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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