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Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells

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ABSTRACT

Chitosan oligosaccharides (COS) have been reported to have anticancer activity, immuno-enhancing effect and antimicrobial activity. However, other biological activities are unknown. Herein, we investigated the protective effects of COS against hydrogen peroxide (H_2O_2)-induced oxidative damage on human umbilical vein endothelial cells (HUVEC, ECV304 cells). After 24 h pre-incubation with COS (25–200 μg/ml), the viability loss in ECV304 cells induced by H_2O_2 (300 μM) for 12 h was markedly restored in a concentration-dependent manner as measured by MTT assay. This effect was accompanied by a marked decrease in intracellular reactive oxygen species (ROS) by measuring intensity of DCFH fluorescence. COS also exerted preventive effects on suppressing the production of lipid peroxidation such as malondialdehyde (MDA), restoring activities of endogenous antioxidants including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), along with the capacity of increasing levels of nitric oxide (NO) and nitric oxide synthase (NOS), as were determined by commercial regent kits. In addition, pre-incubation of COS with ECV304 cells for 24 h resulted in the reduction of apoptosis and the induction of cell cycle arrest in $G_1/S+M$ phase as assayed quantitatively by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit using flow cytometry. Taken together, our findings suggest that COS can effectively protect HUVECs against oxidative stress by H_2O_2, which might be of importance in the treatment of cardiovascular diseases.

1. Introduction

Enhanced oxidative damage after diverse stimuli has been confirmed to be an initial event in the development of cardiovascular diseases [1]. Generation of reactive oxygen species (ROS) by vascular endothelial cells is involved in several clinical conditions associated with atherosclerosis, hypercholesteremia and disseminated intravascular coagulation [2,3]. The up-regulation of ROS in vascular lesions will exert detrimental effects including peroxidation of membranes lipids, endothelium-derived enzymes inactivation and apoptotic occurrences, etc. [1]. In contrast, the oxidative insults can be partially prevented by some free radical scavengers such as Vitamin E, flavonoids and superoxide dismutase [4–6]. Thus, antioxidants that react preferentially with ROS to inactivate them might have therapeutic applications in treating ROS-induced endothelial injury.

Chitosan, the linear polymer of D-glucosamine in β (1,4) linkage, is cellulose-like biopolymer present in the exoskeleton of crustaceans and in cell walls of fungi and insects [7]. Chitosan oligosaccharides (low-molecular weight chitosan, COS) are depolymerized products of chitosan and can be obtained by chemical and enzymatic hydrolysis of chitosan [8]. During the past decades, COS were known to have various biological activities including immunopotentiating, antitumor, antihypertensive, and antimicrobial actions [9–12]. Moreover, COS may affect the mitogenic response as well as the chemotactic activities of animal cells [13]. In addition, increasing attention has now been paid to the use of COS as an antioxidant. By using electron spin resonance (ESR) spin-trapping technique, COS and their derivatives can show high scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and carbon-centered radicals [14–16]. Studies also showed that COS with different molecular weight (1–10 kDa) could inhibit free radical damages to murine melanoma cell line (B16F1) in a dose-dependent manner [17]. However, no direct evidence was provided about the protective effects of COS on oxidative stress-induced vascular endothelium dysfunction, which is an important contributor to the development of cardiovascular diseases.

Human umbilical vein endothelial cells (HUVECs) are commonly accepted as a tool in exploring the mechanisms involved

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in the pathogenesis of cardiovascular diseases [18]. And hydrogen peroxide (H_2O_2) is extensively referred to as one of the major oxidative stimuli in antioxidative studies [19–21]. In the present study, ECV304, a spontaneously transformed HUVEC isolate [22], was applied to explore whether COS is capable of attenuate H_2O_2-induced stress injury, which was performed by measuring the cell viability, the release of lactate dehydrogenase (LDH), the production of intracellular ROS, the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA), as well as the levels of nitric oxide (NO) and nitric oxide synthase (NOS). Furthermore, the changes of apoptotic index and cell cycle were also assessed by flow cytometric analysis. It was found that COS could effectively exert protective effects in ECV304 cells against oxidative stress by H_2O_2.

2. Materials and methods

2.1. Chemicals and reagents

COS was prepared from enzymatic hydrolysis of chitosan and separated with membrane (the degree of *N*-acetylation is below 5%) [23], and the weight percentages of COS with DP (degree of polymerization) 2–6 in oligomixture were: 3.7%, 16.1%, 28.8%, 37.2% and 14.2%, respectively. Dulbecco's-modified Eagle's medium F12 (DMEM-F12) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). H_2O_2, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT), Vitamin C, bovine serum albumin (BSA) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma (St. Louis, MO, USA). The reagent kits for measurement of levels of LDH, MDA, SOD, GSH-Px and NOS were purchased from Nanjing Institute of Jiancheng Bioengineering (Nanjing, China). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Nanjing Key-Gen Biotech Co., Ltd. (Nanjing, China), and 3-Amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate (DAF-FM DA) from Beyotime Institute of Biotechnology (Jiangsu, China). All the other reagents were of analytical grade.

2.2. Cell culture and treatment

ECV304 cell line was obtained from Shanghai Institute of Cell Biology and maintained in DMEM-F12 supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells reached sub-confluence, they were pretreated for 24 h with culture medium containing different concentrations of COS (25, 50, 100 and 200 μg/ml) or Vitamin C (250 μg/ml) that were tested in the experiments. Next, the culture supernatant was collected and the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4). Subsequently, the cells were exposed to H_2O_2 (300 μM) diluted in culture medium for 12 h at 37 °C until further assay.

2.3. Cell viability measurement

ECV304 cells were seeded at density of 5×10^4 cells/ml in 96-well plates and the cell viability was measured using the MTT assay [24]. Briefly, at the indicated time after the treatment as before, the culture supernatant was removed. The cells were washed with PBS and incubated with MTT (5 mg/ml) in culture medium at 37 °C for another 3 h. After MTT removal, the coloured formazan was dissolved in 100 μl of DMSO. The absorption values were measured at 490 nm using a Sunrise Remote Microplate Reader (Grodlg, Austria). The viability of ECV304 cells in each well was presented as percentage of control cells.

2.4. Measurement of intracellular ROS

Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2′,7′-DCFH-DA into DCFH [17]. The intensity of fluorescence reflects enhanced oxidative stress. After the incubation studies as before, ECV304 cells, which had been seeded in black 96-well plates, were washed with PBS (pH 7.4) and then incubated with DCFH-DA (20 μM) in PBS at 37 °C for 2 h. At the end of incubation, the DCFH fluorescence of the cells from each well was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm using a FLX 800 microplate fluorescence reader (Biotech Instruments Inc., USA). The background was from cell-free conditions. Results were expressed as percentage of control (non-stimulated ECV304 cells) fluorescence intensity.

2.5. Evaluation of NO production in fixed cells

Production of NO in ECV304 cells was assessed using the NO-specific fluorescent dye DAF-FM DA as described previously [25]. After incubation studies, the cells, seeded on 25-cm^2 culture flasks, were detached from the flasks by trypsin treatment, followed by being loaded with DAF-FM DA (5 μM) in PBS (pH 7.4) for 20 min at 37 °C. Thereafter, cells were washed thrice and resuspended in PBS. The fluorescence in fixed cells was quantitatively analyzed by a FACS vantage SE flow cytometer (Becton Dickinson, San Jose, CA, USA) at an emission wavelength of 515 nm and an excitation wavelength of 495 nm.

2.6. Preparation of cell lysates

The cells were seeded at a density of 1×10^5 cells/ml in 24-well plates and were allowed to attach for 24 h before treatment. Upon completion of the incubation studies, the culture supernatant was collected for analysis of LDH and NO release. The cells were scraped from the plates into ice-cold RIPA lysis buffer (50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 0.05 mM EDTA) and protein concentration was determined by the bicinchoninic acid (BCA) method, using BSA as a reference standard. Aliquots were was stored at −80 °C until detection for MDA, SOD, GSH-Px and NOS activity.

2.7. Measurement of LDH and nitric oxide (NO) release

LDH, an indicator of cell injury, was detected after the exposure to H_2O_2 with an assay kit according to the manufacturer's protocol. The activity of enzyme was expressed as units per litre and the absorbance was read at 440 nm.

The concentration of nitrites (NO_2^-) and nitrates (NO_3^-), stable end products of nitric oxide (NO), were determined by the Griess reaction as previously described [8]. NO production was determined by measuring the optical density at 550 nm and expressed as units per milligram protein.

2.8. Assay for intracellular contents of SOD, GSH-Px, NOS and MDA

The activities of SOD, GSH-Px, NOS as well as the concentration of MDA were all determined by using commercially available kits. All procedures completely complied with the manufacture's instructions. The activities of enzymes were expressed as units per milligram protein. The assay of SOD activity was based on

its ability to inhibit the oxidation of hydroxylamine by O^{2-} produced from the xanthine–xanthineoxiase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The assay for GSH-Px activity was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2 catalyzed by GSH-Px. One unit of GSH-Px was defined at 412 nm as the amount that reduced the level of GSH by 1 μM in 1 min/mg protein. NOS activity was determined at a wavelength of 530 nm by the reaction of nitric oxide with nucleophiles to form chromophoric production. The activity of NOS was expressed as units per milligram protein. MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric production. Values of MDA level were expressed as nanomoles per milligram protein.

2.9. Flow cytometric evaluation of apoptosis

ECV304 cells growing in 25-cm^2 culture flasks were harvested, washed and double-stained by using an Annexin V-FITC apoptosis detection kit. Annexin V has a strong, Ca^{2+}-dependent affinity for phosphatidylserine (PS), which translocates from the internal to the external surface of the plasma membrane as a probe for detecting apoptosis [26]. Cells that have the loss of membrane integrity will show red staining (propidium iodide, PI) throughout the nucleus and therefore will be easily distinguished between the early apoptotic cells and the late apoptotic cells or necrotic cells. Samples were incubated at room temperature for 15 min in the dark with Annexin V and PI and quantitatively analyzed by a FACS vantage SE flow cytometer.

2.10. Flow cytometry for cell cycle analysis

ECV304 cells growing in 25-cm^2 culture flasks were harvested, washed and fixed with ice-cold alcohol (75%) for more than 24 h. After further being washed twice, cells were incubated with PBS (pH 7.4) containing RNase (5 U) and PI (50 μg/ml) for 15 min at 37 °C. Flow cytometry was performed using a FACS vantage SE flow cytometer.

2.11. Statistics

Statistical analyses were performed using the SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± S.D. of 3–5 independent experiments. ANOVA and Student's *t*-test were performed to determine statistical significance. Differences between groups were considered to be significant at $P < 0.05$.

3. Results

3.1. Time-dependent and concentration-dependent viability losses in ECV304 cells induced by H_2O_2

We first carried out the time-dependent and concentration-dependent studies of viability losses in ECV304 cells induced by H_2O_2. After treatment with increasing concentrations of H_2O_2 for 12 h, cell viability was then examined by MTT method. As shown in Fig. 1A, gradual losses of cell viability were observed with increased concentrations of H_2O_2. The degree of cell injury was maximum at 750 μM among the concentrations tested and the cell viability was close to zero as compared with the vehicle-treated control group. Fig. 1B shows the results of time–response study in which cells were exposed to 300 μM of H_2O_2 up to 24 h this concentration of H_2O_2 started to increase oxidative injury in ECV304 cells 4 h after treatment with H_2O_2. The magnitude of cell injury peaked at 12 h after H_2O_2 exposure and the cell viability was about 67.0 ± 4.7%. In the preliminary studies by flow cytometric analysis, COS (50–200 μg/ml) showed the most markedly preventive effects on cell injury induced by H_2O_2 at 300 μM as compared to other concentrations tested. Based on theses results, ECV304 cells were treated with 300 μM of H_2O_2 for 12 h or vehicle as control in the extensive studies.

3.2. Effects of COS on the viability of H_2O_2-induced ECV304 cells

Effects of COS on the viability of H_2O_2-induced ECV304 cells were evaluated by MTT analysis. The results in Fig. 2A show that, the survival rate of ECV304 cells was about 71.9 ± 1.5% after exposure to 300 μM of H_2O_2 for 12 h. However, pre-incubation of ECV304 cells with different concentrations of COS (25, 50, 100 and 200 μg/ml) for 24 h markedly increased the viability of H_2O_2-induced ECV304 cells in a dose-dependent manner. The treatment with 50, 100 and 200 μg/ml concentration of COS increased the viability of ECV304 cells in a statistically significant fashion to 83.5 ± 7.8%, 92.7 ± 5.9% and 88.2 ± 4.9%, respectively. In addition, no difference was seen in cell viability between cells treated with COS (25–200 μg/ml) alone and controls (data not shown). Apparently, COS were effective for the protection of ECV304 cells against H_2O_2-induced injury.

To further investigate the protective effects of COS, LDH assay, another indicator of cell toxicity, was performed. As shown in Fig. 2B, LDH release in ECV304 cells was minimal in the vehicle-treated control group (38.1 ± 4.1 U/L) and a dramatic increase (109.9 ± 5.7 U/L) was observed after 12 h exposure to 300 μM of H_2O_2. Contrary to this, pre-treatment with COS for 24 h at concentrations above 50 μg/ml attenuated markedly the H_2O_2-induced

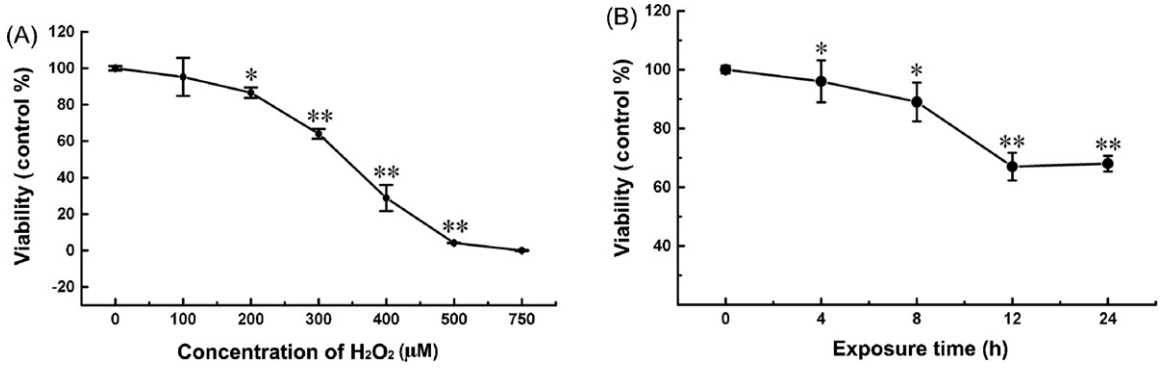

Fig. 1. Viability losses in ECV304 cells induced by H_2O_2. Cell viability was estimated by MTT method as described in Section 2.3. (A) ECV304 cells were treated for 12 h using various concentrations of H_2O_2. (B) ECV304 cells were treated with 300 μM of H_2O_2 for the times indicated. Values are means ± S.D. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle-treated control group.

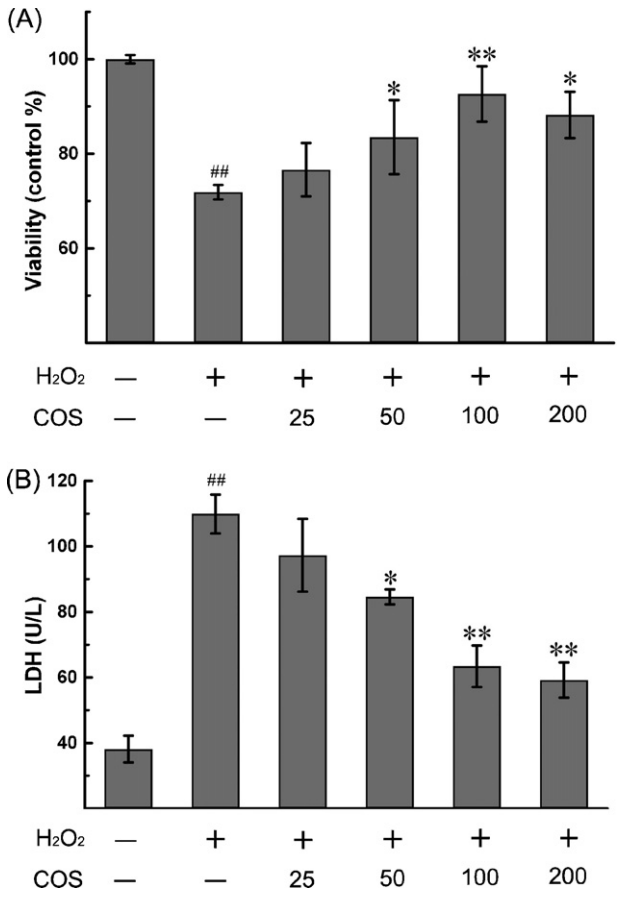

Fig. 2. Protective effects of COS on viability losses in ECV304 cells induced by H_2O_2 (300 μM). ECV304 cells were Pre-incubated with 25–200 μg/ml of COS for 24 h and then exposed to H_2O_2 for 12 h. (A) Preventive effects of COS on cell viability against H_2O_2-induced injury. (B) Inhibition of LDH release in ECV304 cells by COS after exposure to H_2O_2. Values are means ± S.D. ($n = 5$). $^{\#\#}P < 0.01$ compared to the vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

increase in LDH release by 35.4 ± 4.7% or more. The result was consistent with that determined by MTT assay.

3.3. Intracellular radical scavenging ability of COS

The intracellular ROS concentration was determined by measuring the intensity of DCFH fluorescence. When DCFH-DA-labeled cells were incubated in the medium for 2 h, a sudden increment in fluorescence intensity indicated the oxidation of DCFH-DA by intracellular radicals (Fig. 3). The production of DCFH fluorescence in ECV304 cells with H_2O_2 increased significantly to 239.5 ± 16.6% of the vehicle-treated control group, whereas pre-incubation with COS (25–200 μg/ml) significantly reduced the increased fluorescence induced by H_2O_2 in a concentration-dependent manner. Vitamin C (250 μg/ml) showed a similarity to the effects of COS.

3.4. Measurement of SOD and GSH-Px activities as well as MDA content

Treatment of ECV304 cells with 300 μM of H_2O_2 for 12 h caused the decrease in the activities of SOD and GSH-Px by 58.5 ± 6.1% and 29.4 ± 6.5%, respectively. However, pre-incubation with COS (25–200 μg/ml) significantly attenuated the changes of SOD and GSH-Px activities (Fig. 4A and B). At 100 μg/ml of COS, the H_2O_2-induced decrease in SOD and GSH-Px activities was restored separately by about 44.9 ± 5.8% and 23.5 ± 7.3%, which was the

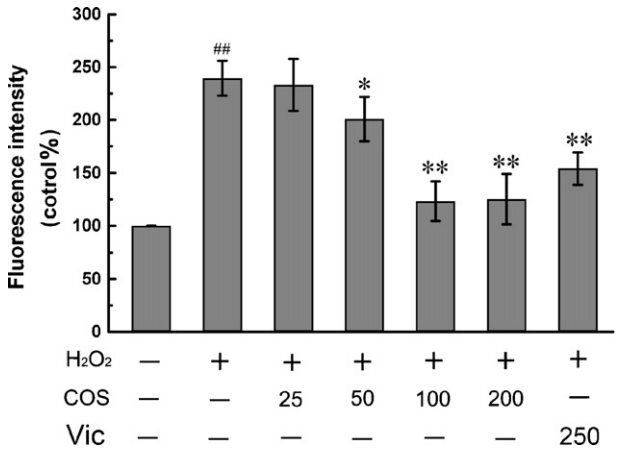

Fig. 3. Inhibitory effects of COS and Vitamin C (Vic) on the production of intracellular reactive oxygen species. ECV304 cells were pre-incubated for 24 h with COS (25–200 μg/ml) or Vitamin C (250 μg/ml), and then exposed to 300 μM of H_2O_2 for 12 h. Values are means ± S.D. ($n = 5$). $^{\#\#}P < 0.01$ compared to the vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

highest among the concentrations of COS tested and higher than that recovered by Vitamin C at 250 μg/ml (19.2% and 15.1%, respectively).

In addition, ECV304 cells treated with 300 μM of H_2O_2 for 12 h caused an increase in the intracellular MDA level by 54.9%, while pre-incubation of cells with 100 and 200 μg/ml of COS or 250 μg/ml of Vitamin C markedly attenuated the increase by about 29.5%, 32.6% and 40.6%, respectively (Fig. 4C).

3.5. Detection of NO release in cell culture medium, intracellular NO and NOS production in ECV304 cells

To determine whether COS can increase the activity of NOS and promote the synthesis and release of NO in ECV304 cells, the content of NO and the activity of NOS were measured. As shown in Fig. 5A, in comparison with the vehicle-treated control group, a slight increase in NO level from cell culture medium was observed after exposure to 300 μM of H_2O_2 for 12 h, whereas pre-incubation with COS (25–200 μg/ml) for 24 h could trigger a comparable increase in a dose-dependent manner.

In parallel, the intracellular NO content in ECV304 cells was also assayed by measuring the intensity of DAF-FM DA fluorescence. The result was roughly similar to that obtained from Fig. 5A. The fluorescence intensity was increased to 112.3 ± 8.7% of the vehicle-treated control group after treatment with 300 μM of H_2O_2 for 12 h. And pre-incubation with COS (25–200 μg/ml) before H_2O_2 exposure gave rise to further increases in fluorescence intensity, which were 104.0 ± 1.1%, 125.9 ± 4.1%, 128.1 ± 1.5% and 124.2 ± 1.9%, respectively, as compared to H_2O_2 treatment alone.

The NOS activity was also directly measured in extracts of ECV304 cells pre-incubated with COS (25–200 μg/ml) prior to H_2O_2 exposure. The result in Fig. 5B shows that a markedly increased NOS activity (141.2 ± 9.2% of control) in ECV304 cells was observed after exposure to 300 μM of H_2O_2 for 12 h, while pre-incubation with COS (25–200 μg/ml) for 24 h resulted in a relatively higher enzymatic activity, which was maximum at 100 μg/ml of COS (125.9 ± 9.1% of H_2O_2 treatment alone).

3.6. Evaluation of H_2O_2-induced apoptosis by flow cytometric analysis

To quantitatively gain insight into anti-apoptotic effects of COS in H_2O_2-induced ECV304 cells, a display of PI versus Annexin V-FITC fluorescence was measured by flow cytometric analysis (Fig. 6). In

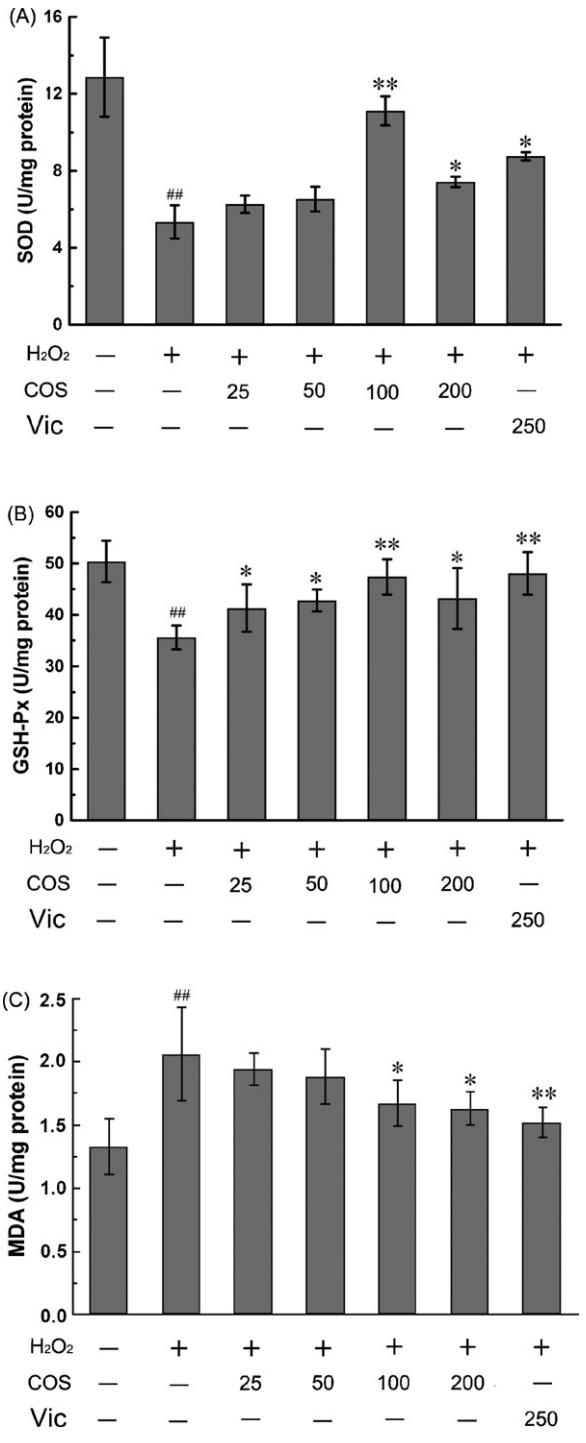

Fig. 4. Effects of COS and antioxidant Vitamin C (Vic) on the production of SOD (A), GSH-Px (B) and MDA (C) in ECV304 cells. ECV304 cells were pre-incubated with COS (25–200 μg/ml) or Vitamine C (250 μg.ml) for 24 h, and then exposed to H_2O_2 (300 μM) for 12 h. Values are means ± S.D. ($n = 5$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

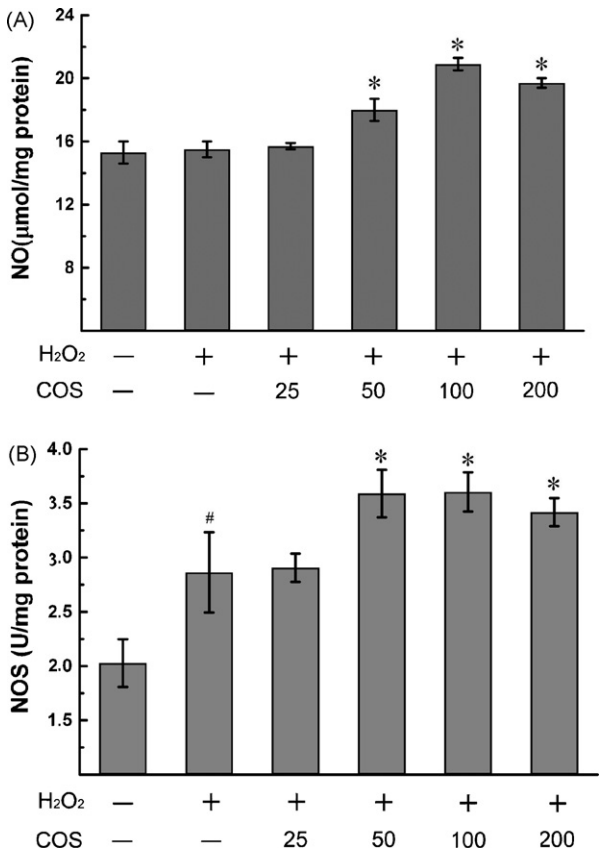

Fig. 5. Effects of COS on NO release in cell culture medium (A) and NOS production in ECV304 cells (B). ECV304 cells were pre-incubated with COS (25–200 μg/ml) for 24 h and then exposed to H_2O_2 (300 μM) for 12 h. Values are means ± S.D. ($n = 5$). $^{\#}P < 0.05$, compared to vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

the vehicle-treated control group (Fig. 6A), 7.6 ± 0.9% cells excluded PI and were positive for Annexin V-FITC binding, which represent apoptotic cells. After exposure to 300 μM H_2O_2 for 12 h (Fig. 6B), the percentage of apoptosis increased to 34.5 ± 5.5%. Nonetheless, pre-incubation with COS (25–200 μg/ml) for 24 h prior to H_2O_2 exposure dose-dependently arrested the apoptosis, and the values of apoptosis were decreased to 33.6 ± 3.2%, 27.8 ± 0.8%, 24.8 ± 2.2% and 21.6 ± 3.0%, respectively (Fig. 6C and D). Moreover, the induction of apoptosis in ECV304 cells treated with COS (25–200 μg/ml) alone was not observed (data not shown).

3.7. Cell cycle assessment of ECV304 cells by flow cytometric analysis

The effects of COS on cell cycle of ECV304 cells induced by 300 μM of H_2O_2 were assessed by measuring the intensity of PI fluorescence. As shown in Fig. 7, in the vehicle-treated control group, 38.2 ± 0.2% of total cells numbered was displayed in the G_0/G_1 phase of the cell cycle (Fig. 7A). After 12 h of H_2O_2 induction, the cell numbers remained in G_0/G_1 was 60.2 ± 1.9%, and only 39.8 ± 2.4% had progressed into $G_2/M + S$ phases of cell cycle (Fig. 7B). Conversely, pre-incubation with COS (25–200 μg/ml) for 24 h definitely ameliorated the delay in cell cycle progression induced by H_2O_2 (Fig. 7C and D). And the cells remained in G_0/G_1 were 60.1 ± 2.4%, 56.9 ± 2.2%, 53.5 ± 0.1% and 48.3 ± 0.5% of the total, respectively (Fig. 7C and D). Additionally, the incubation of COS alone with ECV304 cells for 24 h had no effects on cell cycle progression (data not shown).

4. Discussion

Oxidative stress alters diverse functional responses of endothelial cells and thereby is regarded as a critical pathogenic factor in the development of cardiovascular diseases [27]. High levels of *in vivo* oxidative stress, especially H_2O_2, are associated with increased endothelial cell "turnover" [28]. For example, in pathophysiological situations blood vessels can be exposed to 100 μM

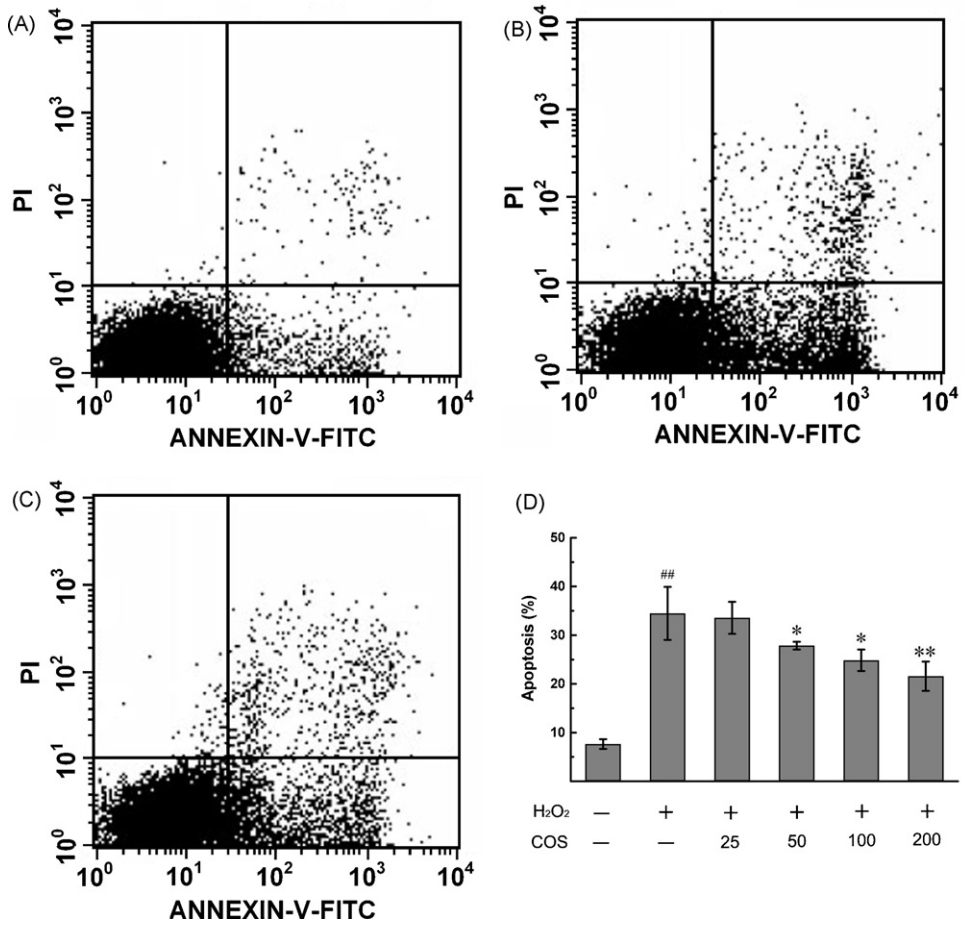

Fig. 6. Effects of COS against H_2O_2-induced apoptosis in cultured ECV304 cells by flow cytometric analysis. ECV304 cells were harvested and labeled with a combination of PI and Annexin V-FITC, and analyzed by flow cytometer. The figures showed representative flow cytometric histograms of vehicle-treated control cells (A), cells exposed to 300 μM of H_2O_2 for 12 h (B) and cells pre-incubated with COS (200 μg/ml) for 24 h prior to 12 h of H_2O_2 exposure. (D) Cells were pre-incubated with indicated concentrations of COS for 24 h prior to 12 h of H_2O_2 exposure. Values are means ± S.D. ($n = 3$). $^{\#\#}P < 0.01$ compared to vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

concentrations of H_2O_2 [29], suggesting a link between oxidative stress and endothelial cell injury. For this reason, there is increasing interest in the use of antioxidant agents against ROS stimulus in cardiovascular diseases, which has been proved in studies of sphingosine 1-phosphate, propofol, Vitamin C [30–32]. As for COS, preliminary studies have proved their positive roles for the antioxidant effects in a wide range of chemical oxidation systems [15,17], indicating the potential role of COS in improving endothelial injury in association with a reduction of oxidative stress. For the first time, here we demonstrated that COS not only prevent oxidative injury in ECV304 cells, but also potently interfere with apoptosis and cell cycle progression due to attenuated exogenous oxidative stress.

As compared to HUVECs, ECV304 has indicated several constitutive difference [22], and was seldom applied to studies such as transendothelial migration assays and cell surface expression of proinflammatory cytokines. In contrast, ECV304 has been commonly used for oxidative stress-induced investigations [33–35]. H_2O_2, when present in excess, can be injurious for ECV304 cells. In this study, exposure of ECV304 cells to 300 μM of H_2O_2 for 12 h showed a significant decrease in cell viability. On the other hand, the cytotoxic effect could be abrogated markedly by COS pre-treatment at 50 μg/ml or higher concentrations. This indicates that COS have an ability to protect ECV304 cells against H_2O_2-induced injury.

Much evidence has revealed that H_2O_2 can cause endothelial cell injury by inducing mitochondrial dysfunction [36,37]. The mitochondrial membrane permeability transition increases formation of ROS by inhibition of respiratory chain [38]. To confirm the ability of COS to scavenge ROS in a cellular environment, ECV304 cells were pre-treated with different concentrations of COS before exposure to H_2O_2. And non-toxic fluorescence probe DCFH-DA was employed for the assessment of oxidative responses considering its suitability for reliable measurement [39]. As we expected, incubation of ECV304 cells with H_2O_2 strikingly increased intracellular ROS, and it can be statistically suppressed by pre-treatment with COS (100–200 μg/ml). The effects of COS were similar to those of Vitamin C (250 μg/ml) in scavenging ROS in ECV304 cells induced by H_2O_2. These facts more strongly suggest that a significant inhibition of H_2O_2-induced ROS production may contribute to restoring the viability of ECV304 Cells. The results were also completely consistent with previous studies, which reported that COS could scavenge cellular free radicals and subsequently inhibit radical mediated cellular oxidation events in human melanoma cells (B16F1) [17]. However, we could not conclude whether COS elicited a direct quenching effect on H_2O_2-induced oxidant species or by some different mechanisms. Hence, further experiments should be performed to support these conclusions.

Lipid peroxidation is one of the primary events in free radical-mediated cell injury [19]. MDA is a by-product of lipid peroxidation induced by excessive ROS and is widely used as a biomarker of oxidative stress [40]. On the other hand, cells are often equipped with several antioxidants for the prevention of free-radical damage. SOD and GSH-Px, along with other enzymatic and non-enzymatic

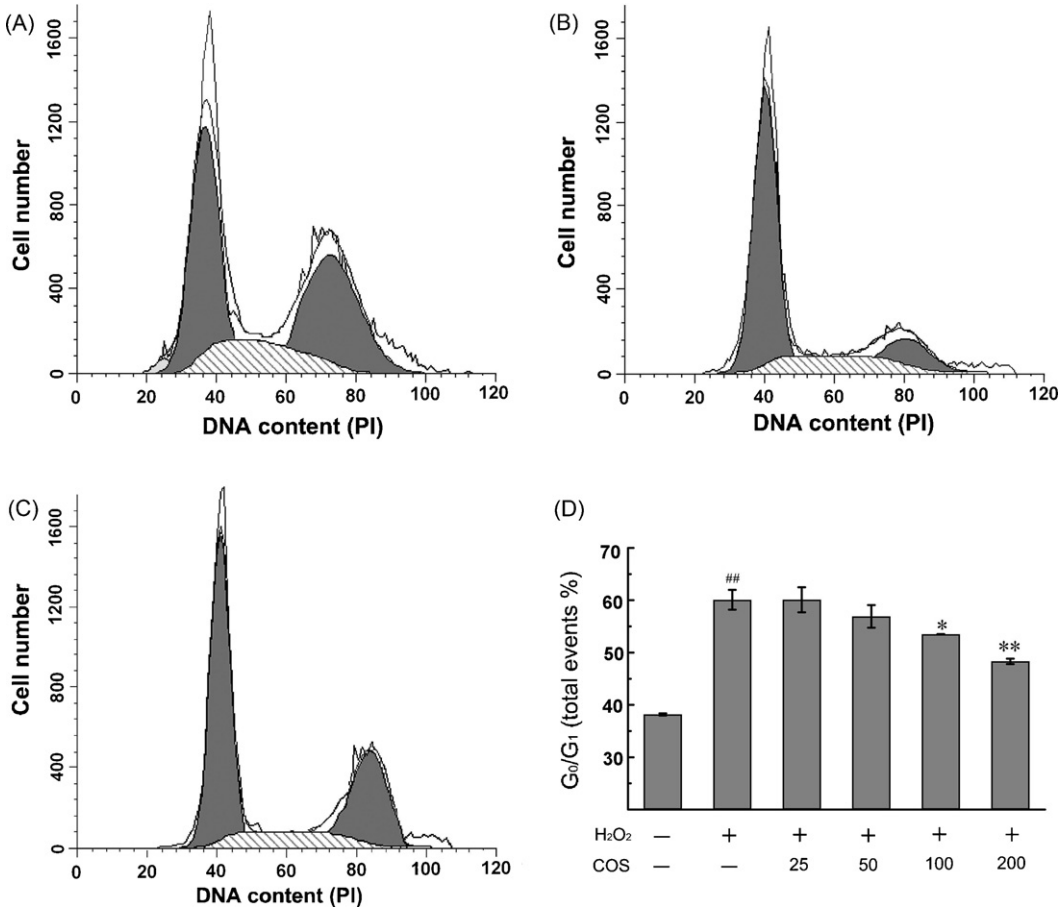

Fig. 7. Flow cytometry for cell cycle analysis. ECV304 cells were harvested and labeled with PI, and analyzed by flow cytometer. The figures showed representative flow cytometric results of vehicle-treated control cells (A), cells exposed to 300 μM of H_2O_2 for 12 h (B) and cells pre-incubated with COS (200 μg/ml) for 24 h prior to 12 h of H_2O_2 exposure. (D) Cells were pre-incubated with indicated concentrations of COS for 24 h prior to 12 h of H_2O_2. Values are means ± S.D. ($n = 3$). $^{\#\#}P < 0.01$ compared to vehicle-treated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the vehicle-treated control group.

antioxidants, play a pivotal role in preventing cellular damage caused by ROS [31]. Therefore, the intracellular ROS can be effectively eliminated by the combined action of SOD, GSH-Px and other endogenous antioxidants, which provide a repairing mechanism for oxidized membrane components. In the present study, significant decreases in SOD and GSH-Px were observed in ECV304 cells after exposure to H_2O_2, indicating the impairment in antioxidant defenses. In addition, an obvious elevation of MDA production was associated with an increase of LDH release. Nonetheless, when ECV304 cells were pre-incubated with COS, these H_2O_2-induced cellular events were blocked to a great extent. Our studies also show a similar efficiency in protecting ECV304 cells against H_2O_2-induced oxidative damage between COS (100–200 μg/ml) and Vitamin C (250 μg/ml). These results jointly suggest that, enhancement of endogenous antioxidant preservation and attenuation of lipid peroxidation may represent a major mechanism of cellular protection by COS.

NO, derived from the action of NOS, is one of the most important mediators in the regulation of endothelial functions [41]. Decreased NO content is associated with increased oxidative flux and accordingly impaired endothelium-dependent vasodilation [42]. Indeed, there have been several reports that down-regulation of NO production is implicated in the pathogenesis and clinical course of all known cardiovascular diseases and is related to future risk of adverse cardiovascular events [43]. In our previous studies, the production of NO in resting neutrophils was significantly increased after incubation with different concentrations of COS [8]. In keeping with previous results, the current studies show that pre-incubation with different concentrations of COS resulted in a further increase in the production of both intracellular NO content and extracellular NO release as compared to H_2O_2-induction alone, and this was accompanied by an up-regulated NOS level. It was apparent that COS may increase the NO content by promoting NOS production. Interestingly, H_2O_2 also directly up-regulated the levels of NO and NOS in ECV304 cells, suggesting that over-production of NO by endothelial cells in response to H_2O_2 stimulation was intended to protect the cells, rather than producing cell injury [18,44]. There have been still some contrary results regarding the effects of COS on the NO synthesis. For example, COS can significantly inhibit NO production in human colorectal adenocarcinoma cells (HT-29) induced by proinflammatory cytokines [45], as is also seen in murine macrophage cells (RAW264.7) induced by LPS [46]. These contradictory effects of COS may depend, at least in part, on different cell types and diverse proinflammatory cytokines.

A role for ROS to mediate apoptosis of endothelial cells has been documented by the demonstration that the radical scavengers Vitamin C and *N*-acetylcysteine inhibit apoptosis of endothelial cells in response to exposure to oxidized LDL, which increase oxidative flux within endothelial cells [47,48]. The importance of oxidative stress in induction of apoptosis has also been suggested by Slater et al. [49]. Recently, H_2O_2 has been suggested as an inducer of apoptosis in several types of cells as a consequence of the disruption of mitochondrial electron transport [37,50]. Our results show that H_2O_2, a major source of ROS, at 300 μM, can markedly increase the permeability in ECV304 cells as demonstrated by LDH leakage and augment the amount of membrane phosphatidylserine

translocating to cell surface as measured by flow cytometry, indicating the presence of apoptotic components in H_2O_2-induced cell injury. Agents that inhibit production of ROS or enhance cellular antioxidant defenses can prevent apoptosis and protect cells from damaging effects of oxygen radicals [51,52]. In agreement with these reports, pre-incubation of COS with ECV304 cells can distinctly protect against H_2O_2-induced apoptosis. Thus, we presume that the abilities of COS to inhibit production of ROS may be responsible for their anti-apoptotic activities. Conversely, a recent study by Wang et al. provided a different observation that addition of high concentrations of COS (250–1000 μg/ml) exhibited the anti-proliferative and pro-apoptotic effects in cultured HUVECs [53], in contrast with our findings in ECV304 cells. It is well known that cells will respond to physical and chemical stimulations that control many aspects of cell function including proliferation, differentiation, and death. Perhaps this difference in observations can be explained by the different doses of COS and the different nature of stimuli.

Suppression of cell growth accompanied by their accumulation in the G_0/G_1 phase of the cell cycle increases the likelihood of any cell damage [54]. The cumulative effects of such an inhibition could be increased propensity for further cell damage, which is often regarded as the triggering event for development of apoptosis [55]. As revealed in our studies by flow cytometric analysis, a higher percentage of ECV304 cells was accumulated in G_0/G_1 phase of the cycle after H_2O_2 exposure, and this can be reversed by pre-incubation with COS. The effects of COS on ECV304 cells, i.e., induction of proliferation and cell cycle arrest in $G_1/S+M$ phase, are compatible with their anti-apoptotic activities. Taken together, these results give credence to the notion that COS, through the cell cycle arrest in $G_1/S+M$ phase, may reduce damage to ECV304 cells and, in turn, the development of apoptosis.

In conclusion, the present studies clearly show that COS can attenuate H_2O_2-induced stress injury in ECV304 cells. The underlining mechanisms of protective effects of COS are partly contributed to a combination of inhibiting intracellular ROS and restoring the activities of endogenous antioxidants, along with the capacity of suppressing endothelial cell apoptosis subsequent to the amelioration of ROS. The antioxidant properties of COS hold great potential for the treatment of oxidative diseases. Since oxidative stress-induced endothelial cell injury plays a key role in atherosclerosis, our findings suggest a novel application for COS in the treatment of cardiovascular diseases.

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