Effect of Taurine on Viability and Proliferation of Murine Melanoma B16F10 Cells

Yon-Suk Kim, Sun Hee Cheong, Jin-Woo Hwang, Gaurav Lodhi, Kwang-Ho Lee, Dong-Kug Choi, Hyuk Song, Sang-Hoon Lee, Dong-June Park, Chang-Bum Ahn, Sang-Ho Moon, Byong-Tae Jeon, and Pyo-Jam Park

1 Introduction

Apoptosis is the process of programmed cell death and dysregulated apoptosis is involved in a variety of diseases such as cancer and neurodegenerative diseases. Agents that suppress the proliferation of malignant cells, and even cause apoptosis, have the potential to both prevent and treat cancer (Parra et al. 2011). Taurine (2-aminoethanesulfonic acid) is a sulfur-containing β-amino acid that is present widely in mammals. It is one of the end-products of cysteine metabolism in mammals and is renally excreted. Taurine exhibits pharmacological actions and various

Y.-S. Kim • J.-W. Hwang • G. Lodhi • K.-H. Lee • D.-K. Choi
Department of Biotechnology, Konkuk University, Chungju 380-701, South Korea

S.H. Cheong • P.-J. Park (✉)
Department of Biotechnology, Konkuk University, Chungju 380-701, South Korea

Nokyong Research Center, Konkuk University, Chungju 380-701, South Korea
e-mail: parkpj@kku.ac.kr

H. Song
Department of Food Bioscience, Research Institute for Biomedical & Health Science, College of Biomedical & Health Science, Konkuk University, Chungju 380-701, South Korea

S.-H. Lee
Food Resource Research Center, Korea Food Research Institute, Republic of Korea
University of Science and Technology, Daejeon 305-350, South Korea

D.-J. Park
Food Resource Research Center, Korea Food Research Institute, Seongnam, South Korea

C.-B. Ahn
Division of Food and Nutrition, Chonnam National University,
Gwangju 500-757, South Korea

S.-H. Moon • B.-T. Jeon
Nokyong Research Center, Konkuk University, Chungju 380-701, South Korea

© Springer International Publishing Switzerland 2015

J. Marcinkiewicz, S.W. Schaffer (eds.), *Taurine 9*, Advances in Experimental Medicine and Biology 803, DOI 10.1007/978-3-319-15126-7_15

beneficial physiological functions. These benefits have encouraged the consumption of seafood containing high concentrations of taurine and the use of taurine in infant formulas, nutritional supplements, and energy drinks (Matsuda and Asano 2012). Moreover, taurine has significant anti-inflammatory properties (Marcinkiewicz 2009) and participates in different physiological processes as it stabilizes cell membranes (Condron et al. 2010) and regulates fatty tissue metabolism (Ueki and Stipanuk 2009) and levels of calcium ions in blood (Ribeiro et al. 2010). The nonmetabolizable β-amino acid taurine suppresses inflammation (Marcinkiewicz and Kontny 2014) and reduces hepatic lipid oxidative stress (Balkan et al. 2002), protecting liver function during ethanol metabolism (Kerai et al. 1998; Yang et al. 2010). Because it is nonmetabolizable, the protective mechanism of taurine is not yet known, but its anti-inflammatory and antioxidative activities (Marcinkiewicz and Kontny 2014) primarily result from its sequestration of HOCl and HOBr (Weiss et al. 1982). Taurine has a protective effect in murine hepatocytes against oxidative stress-induced apoptosis by tert-butyl hydroperoxide (Roy and Sil 2012), exerts a hypoglycemic effect, and suppresses mitochondria-dependent apoptosis in renal and cardiac tissues of alloxan-induced diabetic rats (Das and Sil 2012; Das et al. 2012). There is an increasing interest in studying the additive or synergistic effect of taurine in various diseases including cancer. However, the anticancer activity of taurine in melanoma cells has not been sufficiently studied. In this study, it was determined whether taurine exhibits an anticancer activity that targets proliferation and apoptosis of B16F10 cells.

2 Materials and Methods

2.1 Materials

Taurine, MTT, neutral red, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). β-actin antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). All other reagents were of the highest grade commercially available.

2.2 Cell Culture

The murine melanoma B16F10 cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) under 5 % CO_2 in a humidified incubator at 37 °C.

2.3 MTT Assay

The anti-proliferation assay was performed according to a well-established MTT method with slight modifications (Carmichael et al. 1989). Briefly, B16F10 cells (2.0×10^4 cells/well) were seeded in 96-well culture plates. Cells were treated with samples for 24 h, MTT solution was added into each well, and cells were incubated for 3 h. The medium was discarded and the intracellular formazan product dissolved in 150 μL dimethyl sulfoxide (DMSO) under continuous shaking for 10 min. The absorbance was measured at 540 nm using a microplate reader (Tecan, Austria). Cell viability was expressed as a percentage of the control.

2.4 Neutral Red Assay

The neutral red assay is based on the incorporation of neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) into the lysosomes of viable cells. Briefly, B16F10 cells (2.0×10^4 cells/well) were seeded in 96-well culture plates. Cells were treated with taurine (5, 10, and 20 mM) for 24 h, 200 μL of prepared neutral red solution was added to each well, and the cells were incubated at 37 °C for 24 h. Subsequently, the cells were rapidly washed with a solution of 0.1 % calcium chloride and 0.5 % formaldehyde. To extract the dye from the intact and viable cell, a solubilization solution of 1 % acetic acid and 50 % ethanol was added to the cells. Following 10 min incubation at room temperature, the absorbance (OD) was measured by spectrophotometry at 540 nm. Results were expressed as percentages of the control.

2.5 Cell Cycle Analysis

Cellular DNA content was measured using flow cytometry. Briefly, the cells (1.0×10^5) were seeded in 6-well plates and allowed to adhere overnight. Cells were treated with various concentrations of taurine (5, 10, and 20 mM) for 24 h. The cells were harvested by trypsin treatment, washed with cold phosphate-buffered saline (PBS, pH 7.4), and stained with PI solution (50 μg/mL of propidium iodide, 10 μg/mL RNase, and 0.5 % Tween-20 in PBS). Cell cycle phase distribution and DNA histograms of the stained cells were determined by flow cytometry (FACSCalibur, BD Bioscience). Data from 1.0×10^3 cells per sample were collected and analyzed with CellQuest software (Becton Dickinson).

2.6 Determination of Morphologic Changes

B16F10 cells were seeded in 6-well plates (1.0×10^5 cells/well) and incubated in DMEM at 37 °C under 5 % CO_2 for 24 h. Following a 24 h incubation with taurine (5, 10, and 20 mM), cellular morphology was assessed using a phase-contrast microscope (Nikon, Japan). Images were taken at 200× magnification.

2.7 Hoechst 33342 Staining

B16F10 cells were plated in 6-well plates and treated with taurine (5, 10, and 20 mM) for 24 h, fixed in PBS containing 4 % formaldehyde for 30 min at room temperature. Fixed cells were washed with PBS containing 0.02 % Tween-20 and stained with 1 μg/mL Hoechst 33342 (Sigma, St. Louis, MO, USA) for 20 min at room temperature. The cells were subsequently washed twice with PBS and visualized and photographed using a fluorescence microscope.

2.8 Western Blot Analysis

Equal amounts of total protein (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (PVDF) and blocked with 5 % skimmed milk in TBS-T for 2 h. Blots were incubated with specific primary antibodies and the immune complexes detected using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Incubation with the secondary antibody was followed by triplicate washes with TBS-T and the blots were processed for visualization using an enhanced-chemiluminescence (ECL) detection kit and a Luminescent Image Analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

2.9 Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). Statistical analyses were assessed by Student's *t*-test for paired data and one-way analysis of variance (ANOVA) followed by Duncan's post-hoc multiple range test. Graph Pad Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA) was used. Results were considered significant at $p < 0.05$.

3 Results

3.1 Effect of Taurine on Cell Viability of B16F10 Cells

B16F10 cells were treated with various concentrations of taurine (5, 10, and 20 mM) and the percentage of surviving cells was assessed using both a MTT and neutral red assay. After treatment with taurine, cell viability of B16F10 cells was

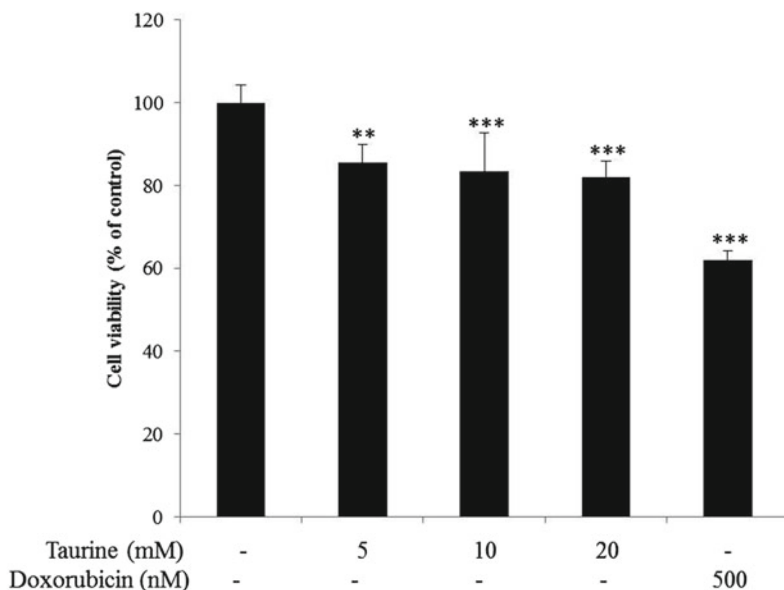

Fig. 1 Effect of taurine on cell viability in the MTT assay. B16F10 cells were treated with taurine for 24 h. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (***$p < 0.001$, **$p < 0.01$ compared with control)

significantly decreased (Fig. 1). Cell viability upon treatment with 5, 10, and 20 mM of taurine was 85.4, 83.3, and 81.9 %, respectively as measured using the MTT assay and 95.9, 92.3, and 84.1 %, respectively as determined with neutral red detection. Treatment of cells with doxorubicin (500 nM) was used as a positive control with cell viability found to be 61.9 and 57.4 % for the MTT and neutral red assay, respectively. Both the MTT and neutral red assay showed that taurine treatment resulted in decreased cell viability in a dose-dependent manner (Fig. 2).

3.2 *Effects of Taurine on the Cell Cycle Distribution of B16F10 Cells*

Table 1 shows the representative histograms of the relative percentage of B16F10 cells in each phase of the cell cycle after incubation in the absence and presence (5, 10, and 20 mM) of taurine for 24 h. As determined by PI staining, the sub-G1 peak of B16F10 cells increased in a dose-dependent manner. Apoptosis was induced with 5.48, 3.90, and 1.66 % after treatment with 5, 10, and 20 mM taurine, respectively (Fig. 3).

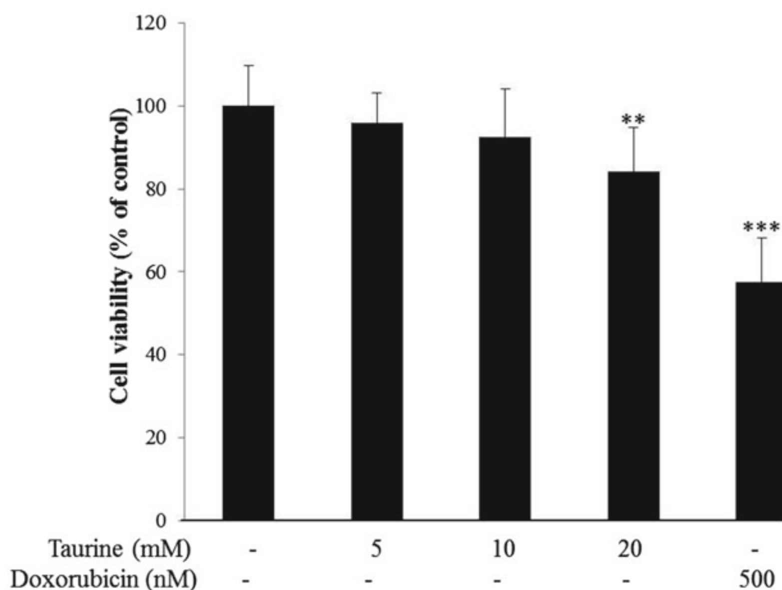

Fig. 2 Effect of taurine on cell viability in the neutral red assay. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (***$p < 0.001$, **$p < 0.01$ compared with control)

Table 1 Effects of taurine on the cell cycle distribution of B16F10 cells

Group	Number of cell (%)			
	Sub G1	G1	S	G2/M
Control	1.47 ± 0.21	52.69 ± 2.52	8.85 ± 1.02	28.91 ± 2.13
Taurine (5 mM)	1.66 ± 0.12	51.98 ± 2.29	10.49 ± 1.13	28.90 ± 2.25
Taurine (10 mM)	3.90 ± 0.24	50.11 ± 3.26	10.24 ± 1.05	27.94 ± 2.19
Taurine (20 mM)	5.48 ± 0.15	45.73 ± 2.11	10.24 ± 1.23	28.68 ± 2.31
Doxorubicin (500 nM)	25.51 ± 2.22	30.04 ± 1.58	10.49 ± 1.21	28.64 ± 2.17

Cells were treated with the indicated concentration of samples for 24 h and stained with PI for flow cytometry analysis. The percentages of cells in each phase of three independent experiments are given

3.3 Apoptotic Effects and Morphological Changes in B16F10 Cells

Morphological changes and cell death in B16F10 cells were observed using an inverted microscope. The morphological changes observed after 24 h exposure to increasing concentrations of taurine are shown in Fig. 4. Control cells were not affected in their proliferation and showed normal cell morphology. However, treatment with taurine caused cell death and decreased cell density. In addition, changes in nuclear morphology were detected by the Hoechst 33342 nuclear staining.

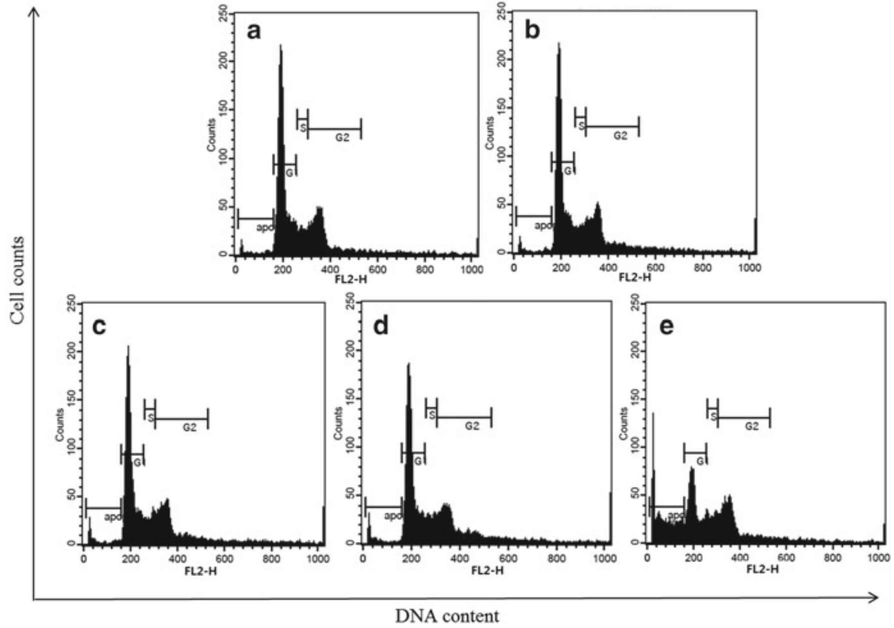

Fig. 3 Cell cycle analysis by flow cytometry. (**a**) Control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

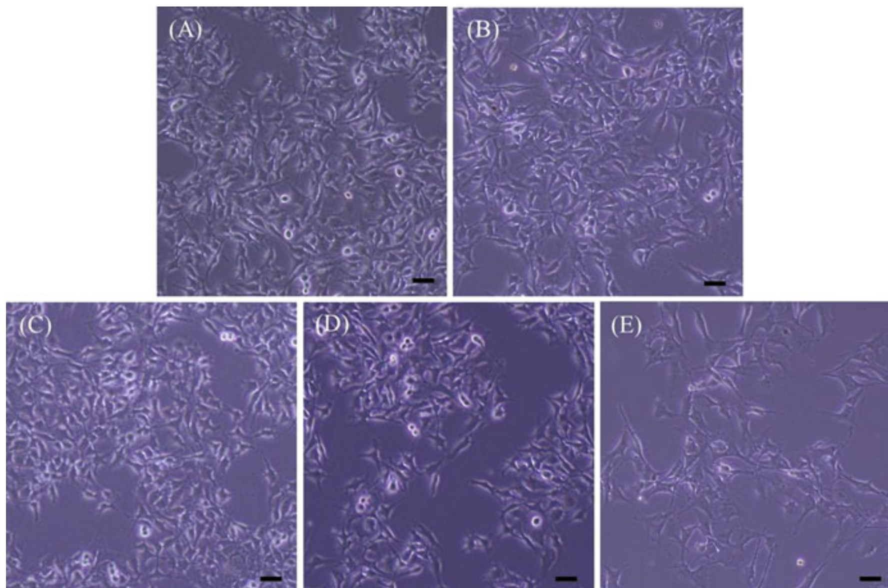

Fig. 4 Morphological changes in B16F10 cells. Cells were incubated with samples for 24 h and photographs were taken using an inverted microscope. Scale bar: 10 μm. (**a**) control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

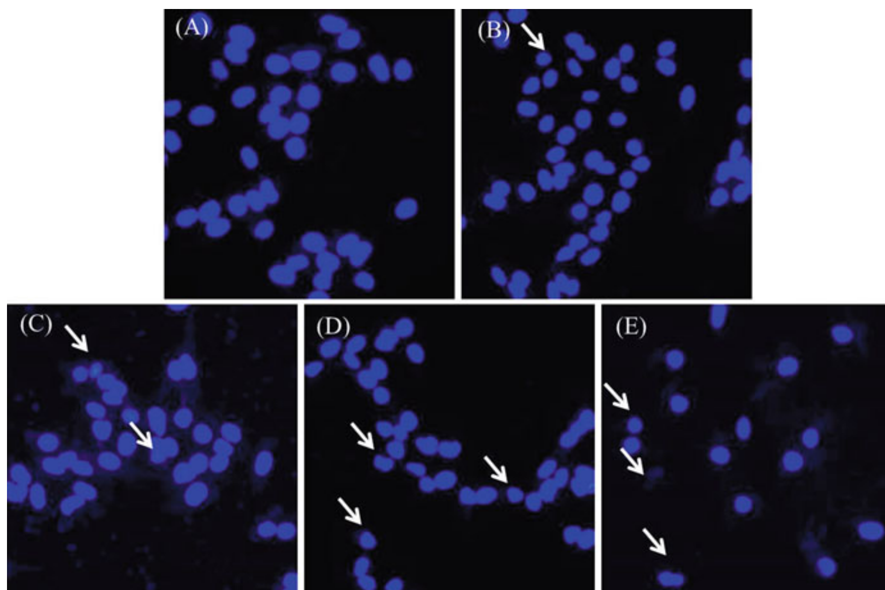

Fig. 5 Nuclear morphological changes in B16F10 cells. Cells were incubated with samples for 24 h, fixed with 4 % paraformaldehyde, and stained with Hoechst 33342. Photographs were taken using a fluorescent microscope (200× magnification). (**a**) Control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

As shown in Fig. 5, the nuclei in the control group were stained as weak homogenous blue, while bright chromatin condensation and nuclear fragmentation were observed in the taurine-treated groups.

3.4 Western Blot Analysis

To confirm apoptosis in response to taurine treatment, the expression levels of Bcl-2 and Bax, an antiapoptotic and proapoptotic protein, respectively, were evaluated by western blot analysis. The expression level of Bcl-2 decreased gradually with increasing taurine concentrations. In contrast, that of Bax did not change (Fig. 6).

4 Discussion

As with many cancers, the development of melanoma is associated with immune suppression (D'Agostini et al. 2005). The capacity to elicit effective T- and B-cell immunity, including anti-tumor cell activity, is ultimately related to stimulation of lymphocyte proliferation and the upregulation of the activity of both NK and CTL cells (Marciani et al. 2000; Zhang et al. 2005). Taurine, one of the most abundant

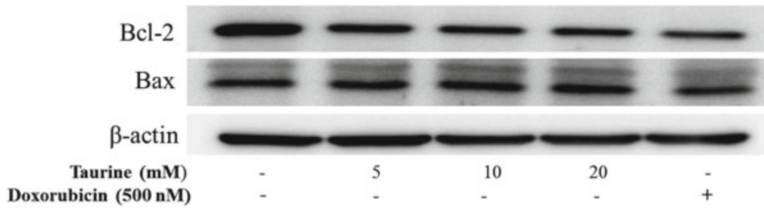

Fig. 6 Effect of taurine on the Bcl-2 family of proteins in B16F10 cells. Cells were treated with the indicated concentration of taurine for 24 h. Cells were subsequently lysed and equal amounts of cell protein (20 μg) were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the indicated antibodies. An ECL detection system was used for visualization of the proteins. β-actin was used as an internal control

free amino acids presents in mammalian tissues, regulates many cellular functions including inflammatory processes (Choi et al. 2006). Beside metabolic regulation, taurine also plays an important role in innate immunity (Nagl et al. 2000) and is directly related to antioxidant properties in clinical (Zulli 2011; Shivananjappa and Muralidhara 2012), toxicological (Turna et al. 2011; Yildirim and Kilic 2011; Shao et al. 2012), and oncological studies (Henderson et al. 2001; Gottardi and Nagl 2010; Shalby et al. 2011). Apoptosis is a regulated process involving changes in the expression of distinct genes. The Bcl-2 family of proteins (e.g., Bcl-2 and Bcl-xL) is a regulator of the apoptotic pathway. Bcl-2 and Bcl-xL are upstream molecules in this pathway and potent suppressors of apoptosis (Hockenbery et al. 1993). These Bcl-2 family genes mainly act in the mitochondrion and are involved in the survival/death pathway, where Bcl-xL and Bcl-2 are responsible for survival and Bak, Bax, and Bad for apoptosis (Basu and Haldar 1998). In this study, we found that taurine inhibits the proliferation of murine melanoma B16F10 cells via apoptosis. Taurine can block melanoma cell proliferation and induce apoptosis through a mitochondrial pathway. In summary, the antiproliferative effect of taurine on B16F10 melanoma cells was investigated. Cell viability was studied using the MTT and neutral red uptake test assays. Moreover, cell cycle analysis and protein expression upon taurine treatment were examined using western blotting and flow cytometry, respectively. These results indicate that taurine suppresses the proliferation of murine melanoma B16F10 cells.

Acknowledgements This study was supported by a grant from the Korea Food Research Institute (Project name: Development of silver foods for the elderly)

References

Balkan J, Kanbagli O, Aykac-Toker G, Uysal M (2002) Taurine treatment reduces hepatic lipids and oxidative stress in chronically ethanol-treated rats. Biol Pharm Bull 25:1231–1233

Basu A, Haldar S (1998) The relationship between BcI2, Bax and p53: consequences for cell cycle progression and cell death. Mol Hum Reprod 4:1099–1109

Carmichael J, Degraff WG, Gamson J, Russo D, Gazdar AF, Levitt ML, Minna JD, Mitchell JB (1989) Radiation sensitivity of human lung cancer cell lines. Eur J Cancer Clin Oncol 25:527–534

D'Agostini C, Pica F, Febbraro G, Grelli S, Chiavaroli C, Garaci E (2005) Antitumour effect of OM-174 and Cyclophosphamide on murine B16 melanoma in different experimental conditions. Int Immunopharmacol 5:1205–1212

Choi HS, Cha YN, Kim CK (2006) Taurine chloramine inhibits PMA-stimulated superoxide production in human neutrophils perhaps by inhibiting phosphorylation and translocation of $p47^{phox}$. Int Immunopharmacol 6:1431–1440

Condron C, Casey RG, Kehoe S, Toomey D, Creagh T, Bouchier-Hayes DJ (2010) Taurine modulates neutrophil function but potentiates uropathogenic E. coli infection in the murine bladder. Urol Res 38:215–222

Das J, Vasan V, Sil PC (2012) Taurine exerts hypoglycemic effect in alloxan induced diabetic rats, improves insulin-mediated glucose transport signaling pathway in heart and ameliorates cardiac oxidative stress and apoptosis. Toxicol Appl Pharmacol 258:296–308

Das J, Sil PC (2012) Taurine ameliorates alloxan-induced diabetic renal injury, oxidative stress-related signaling pathways and apoptosis in rats. Amino Acids 43:1509–1523

Gottardi W, Nagl M (2010) N-chlorotaurine, a natural antiseptic with outstanding tolerability. J Antimicrob Chemother 65:399–409

Henderson JP, Byun J, Williams MV, Mueller DM, McCormick ML, Heinecke JW (2001) Production of brominating intermediates by myeloperoxidase: a transhalogenation pathway for generating mutagenic nucleobases during inflammation. J Biol Chem 276:7867–7875

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ (1993) Bcl-2 function in an antioxidant pathway to prevent apoptosis. Cell 75:241–251

Kerai MD, Waterfield CJ, Kenyon SH, Asker DS, Timbrell JA (1998) Taurine: protective properties against ethanol-induced hepatic steatosis and lipid peroxidation during chronic ethanol consumption in rats. Amino Acids 15:53–76

Marciani DJ, Press JB, Reynolds RC, Pathak AK, Pathak V, Gundy LE, Farmer JT, Koratich MS, May RD (2000) Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. Vaccine 18:3141–3151

Marcinkiewicz J, Kontny E (2014) Taurine and inflammatory diseases. Amino Acids 46:7–20

Matsuda M, Asano Y (2012) A simple assay of taurine concentrations in food and biological samples using taurine dioxygenase. Anal Biochem 427:121–123

Marcinkiewicz J (2009) Taurine bromamine: a new therapeutic option in inflammatory skin diseases. Pol Arch Med Wewn 119:673–675

Nagl M, Hess MW, Pfaller K, Hengster P, Gottardi W (2000) Bactericidal activity of micromolar N-chlorotaurine: evidence for its antimicrobial function in the human defense system. Antimicrob Agents Chemother 44:2507–2513

Parra A, Rivas F, Martin-Fonseca S, Garcia-Granados A, Martinez A (2011) Maslinic acid derivatives induce significant apoptosis in b16f10 murine melanoma cells. Eur J Med Chem 46:5991–6001

Ribeiro RA, Vanzela EC, Oliveira CAM, Bonfleur ML, Boschero AC, Carneiro EM (2010) Taurine supplementation: involvement of cholinergic/phospholipase C and protein kinase A pathways in potentiation of insulin secretion and Ca^{2+} handling in mouse pancreatic islets. Br J Nutr 104:1148–1155

Roy A, Sil PC (2012) Taurine protects murine hepatocytes against oxidative stress-induced apoptosis by *tert*-butyl hydroperoxide via PI3K/Akt and mitochondrial-dependent pathways. Food Chem 131:1086–1096

Shalby AB, Assaf N, Ahmed HH (2011) Possible mechanisms for N-acetyl cysteine and taurine in ameliorating acute renal failure induced by cisplatin in rats. Toxicol Mech Methods 21:538–546

Shao X, Hu ZT, Hu CY, Bu Q, Yan GY, Deng PC, Lv L, Wu D, Deng Y, Zhao JX, Zhu RM, Li Y, Li H, Xu Y, Yang H, Zhao Y, Cen X (2012) Taurine protects methamphetamine-induced developmental angiogenesis defect through antioxidant mechanism. Toxicol Appl Pharmacol 260:260–270

Shivananjappa MM, Muralidhara (2012) Taurine attenuates maternal and embryonic oxidative stress in a streptozotocin diabetic rat model. Reprod Biomed Online 24:558–566
Turna G, Kilic N, Yildirim Z, Sari S (2011) Effects of *Thymus sipyleus* and taurine on hepatic MDA, GSH, AOPP levels and SOD activity in Ehrlich Acide solid tumor model generated mice. Turk Klin Tip Bilim Derg 31:1153–1159
Ueki I, Stipanuk MH (2009) 3T3-L1 adipocytes and rat adipose tissue have a high capacity for taurine synthesis by the cysteine dioxygenase/cysteinesulfinate decarboxylase and cysteamine dioxygenase pathways. J Nutr 139:207–214
Weiss SJ, Klein R, Slivka A, Wei M (1982) Chlorination of taurine by human neutrophils: evidence for hypochlorous acid generation. J Clin Invest 70:598–607
Yang L, Latchoumycandane C, McMullen MR, Pratt BT, Zhang R, Papouchado BG, Nagy LE, Feldstein AE, McIntyre TM (2010) Chronic alcohol exposure increases circulating bioactive oxidized phospholipids. J Biol Chem 285:22211–22220
Yildirim Z, Kilic N (2011) Effects of taurine and age on cerebellum antioxidant status and oxidative stress. Int J Gerontol 5:166–170
Zhang J, Sun R, Wei H, Tian Z (2005) Antitumor effects of recombinant human prolactin in human adenocarcinoma-bearing SCID mice with human NK cell xenograft. Int Immunopharmacol 5:417–425
Zulli A (2011) Taurine in cardiovascular disease. Curr Opin Clin Nutr Metab Care 14:57–60