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Hepatoprotective effect of chitooligosaccharides against *tert*-butylhydroperoxide-induced damage in Chang liver cells

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ABSTRACT

Hepatoprotective effect of chitooligosaccharides (COSs) with different molecular weights was investigated against *tert*-butylhydroperoxide (*t*-BHP)-induced damage in Chang liver cells. For that, we evaluated the cell viability, inhibition of production of reactive oxygen species (ROS), lipid peroxidation inhibition, glutathione (GSH) contents and the levels of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidise (GPx) under oxidative damage by *t*-BHP. COSs does not have any harmful or inhibitory effect against cell growth at concentrations ranging from 0.1 to 1.0 mg/mL. Under oxidative damage, COSs increased the cell viability in Chang liver cells exposed to *t*-BHP and significantly ($p < 0.05$) reduced ROS generation and lipid peroxidation. In addition, COSs assisted to increase the GSH content and antioxidant enzyme activity. Hence, these results indicate that COSs protected Chang liver cells against oxidative damage induced by *t*-BHP via inhibiting production of ROS and lipid peroxidation, and the elevation of the levels of antioxidant enzymes.

1. Introduction

Reactive oxygen species (ROS) such as hydroxyl radicals ($HO^{\bullet}$), super oxide radicals (O_2^-) and H_2O_2 are generated in living systems via aerobic metabolism or via exogenous sources such as ultra violet light, ionizing radiation, pollution systems and drugs (Briviba & Sies, 1994). Hydroxyl radicals are generated from H_2O_2 or from O_2^- radicals in the presence of transition metal ions such as Fe^{2+} and may cause oxidative modifications in biological molecules such as protein, lipid and nucleic acid (Chevion, 1988). In biological systems, there are various endogenous and exogenous defense mechanisms to minimize/or alleviate the overproduction of ROS and the damage caused by them (Ames, Shigenaga, & Hagen, 1993). These include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and many non-enzymatic antioxidant compounds such as polyphenol, glutathione, tocopherol, ascorbic acid, and other thiol protein groups to protect the functional and structural integrity of the biomolecules (Anderson & Phillips, 1999; Tavazzi et al., 2000). It has been reported that the unbalanced between overproduction of ROS and antioxidant defense systems are associated with pathophysiological conditions such as artherosclerosis, aging, arthritis, neurodegenerative cancer and rheumatoid diseases (Briviba & Sies, 1994; Knight, 1997; Rice-Evans & Burdon, 1993).

Chitosan is a natural polymer which is produced by deacetylation of chitin that is a major shell component of crustaceans such as crabs and shrimps. It has been considered as biomaterial because of its biocompatible, biodegradable, and less toxic nature. Chitooligosaccharides (COSs), derivatives of chitosan, can be obtained by either enzymatic or acidic hydrolysis. COSs has been the choice of interest among many researchers due to their potential biological activities such as immunity enhancing and antitumor (Suzuki et al., 1986), antioxidant and radical scavenging activity (Castagnino et al., 2008; Ngo, Kim, & Kim, 2008; Park, Je, & Kim, 2004) and so on. However, there is scanty information with respect to hepatoprotective effect of COSs against *t*-butylhydroperoxide (*t*-BHP)-induced oxidative stress. In this study, as a part of our ongoing investigation on biological properties of COSs, we prepared COSs with different molecular weights and evaluated their hepatoprotective effect against *t*-BHP-induced hepatic damage on Chang liver cells.

2. Materials and methods

2.1. Materials

Fluorescence probes 2′, 7′-dichlorodihydrofluorescin diacetate (DCFH-DA), Diphenyl-1-pyrenylphosphine (DPPP), and monobromobimane (mBBr) were purchased from Sigma Co. (St. Louis, USA). Chang liver cell line (CCL-13) was obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin/streptomycin, and other materials required for culturing of cells were purchased from Gibco

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BRL (Paisley, UK). Enzyme assay commercial kits were supplied by Biovision (CA, USA). Other chemicals used were in analytical grade.

2.2. Preparation of chitooligosaccharides (COSs)

COSs were prepared from 90% deacetylated chitosan as described in our previous report, and further fractionated into three kinds of COSs using an ultrafiltration membrane system (Park et al., 2004). COSs were designated based on their molecular weights as COS I (5–10 kDa), COS II (1–5 kDa) and COS III (below 1 kDa).

2.3. Cell culture and treatment

Chang liver cells purchased from ATCC was cultured in DMEM medium containing heat-inactivated 10% fetal bovine serum, streptomycin (100 μg/mL) and penicillin (100 unit/mL) at 37 °C in an incubator under a humidified atmosphere of 95% air and 5% CO_2. Adherent cells were detached by trypsin-EDTA and plated onto 6- or 96-well plates at 70–80% confluence. COSs were dissolved in PBS to make 1.0, 2.0, 5.0 and 10 mg/mL concentrations, and cells at the confluence were treated with the presence or absence of various COSs at final concentrations of 100, 200, 500 and 1000 μg/mL.

2.4. Cell viability assay

The cell viability was estimated by MTT assay, which is a test of normal metabolic status of cells based on the assessment of mitochondrial activities (Hansen, Nielsen, & Berg, 1989). Chang liver cells were seeded in 96-well plate at a concentration of 4.0×10^5 cells/mL. After 16 h, the cells were treated with different concentrations of various COSs, and incubated in a humidified incubator at 37 °C for 1 h. Then, 150 μM *t*-BHP was added as final concentration, and incubated for 24 h. Thereafter, a 50 μL of MTT stock solution (2 mg/mL) was added and incubated for 4 h. Then, the supernatants were aspirated and the formazan crystals in each well were dissolved in 150 μL of DMSO. Absorbance was measured by ELISA reader (SpectraMax® M2/M2^e, CA, USA) at a wavelength of 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100%.

2.5. Intracellular ROS determination by DCFH-DA

Intracellular formation of ROS was assessed as described previously using oxidation sensitive dye DCFH-DA as a substrate (Takahashi, Shibata, & Niki, 2001). Chang liver cells were seeded in 96-well plate at a concentration of 4×10^5 cells/mL. Cells growing at confluency were labelled with 25 μM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated for 30 min in CO_2 incubator at 37 °C. Non-fluorescent DCFH-DA dye, that is freely penetrate into cells get hydrolyzed by intracellular esterase to 2′,7′-dichlorofluorescin (DCFH), and traps inside the cells. Cells were then treated with different concentrations of various COSs and incubated for 1 h. After washing the cells with HBSS for three times, 150 μM *t*-BHP was added. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of ROS was read after every 30 min at a excitation wavelength of 485 nm and a emission wavelength of 535 nm using a spectroflurometer (SpectraMax® M2/M2^e).

2.6. Lipid peroxidation inhibitory assay

Chang liver cells were seeded into 96-well plate at a concentration of 4.0×10^5 cells/mL. Cells growing at confluency were labelled with DPPP (in DMSO) at a final concentration of 25 μM and incubated in a humidified CO_2 incubator at 37 °C for 30 min in the dark (Takahashi et al., 2001). Cells were then treated with different concentrations of various COSs and incubated another 1 h at 37 °C. Then, cells were washed three times with HBSS and treated with 100 μL of 150 μM *t*-BHP except blank cells and incubated at 37 °C for 30 min. Fluorescence intensity was measured with a spectroflurometer (SpectraMax® M2/M2^e) at an excitation wavelength of 351 and an emission wavelength of 380 nm.

2.7. Determination of intracellular GSH level

Cellular GSH level was determined using monobromobimane (mBBr) as a thiol-staining reagent via the method described by Poot, Verkerk, Koster, and Jongkind (1986) with slight modifications. Chang liver cells were seeded at a concentration of 4.0×10^5 cells/mL and following confluency, treated with different concentrations of various COSs for 1 h. Cells were then labelled with 40 μM mBBr for 30 min in a CO_2 incubator at 37 °C in the dark. After staining, mBBr-GSH fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm using a spectroflurometer (SpectraMax® M2/M2^e). In another set of experiment, cells were exposed to *t*-BHP after treatment with various COSs and followed the same method. The average fluorescence values of cell populations were plotted and compared with blank group in which cells were grown without treatment of COSs.

2.8. Preparation of cell lysate and determination of protein content

Chang liver cells were seeded in 6-well plates at a concentration of 4.0×10^5 cells/mL. At 70–80% confluence, the cells were treated with different concentrations of various COSs except for blank and control cells, and incubated in a humidified CO_2 incubator at 37 °C for 1 h. Then, 150 μM *t*-BHP was added as final concentration to the cells except for blankcells, and incubated for another 12 h at 37 °C. Cells were collected and washed three times in HBSS. Then, HBSS were removed and cells were lysed in 400 μL lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100) for 2 h. The supernatants were obtained by centrifugation at 8000 rpm at 4 °C for 10 min and the protein content was determined via BSA assay kit (BioRad, CA, USA), using bovine serum albumin as a standard.

2.9. Determination of antioxidant enzymes

2.9.1. Catalase (CAT) activity

Catalase (E.C.1.11.1.6) activity of cell lysates was determined using a commercially available kit (Biovision). One unit of catalase was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 1 min. The rate of decomposition of H_2O_2 was measured spectrophotometrically at a wavelength of 570 nm and the enzyme activity was expressed as mU/mg protein.

2.9.2. Superoxide dismutase (SOD) activity

Superoxide dismutase (E.C.1.15.1.1) level in cell lysates was estimated using commercial kit supplied by Biovision (CA, USA). Xanthine and xanthine oxidase were used to generate superoxide anion, which react with tetrasolium chloride to form a yellow color formazan dye. SOD activity was measured at a wavelength of 450 nm and the enzyme activity was expressed as U/mg protein.

2.9.3. Glutathione peroxidase activity (GPx)

The enzyme reaction in the tube, which contains NADPH, reduced glutathione, glutathione reductase, was initiated with the addition of cumene hydroperoxide, and the change in absorbance

at a wavelength of 340 nm was monitored by a spectrophotometer. GPx (E.C. 1.11.1.9) activity was determined using a commercial kit supplied by Biovision and the activity was given as mU/mg protein.

2.10. Statistical analysis

The results were expressed as mean ± SD, and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) followed by Duncan's test. The difference showing a P level of 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Hepatoprotective effect by COSs

COSs were evaluated for its hepatoprotective effect against t-BHP-induced toxicity employed in Chang liver cells. Prior to evaluate hepatoprotective effect, toxicity of COSs was evaluated by MTT cell viability assay, and was not toxic to the cells at the concentration range used (0.1 to 1 mg/mL) (Fig. 1A). In order to determine cytotoxic dose of t-BHP, various concentrations of t-BHP (50–200 μM) were exposed to Chang liver cells, and a 150 μM t-BHP was selected as optimal concentration to make a oxidative damage

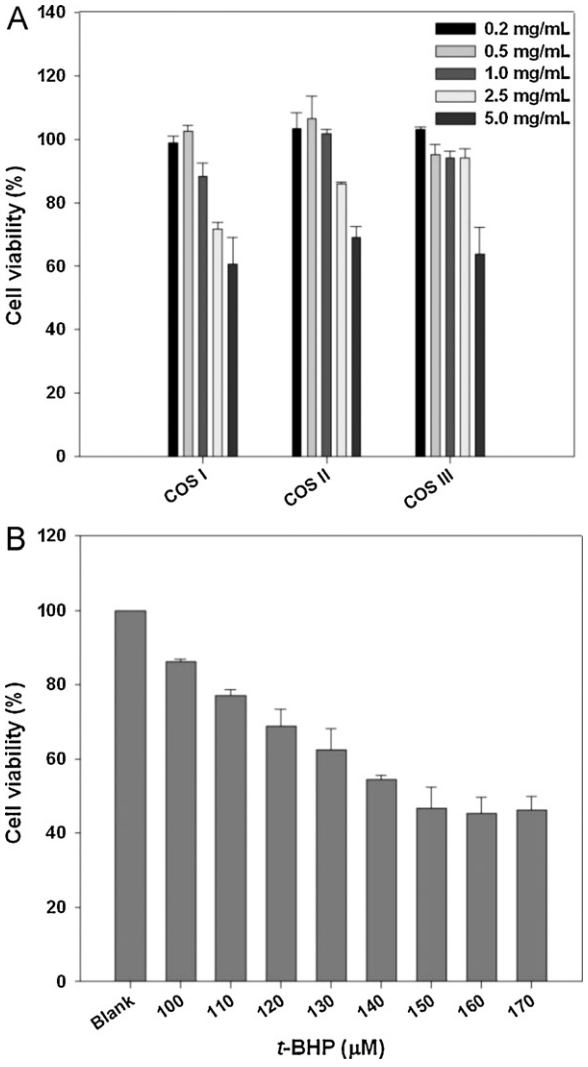

Fig. 1. Effects of cell viability of Chang liver cells by (A) chitooligosaccharides and (B) t-BHP. Cell viability was assessed by MTT assay and data were expressed as the mean ± S.E.

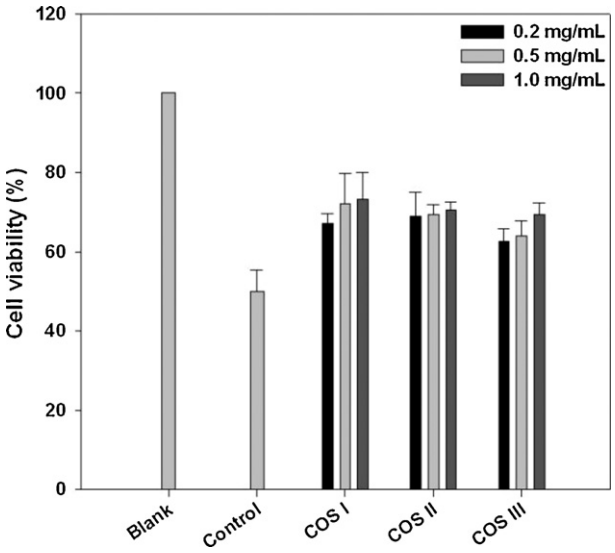

Fig. 2. Increase in viability of Chang liver cells by COSs. Cells (4×10^5 cells/mL) were cultured in DMEM medium for 16 h and exposed to t-BHP for 24 h after pre-incubation with various COSs for 2 h. Control cells treated with 150 μM t-BHP alone for 24 h. Cell viability was assessed by MTT assay and data were expressed as the mean ± S.E.

to Chang liver cells (Fig. 1B). As shown in Fig. 2, cell viability of t-BHP exposed Chang liver cells (control group) decreased to around 50% compared to the blank group. However, pre-treatment of COSs significantly ($p < 0.05$) increased the cell viability up to 70% at all concentrations. Hence, it was found that the reduction of cell viability was greatly mediated by pre-treatment of cells with various COSs. However, no significant effect was observed between COS I, COS II and COS III.

The reaction between superoxide radical and superoxide dismutase as well as some other enzymes such as amino acid oxidase produce H_2O_2 and it can cause membrane damage to release arachidonic acid which in turn responsible for prolonged cell damage (Park et al., 2003). Other than that H_2O_2 can penetrate the cell membrane and react with Fe^{2+} and generate most reactive $HO^{\bullet}$ radicals through Fenton reaction that may cause cell damage leading to various age related diseases (Halliwell & Gutteridge, 1989). In this study, we demonstrated that COSs prevent such deleterious effects and protect cells from oxidative damage.

3.2. Effect of COSs on ROS production and lipid peroxidation

Direct measurement of ROS was performed using a redox-sensitive fluorescent dye, DCFH-DA. DCFH-DA itself is not a fluorescent but in the cells it is converted into DCFH by intracellular esterase. DCFH can react with ROS and convert into the fluorescent DCF which can be measured using spectroflurometer. When DCFH-DA-labelled cells were incubated with t-BHP for 2 h, a sudden increment in fluorescence intensity occurred and it indicated the oxidation of DCFH-DA by intracellular radicals (Fig. 3A). The fluorescence intensity was increased up to 1200 in control cells compared to 100 in blank cells after exposure to t-BHP. However, fluorescence intensity was significantly ($p < 0.05$) decreased in the cells pre-treated with COSs before exposure to t-BHP indicating the scavenging of ROS by COSs. Further, reduction of ROS production was in dose and time dependent fashion (time dependent data not shown). In this case, COS I (MW 5–10 kDa) exhibited superior activity toward the inhibition of ROS production than that of COS II (MW 1–5 kDa) and COS III (MW below 1 kDa).

Many studies have been conducted to investigate antioxidant effect of chitosan and COS compounds, and found that they

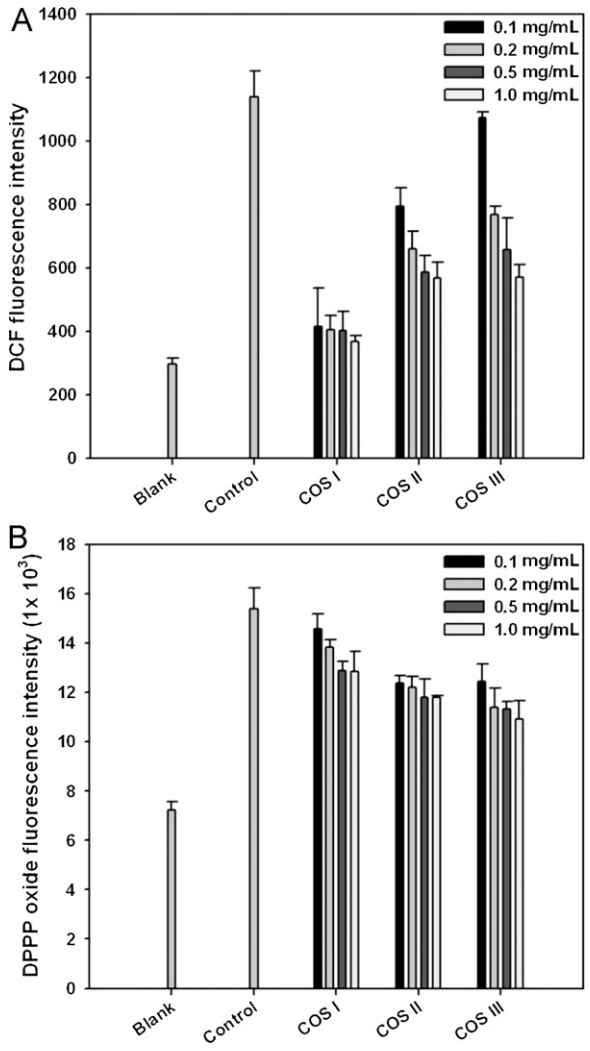

Fig. 3. Effect of COSs on the inhibition of intracellular generation of ROS (A) and lipid peroxidation (B). Intracellular formation of ROS was assessed using oxidation sensitive dye, DCFH-DA. Formation of DCF due to oxidation of DCFH was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Lipid peroxidation was evaluated using oxidative sensitive dye, DPPP. All statistical analysis was performed with three independent experiments and data were expressed as means ± S.E.

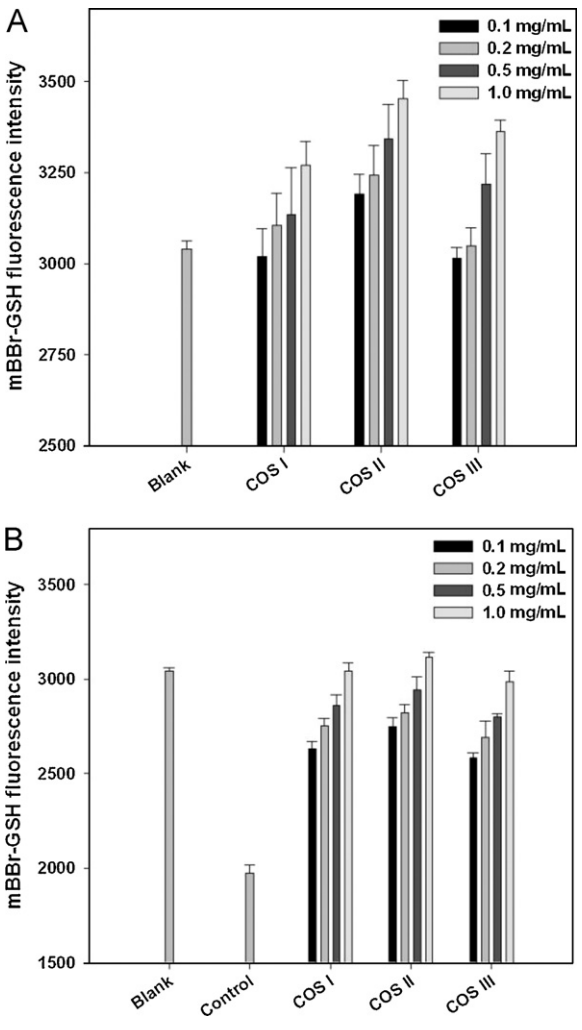

Fig. 4. Effect of COSs on the regulation of GSH level in Chang liver cells. Cells were treated with different concentrations of various COSs and incubated for 30 min. GSH level was determined using mBBr as a thiol-staining reagent. The average fluorescence values of cell populations were plotted and the data were expressed as the mean ± S.E. (A) Without *t*-BHP treatment; (B) with *t*-BHP treatment.

have shown significant results in ROS production (Mendis, Kim, Rajapakse, & Kim, 2007). In this study too, we have proved from the results obtained that COSs are potential source that can be used in medical or pharmaceutical related fields. ROS can readily damage biological molecules such as lipid, protein and DNA which can finally lead to apoptic or necrotic cell death (Jang & Surh, 2001). Therefore, removal of those excess ROS or suppression of their generation by antioxidants defense mechanisms may be effective in preventing oxidative cell death. Hence, these results suggest that a significant inhibition of *t*-BHP-induced ROS production by COSs may contribute to restoring the viability in Chang liver cells. Further, results obtained in this study were in lines with previous studies reported by Mendis et al. (2007) and Liu et al. (2009) on human melanoma cells and human umbilical vein endothelial cells, respectively. However, we could not conclude whether COSs elicited a direct quenching effect on *t*-BHP-induced ROS or by some other mechanisms.

Unsaturated fatty acids and their esters are easily prone to oxidize producing hydroperoxides at the initial stage of lipid peroxidation. Hydroperoxides have attracted much attention as one of the factors that might be associated with some diseases and aging (Meerson, Kagan, Kozlov, Belkina, & Arkhipenko, 1982). Hence, the prevention or reduction of lipid peroxidation enhances the personnel health, and the role of antioxidants has received extensive attention. Inhibitory effect of COSs on oxidative damage in Chang liver cells induced by *t*-BHP was evaluated by lipid peroxidation assay using fluorescence sensitive compound, DPPP (Fig. 3B). DPPP reacts with lipid hydroperoxides selectively and stoichiometrically, and the resulting DPPP oxide can be measured in a solution, cultured cells, and tissues (Takahashi et al., 2001). When cells were exposed to *t*-BHP, fluorescence intensity of DPPP = O, which is the index of lipid peroxidation, was significantly increased but reduced when cells were pre-treated with COSs before exposing to *t*-BHP. Further, activity was doe-dependent, thus, COSs were able to prevent the oxidative damage, indicating that it provides an antioxidant protection for Chang liver cells in the concentration range used.

3.3. Effect of COSs on GSH content

GSH reduce endogenously produced ROS in the presence of selenium-dependent glutathione peroxidase to prevent the oxidative stress and another important function of GSH is detoxification of xenobiotics and their metabolites. GSH plays a crucial role in cell

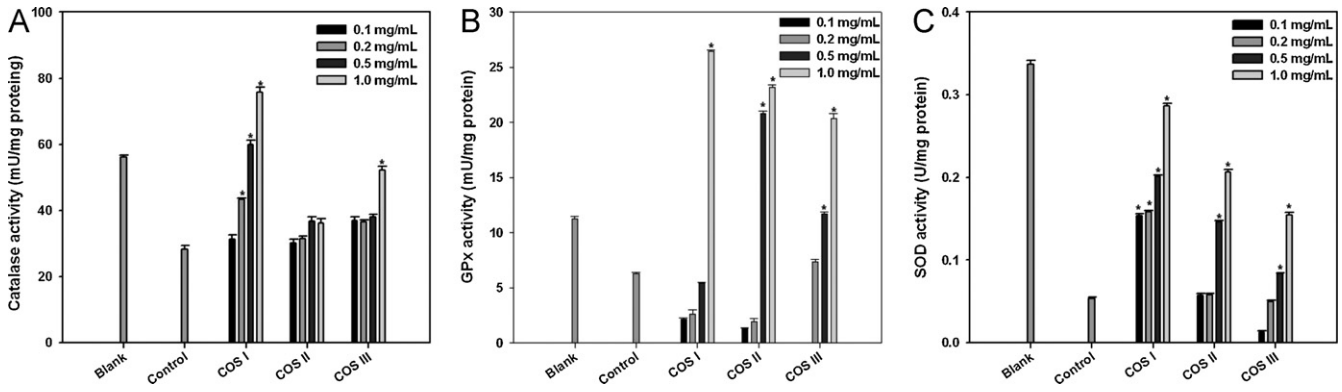

Fig. 5. Effect of COSs on CAT, GPx, and SOD activity in *t*-BHP-induced Chang liver cells. Each bar represents the mean ± S.E. of three independent experiments. $^{*}p < 0.05$ vs. control.

defense against ROS, free radicals and other metabolites (Kedderis, 1996). However, a severe GSH depletion may cause oxidative damage by those radicals and, increase protein thiolation or oxidation of SH groups which may eventually cause for alterations in cellular homeostasis (Estrela, Ortega, & Obrador, 2006). Further, primary mechanism of *t*-BHP-induced toxicity in the cultured cells is decrease of GSH level (Martin et al., 2001). Therefore, the potential of COSs to maintenance the GSH at high levels could be of great importance against *t*-BHP-induced toxicity. In order to evaluate the effect of COSs against *t*-BHP-induced stress, intracellular GSH content was estimated either absence or presence of various COSs and *t*-BHP. Firstly, pre-treatment of COSs with various concentrations without *t*-BHP to Chang liver cells clearly showed an enormous enhancement in the total cellular GSH content (Fig. 4A). We also evaluated GSH levels of Chang liver cells by exposing to *t*-BHP after pre-treatment with various COSs, and found that *t*-BHP depleted the GSH level significantly ($p < 0.05$) compared to that of blank cells but it was effectively restored by pre-treatment with various COSs in a dose-dependent manner (Fig. 4B). Hence, the results obtained in this study revealed that GSH is up-regulated in the presence of various COSs.

3.4. Effect of COSs on antioxidant enzyme activities

Generally, cells respond to oxidative stress with adaptive changes aimed at preventing cellular damage and increasing their survival. The toxicity of ROS varies with cell density, components in the incubation medium, and cell type studied (Weber et al., 1998). Marked cellular damage was observed in response to *t*-BHP as compared to the blank cells. However, pre-treatment of Chang liver cells with COSs elevated the enzyme activity as can be seen in Fig. 5A-C. *t*-BHP treatment reduced the CAT activity to 30 mU/mg protein; however, pre-treatment of cells with COS I restored the CAT activity above 70 mU/mg protein. In regards to SOD activity, COS I pre-treatment exhibited 0.3 U/mg protein compared to 0.35 U/mg protein in blank cells while *t*-BHP treatment reduced the SOD activity to 0.05 U/mg protein. Exposure of cells to *t*-BHP decreased the GPx activity to 7 mU/mg protein but restored the activity to 25 mU/mg protein with the pre-treatment of various COSs which was a higher level than that shown by the blank cells (12 mU/mg protein). Therefore, COSs significantly enhanced the intracellular activities of SOD, CAT and GPx than those of the cells treated only with *t*-BHP. The results showed that COSs enhanced the enzyme activities that may be related to the inhibition of the production of ROS. CAT and SOD are of enormous important in limiting ROS-mediated cell damage, however, needed second line of antioxidant enzymes which include heme oxygenase 1(HO-1) and quinone oxidoreductase 1 (NQO1) to prevent the activity of highly reactive ROS (Siegel et al., 2004). We didn't investigate the activity of those enzymes. Recently it has been reported that COSs function as an antioxidants through the induction of antioxidant enzymes, such as glutathione S-transferases and SOD (Liu et al., 2009). Hence, we can suggest that COSs may help up-regulation of antioxidant enzymes in mediating oxidative stress in Chang liver cells.

4. Conclusions

The results revealed in this study indicate that COSs exhibit antioxidant properties through its ability to enhance the cell viability, mediation of production of ROS, lipid peroxidation and GSH content. Further, this study suggests that increased antioxidant enzyme activity induced by COSs could contribute to defense mechanisms against *t*-BHP induced oxidative damage in Chang liver cells and it may contribute beneficial effect in the treatment of diseases related with oxidative stress through enhancement of antioxidant defense mechanisms after extensive experiments *in vivo*.

References

Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the USA*, *90*, 7915–7922.

Anderson, D., & Phillips, B. J. (1999). Comparative *in vitro* and *in vivo* effects of antioxidants. *Food and Chemical Toxicology*, *37*, 1015–1025.

Briviba, K., & Sies, H. (1994). Non enzymatic antioxidant defense system. In B. Frie (Ed.), *Natural antioxidants in human health and diseases* (pp. 119–121). San Diego, CA: Academic press Inc.

Castagnino, E., Ottaviani, M. F., Cangiotti, M., Morelli, M., Casettari, L., & Muzzarelli, R. A. A. (2008). Radical scavenging activity of 5-methylpyrrolidinone chitosan and dibutyryl chitin. *Carbohydrate Polymers*, *74*, 640–647.

Chevion, M. (1988). A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. *Free radical Biology and Medicine*, *5*, 27–37.

Estrela, J. M., Ortega, A., & Obrador, E. (2006). Glutathione in cancer biology and therapy. *Critical Reviews in Clinical Laboratory Sciences*, *43*, 143–181.

Halliwell, B., & Gutteridge, J. M. C. (1989). Free radical ageing and disease. In B. Halliwell, & M. C. Gutteridge (Eds.), *Free radicals in biology and medicine*. Oxford: University Press, pp. 10–19, 416–508.

Hansen, M. B., Nielsen, S. E., & Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*, *119*, 203–210.

Jang, J. H., & Surh, Y. J. (2001). Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutation Research*, *496*, 181–190.

Kedderis, G. L. (1996). Biochemical basis of hepatocellular injury. *Toxicologic Pathology*, *24*, 77–83.

Knight, J. A. (1997). Reactive oxygen species and the neurodegenerative disorders. *Annals of Clinical and Laboratory Science*, *27*, 11–25.

Liu, H. T., Li, W. M., Xu, G., Li, X. Y., Bai, X. F., Wei, P., et al. (2009). Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. *Pharmacological Research*, *59*, 167–175.

Martin, C., Martinez, R., Navarro, R., Ruiz-Sanz, J. I., Lacort, M., & Ruiz-Larrea, M. B. (2001). *tert*-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. *Biochemical Pharmacology*, *62*, 705–712.

Meerson, F. Z., Kagan, V. E., Kozlov, Y. P., Belkina, L. M., & Arkhipenko, Y. V. (1982). The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. *Basic Research in Cardiology*, *77*, 465–485.

Mendis, E., Kim, M. M., Rajapakse, N., & Kim, S. K. (2007). An in vitro cellular analysis of the radical scavenging efficacy of chitooligosaccharides. *Life Sciences*, *80*, 2118–2127.

Ngo, D. N., Kim, M. M., & Kim, S. K. (2008). Chitin oligosaccharides inhibit oxidative stress in live cells. *Carbohydrate Polymers*, *74*, 228–234.

Park, C., So, H. S., Shin, C. H., Baek, S. H., Moon, B. S., Shin, S. H., et al. (2003). Quercetin protects the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. *Biochemical Pharmacology*, *66*, 1287–1295.

Park, P. J., Je, J. Y., & Kim, S. K. (2004). Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. *Carbohydrate Polymers*, *55*, 17–22.

Poot, M., Verkerk, A., Koster, J. F., & Jongkind, J. F. (1986). De novo synthesis of glutathione in human fibroblasts during in vitro ageing and in some metabolic diseases as measured by a flow cytometric method. *Biochimica et Biophysica Acta*, *883*, 580–584.

Rice-Evans, C., & Burdon, R. (1993). Free radical–lipid interactions and their pathological consequences. *Progress in Lipid Research*, *32*, 71–110.

Siegel, D., Gustafson, D. L., Dehn, D. L., Han, J. Y., Boonchoong, P., Berliner, L. J., et al. (2004). NAD(P)H: quinone oxidoreductase 1: role as a superoxide scavenger. *Molecular Pharmacology*, *65*, 1238–1247.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., & Suzuki, M. (1986). Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydrate Research*, *151*, 403–408.

Takahashi, M., Shibata, M., & Niki, E. (2001). Estimation of lipid peroxidation of live cells using a fluorescent probe, diphenyl-1-pyrenylphosphine. *Free Radical Biology and Medicine*, *31*, 164–174.

Tavazzi, B., Pierro, D. D., Amorini, A. M., Fazzina, G., Tuttobene, M., Giardina, B., et al. (2000). Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress. *European Journal of Biochemistry*, *267*, 684–689.

Weber, H., Roesner, J. P., Nebe, B., Rychly, J., Werner, A., Schroder, H., et al. (1998). Increased cytosolic Ca^{2+} amplifies oxygen radical-induced alterations of the ultrastructure and the energy metabolism of isolated rat pancreatic acinar cells. *Digestion*, *59*, 175–185.