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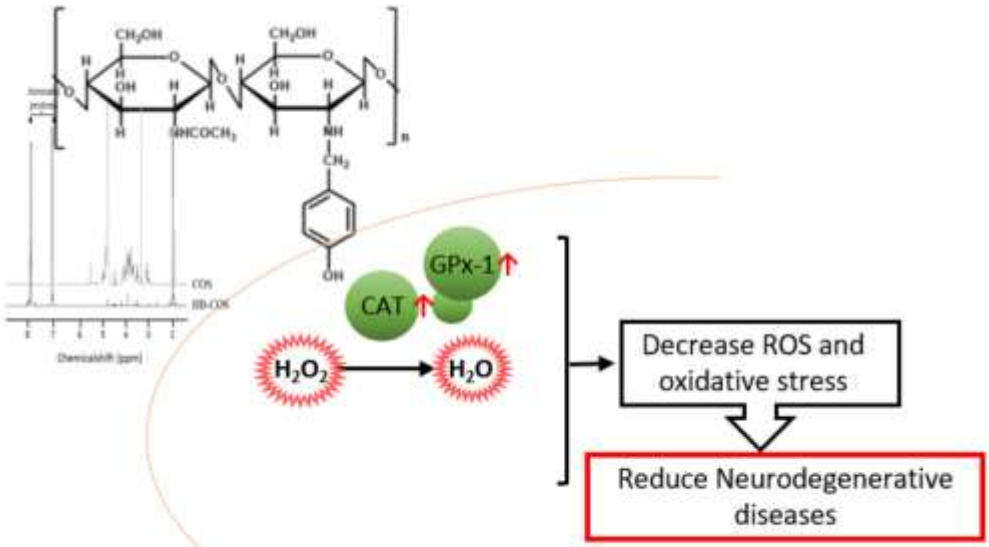
Graphical abstract



4-hydroxybenzaldehyde-chitooligomers suppresses H_2O_2-induced oxidative damage in microglia BV-2 cells

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Abstract

Positive charges of chitooligomer (COS) enable COS to interact with negatively charged anionic groups on the cell surface resulting in an improvement in the biological activity of COS and its derivatives. In this study, 4-hydroxybenzaldehyde-COS (HB-COS) was synthesized and investigated for its abilities against H_2O_2-induced oxidative stress in microglia BV-2 cells. Under oxidative stress, HB-COS significantly attenuated reactive oxygen species (ROS) generation and DNA oxidation, and upregulated the protein levels of antioxidative enzymes. HB-COS is therefore proposed as a potential protective agent against neuronal damage.

Keywords: 4-hydroxybenzaldehyde-COS (HB-COS); oxidative stress; microglia BV-2 cells

Introduction

Inflammation in the brain results from the activation of astrocytes and microglia, and is associated with the pathogenesis of numerous neurodegenerative diseases. Microglia which are the main inflammatory-reacting cells in the brain secrete a variety of proinflammatory and cytotoxic factors, and reactive oxygen species (ROS) [1, 2]. The free radicals generated during the inflammatory process can directly damage neurons involved in the pathogenesis of various neurodegenerative diseases [3].

Chitooligomers (COS) which are oligomers form of chitosan, have positive charges resulting from removal of the acetyl units from D-glucosamine residues, enabling it to interact with negatively charged polymers, macromolecules and polyanions [4, 5]. COS are easily soluble in water due to their shorter chain and free amino groups in D-glucosamin units and are easily absorbed through the intestine, and can quickly get into the blood flow and have a systemic biological effect in the organism [6]. COS and their derivate have attracted increasing attention as promising agents in many different formulations for drug and gene delivery [7, 8]. Furthermore, COS and their derivate have been also evaluated for their neuroprotective properties such as suppression of β-amyloid formation, acetylcholinesterase inhibitors (AChEIs), anti neuro-inflammatory activity and anti-oxidant activity [9-11].

4-hydroxybenzaldehyde (HB) is used as an introduced group to improve the biological properties of HB-COS, and is studied for its effect on antioxidant and positive modulation of GABA action in neurodegenerative diseases [12, 13]; however, the influence of HB-COS on the oxidative stress of microglia cells and the related mechanistic pathway still remain elusive. In this study, we examined the protective effect of HB-COS on H_2O_2-induced oxidative stress in microglia BV-2 cells and addressed the potential of HB-COS in prevention of H_2O_2-induced neuronal damage.

2. Results and discussion

2.1. Structural characterization of 4-hydroxybenzyl-chitooligomer

4-hydroxybenzyl-chitooligosaccaride (HB-COS) were synthesized with over 90% yield as white, fluffy, water-soluble powder. As the synthetic pathway is shown in Fig. 1, amino group in COS reacts with 4-hydroxybenzaldehyde under acidic condition followed by reduction of the Schiff base intermediate ($R_2C=NR$) with $NaBH_4$ by the Borch reduction [14]. By changing the structure of COS via addition of various reactive functional groups, COS derivate may possess differences in their structures and physico-chemical properties. COS was chemically modified to improve proton donation by introducing hydroxyl

group of 4-hydroxybenzaldehyde to the amino group at C-2 position of pyranose unit to produce HB-COS [15, 16].

The FT-IR spectra of HB-COS exhibited characteristic FT-IR pattern of COS, i.e., the absorption bands at wavenumbers 3901 and 3053 cm^{-1} due to OH and NH_2 groups, 2983 and 1652 cm^{-1} corresponded to the C-H bond and C=O bonds of N-acetyl groups and 1593 cm^{-1} due to N–H deformation of amino groups (Fig. 2a) [17, 18]. The FT-IR spectrum of HB-COS was similar to COS except for the increasing intensity of the absorption band at wavenumber 3195 cm^{-1} due to C-H stretch bonds of the pendant methylene groups. The additional absorption bands at wavenumbers 1506-1462 and 895-763 cm^{-1} were observed which were assigned to C=C stretching and C–H deformation (out of plane) of the aromatic group, respectively.

Fig. 2b shows the ^1H NMR spectra of COS and HB-COS. The spectra exhibited the characteristic ^1H NMR pattern of COS, i.e., the multiplets at δ 4.2–3.3 ppm and δ 3.2-3.0 ppm due to H2 and H3, H4, H5 and H6 of deacetylated monomer linked to carbon of the glucopyranose ring and a singlet at δ 2 ppm due to the H2 proton of acetyl proton (H-Ac) of methyl groups of acetylated monomer, respectively. The ^1H NMR spectra of HB-COS was similar to the COS except for the additional signals of the aromatic group of 4-hydroxybenzaldehyde which exhibited typical signals in the aromatic region, δ 8.0-6.8 ppm. These FT-IR and 1H NMR results implied that HB-COS have been successfully synthesized from COS.

2.2. HB-COS protected the DNA oxidation induced by H_2O_2 in BV-2 cells

To explore the cytotoxicity of HB-COS in BV-2 cells, cells were treated with HB-COS (10, 50 and 100 µg/mL) for 24 h and assessed by MTT assay. The L-ascorbic acid (10, 50 and 100 µg/mL) which is a well-known antioxidant compound was used as a positive control to compare the effect of HB-COS [19]. As shown in Fig. 3a, cells with 10 and 50 µg/mL of HB-COS and ascorbic acid did not cause any significant difference in cell viability compared to the negative control group, and highest concentration (100 µg/mL) of HB-COS and ascorbic acid showed more than 80 % cell viability with p value of < 0.01 which is considered as safe to be used in *in vitro* experiments [20, 21]. Therefore, non-toxic concentrations of HB-COS and ascorbic acid were used in further experiments.

Excess oxidative stress accumulation causes damage to the surrounding molecules within the cell and thus cleaves the membrane, altering enzyme activities, and eventually leading to DNA damage [9]. In this study, DNA oxidation was carried out by the combined effect of 300 uM Fe (II) and 500 uM H_2O_2 on genomic DNA isolated from BV-2 cells. As shown in Fig. 3b, DNA in the positive control group treated with Fe(II)–H_2O_2 was degraded, however the DNA with HB-COS exerted protective effect against the stress in a concentration dependent manner, and 100 µg/mL of HB-COS showed the inhibitory effect on DNA damage by up to 82 % ($p < 0.001$) which was close to the protective effect by 100 µg/mL of

ascorbic acid (87.5 %, $p < 0.01$) compared to the non-damaged blank group. This data suggested that HB-COS treatment inhibited the stress of BV-2 cells induced by H_2O_2.

2.3. HB-COS protected the oxidative stress induced by H_2O_2 in BV-2 cells

To confirm the protective effect of HB-COS on the stress of BV-2 cells induced by H_2O_2, intracellular oxidative stress was determined by measuring the intracellular oxidation of 2,7 - dichlorodihydrofluorescein ($DCFH_2$) levels [9, 22]. Exposure of $DCFH_2$-loaded BV-2 cells to H_2O_2, induced oxidative stress assessed by DCF formation indicating ROS production (Fig. 4a). Monitoring DCF fluorescence intensities for 3 h revealed that radical-mediated oxidation increased during incubation. As shown in Fig. 4a, similar to the effect of ascorbic acid (10-100 µg/mL), the treatment of cells with increasing concentrations of HB-COS, protected cells from H_2O_2-induced oxidative stress in a concentration- and time- dependent manner. The effect of HB-COS was also assessed using flow cytometer in where 100 µg/mL of HB-COS showed significant inhibition of ROS formation (58.13 %) compared to 86.20 % of H_2O_2 damaged Control or 50.20 % inhibition of 100 µg/mL of ascorbic acid (Fig. 4b).

2.4. HB-COS induced the expression of antioxidant enzymes

The imbalance in the redox state of a cell or tissue implies a change in ROS generation or metabolism [22]. Excess accumulated ROS from over-activated microglia causes damage to surrounding molecules within the cell, and may initiate neurodegenerative onset via several signaling pathways [21, 23]. Recently, interest has increased in the role of NF-κB and Nrf2 in redox balance maintenance to regulate a pivotal defense mechanism [24, 25]. The crosstalk pathways between NF-κB and Nrf2 has been found in a rat model of brain ischemic injury with increased superoxide dismutase (SOD) and heme oxygenase-1 (HO-1), where Nrf2 activity has been related with NF-κB down-regulation [23]. Hence, to investigate the NF-κB and Nrf2 expression in H_2O_2-exposed BV-2 cells and treated with HB-COS, we examined the changes of nuclear translocation of NF-κB and Nrf2 followed by HB-COS treatment using western blot analysis (Fig. 5a). We found that treatment with HB-COS, decreased the levels of p50 and p-IκB-α which are part of the Nf-κB transcription factors in the nucleus and cytoplasm while increased the DNA-binding activity of Nrf2. These results indicate that HB-COS suppresses NF-κB signaling and activates the Nrf2 pathway.

Additionally, the expression of several enzymes including SOD, catalase (CAT), HO-1, glutathione peroxidase (GPx), glutathione reductase (GRed) and Glutathione (GSH) were assessed. These enzymes play crucial roles in the removal of excess oxygen radicals by catalyzing the dismutation of $O_2^{\cdot -}$ to produce H_2O_2 or reduction of H_2O_2 to H_2O, and are responsible for the synthesis of the main cellular antioxidant [2, 24, 26]. To determine the antioxidant potency of HB-COS in BV-2 cells, the expression of SOD-1, CAT, HO-1, GPx, GRed and GSH were assessed at protein levels. As shown in Fig. 5b, the protein expression of SOD-1, CAT, HO-1 and GSH were induced to above 90 % by 100 μg/mL of HB-COS treatment compared with the Control group, which is similar with the group of 100 μg/mL of ascorbic acid treatment, that is the positive control. The data suggests that HB-COS upregulates the levels of enzymatic antioxidants, that breaks H_2O_2 into H_2O and oxygen. HB-COS could reduce the process of the redox reaction excessively and scavenge oxidants such as H_2O_2, preventing cells from oxidative damage, resulting in a protective effect against the oxidative damage of H_2O_2 in BV-2 cells.

3. Conclusion

In this study, we suggested the protective effects of HB-COS from H_2O_2-induced oxidative stress in BV-2 cells via controlling molecules associated with antioxidant mechanistic pathway. Our study helps to expand understanding of the protective activity of HB-COS on H_2O_2-induced oxidative stress in BV-2 cells, providing potential of HB-COS in prevention of neurodegenerative diseases via suppression of oxidative stress.

4. Experimental

4.1. Chemicals and reagents

Chitooligosaccharides with an average molecular weight of 3-5.0 kDa was obtained from Kitto Life Co. (Seoul, Korea). 4-hydroxybenzaldehyde (98 %), hydrogen peroxide (30 %, w/w) solution, L-Ascorbic acid, dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2yl)-2,5-diphenyltetrazolium bromide (MTT) and 2´,7´-dichlorofluorescien diacetate (DCFH-DA) were purchased from Sigma- Aldrich (Saint Louis, MO, USA).

Murine microglial (BV-2) cells were kindly provided by Prof. Il-Whan Choi (Inje University, Korea). Cell culture media (DMEM), Trypsin-EDTA, penicillin/streptomycin were purchased from Lonza Co. (Walkersville, MD, USA). Nuclear/cytosol fractional kit for Western blot was purchased from BioVision Inc. (Milpitas, CA, USA). Primary and secondary antibodies used for Western blot analysis were

purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals and reagents used in this study were of analytical grade.

4.2. Preparation and identification of 4-hydroxybenzyl-chitooligomer

The 4-hydroxybenzyl-chitooligomer (HB-COS) were obtained as described by Trinh et al (2014) [8]. Briefly, chitooligosaccharides (1g) dispersed in 100 ml of 69 % ethanol (v/v), 1 % acetic acid (v/v) solution (pH 5) and was stirred at room temperature for 12 h with 163.8 mM of 4-hydroxybenzaldehyde. The reaction was added with sodium borohydride ($NaBH_4$, 26.4 mM) when schiff base formed and stirred for another 12-14 h. subsequently, the reaction mixture was quenched with 15% (w/v) sodium hydroxide and centrifuged at 6000 rpm for 30 min. The precipitate was dialyzed against distilled water using dialysis membranes, washed by diethyl ether several times for removing remained aldehyde and dried at room temperature. The product was characterized as HB-COS in the Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum GX, Beaconsfield Bucks, England) with a frequency range of 4000-400 cm^{-1} and proton (^1H NMR) nuclear magnetic resonance with a JEOL JNM-ECP-400 spectrometer (JEOL, Tokyo, Japan).

4.3. Cell culture and cell viability assessment

BV-2 cells were cultured in DMEM supplemented with heat-inactivated 5% fetal bovine serum and 100 μg mL^{-1} penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37 ℃. Cells were sub-cultured at 3 day intervals (80% confluence) using trypsin-EDTA. Cell viability was determined by the ability of mitochondria to convert MTT to insoluble formazan product. Briefly, cells were maintained in 96-well plates at a density of 5×10^3 cells/well for 24 h and subjected to different concentration of HB-COS (10, 50, 100 μg/ml) and ascorbic acid (10, 50, 100 μg/ml) for another 24 h. Subsequently, 100 μL of 1 mg mL^{-1} MTT reagent was added to each well, and incubated for another 4 h. The cell density was determined by measuring optical density (OD) at 540 nm using a microplate reader (Tecan Austria GmbH, Salzburg, Austria). Viable cells were quantified as a percentage and compared with the negative control (no addition of H_2O_2, HB-COS and ascorbic acid).

4.4. Genomic DNA oxidation assay

Genomic DNA was extracted by a method described previously with slight modifications [27]. Cells were suspended into RNase (0.5 mg/ml), sodium acetate (0.2 M), proteinase K (10 mg/ml) and SDS (10%) and were incubated for 30 min at 37 ℃ and for 1 h at 55 ℃. The genomic DNA was subsequently purified by phenol:chloroform:isoamylalcohol (25:24:1) and 100 % ice cold ethanol.

The purified DNA was oxidized with 300 μM of $FeSO_4$, 500 μM of H_2O_2 and different concentration of HB-COS (10, 50, 100 μg/ml) and 100 μg/mL of ascorbic acid in 50 mM phosphate buffer (pH 7.4) at 25 ℃ for 10 min. The mixture was electrophoretically analyzed on a 1% agarose gel and stained with 1 mg/ml ethidium bromide before visualization. The intensity of DNA bands was calculated as a percentage and compared with the non-damaged blank group using AlphaEase® gel image analysis software (Alpha Innotech, CA, USA).

4.5. Determination of intracellular levels of ROS

Intracellular levels of ROS was assessed as described previously, by employing oxidation sensitive dye DCFH-DA as the substrate [7]. Cells were loaded with 20 μM DCFH-DA in Hanks balanced salt solution (HBSS) and incubated for 30 min in the dark. After DCFH-DA staining, cells were washed with PBS three times and treated with different concentrations (100, 50, 10 μg/mL) of HB-COSs and ascorbic acid for another 1h. The formation of fluorescent DCF due to oxidation of DCFH in the presence of several ROS was read after every 30 min at the excitation wavelength of 485 nm and the emission wavelength of 528 nm using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Grodig/Salzburg, Austria). The fluorescence intensity and the phase contrast images of stimulated cells were captured with a Leica inverted microscopy (DM IRB) with a digital CCD camera (CTR 6000, Leica, Wetzlar, Germany) under fluorescence.

In a parallel experiment, the stained cells were harvested and re-suspended into 500 μM H_2O_2 for 45 min to stimulate the ROS generation, and were analyzed by flow cytometry (BD Diagnostic Systems, Cockeysville, MD, USA). The numbers in histograms indicate the percentage of DCF formation due to oxidation of DCFH in the presence of ROS. 10,000 events/sample were acquired using XL-MCL™ flow cytometer equipped with EXPO™ 32 software (Beckman Coulter, Inc., CA, USA).

4.6. Western blot analysis

For separate extraction of nuclear and cytoplasm proteins, nuclear/cytosol fractional kit (BioVision Inc., Milpitas, CA, USA) was used following manufacturer's instructions, in brief, Equal amounts (20 μg) of protein determined by BCA protein assay (Thermo Fisher Scientific, Grand Island, NY, USA) were separated on 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and then blocked in TBS-T buffer (20 mM Tris, pH 7.6, 0.1% Tween 20) containing 5% (w/v) bovine serum albumin. After incubation with the appropriate primary antibody, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase which was used to detect the respective proteins using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences), according to the manufacturer's instructions. Band densities were quantified by a LAS3000®

Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan) and the relative amounts of proteins associated with each specific antibody were normalized to the respective Lamin B and β-actin bands.

4.7. Statistical Analysis

Data were expressed as mean ± SD (n = 3) and analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS v9.1, SAS Institute Inc., Cary, NC, USA). Significant differences between treatment means were determined using Duncan's multiple range tests at the $P < 0.05$ levels.

Acknowledgments

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Figure legend

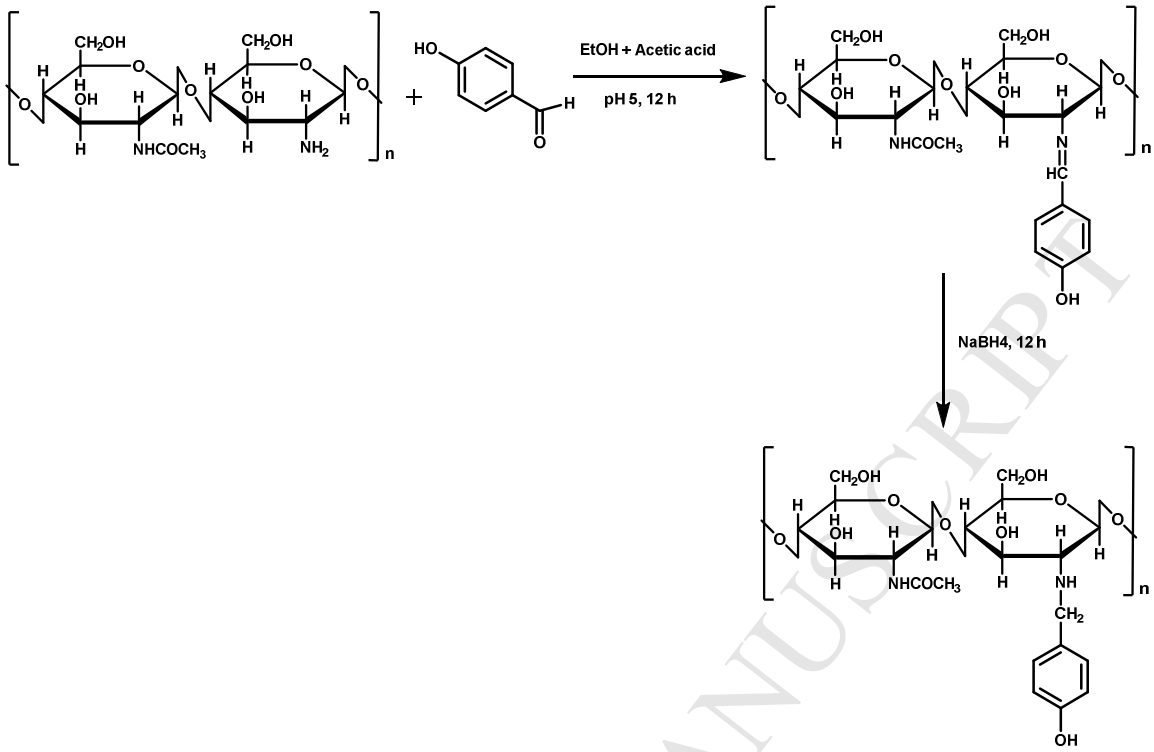
Fig. 1. Synthetic pathway of HB-COS

Fig. 2. FT-IR spectra (a) and ^{1}H NMR spectra (b) of COS and HB-COS.

Fig. 3. The effects on cell viability (a) and DNA oxidation (b) by HB-COS and ascorbic acid in BV-2 cells. Cell viability was measured by the MTT assay after treatment of cell with HB-COS and ascorbic acid for 24 hr. DNA protection of HB-COS on oxidative stress in BV-2 cells by hydroxyl radical generated Fenton reaction. Lane 1: DNA alone (Blank); Lane 2: damaged DNA (500 μM H_2O_2 and 300 μM $FeSO_4$; Control); Lane 3: damaged DNA with 100 μg/ml of ascorbic acid (500 μM H_2O_2 and 300 μM $FeSO_4$; Positive Control); Lane 4-6: damaged DNA with 10, 50 and 100 μg/mL of HB-COS (500 μM H_2O_2 and 300 μM $FeSO_4$). Values are expressed as the means ± SD of three independent experiments. $^{a}p < 0.1$, $^{b}p < 0.01$, $^{c}p < 0.001$ and $^{d}p < 0.0001$ compared with the Blank.

Fig. 4. Effects of HB-COS on intracellular ROS level in BV-2 cells treated with HB-COS and ascorbic acid. Cells were treated with HB-COS (10, 50 and 100 μg/mL) followed by labelling with DCFH- DA for measuring ROS production in H_2O_2 damaged BV-2 cells. Fluorescence microscopic images of activated BV-2 cells loaded with DCFH-DA (a) and flow cytometry analysis of DCF fluorescence in BV-2 cells (b). M1 refers to fluorescence histogram regions corresponding to fluorescence due to DCF. (Negative blank: cells only; Blank: cells + DCFH-DA; Control: cells + DCFH-DA + H_2O_2 (500 μM); ascorbic acid (100 μg/mL): cells + DCFH-DA + ascorbic acid+ H_2O_2; HB-COS: cells + DCFH-DA + HB-COS + H_2O_2).

Fig. 5. Effect of HB-COS and ascorbic acid (100 μg/mL) on levels of p50 (nucleus), p-IκB-α (cytosol) and Nrf2 (nucleus) (a) and levels of antioxidative enzymes (b). Cells were treated with HB-COS (10, 50 and 100 μg/mL) for 24 h and were stimulated by 500 μM H_2O_2 for 1 h. $^{a}p < 0.1$, $^{b}p < 0.01$, $^{c}p < 0.001$ and $^{d}p < 0.0001$ compared with the Control. Lamin B and β-actin were used as an internal control.



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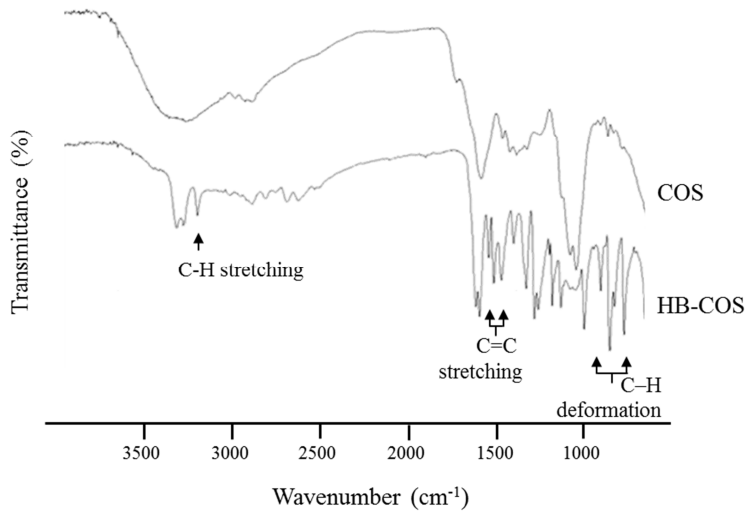

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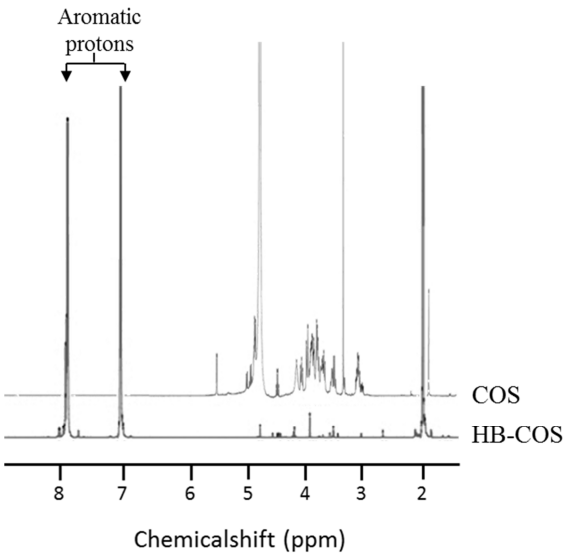

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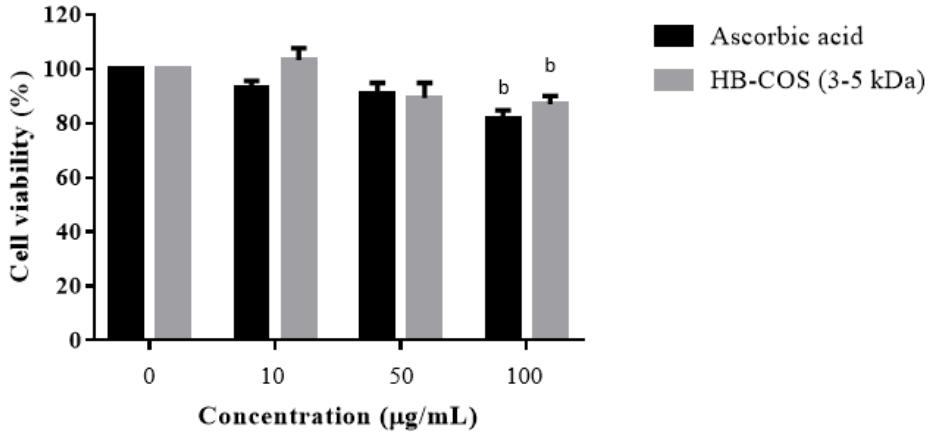

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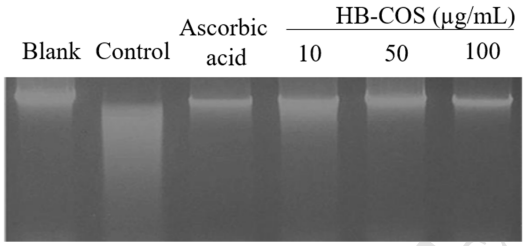
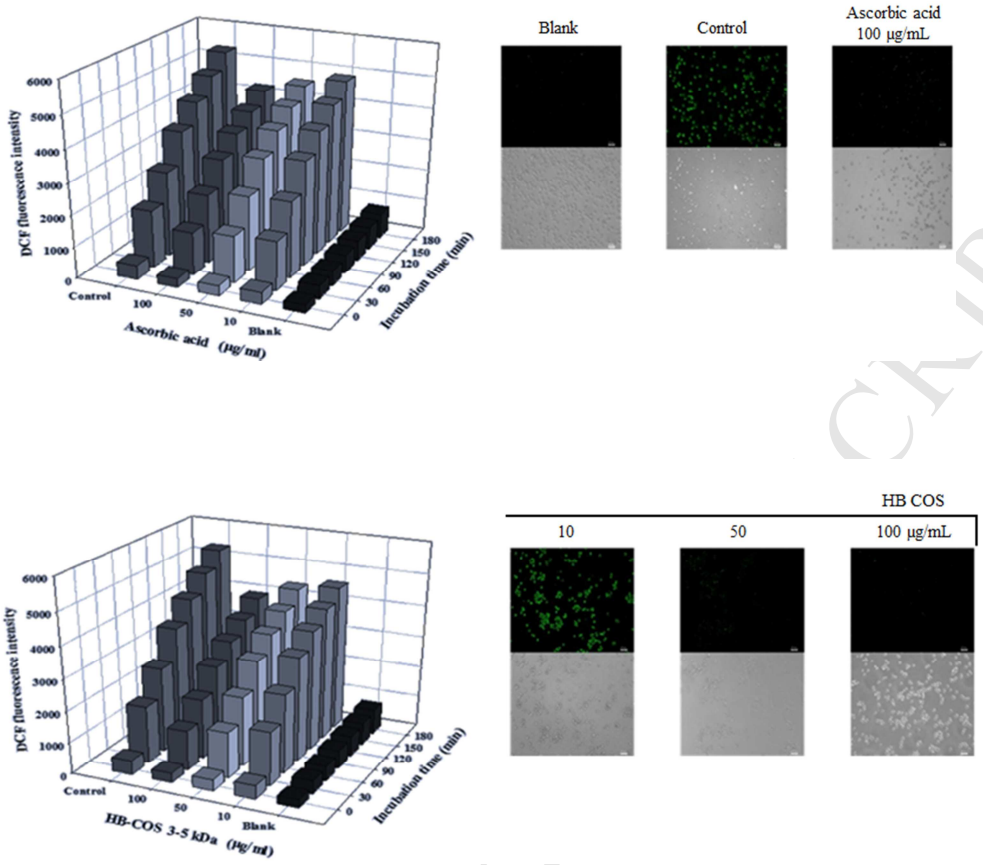


Figure 3. Oh and Ryu et al (2016)

(a)



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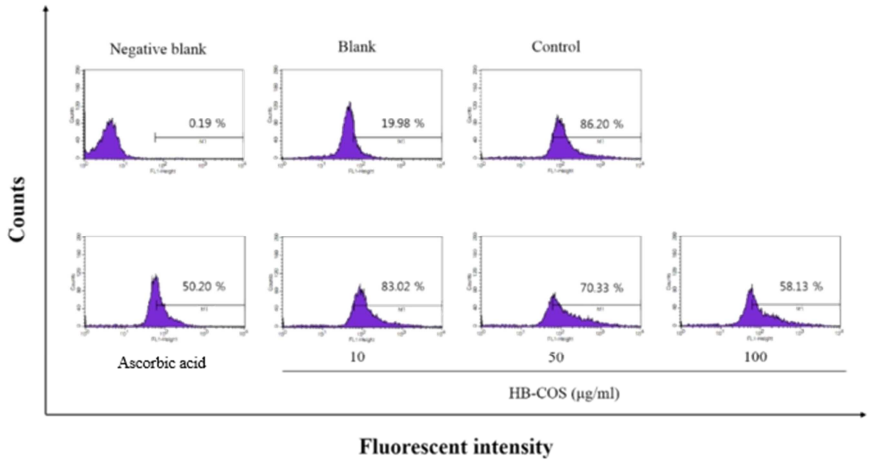
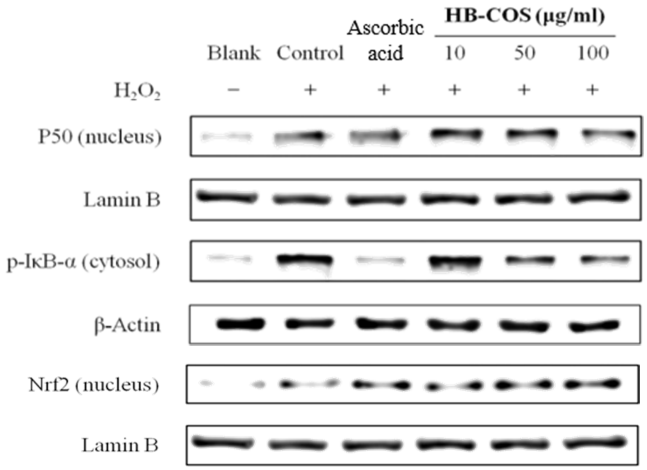
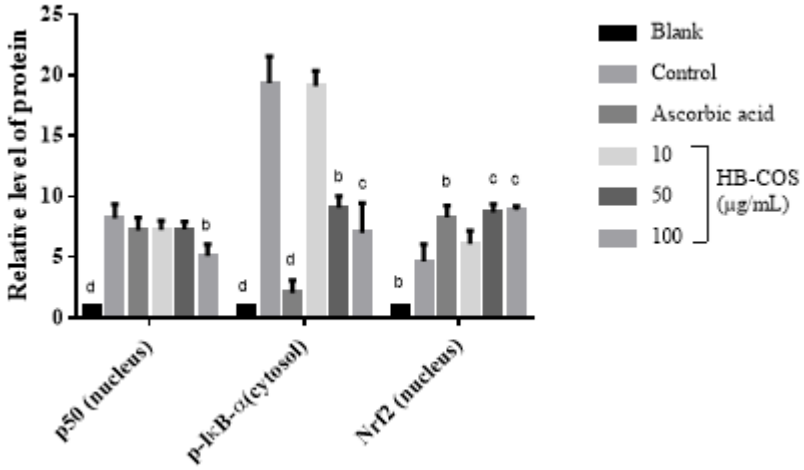


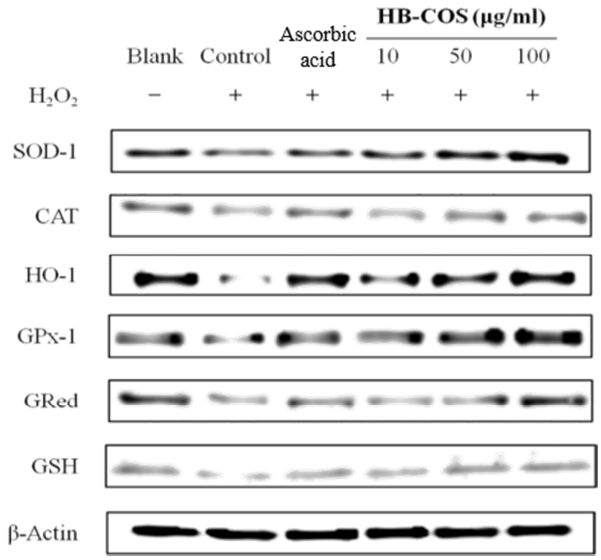
Fig. 4. Oh and Ryu et al (2016)

(a)





(b)



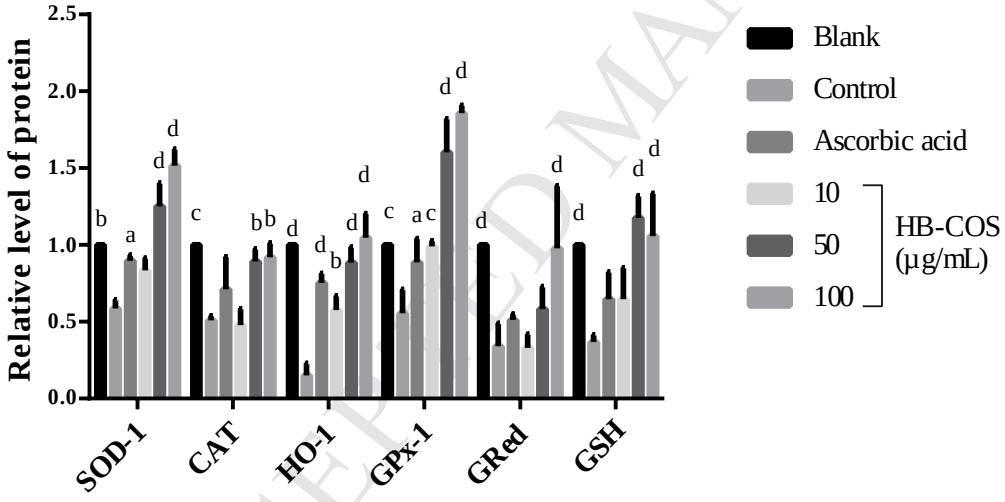


Fig. 5. Oh and Ryu et al (2016)

Highlights

- 4-hydroxybenzaldehyde-COS (HB-COS) was synthesized and characterized.
- Abilities of HB-COS in H_2O_2-induced oxidative stress was compared with ascorbic acid.
- HB-COS has potency as a protective agent *against* neuronal damage.