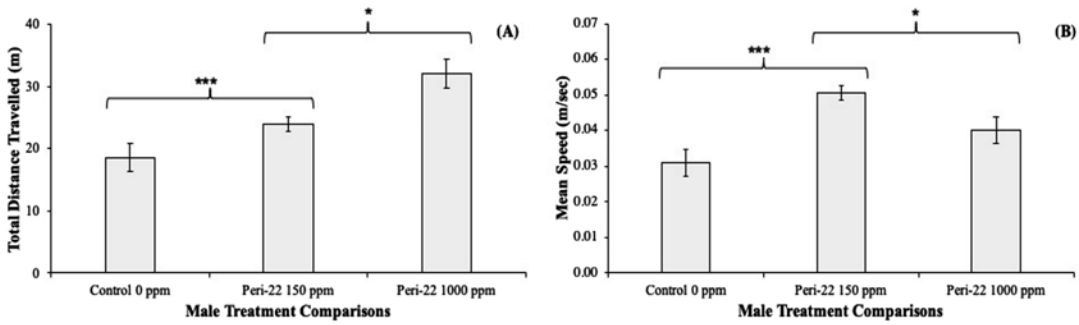

Fig. 3 Effect of Pb^{2+} exposure on male locomotor activity in the OF. Data shown for both *Total Distance Traveled* (*m*) (**a**) and *Mean Speed* (*m/s*) (**b**) and the Peri-22 150 ppm male rats covered more ground (**a**) and moved faster (**b**) than the Control male rats ($p < 0.001$***). The Peri-22 1,000 ppm male rats covered more ground (**a**) but moved slower (**b**) than the Peri-22 150 ppm male rats ($p < 0.05$*). Data are presented as the mean ± SEM

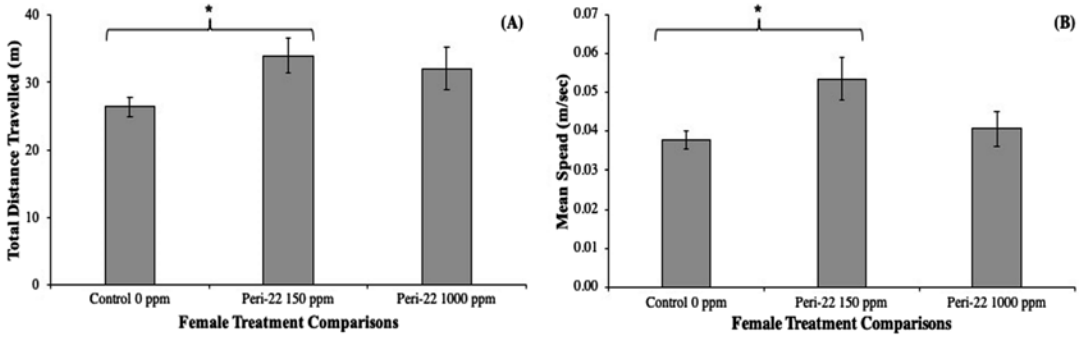

Fig. 4 Effect of Pb^{2+}-exposure on female locomotor activity in the OF. Data shown for both *Total Distance Traveled* (*m*) (**a**) and *Mean Speed* (*m/s*) (**b**) and the Peri-22 150 ppm female rats covered more ground (**a**) and moved faster (**b**) than the Control female rats ($p < 0.05$*). The Peri-22 1,000 ppm female rat's locomotor activity was not different from the Control female rats. Data are presented as the mean ± SEM

nor *Mean Speed* (*m/s*) for female rats (Fig. 4a–b) in the OF. However, the multiple comparisons revealed that the Peri-22 150 ppm female rats covered more ground ($p < 0.05$*; Fig. 4a) and moved faster ($p < 0.05$*; Fig. 4b) in the OF when compared to Control female rats.

3.3 Neurodevelopmental Pb^{2+} Exposure Induced Sex-Dependent Differences in Anxiogenic Behaviors

Considering the baseline differences in locomotor activity as a function of sex and Pb^{2+} exposure in the OF, the same rats were then examined for anxiety-like behaviors in the EPM. First, the Control male and female rats were evaluated for any sex-dependent effects in anxiety-like behaviors in the EPM. There were no sex-dependent differences observed for open arms time mobile (sec; Fig. 5a) nor closed arms time mobile (sec; Fig. 5b). This suggests that both Control male and female rats exhibited similar anxiety-like behaviors in the EPM, and the hyperactivity behavioral phenotype from the OF in Control females did not influence their behaviors in the EPM.

In contrast, the effects of Pb^{2+} exposure did not influence the male rats, regardless of dose in the open arms time mobile (sec; Fig. 6a) nor the closed arms time mobile (sec; Fig. 6c). Thus,

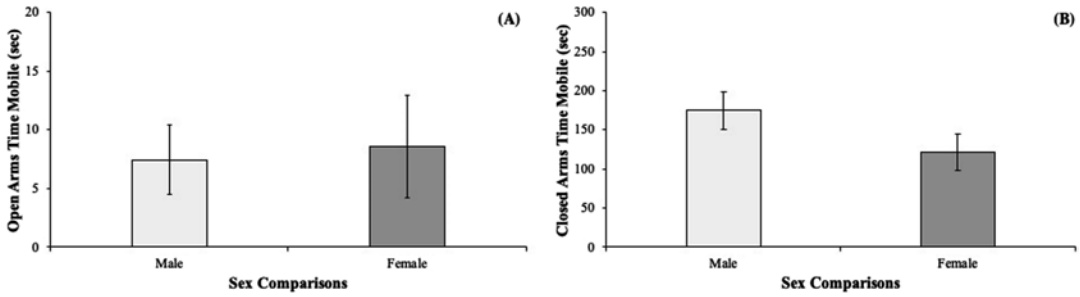

Fig. 5 Effect of *Sex* on locomotor and anxiety-like activity in the EPM. Data show both *Open Arm Time Mobile* (*sec*) (**a**) and *Closed Arm Time Mobile* (*sec*) (**b**), and there were no sex-dependent differences in anxiogenic behaviors in the Control rats. Data are presented as the mean ± SEM

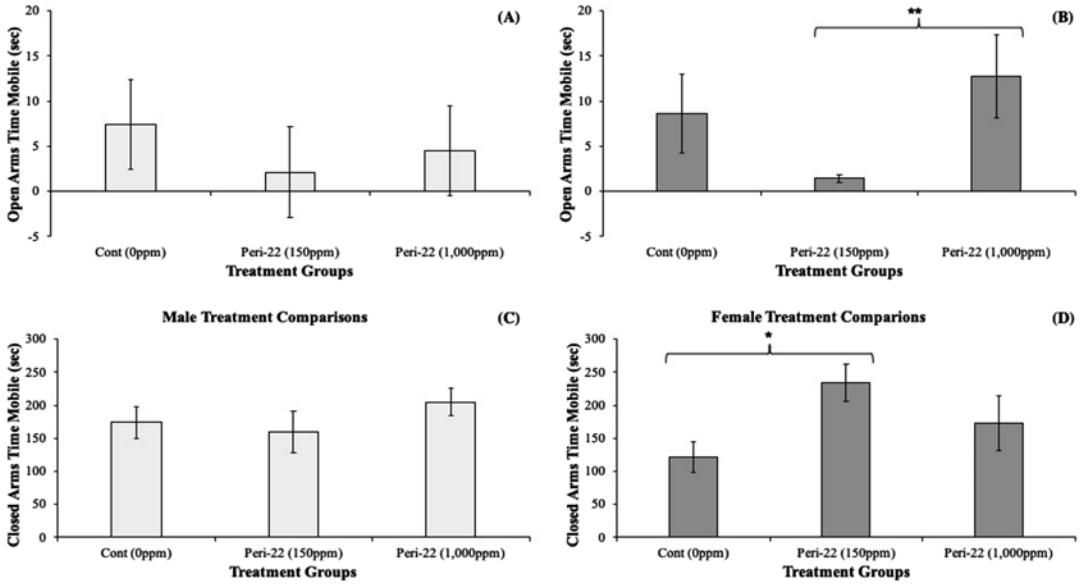

Fig. 6 Effect of Pb^{2+} exposure on locomotor and anxiety-like activity in the EPM. Data show both *Open Arm Time Mobile* (*sec*) (**a–b**) and *Closed Arm Time Mobile* (*sec*) (**c–d**), and there were no effects of Pb^{2+} exposure on anxiogenic behaviors among males. However, among females, the Peri-22 150 ppm rats had an anxiogenic phenotype by spending more time in the closed arms mobile ($p < 0.05$*), which is a sign of hyperactivity. Further, in the open arms, the Peri-22 150 ppm spent more time freezing in then the other treatment groups as trend ($p = 0.59$), but the Peri-22 1,000 ppm female rats were more hyperactive in the open arms when compared to the Peri-22 150 ppm female rats ($p < 0.01$**). Data are presented as the mean ± SEM

male rats that were Pb^{2+}-exposed had similar anxiogenic behaviors as Control male rats. Interestingly, a sex-dependent effect of Pb^{2+} exposure was observed specifically in female rats. The Peri-22 150 ppm female rats had increased hyperactivity and time mobile with the closed arms (Fig. 6d), whereas the Peri-22 1,000 ppm female rats had increased hyperactivity in the open arms when compared to the Peri-22 150 ppm female rats (Fig. 6b). Lastly, a trend was observed ($p = 0.59$) in the Peri-22 150 ppm female rats exhibiting hypoactivity in the open arms when compared to the Control female rats. Taken together, the data suggest that Pb^{2+} exposure causes sex-specific differences in anxiety-like behaviors in female rats.

3.4 Neurodevelopmental Pb^{2+} Exposure Induced Sex-Dependent Differences in Encephalization Quotients and Taurine Influences

Following behavioral testing, the rat's EQ was examined, and an effect of *Sex* was observed between Control males and females $t_{(7.153)}$ = -5.696, $p < 0.001$***, whereby females have greater EQs (Fig. 7). Subsequently, the effects of neurodevelopmental Pb^{2+} exposure revealed a significant effect of *Treatment* $F_{(4)} = 14.311$, $p < 0.01^{\#\#}$, $\eta_p^2 = 0.656$ (Fig. 7). Moreover, there was a significant effect of *Sex* $F_{(1)} = 46.814$, $p < 0.001$***, $\eta_p^2 = 0.474$ and a significant *Treatment X Sex* interaction $F_{(4,1)} = 4.590$, $p < 0.01^{\dagger\dagger}$, $\eta_p^2 = 0.261$ (Fig. 7). The *multiple comparisons* revealed that taurine specifically influenced the female Peri-22 (1,000 ppm + Tau) rats, thereby increasing their EQs, when compared to the Peri-22 (1,000 ppm; $p < 0.01^{\dagger\dagger}$) and the Peri-22 (150 ppm +Tau; $p < 0.05^{\dagger}$) rats, respectively (Fig. 7).

3.5 Neurodevelopmental Pb^{2+} Exposure and Taurine Did Not Influence Total Nuclei Counts Examined by Isotropic Fractionation Methods

To further examine how sex influences the EQ of rats, ITF methods were done on the PFC, HP, and DE brain regions of the rats. The results showed that there were no sex-dependent differences in the total cell counts across the PFC, HP, and DE (Fig. 8a–f). Next, the effects of developmental Pb^{2+} exposure and taurine's influence on ITF total cell counts were examined. In the PFC, HP, and DE, there were no effects of Pb^{2+} exposure or taurine's influence on ITF total cell counts for male rats (Fig. 8a–f). In contrast, for female rats, there were no effects of Pb^{2+} exposure or taurine's influence on ITF total cell counts in the PFC and DE. Similarly, no differences between neuronal and non-neuronal cell populations were observed as a function of Pb^{2+} exposure, nor was taurine's influence, and approximately a 3:1 ratio of non-neuronal to neuronal cells was observed (see Table 1).

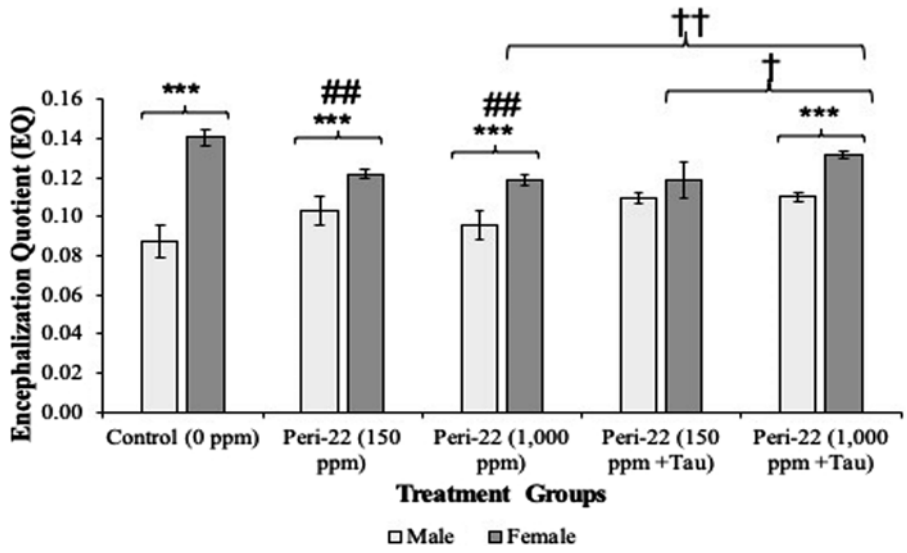

Fig. 7 Effects of neurodevelopmental Pb^{2+} exposure on rat's encephalization quotient (EQ) and the influence of taurine. In all treatment comparisons, except for the Peri-22 (150 ppm +Tau), there was an effect of Sex ($p < 0.001$***). Interestingly, there was a significant Sex X Treatment interaction ($p < 0.01^{\#\#}$), and further taurine increased the EQ specifically in females in the Peri-22 (1,000 ppm +Tau), when compared to the female Peri-22 (150 ppm; $p < 0.05^{\dagger}$) and the female Peri-22 (1,000 ppm; $p < 0.01^{\dagger\dagger}$) rats, respectively. Males (white bars) and females (gray bars). Data are presented as the mean ± SEM

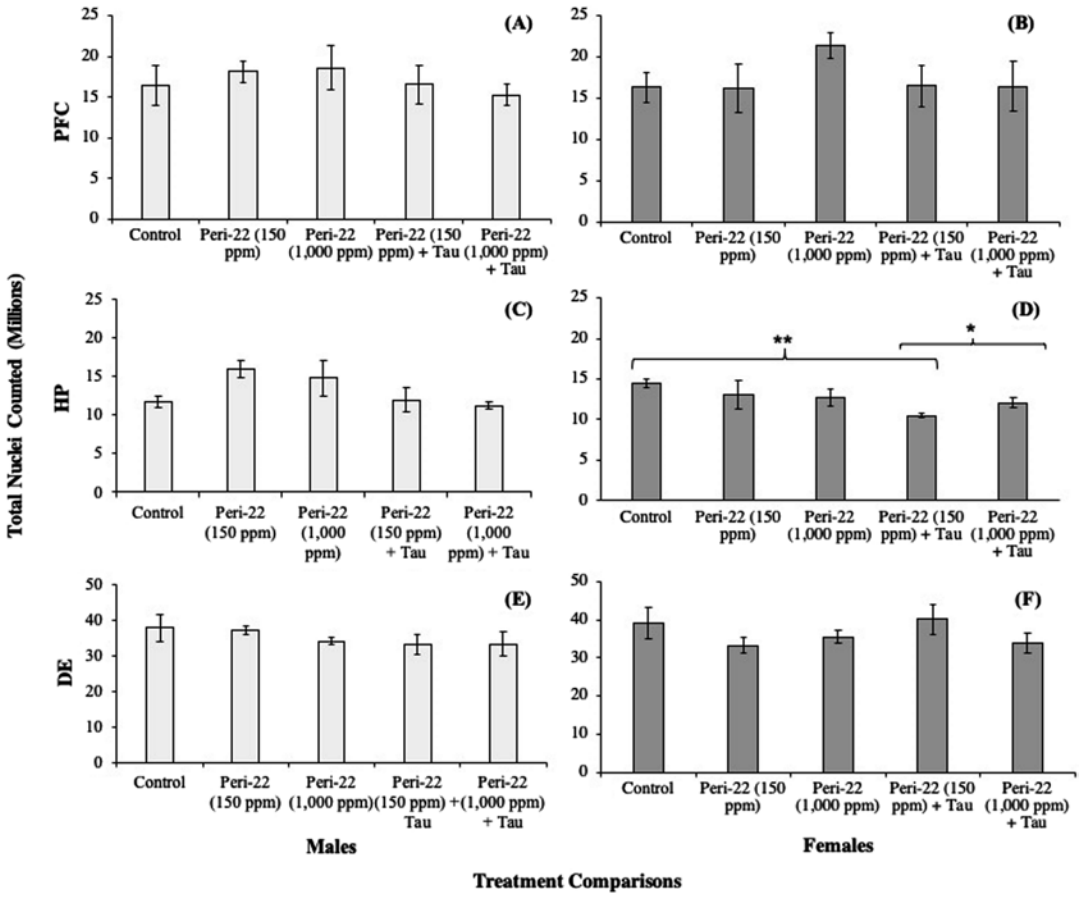

Fig. 8 The effects of Pb^{2+}-exposed and/or co-treated with taurine during neurodevelopment on isotropic fraction methods: total cell counts within the rats' PFC (**a–b**), HP (**c–d**), and DE (**e–f**). There were no significant differences observed, with the exception for the HP in females (**d**), where the female Peri-22 (150 ppm + Tau) is significantly different when compared to female Control ($p < 0.01$**) and the female Peri-22 (1,000 ppm + Tau; $p < 0.05$*) rats. Males (white bars) and females (gray bars). Data are presented as the mean ± SEM

Table 1 Percentage of neuronal and non-neuronal cell counts determined by isotropic fractionation methods

		Control (0 ppm)		Peri-22 (150 ppm)		Peri-22 (1,000 ppm)	
		Male	Female	Male	Female	Male	Female
Brain regions	Cell population	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
PFC	% Neuronal	28.90 (6.59)	31.46 (5.38)	25.41 (6.06)	25.59 (3.17)	27.57 (8.93)	19.28 (1.50)
PFC	% Non-neuronal	71.1 (6.59)	68.52 (5.38)	74.59 (6.06)	74.41 (3.17)	72.43 (8.93)	74.85 (1.50)
HP	% Neuronal	34.15 (8.50)	30.47 (6.35)	31.83 (8.94)	30.53 (7.35)	32.79 (3.36)	26.35 (9.25)
HP	% Non-neuronal	65.85 (8.50)	69.53 (6.35)	68.17 (8.50)	69.47 (7.34)	67.21 (8.94)	73.65 (9.25)
DE	% Neuronal	19.20 (4.73)	21.51 (3.79)	23.05 (14.18)	30.16 (11.22)	20.44 (4.60)	17.67 (0.63)
DE	% Non-neuronal	80.80 (4.73)	78.49 (3.79)	76.95 (14.18)	69.84 (11.66)	79.56 (4.60)	82.33 (0.63)

Note: The SD values are the same for both % neuronal and % non-neuronal cell populations as the datasets are reciprocals.

4 Discussion

The present 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 to better understand the neurobehavioral consequences of Pb^{2+} exposure. The OF revealed that female Control rats are more hyperactive than male Control rats. These sex-dependent effects are differentially altered by Pb^{2+} exposure, whereby in males Pb^{2+} exposure dose-dependently increases hyperactivity in the total distance traveled for both Pb^{2+} exposures and only for Peri-22 150 ppm for mean speed (i.e., velocity). In contrast, only the Peri-22 150 ppm Pb^{2+} exposure increased hyperactivity in female rats for both total distance traveled and mean speed. These phenotypes suggest that lower Pb^{2+} exposures produce aberrant locomotor behaviors in low-light OF test conditions (i.e., non-anxiogenic lighting) that would be predictive of influencing the anxiety-like behaviors in the EPM (for review, see Walf and Frye 2007).

Interestingly, there were no sex differences observed between Control male and female rats in the EPM as measured by the open arms and closed arms time mobile, suggesting similar levels of anxiogenic behaviors. Thus, the sex-dependent differences in locomotor activity (i.e., under low light conditions) did not transfer/carryover into the EPM (i.e., under bright light conditions). What can be inferred is that the Control males and females suppressed their locomotor activity substantially from the OF in the EPM due to the anxiogenic light conditions. Additionally, there could also be a compounding factor in that the rat's might be shifting their inhibitory control processes. For example, the Control female rats were tested in the OF and then the EPM, which could have contributed to the differences in their OF hyperactivity to EPM similar to anxiety-like behaviors when compared to Control male rats. In male rats, Pb^{2+} exposure did not influence anxiety-like behaviors in the EPM for either measure of open arms and closed arms time mobile. Similarly, Pb^{2+}-exposed male rats exhibited a substantial amount of behavioral inhibition of their locomotor activity from the OF to the EPM. In contrast, Pb^{2+}-exposed female rats exhibited anxiety-like behaviors that showed hyperactivity in the closed arms and hypoactivity in the open arms (i.e., Peri-22 150 ppm) and hyperactivity in the open arms (i.e., Peri-22 1,000 ppm). These findings reveal sex-specific effects of Pb^{2+} exposure that show subtle differences in anxiety-like behaviors when considering the location of the EPM zone (i.e., open arms vs. closed arms) and evaluating time mobile as an index of either hyper- or hypoactivity. This is important as rats often freeze within the EPM in either zone and activity is far less frequent. Given that Pb^{2+} exposure increases locomotor activity in low-light conditions in the OF and increases locomotor activity in the EPM under high-light conditions that have subtle differences in zones provides researchers with the unique ability to evaluate and screen novel anxiolytic drugs that may ameliorate neurodevelopmental Pb^{2+} exposure (Neuwirth and Emenike 2021; Vasquez et al. 2021; Neuwirth et al. 2017, 2019a, b, 2021; Neuwirth 2014).

In order to evaluate how these neurobehavioral observations might alter the neurobiology of the brain, an EQ was examined across treatment groups. In particular, an add-on study was conducted using rats that were co-treated with taurine supplementation (0.05% in the drinking water) during the same neurodevelopmental exposure paradigms (i.e., Peri-22 150 ppm and Peri-22 1,000 ppm) that were used to evaluate any neuroprotective against Pb^{2+}-induced brain damage. Given the literature on adults that were Pb^{2+}-poisoned as children having loss of brain volume that persisted across their lifespan, the EQ was theorized to provide a similar metric of brain mass loss as a function of Pb^{2+}-exposure. The data showed that male rats did not lose substantial brain mass in response to Pb^{2+} exposure regardless of dose, but interestingly female rats that were Pb^{2+}-exposed did lose brain mass. This female loss in brain mass was similar for both Pb^{2+} exposures and may be a specific phenomenon in rats as an explanation as to why the findings differ from the human literature. Interestingly, the taurine co-treated rats that were exposed to

Peri-22 1,000 ppm had recovered more brain mass comparable to a Control female rat. This suggests that moderate- to higher-level Pb^{2+} exposures can be protected against by taurine, but lower-level Pb^{2+} exposures may not be prevented by the same level of neuroprotection. Further, the EQ, although a valuable marker for brain mass as it relates to cognitive function (i.e., larger mass equals greater intellectual capacity, whereas lower mass equals greater cognitive deficits; for review see, Gibson et al. 2001), is limited due to its reliance on body weight (Herculano-Houzel 2011a, b), and it does not provide information regarding differences in gray vs. white matter losses/gains nor cell populations that may have changed in response to Pb^{2+} exposure and taurine treatment.

To address this question, the ITF method was employed on brain region samples from the PFC, HP, and DE. Interestingly, the ITF method is able to parse total cell counts and subsequently the percentage of neuronal and non-neuronal cell populations, with the greater quantity of neurons often correlating with the organism's cognitive capacity for learning that changes across stages of development (Marhounová et al. 2019; Harrigan and Commons 2014; Jacon et al. 2021; Herculano-Houzel 2017). The results showed that there were a greater number of cells obtained from the DE relative to the PFC and HP regardless of sex and Pb^{2+} exposures with the exception of the HP for females. In the HP, the females that were Peri-22 (150 ppm + Tau) had lower cell counts relative to Control and Peri-22 (1,000 ppm + Tau) female rats. Further, as Pb^{2+} exposure causes more social-emotional disturbances that are related to the anxiety-like behaviors observed in this study, the HP is known as an influential brain region involved in regulating these emotional traits. The HP regulates the gating of emotions in response to stimuli as behavioral outputs. These behavioral outputs emanate from the coordinated activity of the limbic system that include the PFC (i.e., decision making circuity) and DE (i.e., hormonal regulatory circuitry), hence why they were examined herein.

Moreover, the ITF method did not show any differences between neuronal and non-neuronal cell populations, limiting the ability to pinpoint a specific mechanism for Pb^{2+}-induced brain damage. Thus, despite the advantage of ITF methods to compare total cell, neuronal, and non-neuronal counts across species (von Bartheld et al. 2016; Herculano-Houzel 2009, 2011a, b), these models have been limited to wild or standard laboratory animal strains, and this report appears to be the first to use this method in a neurotoxicology model of Pb^{2+} exposure. Thus, it is plausible that given the diffuse neurotoxicant insults of low-level Pb^{2+} exposure (for review, see Needleman 2004; Needleman and Gatsonis 1990), ITF methods may be insensitive to detect persistent effects of early neurodevelopmental insults. Further, perhaps the ITF method (Morterá and Herculano-Houzel 2012; Bandeira et al. 2009) may be more appropriate to evaluate different neurodevelopmental time-points (i.e., PND 2, 7, 14, 22) during the exact time-period of Pb^{2+} exposure to capture the changes in real-time to evaluate the percentage of neuronal and non-neuronal populations that may change as a function of Pb^{2+} exposure and taurine co-treatment. Future studies should consider evaluating these points before one can deem ITF methods to be incompatible with neurotoxicology studies/models. Based on the current findings, however, neurodevelopmental Pb^{2+} exposure was associated with aberrant anxiety-like behaviors that persisted across the lifespan as an effect that requires further investigation to more accurately infer changes to the associated neurobiology structure-function relationships.

5 Conclusion

In summary, this study shows that neurodevelopmental Pb^{2+} exposure can have persistent neurobehavioral and neurobiological correlates of anxiety-like traits. These observations are consistent with Pb^{2+} exposure causing altered GABAergic signaling through development that may cause deficits in the inhibitory systems that are important for both cognitive and affective regulation. Further, taurine as a partial agonist for the $GABA\text{-}_{AR}$ may prove useful in counteracting against neurodevelopmental Pb^{2+} exposure and,

accordingly, may still prove useful in regaining a proper E/I balance in CNS function long after an organism is removed from the source of Pb^{2+} exposure(s). Although the Pb^{2+}-induced structural changes to the brain may not be directly corrected by taurine supplementation and treatment, it may provide indirect symptom relief and/or recovery by mitigating GABAergic tone across synaptic networks and (re)establish improvements in inhibitory neural networks. This latter point is critical as neurodevelopmental Pb^{2+} exposure may cause earlier forms of neurodegeneration that may accelerate the effects of senescence, and the increase in GABAergic activity may prove useful in forestalling such compounding consequences due to the widespread neurotoxicant actions of Pb^{2+} exposure.

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In Vivo Sex-Dependent Effects of Perinatal Pb^{2+} Exposure on Pilocarpine-Induced Seizure Susceptibility and Taurine Neuropharmacology

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Keywords

Developmental lead exposure · Lead poisoning · Taurine derivatives · Anxiety-like behaviors · Anxiolytic drugs · GABAergic system

Abbreviations

ASV	anodic stripping voltammetry
BLL	blood lead levels
E/I	excitation-to-inhibition
EDTA	ethylenediaminetetraacetic acid
GAD	glutamic acid decarboxylase
Pb^{2+}	lead
PDS	paroxysmal depolarization shift (PDS)
PND	postnatal day

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1 Introduction

Neurodevelopmental disorders have been shown to either cause or contribute to epilepsy (Crino et al. 2006; Tuchman et al. 2005; Chow et al. 2019; Parenti et al. 2020) due to *de novo* variants from specific neuropathologies (Heyne et al. 2018), psychiatric comorbidities (Berg et al. 2011; Chow et al. 2019), as well as autophagy/lysosomal degradative pathway perturbations (Fassio et al. 2020). Further, the relationships between neurodevelopmental disorders and epilepsy are also associated with long-term language (Pal 2011) and social cognition disorders (Pastorino et al. 2021) that persist across the lifespan, increasing the economic burden of society for many years (Hussain et al. 2020). Despite the advances in gene therapies designed to ameliorate a range of these disorders (Turner et al. 2020), rodent genetic models have become invaluable preclinical tools for devising proof-of-concept treatments with face, construct, internal, and external validity in an effort to identify ways to reduce and/or eliminate neurodevelopmental disorders in humans (Animal Research And The Search For Understanding 2006; Gonzalez-Sulser 2020). These preclinical animal models of epilepsy have also proved useful in advancing the field's understanding of rare neurodevelopmental disorders (Fallah & Eubanks 2020); thus, attesting to their scientific value in developing personalized medical psychopharmacotherapies (Tarlungeanu and Novariona 2018).

Moreover, environmental lead (Pb^{2+}) poisoning since antiquity (Rabin 1989; Hernberg 2000; Taylor et al. 2011) remains as an elusive public health problem for many developed and underdeveloped countries (Shinkuma and Huong 2009; Chen et al. 2011; Eneh and Agunwamba 2011; Laidlaw and Taylor 2011; Yan et al. 2013). As early as the late 1800s, case studies of Pb^{2+} poisoning causing epilepsy were reported (Reynolds 1862; Hopkins 1867; Ruxton 1886). Blackfan (1917) reported the vulnerability of children to Pb^{2+}-induced seizures. Remarkably, in the present day, case studies of children with Pb^{2+}-induced seizure disorders are still reported (Rahman et al. 2016; Lockitch et al. 1991; Kumar et al. 1998; Shannon 2003; Hughes 2005; Burki 2012; Rao et al. 2014; Kosravi et al. 2014; Farhat and Khademi 2015). Interestingly, arguments have been made due to pregnant women in some cases having Pb^{2+} poisoning (Forsyth et al. 2018; Shannon 2003) that it would be most proactive to have a BLL screening for pregnant women prior to conception and during each trimester rather than waiting until the child is 2 years old to screen them (Neuwirth 2018). These original case reports point to research focusing on γ-aminobutyric acid (GABA) and valproate in elucidating the mechanisms for Pb^{2+}-induced convulsions in infants as a potential pharmacotherapy (Healy et al. 1984). It is also important to note that taurine has been shown to influence the GABA-$_{AR}$ on the blood vessels which can regulate global blood pressure in response to hypertension (El Idrissi et al. 2013). Notably, in the 1970s, animal models were used to evaluate the mechanisms by which Pb^{2+} poisoning might induce epilepsy in humans (Silbergeld and Goldberg 1974; Silbergeld et al. 1979). However, as this work started, concurrent work revealed the relationship between the glutamatergic *N-methyl-D-aspartate* ($NMDA_R$) and the Pb^{2+}/Ca^{2+} competition through the ion channel, which became a clear mechanism that served to explain the relationship between increased Pb^{2+} exposures and decreased intellectual functions (for review see, Cory-Slechta et al. 1997; Guilarte 1997; Guilarte and McGlothan 1998; Marchetti 2003; Guilarte 2004; Zhi-wei et al. 2005; Neal and Guilarte 2010). Thirty to 50 years later, researchers began to reestablish connecting these early reports of Pb^{2+} poisoning to neurodevelopmental disruption of the GABA-$_{AR}$ (Winder et al. 1982; Drew et al. 1989, 1990; Kumar and Desiraju 1990; Leret et al. 2002; Olympio et al. 2009; Neuwirth 2014; Duan et al. 2017; Neuwirth et al. 2017, 2018, 2019a, b, c, 2022; Nam et al. 2018; Neuwirth and El Idrissi 2021; Neuwirth and Emenike 2021; Cruz et al. 2022). The long-term effects of Pb^{2+}-induced neurodevelopmental disorders manifest into fronto-executive dysfunctions that persist across the lifespan (Neuwirth et al., 2020). These fronto-executive functions can be modeled in rodents with neuro-

developmental Pb^{2+} exposures, providing good models of construct, face, and internal validity (for review, see Tait et al. 2018; Neuwirth et al. 2019a, b, c; Neuwirth and Kolb 2020).

It was not until the GABA-shift (Ben-Ari 2002; Ben-Ari et al. 2012) was fully described (i.e., from early immature excitatory GABA to later mature inhibitory GABA) that the neurodevelopmental effects of Pb^{2+} exposure on the GABAergic system regained relevance. In particular, a few reports have shown that neurodevelopmental Pb^{2+} exposure causes selective cholinotoxicity from septal nerve fibers that project to the entorhinal cortex (Bourjeily and Suszkiw 1997). More recently, nerve growth factor (NGF) was shown to be neuroprotective against Pb^{2+}-induced cholinotoxicity of the septum (Zhou et al. 2000). To date, no reports have investigated the possible relationship of the neurodevelopmental loss of cholinergic neurons that innervate the hippocampus, which would be predictive of altering hippocampal network activity, and possibly contribute to the pathogenesis of seizures. Pilocarpine has been shown to be a selective agonist for *muscarinic 1* (M1) receptors (Caulfield and Stubley 1982). Furthermore, pilocarpine-induced seizures have been a well-established animal model for epilepsy (Turski et al. 1989). Consistent with these reports, pilocarpine acts as a selective M1 receptor agonist increasing cholinergic neural activity emanating from the septal nucleus and projecting to the olfactory bulb, hippocampus, amygdala, as well as the perirhinal, entorhinal, prefrontal, visual, and somatosensory cortices, thereby inducing both local and generalized seizure activity that can result in cholinergic excitotoxicity (i.e., cholinotoxicity; Boskovic et al. 2019). Thus, the present study sought to address the gap in the literature on how neurotoxicants, such as Pb^{2+}, can contribute to early disruption of the GABA-shift, thereby reducing inhibition across the brain. In turn, this would then increase the overall brain excitability to multiple neurotransmitter systems, and in particular, the evaluation of M1 receptor-induced cholinotoxicity could be done through pilocarpine-induced seizures in the rat model. Additionally, the ability for the rats to or fail to exhibit the paroxysmal depolarization shifts (PDS) for their interictal time (IIT; Browne and Holme 2008) would be used to evaluate their GABAergic reserves in protecting against pilocarpine-induced cholinotoxicity. Lastly, the pharmacological administration of taurine would be used to assess its neuroprotective potential against pilocarpine-induced cholinotoxicity. Subsequently, picrotoxin would be used to determine the selectivity of taurine to the $GABA\text{-}_{AR}$ and serve as a pharmacological control for the M1 receptor and $GABA\text{-}_{AR}$ mechanisms of action for the predicted cholinotoxicity in the rat model used herein.

2 Methods

2.1 Subjects

In accordance with the SUNY Old Westbury (SUNY-OW) IACUC approval guidelines, Long-Evans Norwegian hooded male (N = 15) and female rats (N = 30) (Taconic, N.J.) were paired for breeding, and their male F1 generation was used for future experimentation. This breeding paradigm was conducted over a 3-year time period for this study. Whenever possible, rat litters were culled to 8–10 pups to control for maternal socialization that might influence neurodevelopment and its associated electrophysiological and molecular biological outcomes. Rats were fed ad libitum with Purina rat chow (RHM1000 # 5P07). Notably, Control rats were provided regular water, while the Pb^{2+}-exposed rats were provided 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 Pb^{2+} developmental exposure model). At PND 22, Pb^{2+} exposures ceased and all rats returned to a regular water regimen. Rats that were 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.

2.2 Blood Pb^{2+}-Level Analyses

Blood samples were collected and analyzed using the same methods as prior studies (Neuwirth 2014; Neuwirth et al. 2017, 2018, 2019a, b; Neuwirth and El Idrissi 2021). Briefly, a first cohort of (n = 4) male and (n = 4) female pups at PND 22 was randomly selected and sacrificed from the Peri-22 150 ppm and the Peri-22 1000 ppm treatment groups. The blood samples were collected within 2 mL anti-coagulant *ethylenediaminetetraacetic acid* (EDTA) coated syringes (Sardstedt, Germany), mixed to prevent coagulation, and temporarily stored at −80 °C. Blood samples were analyzed using a commercial ESA LeadCare II Blood Lead Analyzer system (Magellan Diagnostics, North Billerica, MA) through electrochemical anodic stripping voltammetry (ASV). The ASV method was conducted by taking 50 µL of whole blood mixed with 250 µL of hydrochloric acid solution (0.34 M). The final mixture was applied 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 Stereotaxic Surgery for Inducing In Vivo Hippocampal CA1 Seizures

Naivë rats that were tested between PND 55–95 from the F1 generation offspring (N = 102) were comprised of both males (n = 46) and females (n = 56) that were subjected to stereotaxic surgery to induce in vivo hippocampal seizures through CA1 consistent with the procedures of Neuwirth and Ros (2021). Briefly, the second cohort of the treatment groups was as follows: Control males (n = 4), Peri-22 150 ppm Pb^{2+} males (n = 5), and Peri-22 1000 ppm Pb^{2+} males and Control females (n = 7), Peri-22 150 ppm Pb^{2+} females (n = 5), and Peri-22 1000 ppm Pb^{2+} females (n = 7), respectively. A third cohort of rats was subjected to taurine treatment as follows: Tau+Control males (n = 5), Tau+Peri-22 150 ppm Pb^{2+} males (n = 7), and Tau+Peri-22 1000 ppm Pb^{2+} males (n = 5) and Tau+ Control females (n = 7), Tau+Peri-22 150 ppm Pb^{2+} females (n = 4), and Tau+Peri-22 1000 ppm Pb^{2+} females (n = 8), respectively. A fourth cohort of rats was subjected to picrotoxin+taurine treatment as follows: Picro+Tau+Control males (n = 5), Picro+Tau+Peri-22 150 ppm Pb^{2+} males (n = 6), and Picro+Tau+Peri-22 1000 ppm Pb^{2+} males (n = 3) and Picro+Tau+Control females (n = 7), Picro+Tau+Peri-22 150 ppm Pb^{2+} females (n = 3), and Picro+Tau+Peri-22 1000 ppm Pb^{2+} females (n = 8), respectively. All rats were weighed prior to being anesthetized using Ketamine 9 mg/Kg:Xylazine 91 mg/Kg (ShopMedVet, Mettawa, IL) that was administered as a 100 mg/Kg cocktail i.p. to place the rat under general anesthesia for the duration of the stereotaxic surgery. Once under anesthesia and absent of observable reflexes, rats were installed into a David Kopf Instruments stereotaxic apparatus (Tujunga, CA), and the following coordinates (Paxinos and Watson 2007; Fig. 1) were used to install a recording electrode into the *cornu ammonis* 1 (CA1) perforant pathway of the hippocampus: M/L + 2.5 mm, A/P + 2.5 mm, and D/V - 2.5 mm. Additionally, three ground stainless steel screws were installed −1.5 mm A, + 1.5 mm P, and − 1.5 mm L of the recording electrode, respectively. The rats were then thermoregulated using a warm blanket, and their eyes were periodically hydrated with GenTeal® tears over-the-counter eye solution.

2.4 Stereotaxic Surgery for Inducing In Vivo Hippocampal CA1 Seizures

Once the recording electrode was installed and the rat was grounded into the electrophysiology rig, a baseline brain activity recording was conducted for 15 min. This was followed by an i.p.

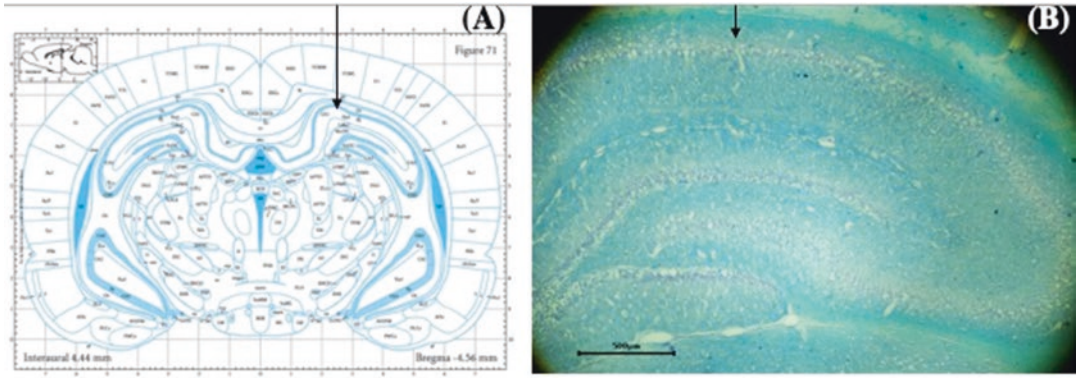

Fig. 1 Depiction of the recording electrode placement within the right hippocampal CA1 region using the stereotaxis atlas (Paxinos & Watson, 2007; **a**) and a histological H and E stained coronal rat brain 10 μm thin slice, imaged at 4X (scale bar = 500 μm), providing evidence of post-surgical recording electrode placement (black arrow; **b**)

injection of atropine 1 mg/Kg (Sigma Aldrich, St. Louis, MO) to block the peripheral nicotinic receptors to prevent the rat from having cardiac arrest when pilocarpine would be later injected into the rat. Following the atropine injection, the rat's brain activity was recorded for another 15 min. Next, 380 mg/Kg of pilocarpine (Fischer Scientific, Hampton, NH) was injected i.p., and the rat's brain activity was recorded for 1 hr. to evaluate them for seizures. For the second rat cohort that was assigned to the taurine treatment group, they followed the same experimental procedure except that they were injected with 43 mg/Kg taurine (Sigma Aldrich, St. Louis, MO) 15 min after the atropine and prior to pilocarpine injection. Likewise, in the third rat cohort that was assigned to the picrotoxin treatment group, they followed the same experimental as the taurine treatment group except that they were injected with picrotoxin 5 mg/kg (Fischer Scientific, Hampton, NH) 15 min after the atropine and prior to the taurine injection. All drugs were dissolved in physiological buffered saline with a pH of 7.4 and sterilized through a 0.2 μm syringe filter prior to injecting any drug into a rat.

2.5 Stereotaxic Surgery for Inducing In Vivo Hippocampal CA1 Seizures

Data were recorded in real time and analyzed using an AD Instruments Power Lab 4/30 data acquisition system with LabChart software (St. Paul, MN). Raw brain activity data were recorded as a full bandwidth. Seizures were manually separated by the five typographies observed (Fig. 2). The *frequency, duration (msec), and peak amplitudes (*μV) were used to evaluate the excitability profiles of the rat's brain activity. In contrast, the rat's *interictal time (IIT; sec)* was used to evaluate the inhibitory profiles of the rat's brain activity as they represent the paroxysmal depolarization shift (PDS) from excitation-to-inhibition for all seizures that were analyzed.

2.6 Statistical Analyses

All behavioral data were collated in Microsoft Excel and later analyzed in IBM SPSS V. 24 (IBM, Inc. Armonk, NY). A *t*-test was used to compare *Sex* differences in the Control rats. Next, an *ANOVA* was used to assess *Treatment* and *Drug Treatment* effects for both male and female rats. The criteria for significance were set at $\alpha = 0.05\%$ with a 95% ± 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 *effect sizes* where applicable.

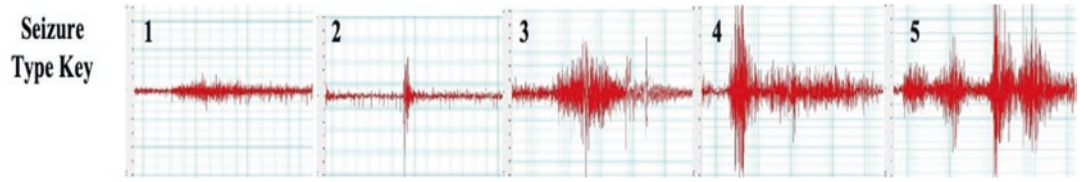

Fig. 2 Raw data graphical representation of the five seizure typographies observed from least severity (1) to most severe (5)

3 Results

3.1 BLLs as a Function of Sex and Pb^{2+} Exposure

The fourth cohort of rats was used to determine the BLLs for the Peri-22 150 ppm and the Peri-22 1000 ppm treatment groups. The BLL data revealed no sex-dependent differences, and BLLs were less than the 39 µg/dL chelation therapy limit. Each Pb^{2+} treatment at the time of sample collection resulted in BLLs ranging from 4.1 to 13.3 µg/dL (SD ± 1.74) for Peri-22 150 ppm rats ($p < 0.001$***) and from 12.5 to 26.2 µg/dL (SD ± 2.91) for Peri-22 1000 ppm ($p < 0.001$***), respectively. The control rats were Pb^{2+} negative as their BLL samples obtained in this study were less than lower detection limit of 3.33 µg/dL. The BLL samples from the rats subjected to seizures were also drawn following the conclusion of the study. However, their BLLs were below the 3.3 µg/dL detection limit consistent with reports from the US Agency for Toxic Substances and Disease Registry (ATSDR, 2007) that Pb^{2+} is not uniformly distributed in bone, blood, and soft mineralizing tissues.

3.2 Gender-Specific Susceptibility to Pilocarpine-Induced Seizures

The Control male and female rats were evaluated for any differences in pilocarpine-induced seizures. In the excitatory brain activity profiles, the data revealed for seizure *frequency* a significant effect of *Sex* $F_{(1)} = 10.297$, $p < 0.05$*, $\eta_p^2 = 0.238$ was observed, but no *Seizure Type* effect $F_{(4)} = 0.891$, $p = 0.480$, nor any *Sex X Seizure Type* interaction $F_{(1,4)} = 0.571$, $p = 0.685$ (Fig. 3a). For the seizure *duration (msec)*, a significant effect of *Sex* $F_{(1)} = 39.730$, $p < 0.001$*** $\eta_p^2 = 0.238$ was observed, but no *Seizure Type* effect $F_{(4)} = 2.444$, $p = 0.666$, $\eta_p^2 = 0.229$ (trend), nor any *Sex X Seizure Type* interaction $F_{(1,4)} = 0.889$, $p = 0.481$ (Fig. 3b). For the seizure *peak amplitude* (µV), a significant effect of *Sex* $F_{(1)} = 32.366$, $p < 0.001$***, $\eta_p^2 = 0.495$ was observed, but no *Seizure Type* effect $F_{(4)} = 1.027$, $p = 0.408$, nor any *Sex X Seizure Type* interaction $F_{(1,4)} = 0.823$, $p = 0.520$ (Fig. 3c). For the inhibitory brain activity profiles using PDS, the IIT (sec) data revealed a significant *Sex* effect $t_{(9)} = 3.004$, $p < 0.01$** (Fig. 3d).

3.3 Pilocarpine-Induced Seizures in Rats Exposed to Pb^{2+}

Following the sex-dependent assessment of pilocarpine-induced seizure 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 male's excitatory brain activity profiles, the data revealed for seizure *frequency* a significant effect of *Treatment* $F_{(2)} = 5.536$, $p < 0.05$*, $\eta_p^2 = 0.156$, and a significant *Seizure Type* effect $F_{(4)} = 2.588$, $p < 0.05^{\#}$, $\eta_p^2 = 0.165$ was observed, but no *Treatment X Seizure Type* interaction $F_{(2,4)} = 0.625$, $p = 0.753$ was observed (Fig. 4a). For the seizure *duration (msec)*, a significant effect of *Treatment* $F_{(2)} = 36.600$, $p < 0.001$***, $\eta_p^2 = 0.567$ was observed, but no *Seizure Type*

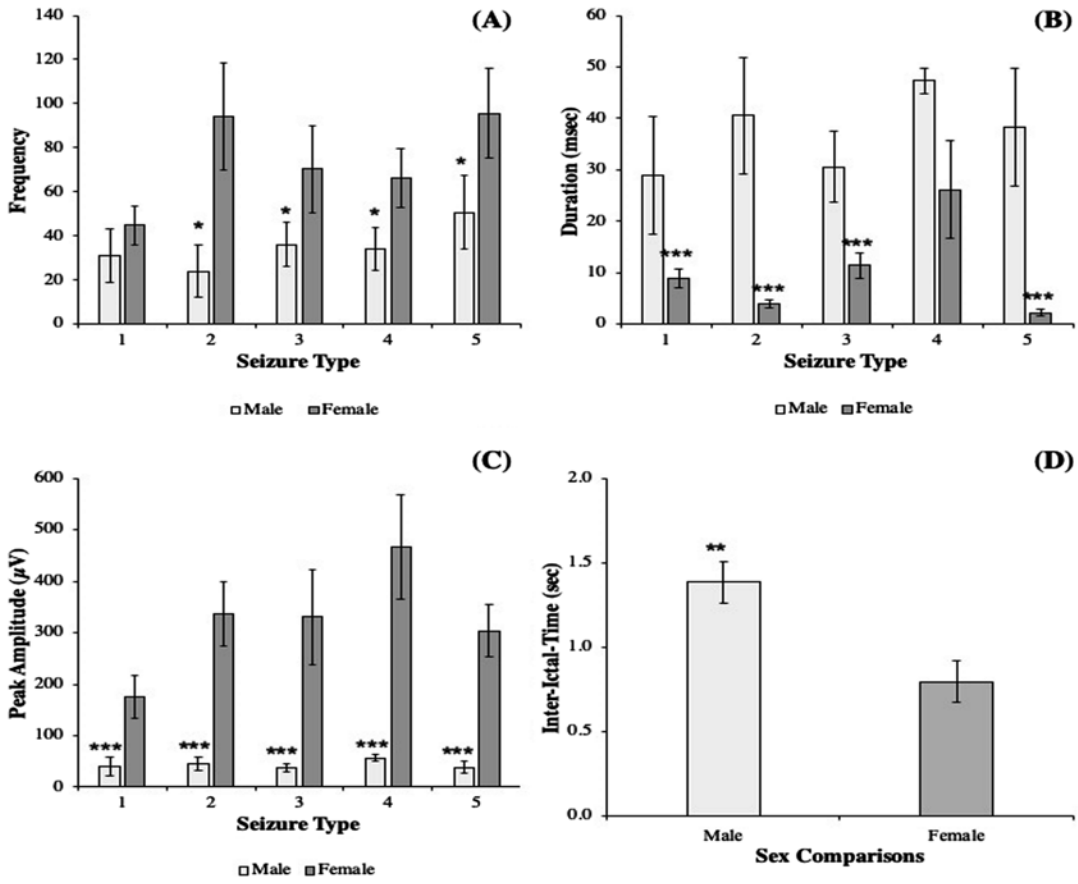

Fig. 3 Comparisons between Control male and female rat's pilocarpine-induced seizures. Females have increased brain excitability in seizure *Frequency* ($p < 0.05$*; **a**), *Duration* (*msec*; $p < 0.001$***; **b**), and peak amplitude (μV; $p < 0.001$***; **c**), as well as reduced inhibition through PDS IIT (*sec;* $p < 0.01$**; **d**). Data are presented as the mean ± SEM

effect $F_{(4)} = 0.708$, $p = 0.666$. However, there was a significant *Treatment X Seizure Type* interaction $F_{(2,4)} = 2.957$, $p < 0.01$††, $\eta_p^2 = 0.297$ (Fig. 4b). For the seizure *peak amplitude (*μV*)*, there was a significant effect of *Treatment* $F_{(2)} = 113.644$, $p < 0.001$***, $\eta_p^2 = 0.802$ and a significant *Seizure Type* effect $F_{(4)} = 2.392$, $p < 0.05^{\#}$ and a significant *Treatment X Seizure Type* interaction $F_{(2,4)} = 2.091$, $p < 0.05$†, $\eta_p^2 = 0.230$ (Fig. 4c). For the inhibitory brain activity profiles using PDS, the IIT (sec) data revealed a significant *Treatment* effect $F_{(2)} = 22.019$, $p < 0.001$**, $\eta_p^2 = 0.800$ (Fig. 4d).

In contrast, for the female's excitatory brain activity profiles, the data revealed for seizure *frequency* a significant effect of *Treatment* $F_{(2)} = 5.424$, $p < 0.01$**, $\eta_p^2 = 0.141$ was observed, but no *Seizure Type* effect $F_{(4)} = 1.089$, $p = 0.369^{\#}$, nor a *Treatment X Seizure Type* interaction $F_{(2,4)} = 0.513$, $p = 0.842$ was observed (Fig. 5a). For the seizure *duration (msec)*, a significant effect of *Treatment* $F_{(2)} = 7.289$, $p < 0.001$***, $\eta_p^2 = 0.181$, a *Seizure Type* effect $F_{(4)} = 8.270$, $p < 0.001$***, $\eta_p^2 = 0.334$, and a significant *Treatment X Seizure Type* interaction $F_{(2,4)} = 2.550$, $p < 0.01$**, $\eta_p^2 = 0.236$ were observed (Fig. 5b). For the seizure *peak amplitude (*μV*)*, there was no effect of *Treatment* $F_{(2)} = 2.223$, $p = 0.916$, nor a *Treatment X Seizure Type* interaction $F_{(2,4)} = 0.684$, $p = 0.704$ was

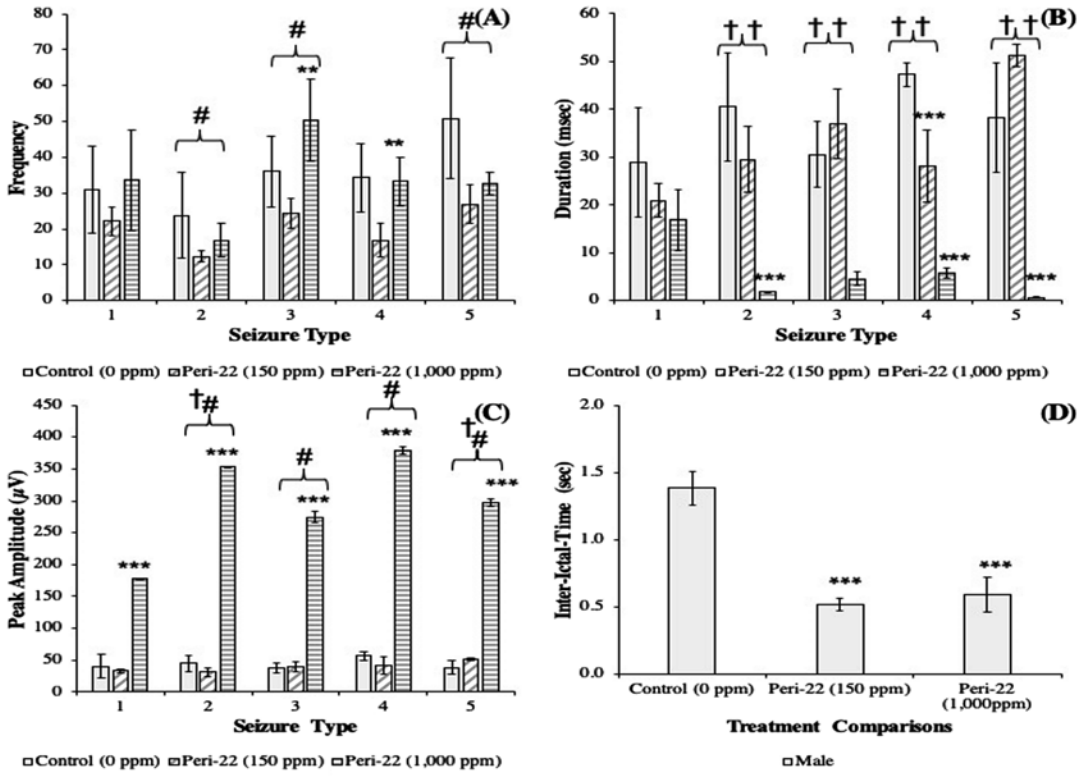

Fig. 4 Comparisons between male Control, Peri-ppm, and Peri-22 1000 ppm rat's pilocarpine-induced seizures. In male rats, Peri-22 1000 ppm increased seizure *Frequency* ($p < 0.05$*; **a**), Peri-22 150 ppm and Peri-22 1000 ppm decreased seizure *Duration* (*msec*; $p < 0.001$***; **b**), and Peri-22 1000 ppm increased seizure peak amplitude (μV; $p < 0.001$***; **c**), as well as Peri-22 150 ppm and Peri-22 1000 ppm reduced inhibition through PDS IIT (*sec;* $p < 0.01$**; **d**). Data are presented as the mean ± SEM

observed (Fig. 5c). However, there was a significant effect of *Seizure Type* $F_{(4)} = 3.422$, $p < 0.01^{\#\#}$ (Fig. 5c). For the inhibitory brain activity profiles using PDS, the IIT (sec) data revealed no *Treatment* effect $F_{(2)} = 2.500$, $p = 0.143$ (Fig. 5d).

3.4 The Developmental Pb^{2+}-Exposure Sex-Dependent Effects in Brain Excitability Were Recovered by Taurine in Males But Not Females

To further test whether the neurodevelopmental Pb^{2+}-exposure sex-dependent effects revealed through pilocarpine-induced seizures could be recovered, an add-on experiment of rats was used to assess taurine's neuroprotection through modulating the GABAergic inhibitory tone prior to pilocarpine-induced seizures on PDS IIT (sec). In male rats, there was a significant effect of *Taurine* $F_{(5)} = 10.67$, $p < 0.001$***, $\eta_p^2 = 0.557$ for the PDS IIT (sec) data and a significant effect of *Picrotoxin* $F_{(8)} = 7.862$, $p < 0.001$###, $\eta_p^2 = 0.560$ for the PDS IIT (sec) data (Fig. 6a). In contrast, for female rats, there were no significant effects of *Taurine* $F_{(5)} = 1.174$, $p = 0.341$ for the PDS IIT (sec) data, nor any significant effects of *Picrotoxin* $F_{(8)} = 1.981$, $p = 0.102$ for the PDS IIT (sec) data (Fig. 6b).

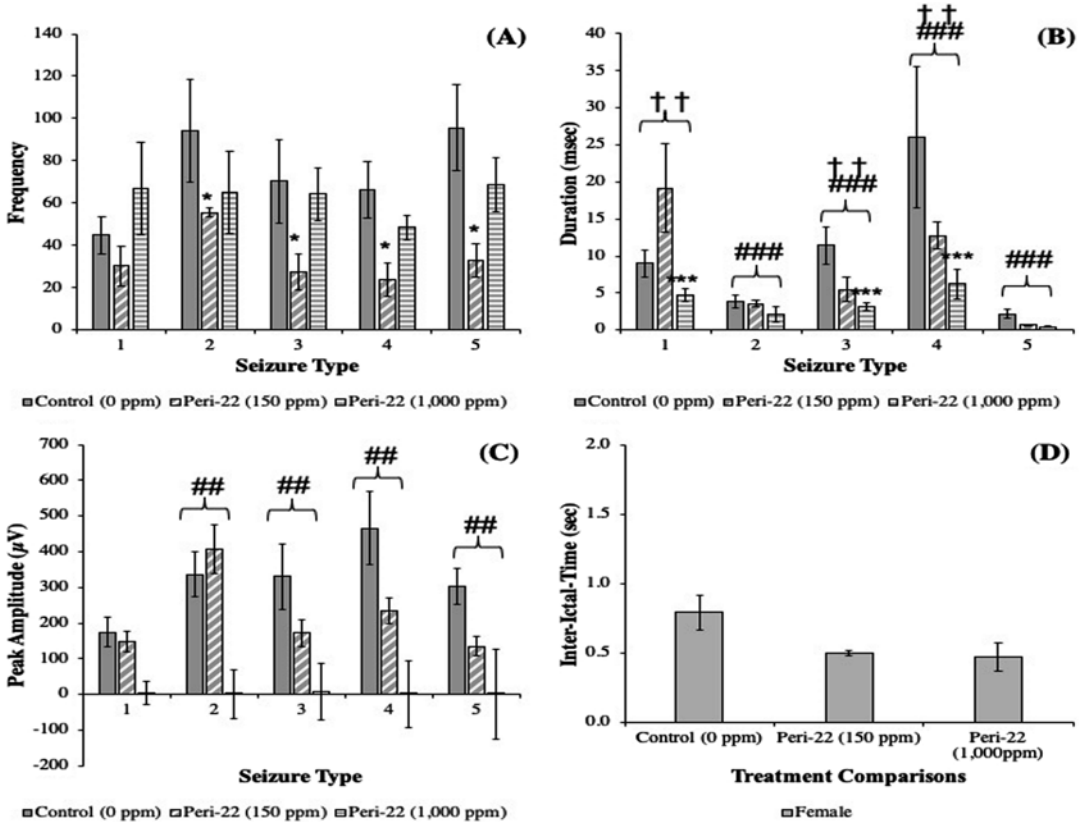

Fig. 5 Comparisons between female Control, Peri-22150 ppm, and Peri-22 1000 ppm rat's pilocarpine-induced seizures. In female rats, Peri-22 150 ppm decreased seizure *Frequency* ($p < 0.05$*; **a**), Peri-22 150 ppm and Peri-22 1000 ppm decreased seizure *Duration* (*msec*; $p < 0.001$***; **b**), and Peri-22 1000 ppm decreased seizure peak amplitude (μV; **c**), and no differences were observed in inhibition through PDS IIT (*sec;* **d**). Data are presented as the mean ± SEM

4 Discussion

The present study sought to evaluate whether neurodevelopmental Pb^{2+} exposure alters the cholinergic and GABAergic systems through pilocarpine-induced seizures in the rat model. The BLLs for both the Peri-22 150 ppm rats and the Peri-22 1000 ppm rats did not differ by sex, but rather were within the clinically relevant range normally observed in children at low-levels of Pb^{2+} exposure. Thus, the animal model used herein has good construct and external validity. In evaluating the sex-based differences in seizure cholinergic-dependent excitability, Control male rats had decreased frequency of seizures, increased duration of seizures, and lower overall peak amplitude of seizures when compared to Control female rats. In addition, Control male rats had greater inhibitory GABAergic responsivity to the pilocarpine-induced seizures as evidenced by increased PDS for their IIT when compared to Control female rats. Thus, Control female rats had greater brain excitability for the overall seizure typographies, but exhibited higher peak amplitudes and lower seizure durations, which were the opposite phenomena observed in Control males defining the sex-based differences.

In the males, neurodevelopmental Pb^{2+} exposure caused differential effects of their cholinergic seizure susceptibility dependent upon their dose of exposure. The Peri-22 150 ppm

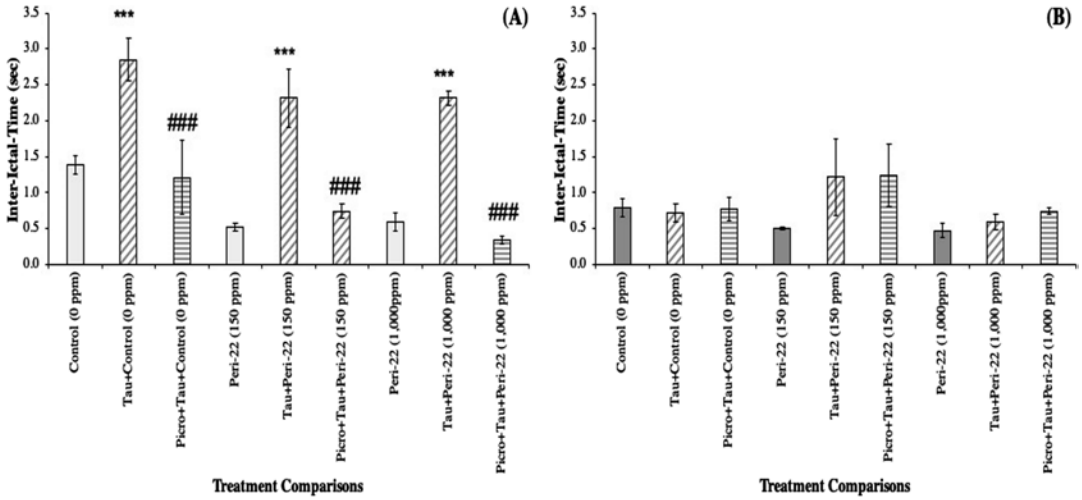

Fig. 6 The effects of taurine and picrotoxin on GABAergic inhibitory tone and neuroprotection of pilocarpine-induced seizures in rats neurodevelopmentally exposed to Pb^{2+}. Assessment of rat's *inter-ictal-time* (IIT) for males (**a**) and females (**b**). The lead treated males had a lower IIT (**a**). In all males treatment groups, taurine increased the IIT, but only in the Peri-22 150 ppm and 1000 ppm treatments did picrotoxin reduce the IIT (**a**). In contrast, the female rats showed little differences in the IIT regardless of treatment (**b**). Data are presented as the mean ± SEM

male rats had lower seizure frequencies, whereas the Peri-22 1000 ppm male rats had lower duration of seizures with greater peak amplitudes defining their treatment-based differences in their cholinergic excitatory traits. In contrast, irrespective of the dose of exposure, both Peri-22 150 ppm and Peri-22 1000 ppm male rats had lower inhibitory GABAergic responsivity to pilocarpine-induced seizures as evidenced by their PDS for their IIT when compared to Control male rats. Interestingly, for female rats, the Peri-22 150 ppm rats reduced their seizure frequency for the overall typographies and had increase seizure duration at lower typographies and decreased seizure duration at higher typographies for their cholinergic excitatory profiles. The Peri-22 1000 ppm female rats had lower peak amplitudes evidencing a low probability or failure to increase excitability when challenged by pilocarpine-induced seizures. The Peri-22 150 ppm and Peri-22 1000 ppm female rats did not differ in their PDS for their IIT when compared to Control female rats indicating similar levels of inhibitory GABAergic responsivity.

After characterizing the sex- and treatment-dependent effects of pilocarpine-induced seizures on neurodevelopmentally Pb^{2+}-exposed rats, to further confirm and describe the physiological and pharmacodynamics of the GABAergic system, two cohorts of rats were employed: 1) a pre-taurine-treated group and 2) a picrotoxin and taurine pre-treated group prior to inducing pilocarpine seizures. In male rats, the taurine pretreatment across all groups increased the PDS for their IIT thereby facilitating their inhibitory GABAergic responses. This taurine effect was pharmacologically blocked by the pretreatment of picrotoxin, thereby suggesting that the taurine effects were specific to the $GABA\text{-}_{AR}$ and that Pb^{2+}-exposed male rats would respond well to taurine treatment for recovering increased brain excitability that emanated from the cholinergic system. Notably, the female rats failed to present similar physiological and pharmacological effects. Across all treatment groups, the female rats did not reveal increased inhibition to taurine pretreatment at the levels observed in males, and the picrotoxin pharmacological block did not change the GABAergic responsivity. These find-

ings suggest that females may be more susceptible to cholinotoxicity as well as potentially having indirect and direct reductions in their GABAergic inhibitory regulation in response to Pb^{2+}-induced elevated brain excitability.

Taken together, as a function of dose (i.e., 150 ppm and 1000 ppm) in Pb^{2+} exposed male and female rats exhibit sex-dependent differences in their susceptibility for cholinergic brain excitability and compensatory GABAergic tone to mitigate against elevated brain activity. It remains to be elucidated whether spontaneous or evoked neuronal communications are differentially or similarly contributing to local and/or global brain excitability that, in turn, could elevate seizure susceptibility. Further, the present study showed that taurine may provide selective sex-dependent neuroprotection against cholinergic brain excitability in males.

5 Conclusion

In summary, this study revealed that females exhibit increased susceptibility for cholinergic brain excitability when compared to males. These sex-dependent differences can be altered based upon the dose of neurodevelopmental Pb^{2+} exposure. The early neurodevelopmental effects of Pb^{2+} exposure can have persistent direct effects on cholinergic excitability and indirectly on GABAergic inhibition. The present study provides more insight into the potential for both low-level Pb^{2+} exposure in males and high-level Pb^{2+} exposures in females to increase the probability for cholinotoxicity. This is an important finding as much work has focused on low-level Pb^{2+} exposures and its neurotoxicant impacts on intellect and other learning and performance behaviors (Needleman and Gatsonis 1990; Wong et al. 1991; Bellinger et al. 1992; Bellinger and Dietrich 1994; Schwartz 1994; Bleecker et al. 1997; Rosen and Mushak 2001; Bellinger and Needleman 2003; Canfield et al. 2003; Needleman 2004; Lidsky and Schneider 2003, 2006; Barbosa et al. 2005; Krieg et al. 2005; Lanphear et al. 2000, 2005; Gilbert and Weiss 2006; Bellinger 2008; Bergdhal and Skerfving 2008; Grandjean and Hertz 2015; Eid and Zawia 2016; Neuwirth 2018; Neuwirth et al. 2019a, b, c, 2020, 2022; O'Connor et al. 2020; Cruz et al. 2022). These reports are consistent with the neuroprotection of taurine in the Fragile X Syndrome model in increasing synaptic plasticity as well as learning and memory (El Idrissi et al. 2010; Neuwirth et al. 2013, 2015; Zhu et al. 2022) reducing anxiety-like behaviors, locomotor activity, and seizure susceptibility (El Idrissi and L'Amoreaux 2008; El Idrissi et al. 2003, 2009, 2012, 2013; L'Amoreaux et al. 2010; Santora et al. 2013). Further, taurine treatment was shown to be beneficial in providing neuroprotection against cholinotoxicity in males, but not females, which raises important questions in the investigation of taurine analogues and/or derivatives in treating a range of central nervous system disorders (Messiha 1979; Madura et al. 1997; Della Corte et al. 2002; Frosini et al. 2003; Gupta and Kim 2003; Gupta et al. 2005; Gupta 2006; Bianchi et al. 2006; Palmi et al. 2006; Ward et al. 2006; Chung et al. 2012; Neuwirth et al. 2019b, 2021; Neuwirth and Emenike 2021). Notably, taurine has been reported to alter the gene expression of GABAergic regulator proteins (Shen et al. 2013), and thus novel taurine compounds should be examined for their associated molecular changes consistent with these reports. The current study builds upon prior studies that sought to evaluate the effects of taurine in ameliorating neurodevelopmental Pb^{2+} exposure (Jiang et al. 1956; Zhu et al. 2005; Yu et al. 2007; Neuwirth 2014; Neuwirth et al. 2017, a, b, 2022; Cruz et al. 2022). However, more work is needed in this area and should be evaluated in conjunction with other neurotransmitter systems that can evoke seizures and/or increase the susceptibility for brain excitability.

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Taurine Prevents LPS-Induced Liver Injury in Weaned Piglets

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Keywords

Taurine · LPS · Apoptosis · OS · Inflammation · Liver

Abbreviations

LPS	lipopolysaccharide
OS	oxidative stress
AST	aspartate; aminotransferase
ROS	reactive oxygen species
ALT	glutamic-pyruvic transaminase
CAT	catalase
SOD	superoxide dismutase
HO-1	heme oxygenase-1
Nrf-2	nuclear factor-E2-related factor2
MDA	malondialdehyde
MAPK	mitogen activated protein kinase
Bcl-2	B-cell lymphoma-2
TLR4	toll-like receptors 4
caspase-3	cysteinyl aspartate specific proteinase
ARE	antioxidant response element
GSH-Px	glutathione peroxidase

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1 Introduction

Inflammation, which is often accompanied by oxidative damage, is the cause of many diseases, as well as introducing synergistic effects that can aggravate disease. LPS is an endotoxin produced by gram-negative bacteria, which can induce oxidative stress (OS) and trigger a systemic inflammatory response, causing impairment of systemic vascular endothelial cell function, increasing vascular permeability, destroying mitochondrial function, and finally leading to cell death (Jong et al. 2021). Therefore, LPS was injected intraperitoneally to establish an acute liver injury model. Excessive OS and ROS result in a series of oxidative events that end in cell/tissue damage (Singh et al. 2019). OS can induce apoptosis through the mitochondria, endoplasmic reticulum, and other pathways. It can also induce apoptosis by activating MAPK pathway and activating and upregulating NF-κB, together with activating

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caspases of the aspartate-specific cysteine protein kinase family. OS is considered to be an important factor leading to aging and disease (Goc et al. 2019).

Taurine is one of the most abundant free amino acids in the body (Park et al. 2014) that was found in the liver (El Idrissi. 2019). Taurine is found in high concentrations within mammalian tissue (Ito et al. 2019) and exhibits a variety of biological functions. In recent years, as a nutritional additive, it has been used in many industries including food and medicine. Taurine plays physiological and regulatory roles in the prevention and treatment of a variety of diseases (Gupta et al. 2005) and participates in various physiological reactions and activities (Mekawy et al. 2021). Studies have shown that taurine can play an important role in cell redox homeostasis and resistance to oxidative stress, due to its ability in stabilizing biofilms, scavenging hypochlorous acid, and alleviating peroxidation damage (Abdel-Moneim et al. 2015). Taurine supplementation, or a combination of taurine and other drugs, can also treat damaged tissues (Murakami 2017). Studies have shown that taurine could effectively inhibit hepatic and renal OS injury induced by ethanol in murines (Goc et al. 2019). Based on the above evidence, we speculate that taurine can play an important protective role in hepatic injury induced by LPS in weaned piglets, and this study additionally explores further its mechanism.

2 Methods

2.1 Animal Models

Twenty weaned piglets (28 days old) were provided by the scientific research pig farm of Shenyang Agricultural University (weight 9.0 ± 0.6 kg) and were randomly divided into four groups, with five piglets in each group – group C (basal diet + 0.9% normal saline), group T (0.3% taurine diet + 0.9% normal saline), group L (basic diet + LPS), and group LT (0.3% taurine diet + LPS). Basic diet reference was NRC (1998). Raw materials were purchased from Shenyang aitejie feed factory, and the formula and content of raw materials are illustrated in Table 1. Nutrient composition and level of feed are illustrated in Table 2. In the morning of the 29th day of rearing, L group and LT group were treated with an intraperitoneal injection of 100 μg/Kg of LPS (purity ≥ 99%) [Beijing Jiakangyuan Chemical Co., Ltd™, China]. Group C and group T were injected with equivalent volumes of 0.9% saline. Samples were collected 4 h post-injection.

Table 1 Composition and content of common diet

Ingredients	Content
Corn	30.00
Puffed corn	20.00
Peeling soybean meal	14.00
Expanded soybean meal	5.00
Fermented soybean meal	5.00
Steam fish meal	3.50
sucrose	5.00
Whey powder	10.00
(50%)Choline chloride	1.00
Lysine hydrochloride	0.35

Table 2 Nutrient composition and level

Nutritional ingredients	Nutrient levels
ME/(MJ/Kg)	14.5MJ/Kg
CP	20.00
Ca	0.90
AP	0.45
Lys	1.35
Met	0.38
SμLfur-containing amino acids	0.76
Thr	0.86
Trp	0.22

Note: Compound premix is provided per kilogram of diet. The compound premix provides the / Kg value of feed:VA 12000IU, VD3 3000IU, VE 60 mg, K33 mg, B2 7.5 mg, B6 4.8 mg, B12 0.04 mg, nicotinic acid 45 mg, pantothenic acid 30 mg, folic acid 1.2 mg, biotin 0.22 mg, Fe 120 mg, Cu 15 mg, Zn 120 mg, Mn 50 mg, Se 0.3 mg, I 0.6 mg

2.2 Sample Collection

Piglets underwent anesthesia, and 25 mL blood was collected from the anterior vena cava and stored in a 50 mL sterile centrifuge tube. Following standing at 4 °C for 40 min, it was

placed into a 4 °C centrifuge and centrifuged at 3500 r/min for 15 min. The upper clear liquid was deemed to be serum, which was separately packed in the tube, marked with corresponding identifying number, and stored at – 80 °C. Following collection, the piglets were sacrificed and the individual abdominal cavity was immediately dissected. The liver was quickly separated, divided into several small sections, washed with precooled 0.9% saline, and placed into cryopreservation tubes, followed by storage in liquid nitrogen at – 80 °C. All procedures involving animals were approved and performed in accordance with the ethical standards of the animal ethics committee of Shenyang Agricultural University (NO. 202006045) following standard guidelines for the use of animals in scientific research.

2.3 HE

The liver was fixed in 4% paraformaldehyde solution for 24 h. After embedding in paraffin, the paraffin section was prepared with a thickness of approximately 5 mm and placed into the clip as standby. Following baking at 64 °C for 30 min, xylene I for 15 min, xylene II for 1 min, absolute alcohol for 5 min, 90% alcohol for 3 min, 80% alcohol for 3 min, 75% alcohol for 3 min, hematoxylin staining for 30 min, eosin staining for 15 s, and neutral gum sealing, the individual hepatic tissue sample was observed under light microscopy.

2.4 qPCR

Hepatic tissue total RNA was extracted, with three technical replicates/sample. *Bcl2*, *BAX*, *Caspase-3*, and *NF-κB p65*, *HO-1*, and *Nrf-2* primers were designed using Primer 5® software, and the primer sequences were as follows (18srna was used as the internal reference gene) (Table 3):

2.5 Western Blot Analysis

SDS-PAGE protein electrophoresis was used to separate extracted protein, with 15 μg of sample in each group. Voltage was set at 110 V, with electrophoresis performed for approximately 1 h, followed by observation of bromophenol blue indicator stain movement across the gel – if it reached the bottom of the rubber plate – at 180 mA constant current, and the film was turned for 90 min. The PVDF membrane was consequently removed and incubated in 3% skim milk at 37 °C for 1 h. The primary antibody was incubated at 4 °C for 12 h. Consequently, phosphate buffered

Table 3 Primer sequence

Gene	Sequence
Bcl2 F	CTGGGATGCCTTTGTGGAGC
Bcl2 R	TCTACTGCTTTAGTGAACCTTTTGC
BAX F	CGCTGGACTTCCTTCGAGAT
BAX R	CTTCCAGATGGTGAGCGAGG
Caspase-3 F	ATTGGACTGTGGGATTGAGACG
Caspase-3 R	TTTCGCCAGGAATAGTAACCAG
NF-κB p65 F	TTCCAAGTTCCCATAGAAGAGCA
NF-κB p65 R	GGGCACGGTTGTCAAAGATG
HO-1 F	AGCACTCACAGCCCAACAGC
HO-1 R	GTACAAGGACGCCATCACCAG
Nrf-2 F	GAGGTAATCCGTCCATCCAGC
Nrf-2 R	TGCCTCCAAAGTATGTCAATCAA

saline Tween-20 wash was performed once every 10 min, for three consecutive cycles. The secondary antibody was consequently incubated with the individual sample at 37 °C for 1 h and developed.

2.6 Data Analysis

SPSS 17.0® software was used for data analyses. T-test was employed to compare the differences between the two groups. One way ANOVA was used to analyze the differences between the two groups. The measurement data was expressed as mean ± standard deviation (x ± SD), with statistical significance being $P < 0.05$ and $P < 0.01$.

3 Results

3.1 Taurine Has Protective Effects on LPS-Induced Hepatic Injury in Piglets

As shown in Fig. 1a, in group C, the boundary of hepatic lobules was well-defined and arranged regularly. Hepatocytes and hepatic sinuses surrounding the central vein were arranged radially. Hepatocytes were round and full. There was no obvious abnormality in the portal area between the adjacent hepatic lobules. Staining was uniform, and there was no obvious inflammatory change. In group L, the hepatocytes were arranged in a disorderly manner, with lymphocyte and neutrophil infiltrations around the portal area, while the cytoplasm was lightly stained. Regarding the LT group, hepatocyte arrangement was disorderly and decreased significantly, together with the localized identification of a small number of lymphocytes and neutrophils.

The serum levels of ALT and AST within weaned piglets are important clinical indicators of hepatic injury/function. The results are shown in Fig. 1b and c. In comparison to group C, the activities of ALT and AST in piglet serum within group L were significantly increased ($P<0.05$). In comparison to group L, the activities of ALT and AST in piglet within group LT were significantly decreased ($P<0.05$). Simultaneously, taurine demonstrated the trend of increasing the ratio of ALT/AST in serum ($P<0.05$). The results showed that taurine could improve the liver function within piglets stimulated by LPS.

3.2 Taurine Possibly Increases SOD, CAT, and GSH-Px Activity in LPS-Induced Piglet Liver and Reduced MDA Activity

As shown in Fig. 2a and b, in comparison to group C, SOD activity in group L piglet serum/liver decreased significantly ($P<0.05$). There was no significant difference in SOD activity between group LT and group C. In comparison to group L,

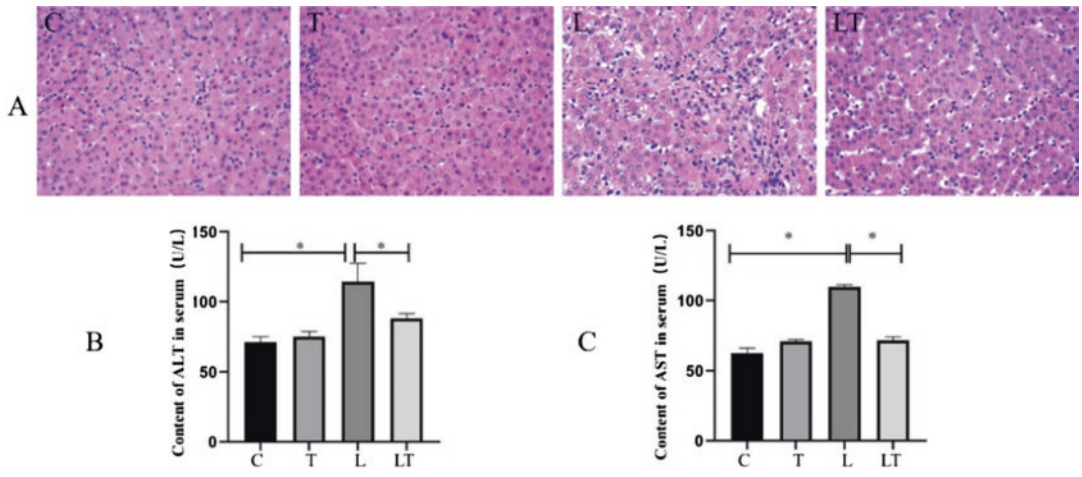

Fig. 1 Following 28 days of feeding, LPS was injected intraperitoneally. Serum was collected after 4 h, and liver tissue (400×) (**a**), serum ALT (**b**), and AST (**c**) were detected (5 samples/group) *$P<0.05$

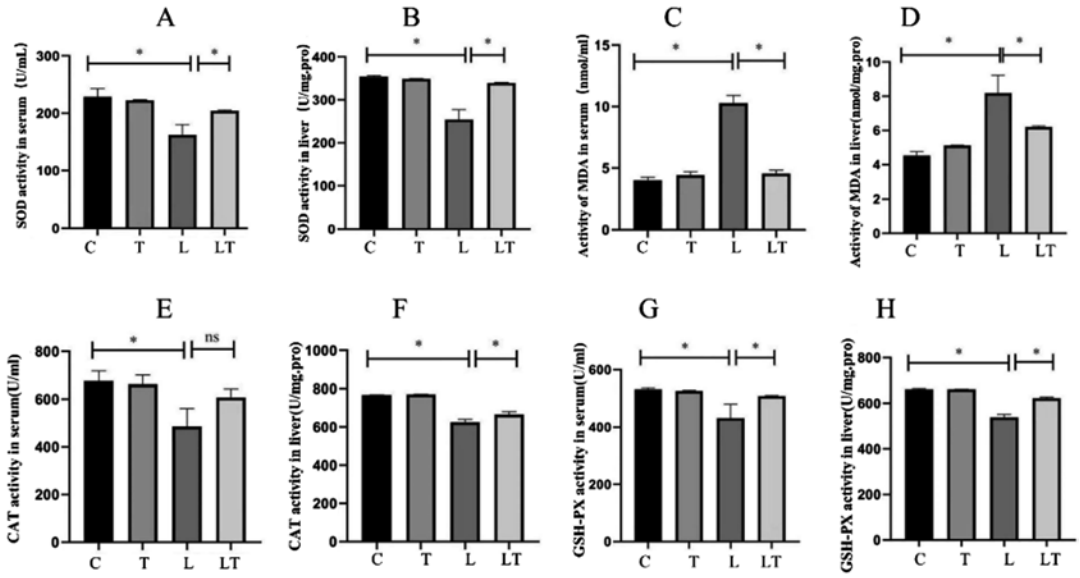

Fig. 2 The activities of SOD (**a–b**), MDA (**c–d**), CAT (**e–f**), and GSH-Px (**g–h**) in serum and liver tissue were detected with five biological replicates in each group (*$P<0.05$)

the SOD activity of group LT increased significantly. The results showed that taurine could reduce LPS-induced SOD activity within piglets. As shown in Fig. 2c and d, in comparison to group C, MDA serum/liver content in group L piglets increased significantly ($P<0.05$), though there was no significant difference between group C and group T. In comparison to group L, the content of MDA in LT decreased significantly ($P<0.05$). The results showed that taurine could reduce the MDA content within serum and liver tissue of LPS-induced piglets. As shown in Fig. 2e and f, in comparison to group C, CAT activity in serum/liver tissue in group L piglets decreased significantly ($P<0.05$). In comparison to group L, CAT activity in group LT increased significantly, eliminating the effect of LPS stimulation. As shown in Fig. 2g and h, In comparison to group C, the activity of GSH-PX in serum and liver of piglets in group L decreased significantly ($P<0.05$). In comparison to group L, the activity of GSH-PX in group LT increased significantly, and there was no significant difference between group C and group T. The results showed that taurine could effectively inhibit the decrease of GSH-PX activity in LPS-stimulated piglets.

3.3 Taurine Can Reduce LPS-Induced Inflammatory Response and Oxidative Stress

As shown in Fig. 3a–b, in comparison to group C, the mRNA expression of *HO-1* and *Nrf-2* in the liver was significantly decreased by LPS stimulation ($P<0.05$). In comparison to group L, the mRNA expression of *HO-1* and *Nrf-2* in the liver was significantly increased in group LT ($P<0.05$), though there was no significant difference between group C and group T. As shown in Fig. 3c, in comparison to group C, the expression of *NF-κB p65* mRNA in group L decreased significantly ($P<0.05$). There was no significant difference in *NF-κB p65* mRNA expression between group C and group T. In comparison to group L, the expression of *NF-κB p65* mRNA within group LT was significantly increased. As shown in Fig. 3d, in comparison to group C, the expression of Nrf-2 protein in group L was significantly lower ($P<0.05$), and there was no significant difference between group C and group T. In comparison to group L, the level of Nrf-2 protein in group LT was significantly higher ($P<0.05$). As

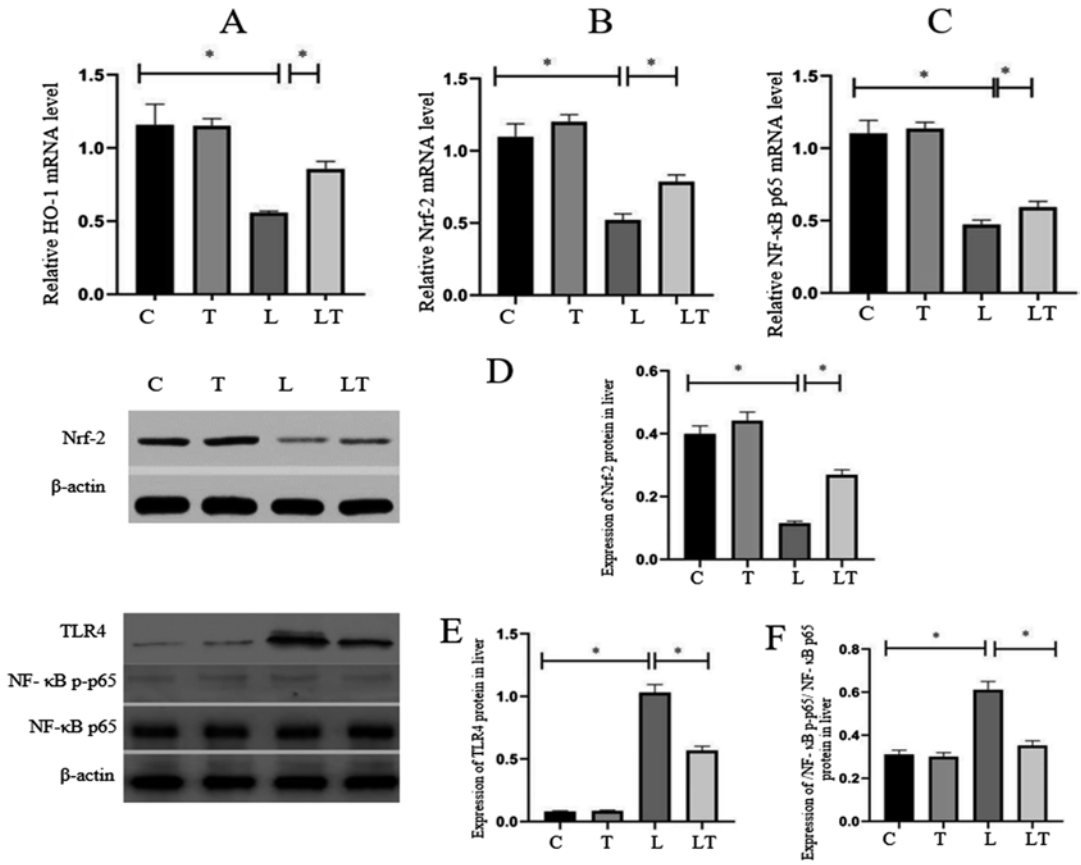

Fig. 3 Expression mRNA of *HO-1*, *Nrf-2*, and *NF-κB p65* was detected in each group. Proteomic expression of Nrf-2, TLR4, and NF-κB p-p65/NF-κB p65 was also detected. There were five biological replicates in each group (* $P<0.05$).

shown in Fig. 3e–f, in comparison to group C, the expression of TRL4 and NF-κB p-p65/NF-κB p65 of group L was increased significantly (*P< 0.05*). There was no significant difference in the expression of between groups C and T. The TRL4, NF-κB p-p65/NF-κB p65 expression between LT group and L group decreased significantly (*P<0.05*). The results showed that taurine modulates the expression of proteins involved in OS and its effects.

3.4 Taurine Can Reduce Liver Cell Apoptosis

As seen in Fig. 4a, the apoptotic rate of hepatocytes in group L was significantly increased compared to that of group C (*P<0.05*), while the apoptotic rate of hepatocytes in group LT was significantly decreased relative to group C (*P<0.05*). Detection of *Bcl2* by qPCR is shown in Fig. 4b. In comparison to group C, the expression of *Bcl2* mRNA was significantly decreased in group L (*P<0.05*). In comparison to group L, the expression of *Bcl2* mRNA was significantly increased in group LT (*P<0.05*). As shown in Fig. 4c and d, mRNA expression of *BAX* and *Caspase-3* in group L was significantly increased compared to those of group C (*P<0.05*), while the mRNA expression of *BAX* and *Caspase-3* in group LT was significantly decreased (*P<0.05*). As shown in Fig. 4e, in comparison to group C, the expression of Caspase-3 in group L was significantly increased (*P<0.05*), and there was no significant difference between group C and group T. In comparison to group L, the expression of

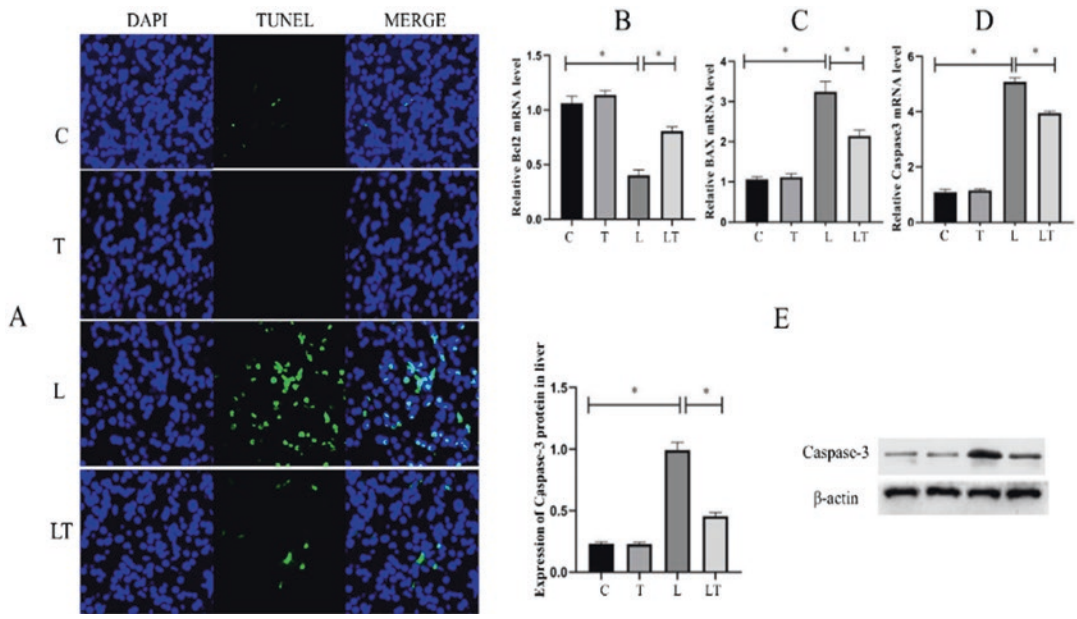

Fig. 4 TUNEL method was used to detect hepatocyte apoptosis; transcriptomic expression of *Bcl2*, *BAX*, and *Caspase-3*; and proteomic expression of Caspase-3 (* $P< 0.05$)

Caspase-3 in group LT was significantly decreased ($P<0.05$). The results show that taurine moderately inhibits apoptosis of LPS-stimulated piglet hepatocytes.

4 Discussion

The liver is an important hub of physiological processes. ALT and AST are a class of enzymes that catalyze amino acid and keto acid amino transfer and are mainly located within hepatocytes. Whenever hepatocytes are damaged, cell membrane permeability increases, which leads to the release of ALT and AST into the bloodstream. Therefore, serum ALT and AST levels are important biomarkers for evaluating hepatic function (Cao et al. 2015).

LPS-induced OS plays an important role in the pathogenesis of hepatic injury in piglets. Oxidants and antioxidants in healthy organisms are usually balanced. The accumulation of oxidants and the consumption of endogenous antioxidants can cause OS. LPS can interfere with the production of oxygen free radicals and inhibit the antioxidant defense system, which is responsible for the elimination of excessive oxygen free radicals. The inhibition of this system leads to hepatic OS (Zhu et al. 2012). Excessive oxygen free radicals react with biomacromolecules, proteins, and DNA, leading to hepatocyte damage (Wu and Cederbaum 2009). SOD can protect hepatocytes from oxidants, while CAT and GSH-PX are a class of important antioxidant enzymes which can catalyze the decomposition of hydrogen peroxide, H_2O_2. One of the important functions of CAT is to decompose H_2O_2 into H_2O and O_2. They can work together to remove lipid peroxide and oxygen free radicals, protecting hepatocytes from ROS damage. Thus CAT plays an important role in protecting the structural and functional integrity of cell membranes (Glade and Meguid 2017). Concomitantly, under stress conditions, it can alleviate ROS-induced damage to biofilms, in order to maintain cellular integrity and function (Xu et al. 2018). When LPS stimulates OS, oxidative damage to CAT, GSH-PX, and SOD can result in a decrease of activity. Furthermore, the levels of CAT, GSH-PX, and SOD directly reflect antioxidant capacity (Lieber 1997). Conversely, MDA is the final product of lipid peroxidation, which is trig-

gered by the reaction of ROS with lipids (or in the present study, it is measure of hepatic oxidative damage due to free radicals). In our study, taurine significantly reduced the level of MDA in LPS-stimulated liver tissue and increased the activities of CAT, GSH-PX, and SOD in piglets, which indicated that taurine could play an antioxidant role in LPS-induced oxidative damage by reducing OS and improving the activities of the antioxidant defense system.

In order to further explore the mechanism of taurine on LPS-induced antioxidant stress and the antioxidant defense system in piglet livers, we detected the mRNA transcription levels of *Nrf-2* and *HO-1*, as well as the protein levels of Nrf-2 in hepatic tissue. Nrf-2 is an important transcription factor and molecular player in the antioxidant stress pathway. It induces detoxification enzymes and antioxidant proteins encoded by downstream genes involved in various OS responses. Under normal conditions, Nrf-2 activity is regulated by binding with Keap1. Under OS, Nrf-2 dissociates from Keap1, transfers itself to the nucleus, and binds with transcription factors. The complex binds to the ARE and promotes the transcription of multiple antioxidant genes, such as HO-1 and GSH. Therefore, Nrf-2 is considered to be a novel target for the treatment of multiple diseases (Cao et al. 2015). HO-1 is a downstream target of Nrf-2 and a powerful antioxidant. It can catalyze the oxidation of heme to antioxidant molecules, carbon monoxide, and biliverdin, together with improving the survival rate of hepatocytes. HO-1 can be activated by Nrf-2 translocation (Surh 2003). In our study, LPS induced a significant downregulation of *Nrf-2* and *HO-1* mRNA levels and reduced Nrf-2 expression, while taurine-treated piglets significantly increased *Nrf-2* and *HO-1* mRNA transcription levels and Nrf-2 content in hepatic tissue. This suggests that the protective mechanism of taurine against LPS-induced liver injury may be partly attributed to potential antioxidant stress, which enhances the oxidative defense system through activation of the Nrf-2/HO-1 signaling pathway.

OS can promote the recruitment and activation of leukocytes and resident cells, thus regulating the expression of pro-inflammatory cytokines and chemokines, leading to inflammation and ultimately apoptosis (Hassanein et al. 2019). Compared with group L, we found that in the LT group, there was no significant difference in the expression of NF-κB p65, but the NF-κB p-p65/NF-κB p65 ratio was significantly decreased. We also studied the effect of taurine on LPS-induced hepatocyte apoptosis. The TUNEL method was used to detect the rate of hepatocyte apoptosis. It was found that, in comparison to the control group, the apoptotic rate of hepatocytes in taurine-fed piglets was significantly decreased after being stimulated by LPS, suggesting that taurine can effectively inhibit LPS-induced apoptosis. Moreover, we detected the mRNA expression of *Bcl-2/BAX/Caspase-3* through real-time quantitative PCR and examined proteomic expression of Caspase-3 by Western-blot analysis. The intrinsic apoptotic pathway mediated by mitochondria is controlled by the pro-apoptotic protein BAX and the anti-apoptotic protein Bcl-2. Caspase-3 is the most critical protease in the cascade of cysteine protein kinases and plays a central role in the process of apoptosis (Domracheva et al. 2017). Therefore, the detection of cleaved Caspase-3 is considered to be a reliable biomarker for apoptotic cells. In this study, taurine significantly downregulated the mRNA expression levels of *BAX* and *Caspase-3* in the liver of LPS-stimulated piglets, upregulated the mRNA expression levels of *Bcl2*, and significantly inhibited the proteomic expression of Caspase-3 post-LPS induction. In conclusion, taurine can reduce LPS-induced apoptosis in piglet liver by alleviating oxidative stress and inflammatory response.

5 Conclusion

Taurine prevents LPS-induced apoptosis in piglet liver. Therefore, taurine can be used as a feed additive to prevent liver injury caused by oxidative stress and inflammation.

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