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Schisandrin B protects rat cortical neurons against $A\beta_{1-42}$-induced neurotoxicity

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In the present study, we investigated the neuroprotective effects of schisandrin B on amyloid-β_{1-42}-induced toxicity and its potential mechanisms in rat cortical neuron cells. Amyloid β_{1-42} significantly reduced cell viability and increased apoptosis. Pretreatment with schisandrin B prior to amyloid-β_{1-42} exposure significantly elevated cell viability and reduced apoptosis. The anti-apoptotic effect of schisandrin B in rat cortical neurons was mediated by up-regulation of the anti-apoptotic protein Bcl-2 and down-regulation of the pro-apoptotic protein Bax. Schisandrin B also reduced the release of mitochondrial cytochrome c into cytosol and decreased caspase-9 and caspase-3 activities. Furthermore, schisandrin B increased activities of anti-oxidant reduced glutathione and decreased production of oxidative glutathione. Taken together, these results suggest that schisandrin B protected primary cultures of rat cortical cells against amyloid-β_{1-42}-induced neurotoxicity through anti-apoptosis involved in a mitochondria-mediated pathway and anti-oxidant action. Schisandrin B may represent a potential treatment strategy for Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive degeneration, loss of neurons in the brain, and two neuropathological hallmarks of the disease: neurofibrillary tangles and senile plaques (Selkoe 1999). Amyloid-β (Aβ) peptide is the major component of senile plaques and is a causal role in the development and progress of AD (Hardy and Higgins 1992).

Apoptosis plays a pivotal role in Aβ-induced cell death (Morais Cardoso et al. 2002). There are two main pathways leading to apoptosis in mature neurons: the extrinsic and intrinsic pathway. The interaction of anti-apoptotic proteins (such as B-cell leukemia/lymphoma-2 (Bcl-2)) and proapoptotic proteins (such as B-cell lymphoma 2-associated protein X (Bax)) plays a crucial role in the intrinsic apoptotic pathway (Antonsson and Martinou 2000). In the intrinsic apoptotic pathway, mitochondrial activation triggers the release of cytochrome c via Bcl-2/Bax and subsequently activates caspase-9 and caspase-3 (Kluck et al. 1997). Evidence has shown that Aβ-induced apoptosis is concomitant with the down-regulation of Bcl-2 (Tamagno et al. 2003), and/or up-regulation of Bax (Paradis et al. 1996).

Reduced glutathione (GSH) plays an important role in numerous cellular functions, including DNA synthesis and regulation of cytosolic Ca^{2+} homeostasis (Meister and Anderson 1983; Reed 1990; Smith et al. 1996). The regeneration of GSH from oxidized glutathione (GSSG) is important in protecting against the free radical-mediated neuron damage induced by Aβ (Recknagel and Glende 1989).

Schisandrin B was shown to protect against hypoxia/reoxygenation-induced apoptosis and inhibit associated changes in Ca^{2+}-induced mitochondrial permeability transition and mitochondrial membrane potential in H9c2 cardiomyocytes (Chiu et al. 2008). Furthermore, schisandrin B enhanced cerebral mitochondrial anti-oxidant status and structural integrity, and protected against cerebral ischemia/reperfusion injury in rats (Chen et al. 2008). The structure of schisandrin B is shown in Fig. 1.

In the present study, we investigated the neuroprotective effects of schisandrin B on Aβ-mediated toxicity in primary cultures of rat cortical neurons. The underlying mechanisms by which schisandrin B conferred its effect were also elucidated.

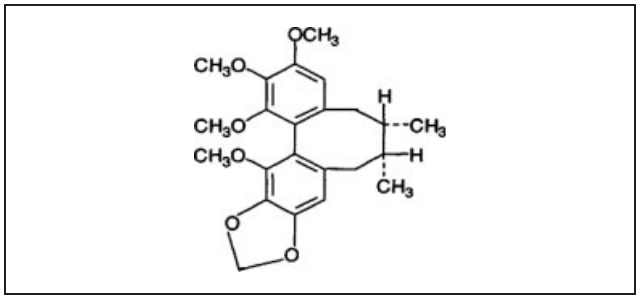

Fig. 1. Structure of schisandrin B

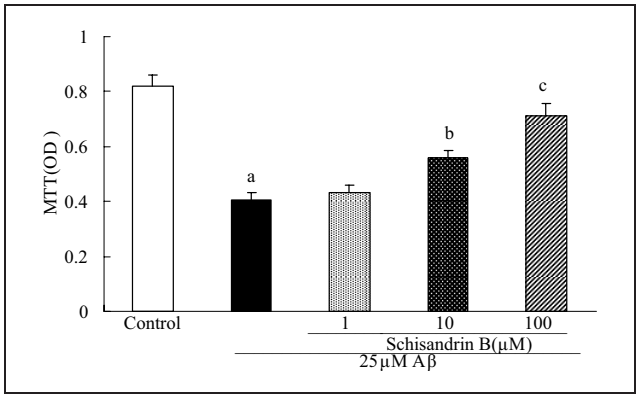

Fig. 2: Effect of schisandrin B on the cell viability in $A\beta_{1-42}$-induced neurotoxicity. Neuron cell cultures were pretreated with schisandrin B (1, 10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. Cell viability was measured by MTT. Each value represents the mean ± S.D. from three experiments. $^{a}p < 0.01$ vs. control, $^{b}p < 0.05$ $^{c}p < 0.01$ vs. $A\beta_{1-42}$ group

2. Investigations and results

2.1. Effect of schisandrin B on the cell viability in $A\beta_{1-42}$-induced neurotoxicity

As shown in Fig. 2, when exposed to $A\beta_{1-42}$, the cell viability of neuron cells significantly decreased. Pretreatment with different concentrations of schisandrin B (10 and 100 μM) before exposed to 25 μM of $A\beta_{1-42}$ showed protecting effects in a dose-dependent manner. Schisandrin B at 1 μM did not cause apparent cytoprotection.

2.2. Effect of schisandrin B on the apoptosis in $A\beta_{1-42}$-induced neurotoxicity

As shown in Fig. 3A, in control group, 6.4% cells represented apoptotic cells. After exposure to 25 μM $A\beta_{1-42}$ for 24 h, the percentage of apoptosis increased to 28.1% (Fig. 3B). Preincubation with schisandrin B (10 and 100 μM) for 2h dose-dependently arrested the apoptosis, and the values of apoptosis were decreased to 17.7% and 11.2%, respectively (Fig. 3C–D).

2.3. Effect of schisandrin B on Bcl-2 and Bax levels in $A\beta_{1-42}$-induced neurotoxicity

As shown in Table 1, treatment of neuron cells with 25 μM $A\beta_{1-42}$ caused a decrease in the intracellular Bcl-2 levels and increased the level of the Bax, while preincubation of cells with 10 and 100 μM schisandrin B markedly reversed these effects in a dose-dependent manner.

2.4. Effect of schisandrin B on the content of cytochrome c in $A\beta_{1-42}$ induced neurotoxicity

As shown in Fig. 4, $A\beta_{1-42}$ caused the accumulation of cytochrome c at 25 μM concentration for 24 h. When the cells were pretreated with 10 and 100 μM schisandrin B, cytochrome c was significantly reduced.

2.5. Effect of schisandrin B on caspase-9 and caspase-3 activities in $A\beta_{1-42}$-induced neurotoxicity

As shown in Fig. 5, the caspase-9 and caspase-3 activities of neuron cells in $A\beta_{1-42}$ group increased significantly. Pretreatment with 10 and 100 μM schisandrin B for 2 h resulted in the decrease of caspase-9 and caspase-3 activity.

2.6. Effect of schisandrin B on GSH and GSSG levels in $A\beta_{1-42}$-induced neurotoxicity

As shown in Table 2, treatment of neuron cells with 25 μM $A\beta_{1-42}$ caused the decrease in level of the GSH

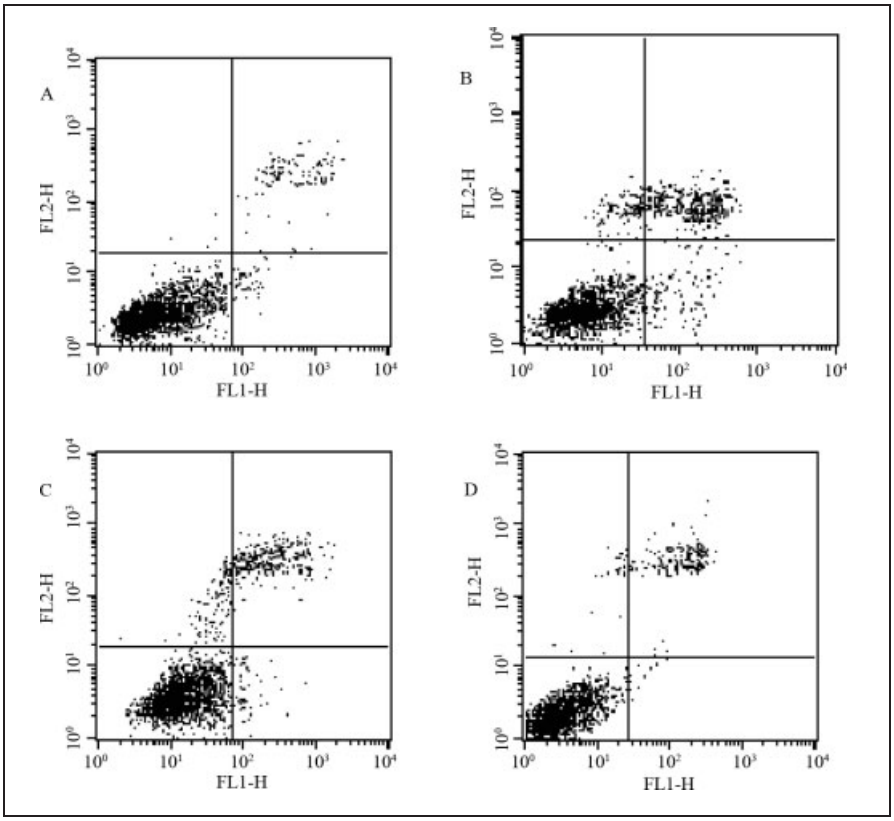

Fig. 3:
Effect of schisandrin B on the apoptosis in $A\beta_{1-42}$-induced neurotoxicity. Neuron cell cultures were pretreated with schisandrin B (10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. A: Control group; B: 25 μM $A\beta_{1-42}$ treated group; C: 10 μM schisandrin B + 25 μM $A\beta_{1-42;}$ D: 100 μM schisandrin B + 25 μM $A\beta_{1-42}$

Table 1: Effect of schisandrin B on Bcl-2 and Bax levels in $A\beta_{1-42}$-induced neurotoxicity

	Concentration (μM)	Bcl-2 (U/mg protein)	Bax (U/mg protein)	Bcl-2/Bax (ratio)
Control		20.43 ± 1.48	23.63 ± 1.32	0.86
$A\beta_{1-42}$		8.57 ± 0.65[a]	44.03 ± 2.55[a]	0.19[a]
Schisandrin B + $A\beta_{1-42}$	1	9.56 ± 0.35	42.13 ± 1.26	0.21
	10	13.36 ± 1.00[b]	28.07 ± 1.70[b]	0.48[c]
	100	18.50 ± 1.05[c]	22.43 ± 2.00[c]	0.82[c]

Neuron cell cultures were pretreated with schisandrin B (1, 10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. Each value represents the mean ± S.D. from three experiments. [a] $p < 0.01$ vs. control, [b] $p < 0.05$ [c] $p < 0.01$ vs. $A\beta_{1-42}$ group

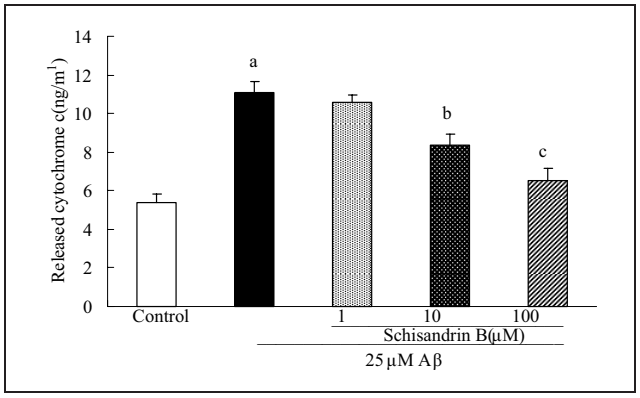

Fig. 4: Effect of schisandrin B on the content of cytochrome c in $A\beta_{1-42}$ induced neurotoxicity. Neuron cell cultures were pretreated with schisandrin B (1, 10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. Each value represents the mean ± S.D. from three experiments. [a] $p < 0.01$ vs. control, [b] $p < 0.05$ [c] $p < 0.01$ vs. $A\beta_{1-42}$ group

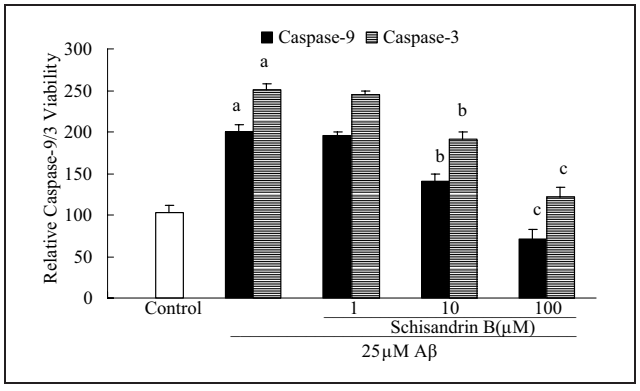

Fig. 5: Effect of schisandrin B on caspase-9 and caspase-3 activities in $A\beta_{1-42}$-induced neurotoxicity. Neuron cell cultures were pretreated with schisandrin B (1, 10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. Each value represents the mean ± S.D. from three experiments. [a] $p < 0.01$ vs. control, [b] $p < 0.05$ [c] $p < 0.01$ vs. $A\beta_{1-42}$ group

and the increase in the intracellular GSSG levels, while preincubation of cells with 10 and 100 μM schisandrin B markedly reversed these effects in a dose-dependent manner.

3. Discussion

The Bcl-2 family of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) and pro-apoptotic proteins (e.g., Bax and Bid) are important modulators in regulating cell death (Boise et al. 1993). In addition, altered ratio of anti-to-pro-apoptotic Bcl-2 family proteins is significant in determining if apoptosis occurs. In the present study, ELISA analysis revealed that compared with the control group, the Bcl-2/Bax ratio was decreased significantly by treatment with $A\beta_{1-42}$ and was attenuated by a pretreatment with schisandrin B. Hence, Bcl-2 and Bax may be critical determinants of apoptosis induced by $A\beta_{1-42}$ in neuron cells and schisandrin B may protect against the $A\beta_{1-42}$-induced apoptosis by enhancing Bcl-2/Bax expression ratio.

Bcl-2 family plays an important role in regulating the release of cytochrome c, and caspases activation. Bcl-2 can protect the cell from the apoptosis through regulating the mitochondrial transition pore opening by opposing the effect of Bax, thereby blocking the release of cytochrome c from the mitochondria and preventing caspases activation (Vander Heiden et al. 1997). Bcl-2 also acts as an antioxidant that could prevent reactive oxygen species production and block the depletion of cytochrome c and caspases activation (Kirsch et al. 1999).

Caspases play a critical role in the apoptosis of neurons (Marin et al. 2000). Caspases transduce the apoptotic cell death signals in a cascade manner, where the initiator caspases cleave and activate the effector caspases, which then degrade other cellular targets leading to cell death (Wolf and Green 1999). Caspase-3 is one of the effector caspases found in apoptotic cells, which is activated by action of upstream signaling, caspase-9, an initiator caspase (Masumura et al. 2000). In this study, we demonstrated that the induction of apoptosis by $A\beta_{1-42}$ was accompanied by a significant increase in caspase-9 and -3 activ-

Table 2: Effect of schisandrin B on GSH and GSSG levels in $A\beta_{1-42}$-induced neurotoxicity

	Concentration (μM)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG (ratio)
Control		45.63 ± 1.35	5.76 ± 0.95	7.92
$A\beta_{1-42}$		23.20 ± 1.90[a]	15.67 ± 0.50[a]	1.48[a]
Schisandrin B + $A\beta_{1-42}$	1	23.80 ± 1.80	15.56 ± 0.90	1.53
	10	28.06 ± 1.70[b]	13.37 ± 0.80[b]	2.10[b]
	100	35.43 ± 1.10[c]	12.50 ± 0.60[c]	2.83[c]

Neuron cell cultures were pretreated with schisandrin B (1, 10, and 100 μM) 2 h before exposure to 25 μM $A\beta_{1-42}$ and then maintained for 24 h. Each value represents the mean ± S.D. from three experiments. [a] $p < 0.01$ vs. control, [b] $p < 0.05$ [c] $p < 0.01$ vs. $A\beta_{1-42}$ group

ities. These effects were attenuated by pretreatment with schisandrin B. Therefore, these findings suggest that caspase-9/-3 might be one of the main effector protein in $A\beta_{1-42}$-induced apoptosis, and schisandrin B protects against the $A\beta_{1-42}$-induced apoptosis by blocking the activities of caspases-9/-3.

GSH is an important intracellular anti-oxidant and essential cofactor for anti-oxidant enzymes that protects against endogenous oxygen radicals. GSSG is referred to as oxidized GSH. Disturbance of GSH/GGSG homeostasis may either lead to or result from oxidative stress in AD (Schulz et al. 2000). Schisandrin B treatment may either lead to enhanced synthesis of GSH or inhibition of its degradation, resulting in a slowing of $A\beta_{1-42}$-induced oxidative stress signaling. These studies show that pretreatment with schisandrin B suppressed $A\beta_{1-42}$-induced GSSG oxidative damage, while improving overall cell survival.

Taken together, the present study suggests that schisandrin B may protect neuron cells from $A\beta_{1-42}$-induced apoptosis by regulating the Bcl-2/Bax ratio, caspases, and effectors involved in the mitochondria-mediated pathway and due to an anti-oxidant action.

4. Experimental

4.1. Preparation of schisandrin B and $A\beta_{1-42}$

Schisandrin B (purity $>99.9\%$, purchased from National Institute for the Control of Pharmaceutical and Biological Products, China) was prepared as stock solution in dimethylsulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS) before the experiment, 0.1% (v/v) DMSO had no toxic effect itself. $A\beta_{1-42}$ (Sigma, USA) was prepared by dissolving in PBS and was incubated at 37 °C for 7 days to allow fibrillation. After incubation, peptide was stored at −20 °C before use.

4.2. Primary cultures of rat cortical neurons and treatment

Primary cortical neuron cultures were prepared from Wistar rats (Peking University Health Science Center Experimental Animal Department) at gestation day 18 as previously described (Jiang et al. 2007). Dissociated neurons were suspended in plating medium (MEM containing 10% fetal bovine serum, 5% heat-inactivated horse serum, 2 mM glutamine, 0.2 μM cystine, 100 IU/ml penicillin and 100 mg/ml streptomycin) and transferred to poly-L-lysine-coated 25 cm^2 flasks at a density of $5.0–5.5 \times 10^5$ cells/ml. After 4–6 h, the plating medium was removed and replaced with maintenance medium (Neurobasal medium, 2% B-27 supplement, and 0.5mM L-glutamine, Gibco BRL).

Cells were plated at an appropriate density according to each experimental scale. After 2 h preincubation with 1, 10 and 100 μM of schisandrin B, the cells were subjected to fibrillar $A\beta_{1-42}$ at a final concentration of 25 μM for 24 h.

4.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were plated at a density of 1.5×10^5 cells/well in 96-well plates. The cell viability was measured using the MTT method as previously described (Schubert et al. 1995). Briefly, after treatment, 10 μL of MTT (Sigma, U.S.A) solutions (0.5 mg/mL in PBS) was added and incubated for 4 h. After the incubation, 100 μL of DMSO was added for 10 min. Absorbance was measured at 570 nm using a plate reader (Bio Rad Laboratories Inc, CA,USA).

4.4. Apoptosis assay

Apoptosis was monitored using a flow cytometer (Becton Dickinson) and the fluorescent dye annexin-V-FITC/ propidium iodide (PI) (Biosea BCL, Beijing, China). After treatment, 1×10^6 cells were centrifugated at $1{,}000 \times g$ for 5 min at 4 °C and then the cells were washed 2 times with ice-cooled PBS. 10 μL of annexin-V-FITC and 500 μL of 1×binding buffer were added and incubated for 30 min at 4 °C, 5 μL of PI were then added before the analysis using Cell Quest software.

4.5. Bcl-2 and Bax assays

Bcl-2 and Bax protein was measured using commercially enzyme-linked immunosorbent assay (ELISA) kits (EMD Chemicals Inc., Darmstadt, Germany). Briefly, cells were adjusted to 1×10^4 cells/well in a microtitre plate. After exposed to 1, 10 and 100 μM of schisandrin B for 2 h, cells were treated with 25 μM $A\beta_{1-42}$ for 24 h. Cells were lysed and the microtitre plate was centrifuged at 250 × g for 10 min at 4 °C. Then, 100 μL of the supernatant were removed and analysed by ELISA, using the instructions recommended by the manufacturer. The optical absorbance of the samples was measured at 450 nm using a microplate reader (Bio Rad Laboratories Inc, CA,USA).

4.6. Cytochrome c assay

The content of cytochrome c was assessed with a solid phase ELISA kit (Chemicon, USA). After exposed to 25 μM $A\beta_{1-42}$ for 24 h, 5×10^5 neuron cells were harvested and resuspended in PBS (pH 7.4). Then the cells were centrifuged at $100{,}000 \times g$ for 30 min. Supernatants (100 μL) were added into the 96-well microplates coated with 100 μL of monoclonal antibody and incubated at room temperature for 2 h. Then 100 μL of diluted streptavidin-enzyme conjugate was added to all wells and incubated for 1 h. At last, 100 μL of 3,3′,5,5′ tetramethyl benzidine (TMB) substrate was added to each well and incubated for 5 min. Absorbance of samples was measured at 450 nm in a microplate reader (Bio Rad Laboratories Inc, CA,USA).

4.7. Caspase-9 and caspase-3 assays

Caspases activities were assayed using caspase-9 and caspase-3 activity assay kits (Keygen Biotec, Nanjing, China) according to the manufacturer's instructions. Briefly, cells were grown on 100 mm dishes and treated with 25 μM of $A\beta_{1-42}$ alone or were pretreated with 1, 10 and 100 μM of schisandrin B prior to the 25 μM of $A\beta_{1-42}$ treatment for 2 h. The media were removed from the culture dishes and the cells were collected and washed with PBS, then resuspended in a cell lysis buffer at 4 °C. After incubation on ice for 10 min, the lysates were centrifuged for 20 min at 12,000 × g at 4 °C, and the supernatants were collected and protein concentrations were determined. Cell lysates (100 μg) were mixed with reaction buffer containing the LEHD-pNA substrate (200 μM) for caspase-9 activity and DEVD-pNA substrate (200 μM) for caspase-3 activity. The absorbance was measured in the wells at 405 nm using an ELISA reader.

4.8. GSH and GSSG assays

The concentrations of total glutathione (T-GSH), GSH and GSSG were measured by an enzymatic method according to the commercial assay kit procedure (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, after exposed to 25 μM $A\beta_{1-42}$ for 24 h, the cells were washed two times with PBS and then homogenized. The homogenate was centrifuged at 4 °C at $10{,}000 \times g$ for 30 min. T-GSH was assayed using the 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. GSSG was measured by measuring 5-thio- 2-nitrobenzoic acid (TNB) which was produced from the reaction of reduced GSH with DTNB. The rate of TNB formation was measured at 412 nm over 3 min. The concentration of reduced GSH in the sample was obtained by subtracting GSSG from T-GSH.

4.9. Protein assay

Protein content was measured according to Bradford (1976), using bovine serum albumin as a standard.

3.10. Statistical analysis

Results are expressed as the mean ± S.D. All results were analyzed by one-way analysis of variance (ANOVA) test using SPSS 13.0 system (SPSS Inc. Chicago, IL, U.S.A.) for Windows. Differences were considered significant at $p < 0.05$.

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