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# Prevention of $H_2O_2$-induced oxidative stress in Chang liver cells by 4-hydroxybenzyl-chitooligomers

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ABSTRACT

In this study, a bioactive derivative of chitooligomers (1.0–3.0 kDa), 4-hydroxybenzyl-COS (HB-COS), was synthesized to enhance antioxidant activity. Hence, HB-COS was evaluated for its capabilities against $H_2O_2$-induced oxidative stress in human Chang liver cells. It was found that HB-COS possessed the free radical scavenging activity via decreasing the intracellular reactive oxygen species production. Furthermore, HB-COS significantly reduced the oxidation of DNA in a dose-dependent manner. Notably, HB-COS treatment upregulated the gene and protein expressions of antioxidative enzymes and thereby enhancing the intracellular antioxidant mechanisms. In addition, HB-COS treatment caused a remarkable blockade on degradation of inhibitory kappa B alpha (IκB-α) protein and translocation of nuclear factor kappa B (NF-κB). The current study demonstrated that HB-COS effectively attenuated hydrogen peroxide-induced oxidative stress in Chang liver cells by increasing levels of antioxidant enzymes and inhibiting reactive oxygen species generation, DNA oxidation and the NF-κB signaling pathway.

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## 1. Introduction

Oxidative stress is one of the major risk factors for chronic diseases, such as arthritis, diabetes, neurodegenerative diseases and cardiovascular complications. Reactive oxygen species (ROS) generated during oxidative stress conditions caused adverse effect to cells. ROS attack macromolecules such as DNA, proteins, and lipids, lead to many health disorders. To protect human body from highly toxic ROS, the body has acquired antioxidative stress mechanisms, or an antioxidant defense system, including intracellular antioxidant enzymes, such as superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) (Dhalla, Temsah, & Netticadan, 2000; Maulik & Das, 2002; Ngo, Wijesekara, et al., 2011; Seven, Guzel, Aslan, & Hamuryudan, 2008).

Chitooligomers (COS) have attracted increasing attention due to absence of toxicity, and superior biocompatibility. COS are chitosan derivatives (polycationic polymers comprised principally of glucosamine units) and can be generated via either chemical or enzymatic hydrolysis of chitosan (Kim, Ngo, & Rajapakse, 2006; Muzzarelli, Stanic, & Ramos, 1999). COS and COS derivatives have important biological properties in medicinal and pharmaceutical applications such as antioxidative (Ngo, Kim, & Kim, 2008; Ngo, Kim, & Kim, 2012; Ngo, Lee, Kim, & Kim, 2009; Ngo, Qian, Ngo, et al., 2011; Ngo, Qian, Vo, et al., 2011), antiallergy (Vo, Kong, & Kim, 2011; Vo, Ngo, & Kim, 2012; Vo, Kim, Ngo, Kong, & Kim, 2012), antiinflammatory (Lee, Senevirathne, Ahn, Kim, & Je, 2009, Pangestuti, Bak, & Kim, 2011; Ngo, Ngo, et al., 2012), anti-human immunodeficiency virus (Vo & Kim, 2010), anticoagulant (Yang et al., 2012), adipogenesis inhibitory (Cho et al., 2008), anticancer (Shen, Chen, Chan, Jeng, & Wang, 2009), antibacterial (Xu, Xin, Li, Huang, & Zhou, 2010), antihypertensive (Qian, Eom, Ryu, & Kim, 2010; Ngo, Qian, et al., 2008) and immuno-stimulant (Jeon & Kim, 2001) properties.

Recently, chemical modifications of COS have attracted a great deal of attention because such approach would enhance or add biological activities while keeping the natural backbone of COS and the biochemical effects of the introduced group. In this respect, 4-hydroxybenzyl-grafted derivative of COS (HB-COS) has been synthesized to improve their bioactivities. In the present study, we further evaluate whether HB-COS enhances the inhibitory effect against oxidative stress in human Chang liver cells.

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## 2. Materials and methods

### 2.1. Materials

COS (molecular weight 1.0–3.0 kDa) were donated by Kitto Life Co. (Seoul, Republic of Korea). MTT reagent (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide), 4-hydroxybenzaldehyde, agarose, fetal bovine serum (FBS) and some other chemicals were products of Sigma Chemical Co. (St. Louis, MO, USA).

Human Chang liver cells were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) medium, penicillin/streptomycin and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA). All other chemicals were of analytical grade or of the highest grade available commercially.

### 2.2. Synthesis of a novel bioactive derivative of chitooligomers (4-hydroxybenzyl-chitooligomers)

COS (1 g) were dissolved in 100 ml of 69% ethanol (v/v), 1% acetic acid (v/v) and adjusted the pH to 5. Thereafter, 4-hydroxybenzaldehyde (2 g) was added to the mixture, and continuously stirred at room temperature for 12 h. After Schiff base was formed, sodium borohydride ($NaBH_4$, 0.1 g) was added to the reaction mixture and stirred overnight (12–14 h) (Jagadish, Divyashree, Viswanath, Srinivas, & Raj, 2012). After that, the reaction mixture was neutralized with 15% (w/v) sodium hydroxide and centrifuged at 6000 rpm for 30 min. Subsequently, the solution was filtered using a filter paper to obtain a precipitate. The precipitate was dialyzed against distilled water using dialysis membranes molecular weight cut off 1 kDa in order to remove salt.

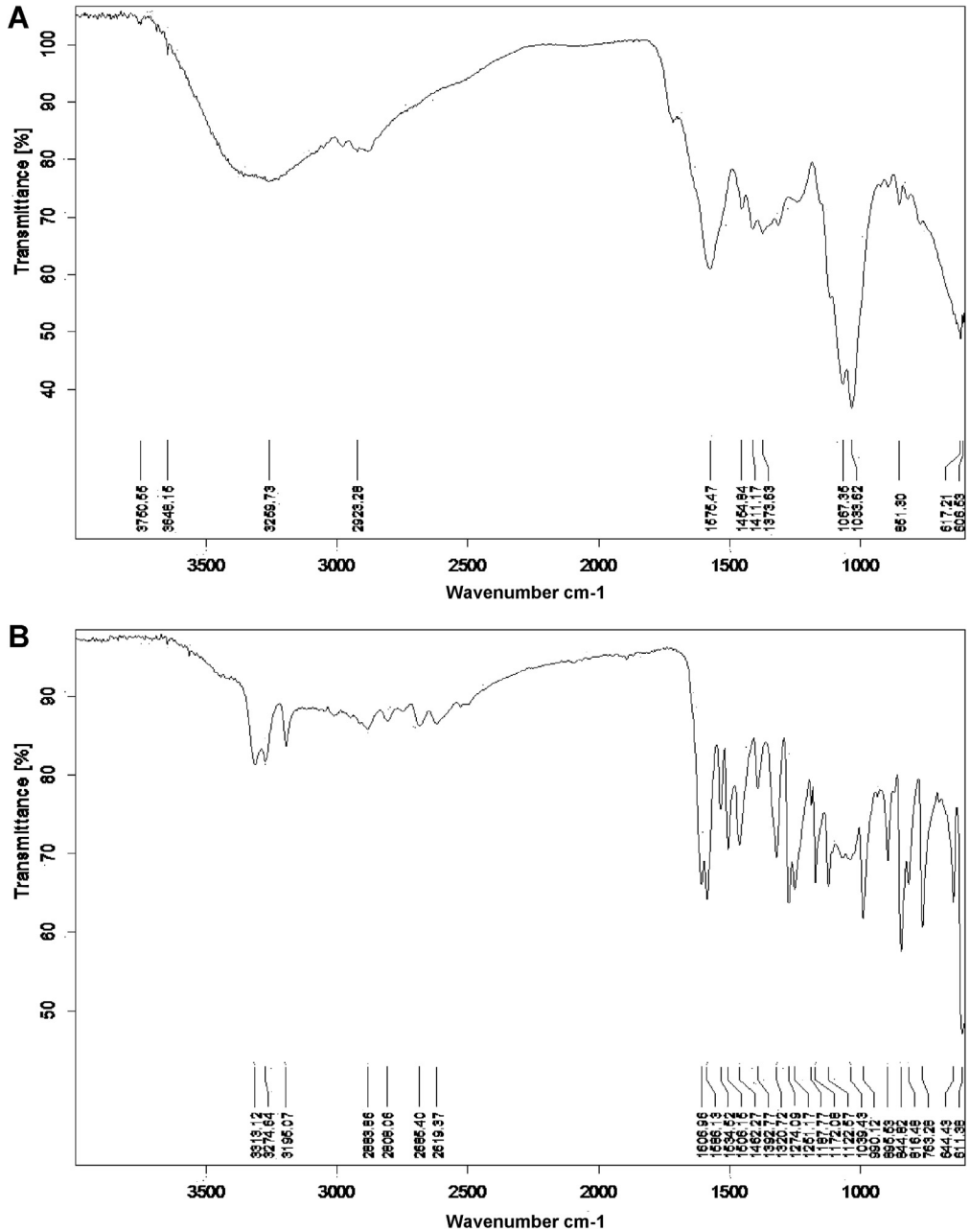


**Fig. 1.** FT-IR spectra of COS (A) and HB-COS (B); $^1H$ NMR spectra of COS in $D_2O$ (C) and HB-COS in $CH_3COOD$ (D).

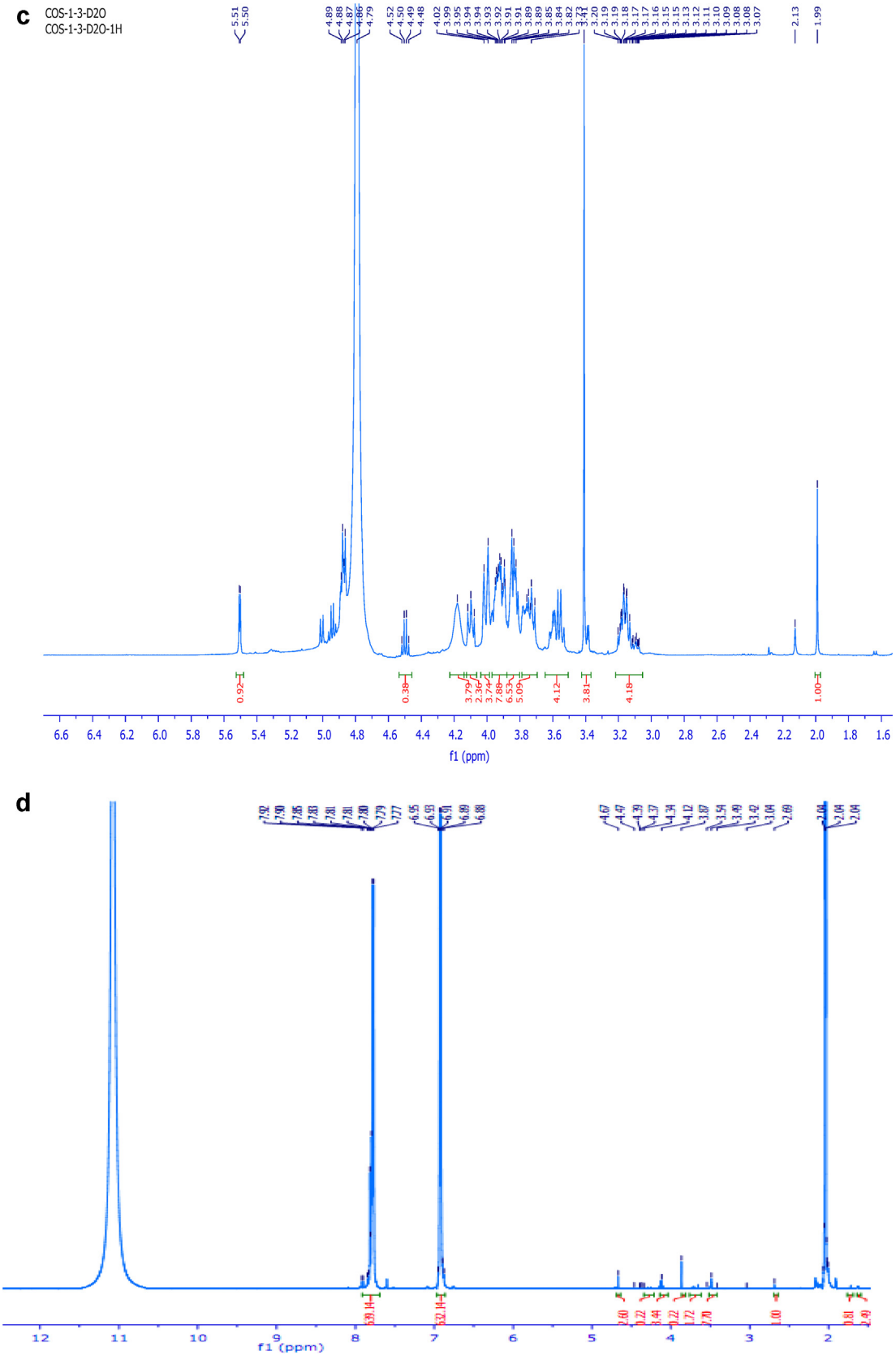


**Fig. 1.** (*Continued.*)

After dialysis, the precipitate was washed with diethyl ether several times to remove the un-reacted aldehyde. The product was dried at room temperature to give HB-COS (0.39 g) (Jagadish et al., 2012; Muzzarelli & Ilari, 1994; Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982; Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994).

The spectra of sample in the forms of KBr disk were obtained using a Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum GX, Beaconsfield Bucks, England) with a frequency range of 4000–400 $cm^{-1}$. Proton nuclear magnetic resonance ($^1H$ NMR) spectra were recorded on a JEOL JNM-ECP-400 spectrometer (JEOL, Tokyo, Japan).

### 2.3. Cytotoxicity and DNA oxidation determination in Chang liver cells

Chang liver cells were grown in DMEM medium containing 10% (v/v) FBS, 100 μg/ml penicillin–streptomycin and 5% $CO_2$ at 37 °C. Cytotoxicity levels of the samples on cells were measured using the MTT method as described by Ngo, Ngo, et al. (2012).

Genomic DNA was extracted from Chang liver cells using AccuPrep® genomic DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer's protocol. Hydrogen peroxide mediated DNA oxidation was determined according to Ngo, Kim, et al. (2012).

### 2.4. Determination of intracellular formation of ROS using fluorescence-activated cell sorting (FACS) and light microscope analyses

Intracellular oxidative stress was assayed by measuring the intracellular oxidation of 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) (Rosenkranz et al., 1992). Chang liver cells were treated with the samples for 24 h and then incubated with 20 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 1 h at 37 °C. Subsequently, the cells were washed, harvested, and re-suspended into phosphate buffered saline solution containing $H_2O_2$ (500 μM) for 1 h. Finally, the cells were analyzed by flow cytometry (BD Diagnostic Systems, Cockeysville, MD, USA). The numbers in histograms indicate the percentage of 2′,7′-dichlorofluorescein (DCF) formation due to oxidation of $DCFH_2$ in the presence of several ROS. 10,000 events/sample were acquired using XL-MCLTM flow cytometer equipped with EXPOTM 32 software (Beckman Coulter, Inc., CA, USA).

The phase contrast images of stimulated Chang liver cells were collected using Leica inverted microscopy (DM IRB) with a digital CCD camera (CTR 6000, Leica, Wetzlar, Germany) under fluorescence. Intracellular ROS was determined by DCFH-DA fluorescent staining (Ngo, Ryu, & Kim, 2014). Chang liver cells were treated with the samples for 24 h and incubated with 20 μM DCFH-DA for 1 h in the dark. Subsequently, the cells were washed and stimulated with $H_2O_2$ (500 μM) for 1 h. Fluorescence images of the specimens were captured with a Leica system.

### 2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed to detect the mRNA expression levels of antioxidant enzymes. Total cellular RNA from treated Chang liver cells were lysed with Trizol® reagent (Invitrogen Corporation, Faraday Avenue, USA) according to the manufacturer's protocol. Briefly, total RNA (2 μg) was converted to cDNA using a Reverse transcription System (Promega, Madison, WI, USA). Primer sequences used to amplify the desired cDNA were as follows: SOD-1 forward and reverse primers: 5′-AGG-GCA-TCA-TCA-ATT-TCG-AG-3′ and 5′-TGC-CTC-TCT-TCA-TCC-TTT-GG-3′; GSH forward and reverse primers: 5′-AGC-ATT-TGG-CAA-AGG-AGA-AA-3′ and 5′-ATC-CGT-GCT-CCG-ACA-AAT-AG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward and reverse primers: 5′-GAG-TCA-ACG-GAT-TTG-GTC-GT-3′ and 5′-GAC-AAG-CTT-CCC-GTT-CTC-AG-3′.

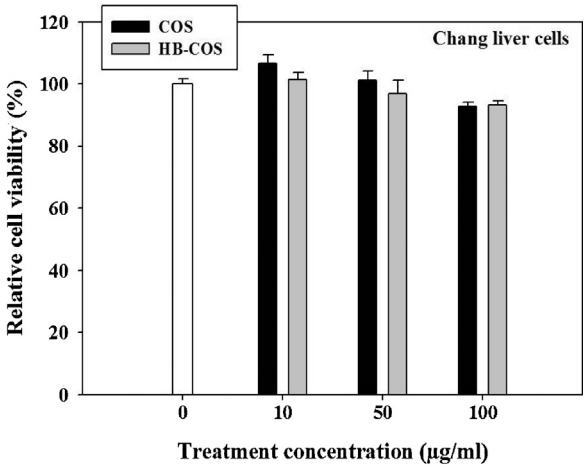


**Fig. 2.** Cell viability was determined by the MTT assay. Cytotoxic effects of COS and HB-COS on Chang liver cells were compared with COS or HB-COS non-treated group. Values are expressed as the means ± standard deviation (SD) of three replicate experiments.

### 2.6. Western blot analysis and extraction of nuclear and cytoplasm proteins

Standard procedures were used for the Western blotting, in brief; Chang liver cells were lysed in RIPA lysis buffer (NP-40, Sigma–Aldrich, USA). Remaining cell debris was removed by centrifugation. Protein concentrations in supernatants were determined with the Bio-Rad protein assay (Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Proteins (20 μg) were diluted in 5× sample buffer [10% sodium dodecyl sulfate (SDS) and 100 mM each dithiothreitol (DTT), glycerol, bromophenol blue and Tris–HCl], separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% (w/v) BSA in TBST (25 mM Tris–HCl, 137 mM NaCl and 0.1% Tween 20, pH 7.4) for at least 1 h at 4 °C. Membranes were incubated with primary antibodies (1:1000) for 1 h at 4 °C. After washing with TBST, the blots were incubated with secondary antibodies (1:5000) for 1 h at 4 °C in the dark. They were then washed again three times with TBST, and developed with an enhanced electrochemiluminescence (ECL) assay kit (Amersham Pharmacia Biosciences, UK) according to the manufacturer's instructions. Western blot bands were visualized using LAS3000® Luminescent image analyzer and protein expression levels were quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan). Detection of β-actin was used as a control for the equal loading of protein.

In a parallel experiment, for separate extraction of nuclear and cytoplasm proteins, CelLytic™ NuCLEAR™ Extraction kit (S26-36-23, Sigma–Aldrich Co., St. Louis, MO, USA) was used following manufacturer's instructions.

### 2.7. Statistical analysis

Data were expressed as means ± standard error of the mean ($n = 3$). Student's $t$-test was used to determine the level of significance at $P < 0.05$.

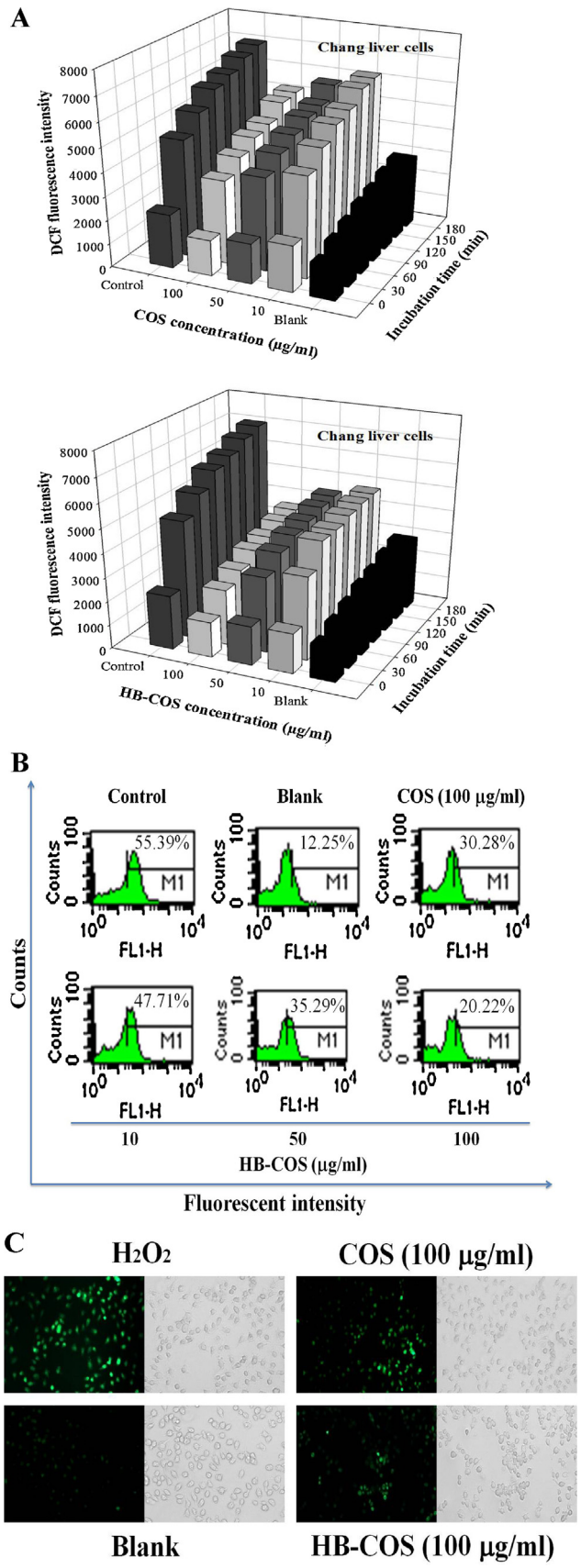


**Fig. 3.** Effect of HB-COS on intracellular ROS level of Chang liver cells (A). The cells were pretreated with HB-COS and then exposed to $H_2O_2$ to produce ROS. Intracellular radical scavenging activity of HB-COS was compared with $H_2O_2$ alone-treated group (control) and $H_2O_2$ non-treated group (blank). The ROS level is represented as DCF fluorescence. (B) Cellular free radical scavenging activity of HB-COS by FACS analysis. Values are expressed as the means ± SD of three replicate experiments. (C) Representative DCFH-DA fluorescent images of Chang liver cells. Fluorescent images showing stronger cellular DCFH-DA staining (green fluorescence; an indicator of production of ROS). Fluorescence images were analyzed with Leica system.

## 3. Results and discussion

### 3.1. Structural characterization of 4-hydroxybenzyl-chitooligomers

The structures of COS and HB-COS were confirmed via FT-IR (Fig. 1A and B) and $^1$H NMR (Fig. 1C and D) data as follows. According to the FT-IR spectrum (Fig. 1A), the peaks of COS were assigned to the glucosamine and N-acetyl glucosamine structures at 3901 and 3053 $cm^{-1}$ (O—H and N—H bonds), 2983 $cm^{-1}$ (CH bond), 1652 $cm^{-1}$ (C=O bonds of N-acetyl groups) and 1593 $cm^{-1}$ (N—H bonds of primary amino groups). In the FT-IR spectrum (Fig. 1B), HB-COS appeared characteristic absorption peaks of bonds inside the benzene ring at 3195 $cm^{-1}$ (C—H stretch bonds), 1506 and 1462 $cm^{-1}$ (C—C bonds), 895, 844, 816, and 763 $cm^{-1}$ (C—H "oop" bonds). Regarding the structure characteristic of benzene ring of 4-hydroxybenzaldehyde, it can be concluded that HB-COS was successfully synthesized.

From $^1$H NMR spectrum (Fig. 1C), COS showed a peak at 2 ppm belonging to the acetyl proton (H-Ac) of methyl groups of acetylated monomer. Furthermore, COS showed the proton H-2 of deacetylated monomer (H-2 D) which linked to C-2 of the glucopyranose at the range 3.2–3.0 ppm. In addition, COS showed the H-3, H-4, H-5 and H-6 protons linked to carbon C-3, C-4, C-5 and C-6 of glucopyranose at 4.2–3.3 ppm, respectively. Moreover, COS showed the proton H-1 of deacetylated monomer (H-1 D) which linked to C-1 of deacetylated monomer at 5.5–5.2 ppm. According to the $^1$H NMR spectrum, HB-COS showed new peaks at 8–6.9 ppm belonging to the protons of aromatic ring of 4-hydroxybenzaldehyde as compared with COS (Fig. 1D). These results confirmed the successful link 4-hydroxybenzaldehyde and COS.

### 3.2. Effect of 4-hydroxybenzyl-chitooligomers on the viability of Chang liver cells

The cytotoxic effects of HB-COS and COS were tested on Chang liver cells via MTT assay. As shown in Fig. 2, HB-COS and COS did not show any significant toxicity toward cell line at the tested concentrations. Therefore, it was confirmed that HB-COS and COS are safe materials in terms of cellular toxicity, and thereby could be used as safe and effective antioxidants having no cytotoxicity and no effect on the proliferation of Chang liver cells. According to these results, the nontoxic concentrations of HB-COS and COS were used for further experiments.

### 3.3. Intracellular radical scavenging effects of 4-hydroxybenzyl-chitooligomers using flow cytometry and light microscope analyses

ROS include a broad range of undesirable radicals such as superoxide anion, hydrogen peroxide, •OH, peroxyl radicals, alkoxyl radicals and singlet oxygen. The unregulated production of ROS leads to damage of cellular macromolecules (Fubini & Hubbard, 2003). Therefore, to assess levels of intracellular ROS, Chang liver cells were labeled with fluorescence probe DCFH-DA, a specific probe for ROS. Substantially, DCFH-DA is hydrolyzed by esterase enzyme to form $DCFH_2$, which subsequently reacts with ROS to form a fluorescent DCF in cells. After cells were incubated with $H_2O_2$ (500 μM) for 30 min, the rapid increment in DCF fluorescence intensity indicated oxidation of $DCFH_2$ by intracellular radicals

(Fig. 3A). The monitoring of DCF fluorescence intensities for 3 h revealed that radical-mediated oxidation increased during incubation. However, the treatment with HB-COS or COS decreased the DCF fluoresce in time-dependent manner. At the concentration of 100 μg/ml, HB-COS and COS exhibited to be effective in the free radical scavenging even only after 30 min of incubation as compared to control group stimulated with $H_2O_2$ alone. Notably, HB-COS was observed to be stronger than COS in free radical scavenging activity.

In addition, ROS production was also determined using flow cytometry and light microscope analyses. The results obtained from flow cytometry analysis showed that the fluorescence intensity was high in $H_2O_2$-stimulated cells. However, the presence of HB-COS alleviated fluorescence intensity in a dose-dependent manner, indicating the scavenging effect of HB-COS on ROS formation (Fig. 3B). The scavenging activity of HB-COS was found to be stronger than that of COS at the same concentration treatment. Likewise, Fig. 3C showed that $H_2O_2$-stimulated cells exhibited high fluorescence intensity. Conversely, the ROS production was strongly scavenged by HB-COS treatments.

ROS and oxidative stress are important features of cardiovascular diseases including hypertension, atherosclerosis, and congestive heart failure. ROS generating in the blood vessel wall may contribute to endothelial dysfunction by oxidation of biomolecules (Sugamura & Keaney, 2011). According to these results, it was suggested that HB-COS scavenged intracellular ROS and thereby protected the cellular macromolecules from ROS-mediated damage.

### 3.4. Protective effects of 4-hydroxybenzyl-chitooligomers on cellular DNA oxidation in Chang liver cells

Highly reactive oxygen radical accumulation cause damage to surrounding molecules within the cell and thus cleaves the membrane, altering enzyme activities, and eventually leads to DNA damage. Cellular DNA is susceptible to oxidation by ROS and free radicals. They induce DNA damage by breaking the DNA strands and DNA–protein cross linking (Uttara, Singh, Zamboni, & Mahajan, 2009). In this study, HB-COS was analyzed for its protective effects against DNA oxidation using genomic DNA isolated from Chang liver cells. The isolated DNA was subjected to oxidation by •OH generated via the Fenton reaction. The effect of HB-COS on the •OH-induced DNA damage was observed after electrophoresing the DNA on agarose gel. The results demonstrated a clear inhibition of DNA oxidation with HB-COS treatment (Fig. 4).

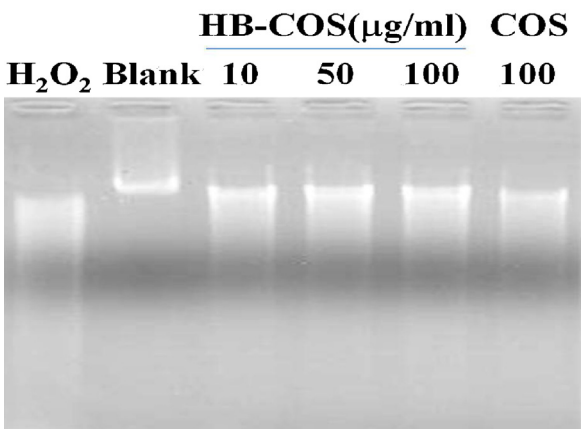


**Fig. 4.** DNA oxidative protection in Chang liver cells by hydroxyl radical generated from Fenton reaction in the presence of HB-COS. Genomic DNA from Chang liver cells was pretreated with HB-COS and exposed to hydroxyl radical. After 10 min, reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 40 min at 100 V and visualized by UV light after being stained with 1 mg/ml ethidium bromide. Line 1: DNA damage control ($FeSO_4$ and $H_2O_2$); Line 2: DNA alone (blank); Lines 3–6: $FeSO_4$ and $H_2O_2$ in the presence of HB-COS or COS.

### 3.5. Effect of 4-hydroxybenzyl-chitooligomers on the expression of antioxidative enzymes using RT-PCR and Western blot analyses

The involvement of HB-COS in regulating the expression of antioxidative enzymes were investigated by RT-PCR (Fig. 5A) and Western blot (Fig. 5B). SOD-1 and GSH expressions were examined after HB-COS treatment in $H_2O_2$-induced Chang liver cells.

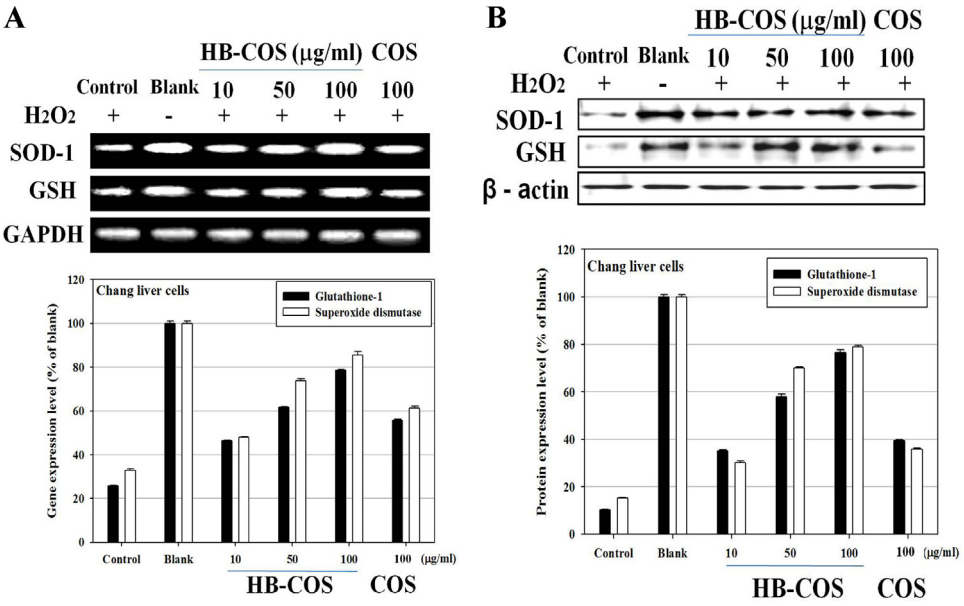


**Fig. 5.** Effect of HB-COS on $H_2O_2$-stimulated antioxidant enzymes in Chang liver cells at the different concentrations compared with $H_2O_2$ non-treated group (blank) and $H_2O_2$ alone-treated group (control). The expression levels of mRNA and protein were determined using RT-PCR (A) and Western blot (B) analysis, respectively. GAPDH and β-Actin expressions were used as internal controls.

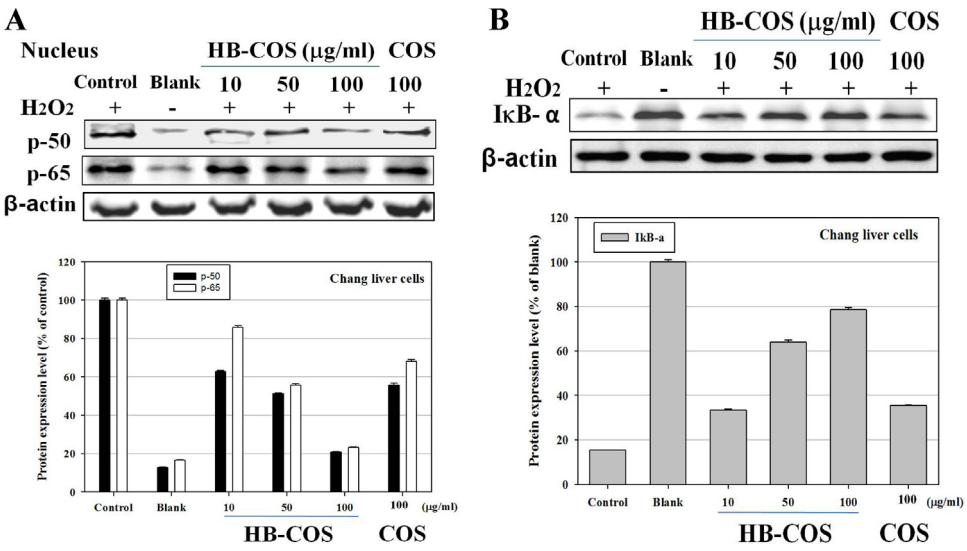


**Fig. 6.** Effects of HB-COS on NF-κB activation in $H_2O_2$-stimulated Chang liver cells. The cells were incubated for 24 h with the indicated doses of HB–COS prior to $H_2O_2$ treatment for 1 h. Cytosolic and nuclear protein extracts were prepared by commercial kit from Sigma (MO, USA). The p50 and p65 subunits of NF-κB in nuclear protein extract (A) and level of IκB-α in the cytosolic protein extract (B) were determined by a Western blot analysis. The β-actin was used as an internal control.

The cellular mechanism of ROS scavenging by HB-COS was understood by studying the enzymes related to ROS production. SOD is a major antioxidative enzyme which breaks down superoxide radicals into hydrogen peroxide and molecular oxygen. Therefore, it reduces the damaging reactions of superoxide and protects cells from the toxicity of superoxide. GSH plays a vital role in regulating intracellular redox status. The increase in GSH level protects all kinds of cells against death either by removing free radicals or by conjugating with toxicants (Blokhina, Virolainen, & Fagerstedt, 2003; Dickinson & Forman, 2002). Interestingly, HB-COS treatment upregulated the gene and protein expression levels of SOD-1, in order to maintain the antioxidation process. Similarly, the gene and protein expression levels of GSH were upregulated by HB-COS treatment. The treatment of HB-COS had a positive effect on the expression levels of antioxidative enzymes, suggesting that HB-COS reduced the oxidative damage through stimulating the expression of antioxidative enzymes.

### 3.6. 4-Hydroxybenzyl-chitooligomers blocked the nuclear factor kappa B (NF-κB) activation and translocation

Evidence is rapidly accumulating to indicate that oxidative stress/free radicals lead to the activation of NF-κB, which in turn induces the expression of genes. NF-κB is a master transcription factor that controls the expression of pro-inflammatory gene products in cells of many different lineages. In normal conditions, p50/p65 dimers of NF-κB (p50/p65) are bound to inhibitory κB (IκB) proteins, and consequently retained within the cytoplasm. Conversely, IκB proteins are rapidly degraded in the activated cells, and the freed NF-κB is translocated to the nucleus, wherein it binds to target sites and induces the transcriptions of genes (Hayden & Ghosh, 2004; Maulik & Das, 2002). Thus, Chang liver cells stimulated by $H_2O_2$ induced a remarkable degradation of IκB-α in the cytoplasm and a rapid increase of p50/p65 subunits in nucleus (Fig. 6**A**). However, the nuclear translocation of p50/p65 subunits was decreased in Chang liver cells exposed to HB-COS. Moreover, HB-COS also blocked $H_2O_2$-induced IκB-α degradation in a concentration-dependent manner (Fig. 6B). These results indicated that HB-COS inactivated NF-κB via blocking the degradation of IκB-α protein, and thus inhibiting the nuclear translocation of p50/p65 subunits.

## 4. Conclusion

Under above described experimental conditions, HB-COS exhibited protective effect against $H_2O_2$-induced cytotoxicity via inhibiting intracellular ROS generation in Chang liver cells. Consequently, HB-COS could inhibit and prevent oxidative damage to DNA. In addition, HB-COS increased intracellular antioxidant enzymes activities and suppress the NF-κB activation under $H_2O_2$-induced oxidative stress. Thus, the possible mechanisms that suppressed oxidative stress of HB-COS in the $H_2O_2$-induced Chang liver cells were suggested to be due to scavenging of ROS and enhancing antioxidant enzymes defense systems. These results indicated that HB-COS might provide beneficial effects in the treatment of disorders related to oxidative stress. Collectively, HB-COS is considered as a potent free radical scavenger to balance between oxidative and anti-oxidative process against damage biomolecules in cellular systems.

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