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Inhibition of free radical-mediated oxidation of cellular biomolecules by carboxylated chitooligosaccharides

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Abstract—The objective of this study was to identify the cellular antioxidant effects of carboxylated chitooligosaccharides (CCOS), a chemically modified derivative of chitooligosaccharides (COS), by assessing oxidation inhibition potential on cellular biomolecules such as lipids, proteins, and direct scavenging of reactive oxygen species (ROS). Radical-mediated oxidation of cell membrane lipids and proteins was dose-dependently inhibited by CCOS, assessed by amount of lipid hydroperoxides and carbonyl carbon content in mouse macrophages, RAW264.7 cells. Further, CCOS inhibited myeloperoxidase (MPO) activity in human myeloid cells (HL60) suggesting indirect possibility of inhibiting generation of reactive oxygen species (ROS) such as superoxide radicals, H_2O_2 and HOCl. Direct radical scavenging studies carried out with DCFH-DA fluorescence probe concluded that CCOS can act as a potent radical scavenger in cells.

1. Introduction

Oxidative stress has been identified as a common endpoint of several chronic diseases such as diabetes, arthritis, neurodegenerative disease, and cardiovascular complications.[1] These disease conditions have direct or indirect relationships with oxidation of cellular biomolecules by reactive oxygen species (ROS) generated excessively in tissues. Specially, leukocytes have been identified to produce large amount of ROS during infections as a part of direct immune response.[2] Therefore, biomolecules in leukocytes have a great risk to get oxidized by ROS during inflammatory events. Natural antioxidant system in human body has an important role in maintaining the balance between oxidants and antioxidants, thereby protecting oxidation of cellular biomolecules. However, during chronic disease conditions bodily antioxidant system cannot function properly and supplementary antioxidants are important mediators.

In the present world, there is an increasing concern about the potential utilization of chitooligosaccharides (COS) and their derivatives in pharmaceutical industry due to their number of reported biological activities such as antifungal activity, antibacterial activity, immunoenhancing effects, and protective effects against infections.[3] Among those biological activities, antioxidant properties of COS have attracted much attention due to their proton donation ability. Previously, we have reported the antioxidant effects of COS in in vitro systems by evaluating radical scavenging potencies using ESR spectroscopy.[4] Moreover, chemically modified COS derivatives have also been studied for antioxidant effects in in vitro systems.[5] However, up-to-date there are no reports on antioxidant effects of COS or their derivatives studied in cellular systems. Therefore, this study was conducted as a continuation of our previous study[5] to identify the antioxidant effects of carboxyl functional group-bonded COS (carboxylated chitooligosaccharides, CCOS) in cell systems. According to our knowledge, this is the first study that discusses effects of a COS derivative on oxidation inhibition of biomolecules in oxidation-induced cultured cell systems.

2. Results and discussion

2.1. Synthesis of carboxylated chitooligosaccharides (CCOS)

Structural features of COS were chemically modified to improve proton donation from free amino group at C-2

Keywords: Carboxylated chitooligosaccharides (CCOS); Reactive oxygen species (ROS); Lipid peroxidation; Radical scavenging; Antioxidant.
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position by introducing a $–COCH_2CH_2COOH$ functional group (Fig. 1A). Synthesis of CCOS was confirmed by NMR (^1H NMR and ^{13}C NMR), elemental analysis (C, N, and H), and FT-IR data as follows. FT-IR (KBr, ν, cm^{-1}): 3408 (s, O–H), 2931 and 2860 (w, C–H), 1723 (w, ester C=O), 1653 (m, amide C=O), 1565 (m, carboxyl C=O), 1408 (m, carboxyl C=O), 1112, 1068, 1030 (s, pyranose ring)[6,7] (Fig. 1B); ^{13}C NMR (400 MHz, D_2O, δ, ppm): 22($N–CH_3$), 32 (CH_2CH_2 close to carboxyl group), 56 (C-2), 61 (C-6), 69, 73 and 76, 78 (C-3, 4, 5), 102 (C-1), 174 (amide C=O), 176 and 180 (C=O); ^1H NMR (400 MHz, D_2O, δ, ppm): 1.9 (CH_3), 2.6, 3.3–3.6, and 4.5(C-1 ~ 6), 2.4($–CH_2CH_2–$).[8] Elemental analysis: C% (39.77), N% (4.610), and H% (4.924). Degree of substitution of carboxyl group per pyranose unit: 0.8989.

2.2. Effects of CCSO and H_2O_2 on leukocyte viability

To study the oxidation inhibitory effects of CCOS, human and mouse leukocyte cell lines (HL60 and RAW264.7) were employed. These cells are commonly used to study ROS-mediated cellular events since they can produce high amount of ROS following stimulation.[9] Prior to the oxidation inhibition experiments, toxicity of CCOS was determined by MTT cell viability assay and any significant ($P < 0.01$) toxic effect was not observed on both cell types under the tested concentrations (Fig. 2). Therefore, based on the above results non-toxic concentrations of CCOS were used for all experiments. In addition to test materials, toxicity of H_2O_2 was also assessed by MTT assay to determine non-toxic concentrations. In many assay systems, maximum of 2 mM final concentration of H_2O_2 was used to generate hydroxyl radicals which is non-toxic to the cells until 6 h of treatment.

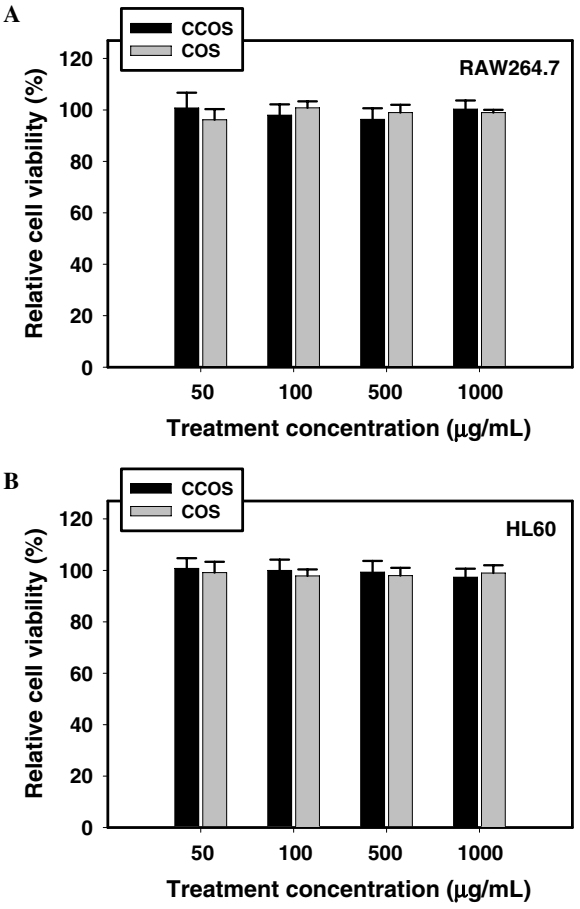

Figure 2. Cytocompatible effects of CCOS and COS on RAW264.7 cells (A) and HL60 cells (B). Different concentrations of CCOS and COS were applied to the cells for 24 h and cell viability was assessed by MTT assay as described in the text. Results are means ± standard error of three independent experiments.

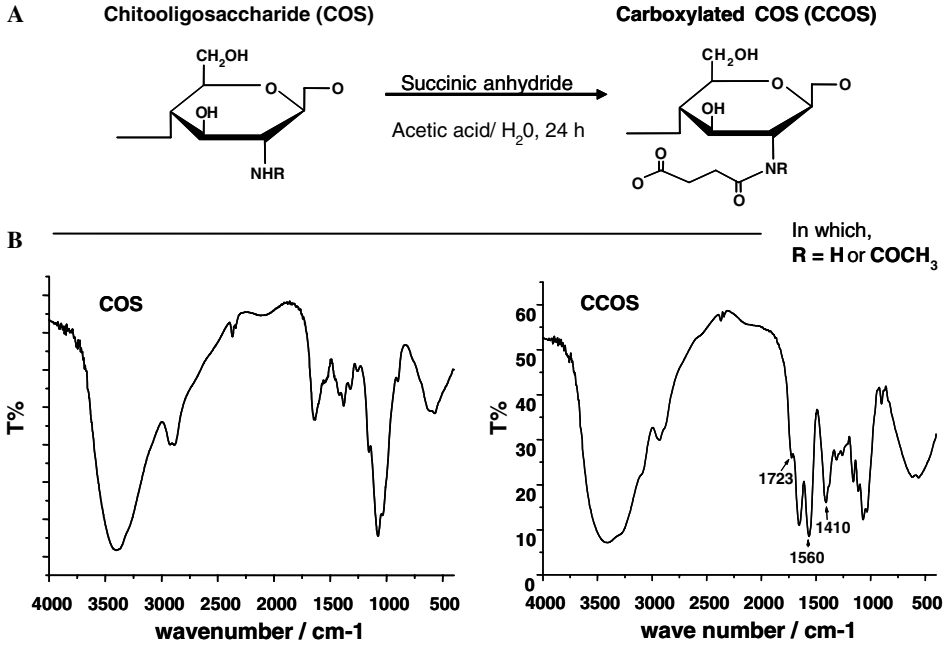

Figure 1. Schematic representation for the synthesis of CCOS from COS (A) and their FT-IR spectra (B). Successful substitution of $–COCH_2CH_2COO^-$ group at the amino position of pyranose unit was confirmed by increased absorption intensities of symmetric and asymmetric stretch absorptions of carboxyl groups (1560 and 1410 cm^{-1}).

2.3. Cell membrane lipid peroxidation inhibition

Unsaturated fatty acids in cell membrane lipids are highly susceptible for the free radical attack during oxidation. To study the effects of CCOS and COS on inhibition of cell membrane lipid peroxidation, two different methods were employed. In the first study, a sensitive fluorescence probe, diphenyl-1-pyrenylphosphine (DPPP), was used to determine the lipid hydroperoxide level of mouse macrophages (RAW264.7 cells) exposed to strong alkyl radical generator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). DPPP itself is not fluorescent, but DPPP oxide, resulting products of the reaction with hydroperoxides, is fluorescent with high fluorescence yield. This is possible because, DPPP molecules are incorporated easily into the cell membranes and hydroperoxides (oxidation products of lipids) preferably react with DPPP.[10] After 6 h of treatment with AAPH, more than threefold increment of DPPP oxide fluorescence was observed in the cells (Fig. 3). This increment clearly indicated accelerated lipid peroxidation in cell membranes and CCOS could reduce DPPP oxide fluorescence dose-dependently by inhibiting lipid peroxidation. However, concentrations of COS from 50 to 500 μg/mL exerted similar inhibitory effects (about 25%) and at 1000 μg/mL its inhibitory effect was doubled (50%). In contrast, CCOS could inhibit membrane lipid peroxidation dose-dependently even at low concentrations and about 63% inhibition was observed at the highest concentration.

In addition to DPPP oxide fluorescence method, thiobarbituric acid reactive substances (TBARS) assay, one of the most popular biochemical analysis methods for the assessment of lipid peroxidation products, was also used to evaluate membrane lipid peroxidation. In this assay, membrane lipid peroxidation was initiated by generating hydroxyl radicals (OH) via Fenton's reaction as described in the Section 3. As we expected, CCOS could reduce TBARS in the cells to a similar pattern observed in the DPPP oxide fluorescence assay (Fig. 4). Moreover, CCOS had significantly ($P < 0.05$) higher inhibitory effects compared to COS at 500 and 1000 μg/mL. The inhibitory effect of CCOS at the highest concentration was about 75% compared to the control. Previously we have reported that COS can scavenge OH and alkyl radicals by donating protons in in vitro systems.[4,5] Based on the results of present study, we can presume that COS inhibit cellular lipid peroxidation by scavenging ROS. In addition, the higher lipid peroxidation inhibitory effect of CCOS can be expected due to improvement of proton donation by the introduced group, $–COCH_2CH_2COOH$.

2.4. Inhibition of membrane protein oxidation

Oxidation of cellular proteins due to ROS attack generates carbonyl moieties and those have been identified as an early marker for protein oxidation.[11] Moreover, oxidation of membrane proteins results in increased membrane fluidity, compromised integrity, and inactivation of membrane-bound receptors and enzymes. Therefore, compounds those can inhibit protein oxidation play a major role in maintaining cellular integrity and normal biological functions. In the present study, oxidation inhibition effects of CCOS on leukocyte membrane proteins were assessed according to the method described in the text. When mouse macrophage membranes were exposed to OH, an increased formation of carbonyl moieties was observed (Fig. 5). Carbonyl protein content expressed in nmol/mg of protein was about 15 times higher in oxidation-stimulated cell membranes com-

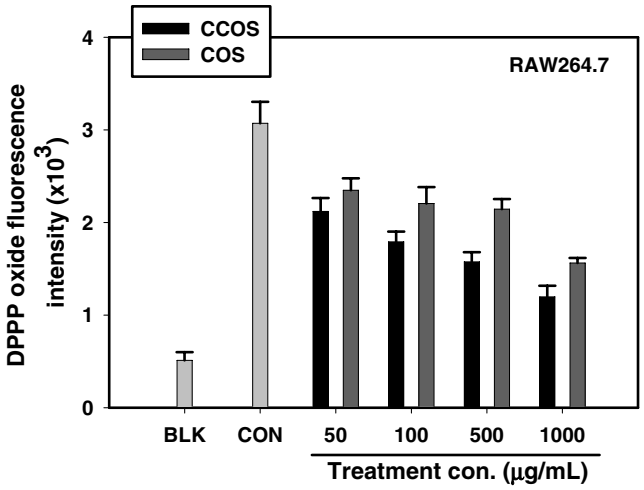

Figure 3. Effect of CCOS and COS on membrane lipid peroxidation inhibition, assessed by DPPP fluorescence assay. RAW264.7 cells treated with different concentrations of CCOS and COS were exposed to alkyl radical-generating agent, AAPH, to initiate membrane lipid peroxidation. DPPP oxide fluorescence emitted due to oxidation of DPPP by lipid hydroperoxides was compared with AAPH non-treated blank group and AAPH alone-treated control. Results are means ± standard error of three independent experiments.

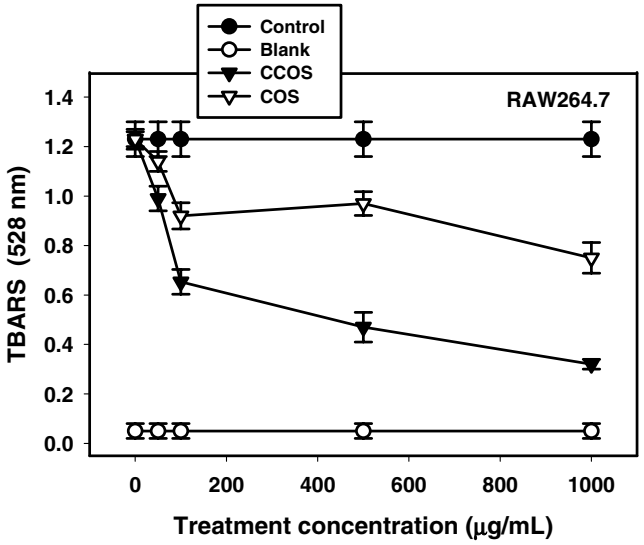

Figure 4. Membrane lipid peroxidation determination by TBARS method. RAW264.7 cells were exposed to ·OH generated via Fenton's reaction and oxidation products of membrane lipids which can react with TBA were determined spectroscopically at 528 nm. Effect of CCOS and COS on oxidation inhibition of membrane lipids was compared with ·OH non-treated blank group and ·OH alone-treated control in three independent experiments and are presented as means ± SE.

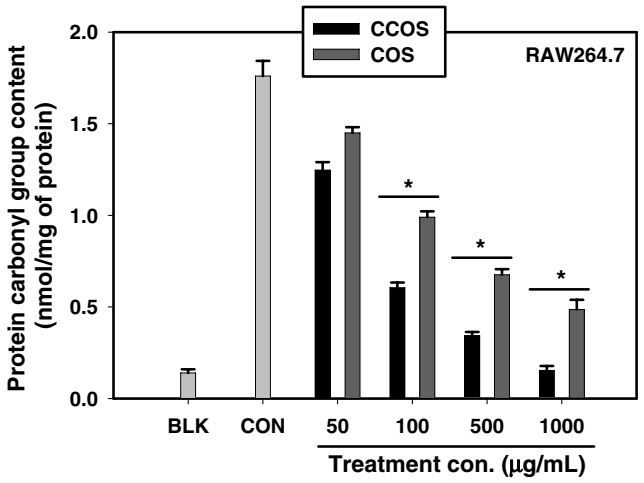

Figure 5. Assessment of cell membrane protein oxidation. RAW264.7 cell membranes were treated with different concentrations of CCOS or COS and protein oxidation was triggered by generating ·OH via Fenton's reaction. Degree of protein oxidation was determined by assessing the amount of carbonyl–carbon content as described in the text and compared with ·OH non-treated blank group and ·OH alone-treated control. The carbonyl group content was expressed in nmol/mg of protein, using a molar absorption co-efficient of 22,000 M^{-1} cm^{-1}. Results are expressed as means ± SE of three independent tests and significance was determined at $P < 0.05$.

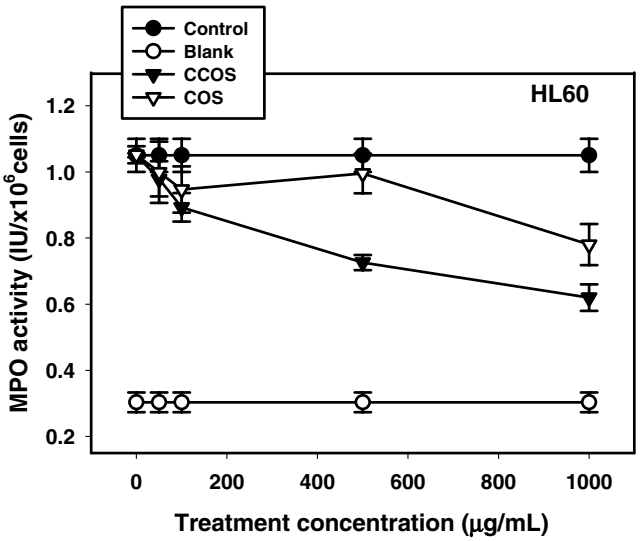

Figure 6. Inhibition of myeloperoxidase (MPO) activity in HL60 cells by CCOS. Cells were treated with different concentrations of CCOS or COS and expression of MPO was stimulated by human TNF-α (0.05 μg/mL). An assay mixture containing 2 mM H_2O_2 and 0.02 M O-dianisidine was added to the cells and amount of MPO released was measured spectrophotometrically at 460 nm. MPO activity was compared with TNF-α non-stimulated blank and sample non-treated control group. Results are means ± standard error of three independent experiments.

pared to that of non-stimulated blank group. When the cell membranes in reaction mixture were pre-treated with different concentrations of CCOS or COS, carbonyl protein formation was clearly decreased. Furthermore, a significant difference ($P < 0.05$) between COS and CCOS was observed to inhibit protein oxidation at concentrations higher than 100 μg/mL. Protein oxidation inhibition results were in line with that of lipid peroxidation and involvement of $–COCH_2CH_2COOH$ group for scavenging of ROS was further confirmed.

2.5. Myeloperoxidase (MPO) inhibitory effects of CCOS

Neutrophils provide first line defense against invading pathogens by generating superoxide radicals and converting them into more reactive secondary oxidants H_2O_2.[12] Myeloperoxidase, the most abundant haem enzyme in neutrophils, converts H_2O_2 into HOCl which is the most powerful oxidant acting against pathogens during infections.[13] The large amount of ROS released into the extracellular medium by neutrophils not only contribute to kill pathogens but also destruct healthy tissues of the host. This causes oxidation of thiole compounds in proteins and unsaturated fatty acids of membrane lipids destabilizing the cell membrane integrity. Therefore, inhibition of MPO activity is an important approach to control free radical-mediated oxidation of biomolecules in neutrophils. In this study, we evaluated whether CCOS can inhibit MPO activity which in turn helps to prevent oxidation of cellular biomolecules. For that, human myeloid cell line HL60 was selected due to its reported high expression of MPO following stimulation.[13] As depicted in Figure 6, CCOS could reduce MPO activity dose-dependently compared to TNF-α-stimulated control in HL60 cells. However, COS, the starting material of CCOS, could not inhibit MPO as efficiently as CCOS. At the highest concentration of CCOS, about 43% inhibition of MPO was observed and it can be speculated that this is an indirect way of acting as cellular antioxidant.

2.6. Cellular radical scavenging effect of CCOS

Finally, we were interested in studying the direct effects of CCOS to scavenge cellular radicals. For that, RAW264.7 cells were labeled with fluorescence probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described in Section 3. During labeling, non-fluorescent DCFH-DA dye that freely penetrates into cells gets hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescein (DCFH), and traps inside the cells.[14] As shown in Figure 7, fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time course increment up to 2 h. Pre-treatment with CCOS decreased the DCF fluorescence dose- and time-dependently. Even after 30 min of incubation, CCOS exerted a considerable radical scavenging effect at 100 μg/mL concentration. More clearly, at the concentration of 1000 μg/mL, CCOS could scavenge radicals significantly ($P < 0.01$) throughout the incubation time. This clearly indicated that the oxidation protection effects exerted by CCOS are due to direct scavenging of cellular radicals. These results are in line with our previous observations that CCOS can donate protons under in vitro conditions to scavenge free radicals. Therefore, for the first time here we confirmed the radical scavenging antioxidant effects of a COS derivative (CCOS) in cell systems. Further, we can suggest that CCOS is a potent antioxidant compound that

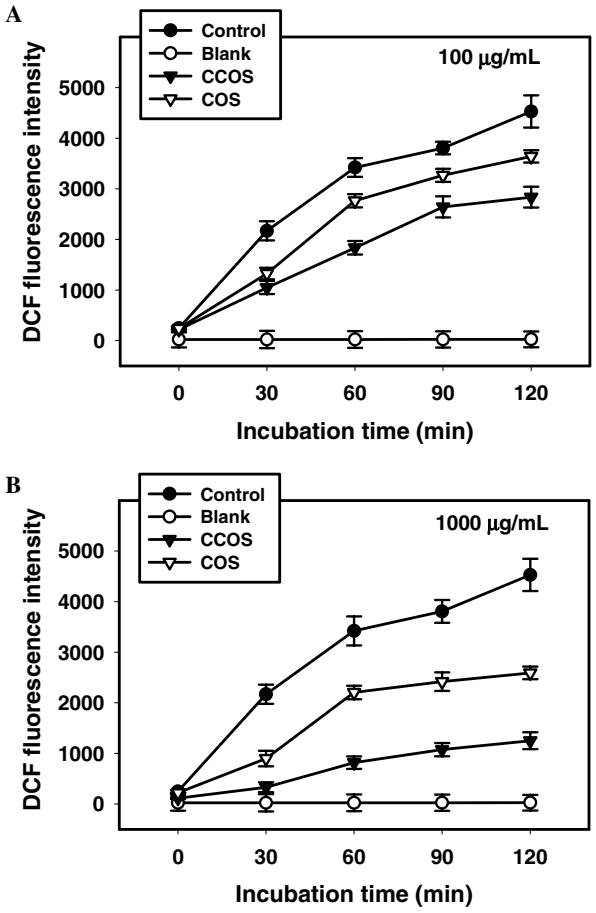

Figure 7. Cellular radical scavenging activity of CCOS at 100 μg/ml (A) and 1000 μg/ml (B). RAW264.7 cells were labeled with non-toxic fluorescence dye, DCFH-DA, and treated with different concentrations of CCOS or COS. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) were detected time-dependently ($\lambda_{excitation} = 485$ nm and $\lambda_{emission} = 528$ nm). Effects of CCOS and COS on the scavenging of cellular ROS were compared with H_2O_2 non-stimulated blank and sample non-treated control group in three independent experiments.

can protect radical-mediated oxidation of cellular biomolecules.

3. Experimental

3.1. Materials

Chitooligosaccharides (acetylation degree, 27.29% and molecular weight, $6.0–7.0 \times 10^3$ Da) were kindly donated by Kitto Life Co. (Seoul, Korea). All the chemicals required for synthesis of CCOS, including succinic anhydride, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RAW264.7 and HL60 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, penicillin/streptomycin, fetal bovine serum (FBS), and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA). Fluorescence probes such as DPPP and DCFH-DA were obtained from Molecular Probes Inc. (Eugene, OR, USA).

3.2. Synthesis of CCOS

For the preparation of CCOS, COS (6.5 g) dissolved in 10% acetic acid aqueous solution (50 mL) were mixed with methanol (15 mL) while stirring and succinic anhydride dissolved in acetone (6.6 g) was added drop by drop at room temperature for 1 h as described by us previously.[5] The reaction mixture was stirred for 4 h and pH was maintained at 9.0–10.0 with sodium carbonate. Resultant CCOS were purified using Micro Acilyzer G3 (Asashi Kasei Corp., Japan) equipped with a 500-Da molecular weight cut-off dialysis membrane and lyophilized.

3.3. Structural confirmation of CCOS

After synthesis, CCOS were chromatographed on a Bio-Gel P-3000 gel-permeation chromatography column incorporated with a TSP P100 instrument and average molecular weights together with their intensities were estimated by standard Pullulan® molecular weight markers (5.9×10^3, 2.28×10^4, and 4.73×10^4 Da) and a refractive index detector (Sulphodex RI-71). Structure of CCOS was determined using proton NMR (^{1}H NMR), carbon NMR (^{13}C NMR) (in a D_2O environment on a JNM-ECP-400 spectrometer (JEOL, Japan), C, N, and H elemental analysis (Elementar Vario, EL, USA), and FT-IR spectrophotometry (Spectrum 2000 spectrophotometer, Perkin-Elmer, USA).

3.4. Culture of cells and viability determination

Leukocyte cell lines, RAW264.7 and HL60, were grown, respectively, in DMEM and RPMI-1640 media containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg/mL penicillin–streptomycin at 5% CO_2 and 37 °C humidified atmosphere.

For cytotoxicity determination studies, cells were cultured in microtiter 96-well plates (1.5×10^5 cells/well) with serum-free media and treated with different concentrations of CCOS or COS for 24 h or H_2O_2 for 6 h. Cell viability was spectrophotometrically (GENios® microplate reader, Tecan Austria GmbH, Austria) assessed by MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as previously described.[15]

3.5. Membrane lipid peroxidation assessment by DPPP fluorescence method

Intracellular lipid hydroperoxide level was determined by the fluorescence probe, DPPP, as described previously with slight modifications to assess the degree of membrane lipid oxidation.[10] For this experiment, RAW264.7 cells growing in culture dishes were washed three times with PBS and labeled with 13 μM DPPP (dissolved in DMSO) for 30 min at 37 °C in the dark. Cells were washed three times with PBS and seeded into fluorescence microtiter 96-well plates at a density of 1×10^8 cells/mL using serum-free media. Following complete attachment, cells were treated with predetermined

concentrations of samples and incubated for 1 h. After incubation, 3 mM AAPH in PBS was added and DPPP oxide fluorescence intensity was measured after 6 h ($\lambda_{excitation}$ = 361 nm, $\lambda_{emission}$ = 380 nm) using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Austria). Fluorescence values were normalized to cell numbers using MTT cell viability assay.

3.6. Membrane lipid peroxidation assessment by TBARS method

Cells were analyzed for the generation of lipid peroxidation products by a modification of the thiobarbituric acid-reactive substances (TBARS) method, as previously described.[16] Briefly, 200 μL of PBS-suspended RAW264.7 cells was added to an eppendorf tube and incubated for 10 min with pre-determined concentrations of test samples or same volume of distilled water as a control. Cellular oxidation was triggered by adding 2 mM H_2O_2 and 0.1 M $FeSO_4$, and incubated for 30 min. Two-fold volume of cold trichloroacetic acid (10% w/v) was added and protein was precipitated by centrifugation. The supernatant was then added to an equal volume of TBA (1% w/v) and heated at 90 °C for 30 min. After cooling, reaction mixture was centrifuged and absorbance of the supernatant was determined at 528 nm.

3.7. Membrane protein oxidation assay

Amount of carbonyl groups in cell membrane proteins was determined to assess the cellular protein oxidation level as described earlier.[17] RAW264.7 cells growing in culture dishes were washed three times with PBS and lysed in lysis buffer without reducing agents (25 mM Tris–HCl, pH 7.8, 2 mM EDTA, 180 mM NaCl, and 1% Triton X-100). Aliquots of cell lysate were separately incubated with indicated concentrations of CCOS and COS for 30 min at 37 °C. Protein oxidation was initiated by adding 0.1 M $FeSO_4$ and 2 mM H_2O_2 to the mixture and incubation was continued for another 1 h. Trichloroacetic acid (400 μL from 20% solution) was added to each reaction mixture and solubilized proteins were precipitated by centrifugation. Precipitated protein was resuspended in 0.2% 2,4-dinitrophenyl hydrazine (in 2 mM HCl) and allowed to stand at room temperature for 40 min. The protein was precipitated again with 20% trichloroacetic acid and the pellet was washed three times with ethanol:ethyl acetate (1:1 v/v) solution. The pellet was then dissolved in 6 N, guanidine hydrochloride (500 μL) and incubated for 15 min at 37 °C. After centrifugation at 1500*g* for 5 min, absorbance of the supernatant was recorded against a complementary blank at 370 nm using a UV/Visible microplate reader. A blank was prepared with a parallel procedure using 2 mM HCl alone instead of 2,4-dinitrophenyl hydrazine reagent. The carbonyl group content was expressed in nmol/mg of protein, using a molar absorption co-efficient of 22,000 $M^{-1}\,cm^{-1}$.

3.8. Myeloperoxidase activity assay

Human myeloid cell line HL60 was used to evaluate cellular MPO activity by *O*-dianisidine method with modifications.[18] Cells cultured in microtiter 96-well plates were pre-incubated with various concentrations of test samples for 30 min at 37 °C and incubation was continued for another 30 min following stimulation with human TNF-α (0.05 μg/mL). An assay mixture containing 0.05 mL of 2 mM H_2O_2 in 0.1 M phosphate buffer (pH 6.0) and 0.05 mL of 0.02 M *O*-dianisidine (freshly prepared) in deionized water was added to the cells and amount of MPO released was measured spectrophotometrically at 460 nm. The total MPO activity was plotted as an absorbance value and it was compared with the control group in which cells were grown in absence of inhibitors.

3.9. Cellular ROS determination by DCFH-DA

Oxidation-sensitive dye DCFH-DA was used to detect formation of intracellular reactive oxygen species (ROS) as described previously.[19] For that, RAW264.7 cells growing in fluorescence microtiter 96-well plates were labeled with 20 μM DCFH-DA in HBSS for 20 min in the dark. Cells were then treated with different concentrations of CCOS or COS and incubated for another 1 h. After washing the cells with PBS for three times, 500 μM H_2O_2 was added. The intensity of fluorescence signal emitted by 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH by cellular ROS was detected time dependently ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 528 nm) using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Austria). Following maximum rate of fluorescence increase, each well was normalized to cell numbers using MTT cell viability assay. Effects of treatments were plotted and compared with fluorescence intensity of control and blank groups.

3.10. Statistical analysis

Data were expressed as means ± standard error of the mean after at least three independent experiments. Student's *t* test was used to determine the level of significance at $P < 0.05$.

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