Chitooligosaccharides inhibit ethanol-induced oxidative stress via activation of Nrf2 and reduction of MAPK phosphorylation

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Abstract. Chitooligosaccharides (COS) are hydrolyzed products of chitosan and have been proven to exhibit various biological functions. The aims of this study were to investigate the mechanisms underlying the hepatoprotective effects of COS against ethanol-induced oxidative stress *in vitro*. Human L02 normal liver cells were pretreated with COS (0.25, 0.5 and 1.0 mg/ml) and then hepatotoxicity was stimulated by the addition of ethanol (80 mM). Pretreatment with COS protected L02 cells from ethanol-induced cell cytotoxicity through inhibition of reactive oxygen species generation. Furthermore, ethanol-induced lipid peroxidation and glutathione depletion was inhibited by COS. The antioxidant potential of COS was correlated with the induction of antioxidant genes including HO-1, NQO1 and SOD via the transcriptional activation of nuclear factor erythroid-2-related factor-2 (Nrf2). Additionally, the protective effects of COS against ethanol were blocked by Nrf2 knockdown. Moreover, signal transduction studies showed that COS was able to suppress the ethanol-induced phosphorylation of p38 MAPK, JNK and ERK. In conclusion, the COS-mediated activation of Nrf2 and reduction of MAPK phosphorylation may be important for its hepatoprotective action.

Introduction

Alcohol is one of the most widely used psychoactive substances worldwide. Alcohol consumption carries a risk of adverse health effects since alcohol cannot be excreted and must be metabolized, primarily by the liver. If alcohol is consistently consumed at high levels, the metabolic byproducts can promote the development of steatosis, which can progress to steatohepatitis, fibrosis, cirrhosis, liver failure and/or hepatocellular carcinoma (1). Although diverse mechanisms are involved in alcohol-induced hepatotoxicity, accumulating evidence has supported that oxidative stress is crucial in the pathogenesis and progression of alcoholic liver disease (2). Oxidative stress refers to the enhanced generation of reactive oxygen species (ROS) and/or depletion of the antioxidant defense system, causing an imbalance between pro-oxidants and antioxidants (3). Thus, antioxidant therapy, as a promising strategy for alcoholic liver disease treatment, has been gaining attention.

Chitosan is derived from the deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi (4). Chitooligosaccharides (COS) can be generated by the chemical or enzymatic hydrolysis of chitosan (5). COS has attracted interest in pharmaceutical and medicinal applications due to high solubility and non-toxicity. In a previous study, COS significantly attenuated alcohol-induced cytotoxicity in human HepG2 cells (6). However, the molecular mechanisms for the cytoprotection of COS against alcohol are poorly understood (6).

Nuclear factor erythroid-2-related factor-2 (Nrf2) is a basic leucine zipper transcription factor that binds to antioxidant response element (ARE) sequences in the promoter regions of specific genes (7). The levels of intracellular antioxidants and antioxidant enzymes are regulated by Nrf2. Under normal conditions, Nrf-2 is combined with Kelch-like ECH-associated protein 1 (Keap-1) in the cytosol. Under oxidative stress, Nrf-2 dissociates from Keap-1 and the unbound Nrf-2 translocates into the nucleus where it binds to the ARE of antioxidant genes (8). These genes include the rate-limiting enzyme in the glutathione (GSH) synthesis pathway, superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (9). Findings of recent studies identified that Nrf-2 activation protected against alcohol-induced liver damage (10,11). However, whether the beneficial effects of COS are associated with the activation of Nrf-2 remains to be clarified.

Mitogen-activated protein kinase (MAPK) cascades are another major signaling pathway that is activated in response to various cellular stimuli. Evidence has shown that the MAPK family is essential in the initiation of cell processes such as proliferation, differentiation, development, apop-

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tosis, oxidative stress and inflammatory responses (12). The major MAPK subfamilies identified are p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (13). One type of stress that induces the potential activation of MAPK pathways is oxidative stress caused by ethanol (14). Therefore, drugs, which exert effects against the ethanol-induced activation by attenuating MAPK, may be candidates for therapeutic use in liver diseases.

In the present study, we investigated whether COS could confer protection against ethanol-induced oxidative damage in human L02 normal liver cells. To determine the underlying mechanisms of COS, we assessed the effects of COS on Nrf2 activation and MAPK phosphorylation following ethanol exposure.

Materials and methods

Cell culture and drug treatment. COS (degree of deacetylation ≥95%; average molecular weight, <1,000 Da) was obtained from the Dalian Institute of Chemical Physics (Dalian, China). Human L02 normal liver cells were obtained from the Chinese Academy of Science (Shanghai, China), and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sijiqing, Hangzhou, China) at 37˚C in a 5% CO_2 incubator. Cultures were allowed to reach 80-90% confluence prior to experiments being initiated. The cells were pretreated with different concentrations of COS (0.25, 0.5 and 1.0 mg/ml) and incubated in a humidified incubator at 37˚C for 1 h. Ethanol (80 mM) was then added as a final concentration and incubated for 24 h. Untreated cells served as the control.

MTT assay. L02 cells were seeded in a 96-well plate at a density of $5.0x10^3$ cells/ml. Following treatment with the above-mentioned methods, the medium was removed and 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml; Sigma, St. Louis, CA, USA) was added to each well. The cells were then incubated in the dark at 37˚C for an additional 4 h. The reaction was stopped by the addition of 150 μl DMSO (Sigma) and the absorbance of samples at 570 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100%. The viability of L02 cells in other groups was presented as a percentage of the control cells.

Detection of cell apoptosis by flow cytometry. Cells were collected, followed by the addition of 5 μl FITC-labeled Annexin V and 2 μl propidium iodine (PI) (both from Beyotime Biotechnology, Nantong, China). The cells were mixed well and bathed in warm water for 15 min at 37˚C in the dark, followed by the addition of 400 μl buffer solution to detect cell apoptosis. Stained cells were filtered through a nylon-mesh sieve to remove cell clumps and analyzed by a FACScan flow cytometer and CellQuest analysis software (Becton-Dickinson, Franklin Lakes, CA, USA).

Measurement of ROS production. 2'7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is able to diffuse through the cell membrane and become enzymatically hydrolysed by intracellular esterases to produce non-fluorescent DCFH. The oxidation of DCFH by intracellular ROS results in fluorescent DCF which stains the cells. Thus, the intracellular ROS generation of cells can be detected and quantified by DCFH-DA. Cell samples were incubated in the presence of 10 μM DCFH-DA (Sigma) in phosphate-buffered saline (PBS) at 37˚C for 30 min, washed two times with PBS and centrifuged at 1,200 rpm to remove the extracellular DCFH-DA. Stained cells were examined under a fluorescence spectrophotometer (Hidex Oy, Turku, Finland) at 498/530 nm. The percentage of DCF fluorescence was compared with the control, which was arbitrarily assigned as 100%.

Determination of lipid peroxidation and reduced GSH. Accumulated intracellular lipid peroxidation was measured as MDA equivalent generated, as an indicator of lipid peroxidation in cultured cell lysates (10). The levels of MDA and GSH were detected using commercial kits (Jiancheng, Nanjing, China). The MDA levels were measured at 535 nm based on the reaction of thiobarbituric acid (TBA) with MDA, while the GSH levels were measured at 412 nm following the reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Transfection of small interfering RNA. The small interfering RNA (siRNA) directed against Nrf2 (Nrf2-siRNA) or non-targeting negative control siRNA (NC-siRNA) was purchased from GenePharma (Shanghai, China). L02 cells were transfected with the Nrf2- or NC-siRNA with Lipofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, the cells were seeded in 6-well plates at a density of $2x10^5$ cells/well in 2 ml of complete DMEM. The cells were allowed to grow to 60-80% confluence prior to transfection with siRNA. For each well, 100 pmol of siRNA were mixed with 2 μl of Lipofectamine. Serum- and antibiotic-free DMEM medium (500 μl) was subsequently added. The cells were exposed to the transfection mixture for 6 h. At the end of the incubation period, 1.5 ml of antibiotic-free complete medium was added and the cells were cultured for an additional 18 h. Following treatment with COS and/or ethanol, the cells were harvested and Nrf2 expression was determined by quantitative PCR (qPCR) and western blotting.

qPCR analysis. Total RNA of cells was extracted using TRIzol reagent (Invitrogen). Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μl containing 200 units of SuperScript II RNase H Reverse Transcriptase (Invitrogen) at 42˚C for 50 min. This was followed by inactivation at 70˚C for 15 min. The products were amplified for 40 cycles for 10 sec at 95˚C for denaturation and 30 sec at 56˚C for annealing. GAPDH expression was used as endogenous control for the normalization of gene expression. The primer sequences for the genes are shown in Table I and were synthesized by Sangon (Shanghai, China).

Western blot analysis. The treated cells were lysed and centrifuged at 12,000 x g for 5 min at 4˚C. The supernatant was collected and 5X protein loading buffer was added followed by boiling for 10 min. After SDS-PAGE, the cells were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The

Table I. Oligonucleotide sequences for qPCR of Nrf2 and antioxidative enzymes.

Gene	Sequences (5'-3')
HO-1	F: CTGACCCATGACACCAAGGAC
	R: AAAGCCCTACAGCAACTGTCG
SOD	F: CGGATGAAGAGAGGCATGTT
	R: CACCTTTGCCCAAGTCATCT
NQO1	F: TTCTCTGGCCGATTCAGAGT
	R: GGCTGCTTGGAGCAAAATAG
Nrf2	F: ACACGGTCCACAGCTCATC
	R: TGTCAATCAAATCCATGTCCTG
GAPDH	F: CCAACCGCGAGAAGATGA
	R: CCAGAGGCGTACAGGGATAG

qPCR, quantitative PCR; Nrf2, nuclear factor erythroid-2 related factor-2; HO-1, heme oxygenase-1; SOD, superoxide dismutase; F, forward; R, reverse.

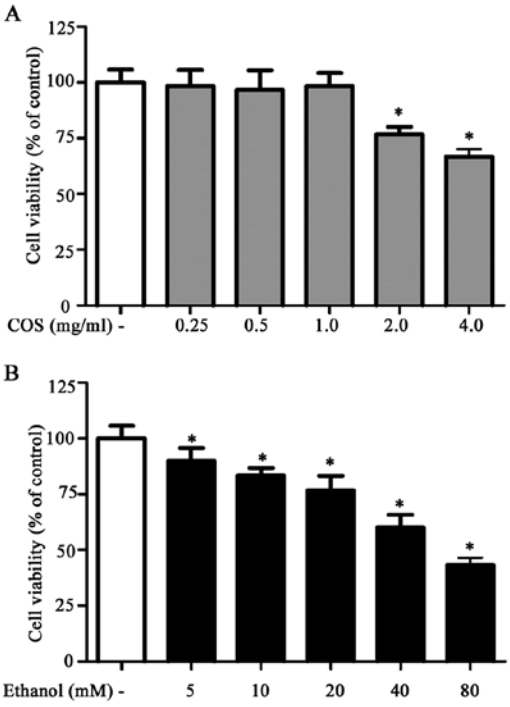

Figure 1. Cytotoxic effects of COS and ethanol on cultured human L02 normal liver cells. Cell viability was measured by MTT assay. (A) Cells were treated with various concentrations of COS for 24 h. (B) Cells were treated with the indicated concentrations of ethanol for 24 h. Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. COS, chitooligosaccharides.

membranes were blocked with 5% non-fat milk solution for 1 h at room temperature, and then incubated with the specific primary antibodies overnight at 4˚C. After washing three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotechnology) for 1 h at room temperature. The ECL (Beyotime Biotechnology) chemiluminescence method was used to visualize the protein bands. Antibodies directed against phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-ERK, p38 MAPK, JNK and ERK were obtained from Cell Signaling Technology (Beverly, MA, USA). SOD, NQO1, HO-1, Nrf2, histone H3 and GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software. Each assay was performed at least 3 times. Data are presented as means ± SD. The Student's t-test was used to evaluate the significance of data. P<0.05 was considered to indicate a statistically significant result.

Results

COS effectively suppresses ethanol-induced cytotoxicity in L02 cells. Prior to *in vitro* hepatoprotective studies, the cytotoxic effect of COS was examined in human L02 normal liver cells. Results of the MTT assay showed that L02 cells treated with increasing concentrations of COS (0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) for 24 h did not show any cytotoxic effect up to the concentration of 1.0 mg/ml. The concentration of ethanol required to induce oxidative stress and cell death in L02 cells was also examined. Among the various concentrations tested (5, 10, 20, 40 and 80 mM), ethanol caused loss of cell viability at a concentration of 80 mM over a period of 24 h incubation (Fig. 1B). Therefore, the non-cytotoxic concentrations of COS (0.25, 0.5 and 1.0 mg/ml) and cytotoxic concentration of ethanol (80 mM) were selected as the standard concentrations for the subsequent assessments.

Different concentrations of COS were evaluated for its protective effect against ethanol-induced toxicity employed in L02 cells. As shown in Fig. 2A, ethanol treatment markedly decreased L02 cell viability to 46.3±3.2% compared with the control group (P<0.05). However, pretreatment with COS significantly attenuated the decrease of cell viability, which was 66.1± 2.1, 70.5±4.3 and 78.6±2.5% for COS (0.25, 0.5 and 1.0 mg/ml) (P<0.05). Furthermore, the apoptosis triggered by ethanol with or without pre-incubation of COS was detected by flow cytometry (Fig. 2B). The results showed that the apoptosis ratio of L02 in the control group was 6.22%, and it increased to 18.47% (P<0.05) following induction by ethanol for 24 h. After the cells were pretreated with COS (0.25, 0.5 and 1.0 mg/ml) for 1 h and induced by ethanol for 24 h, the cell apoptotic ratios were 16.15, 12.91 and 9.73%, respectively (P<0.05). These results suggested that COS was able to efficiently protect L02 cells against oxidative stress induced cell damage in a dose-dependent manner.

COS pretreatment prevents ethanol-induced ROS generation in L02 cells. Sustained intracellular ROS production has been recognized as a crucial step for ethanol-induced oxidative stress (15). Fig. 3 shows the mean values of DCF fluorescence, an indicator of intracellular ROS generation. L02 cells exposed to ethanol at 80 mM exhibited a significant increase in the intracellular level of ROS as compared with that in the control cells (P<0.05). However, the increase in intracellular ROS (235%) caused by ethanol was significantly reduced to 181, 157 and 129% by 0.25, 0.5 and 1.0 mg/ml of COS, respectively (P<0.05).

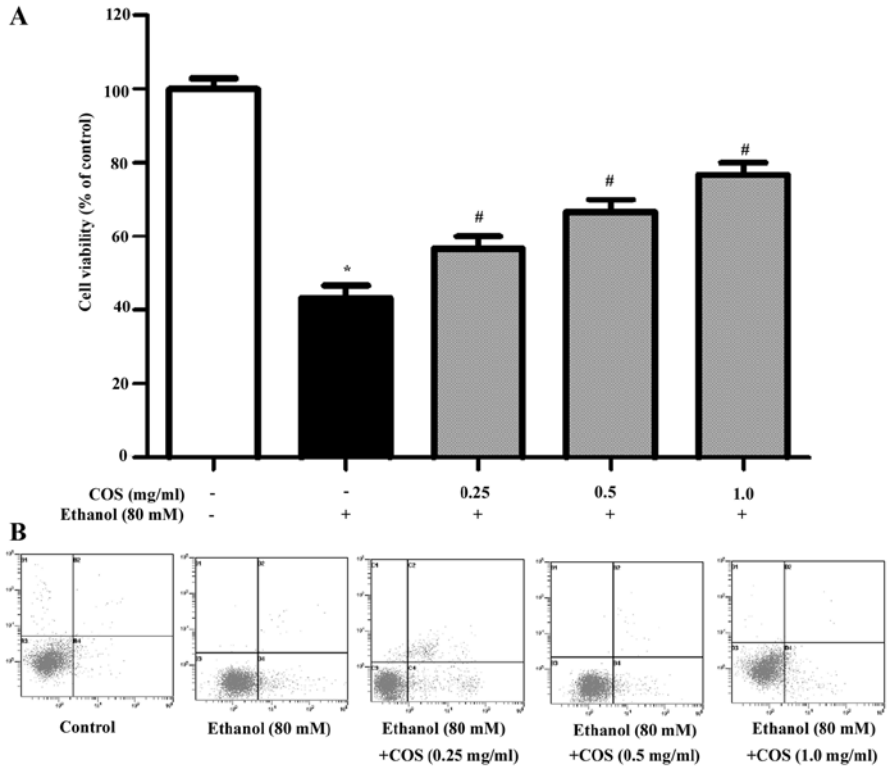

Figure 2. Protective effects of COS on ethanol-induced cell death in L02 cells. Cells were pretreated with increasing concentrations of COS for 1 h, then oxidative stress was induced by ethanol for 24 h. (A) Cell viability was measured by MTT assay. (B) Cell apoptosis was detected by flow cytometry. Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. #P<0.05 was considered significant for sample vs. ethanol COS, chitooligosaccharides.

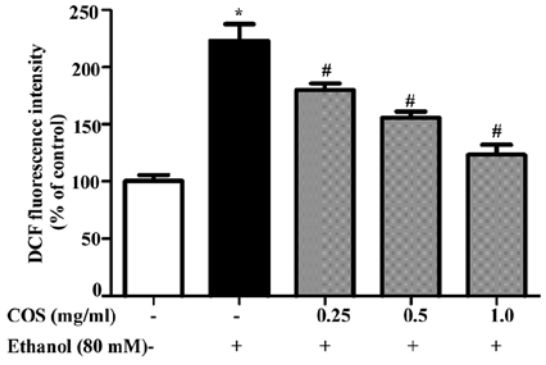

Figure 3. Protective effects of COS on ethanol-induced ROS generation in L02 cells. The percentage of fluorescence intensity of the DCF-stained cells was quantified using a fluorescence spectrophotometer. Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. #P<0.05 was considered significant for sample vs. ethanol. COS, chitooligosaccharides; ROS, reactive oxygen species.

COS inhibits ethanol-induced lipid peroxidation and GSH depletion in L02 cells. Inhibitory effect of COS on oxidative damage in L02 cells induced by ethanol was evaluated by lipid peroxidation assay. As shown in Fig. 4A, ethanol-exposure significantly (P<0.05) increased the intracellular MDA level (45±1.2 μM) compared to the control group (4±0.8 μM). However, the elevated MDA levels were significantly (P<0.05) decreasesd to 33±0.5, 23±1.7 and 14±0.6 μM at 0.25, 0.5 and 1.0 mg/ml of COS, respectively. By contrast, the effect of COS on ethanol-induced GSH depletion was investigated. Fig. 4B

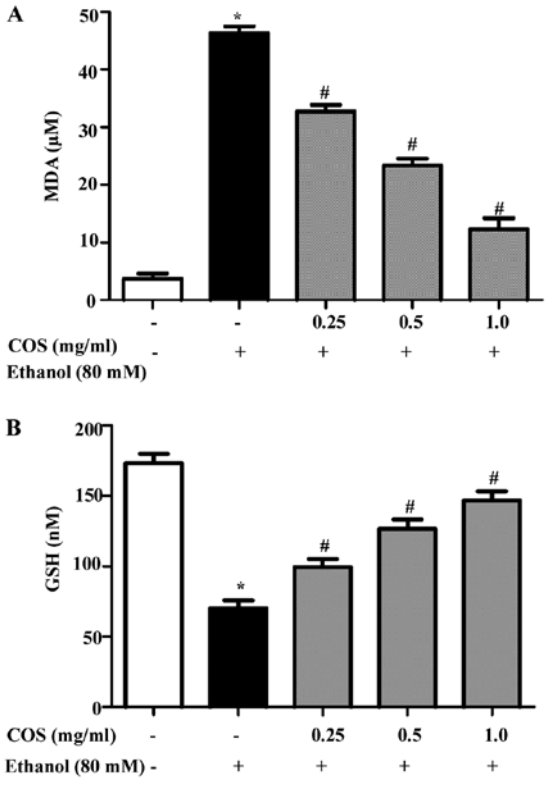

Figure 4. Protective effects of COS on ethanol-induced (A) MDA and (B) GSH depletion in L02 cells. Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. #P<0.05 was considered significant for sample vs. ethanol. COS, chitooligosaccharides; GSH, glutathione.

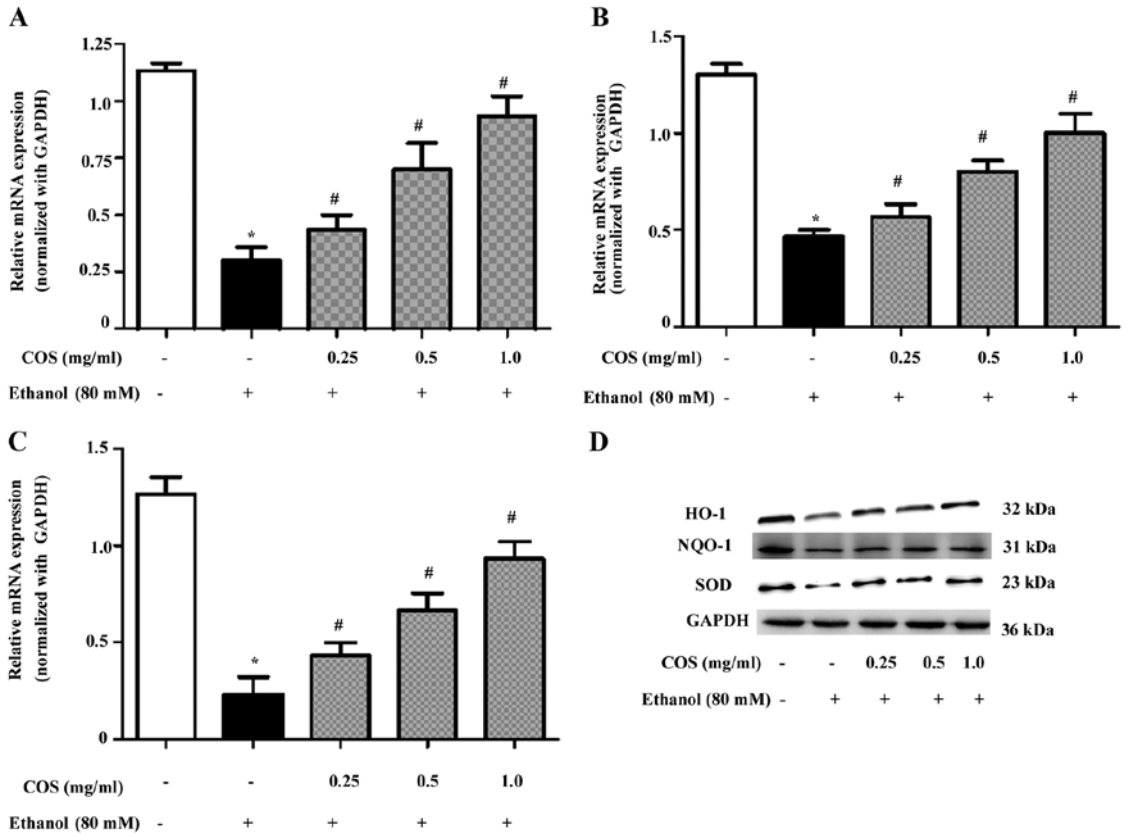

Figure 5. Effects of COS on ethanol-induced antioxidant genes expression in L02 cells. qPCR analysis was performed to monitor the mRNA expression levels of (A) HO-1, (B) NQO-1 and (C) SOD. Protein expression levels of HO-1, NQO-1 and SOD were monitored by (D) western blot analysis. Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. #P<0.05 was considered significant for sample vs. ethanol. COS, chitooligosaccharides; HO-1, heme oxygenase-1; SOD, uperoxide dismutase.

shows that ethanol depleted the GSH levels (71±2.1 nM) significantly (P<0.05) compared to that of the control cells (169±3.6 nM). However, the levels were effectively restored by pretreatment with COS in a dose-dependent manner (99±2.3, 127±1.9 and 145±2.8 nM at concentrations of 0.25, 0.5 and 1.0 mg/ml, respectively).

COS upregulates antioxidant gene expression in ethanol-induced L02 cells. To determine whether the inhibitory effect of COS on ethanol-induced oxidative stress was associated with the induction of antioxidant genes, we examined the mRNA and protein expression of HO-1, NQO-1 and SOD. The qPCR analysis showed that pretreatment with COS significantly (P<0.05) increased the mRNA expression levels of HO-1 (Fig. 5A), NQO-1 (Fig. 5B) and SOD (Fig. 5C) in a dose-dependent manner. In addition, the western blot analysis confirmed that pretreatment with COS significantly (P<0.05) increased HO-1, NQO-1 and SOD protein expression in ethanol-induced L02 cells (Fig. 5D).

COS stimulates the nuclear translocation of Nrf-2 in L02 cells. It is well known that antioxidant genes including HO-1, NQO-1 and SOD are transcribed by Nrf2, a major transcription factor regulating ARE-driven phase II gene expression (9). To investigate the effect of COS on the nuclear translocation of Nrf-2, cytosolic and nuclear fractions were prepared, and the protein levels of Nrf-2 were measured by western blot

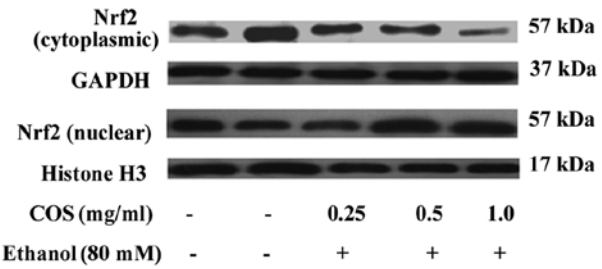

Figure 6. COS enhanced Nrf-2 nuclear translocation in ethanol-induced L02 cells. Nuclear and cytoplasmic lysates were prepared and subjected to western blot analysis. Histone H3 for nuclear fraction and GAPDH for cytoplasmic fraction was used as an internal control. COS, chitooligosaccharides.

analysis. The results indicated that the nuclear protein levels of Nrf-2 were dose-dependently increased in L02 cells treated with COS, while the cytosolic protein levels of Nrf-2 were decreased (Fig. 6).

Nrf2 knockdown decreases the protective effects of COS in L02 cells. To investigate whether the protection of COS against ethanol-induced toxicity was attributed to the activation of Nrf2, we developed an Nrf2 gene knockdown model in L02 cells using siRNA transfection. As shown in Fig. 7A and B, L02 cells transfected with Nrf2 siRNA exhibited a direct reduction at the levels of Nrf2 mRNA and protein (P<0.05),

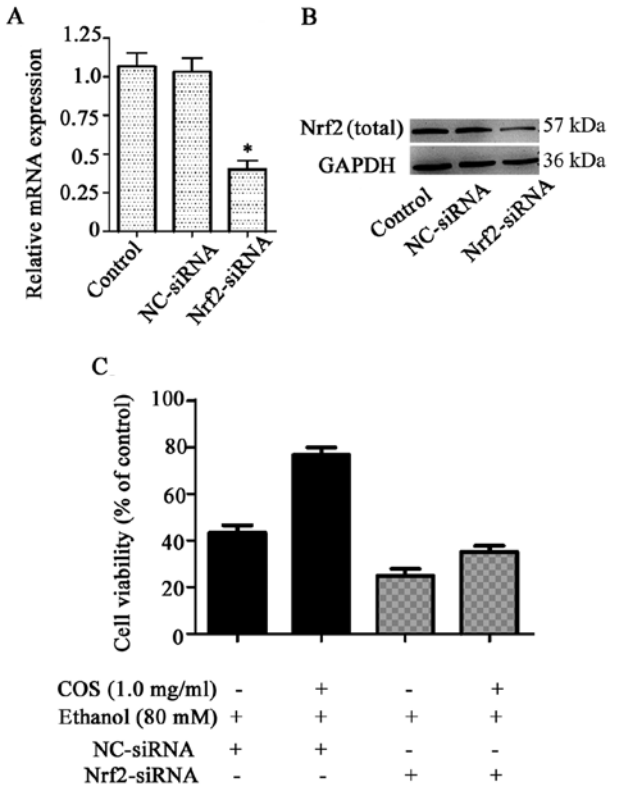

Figure 7. Nrf2 knockdown decreased the protective effects of COS in ethanol-induced L02 cells. L02 cells were transfected with Nrf2- or NC-siRNA. (A) qPCR results of Nrf2 mRNA. (B) Western blot results of Nrf2 protein. After 24 h of transfection, the cells were incubated with or without COS (1.0 mg/ml) for 1 h and were induced by ethanol (80 mM) for 24 h. Cell viability was determined by MTT assay (C). Results are presented as means ± SD of three independent experiments. *P<0.05 was considered significant for sample vs. control. Nrf2, nuclear factor erythroid 2-related factor-2; COS, chitooligosaccharides.

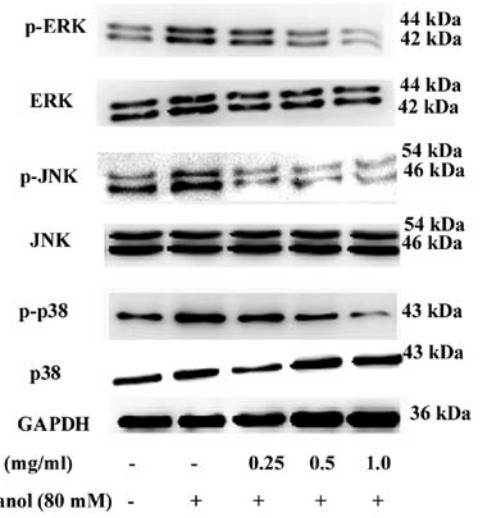

Figure 8. Effects of COS on ethanol-induced MAPK activation in L02 cells. Cells were pretreated with different concentrations of COS for 1 h and then exposed to ethanol for 24 h. Total cell lysates or the nuclear fraction were prepared and subjected to western blot analysis. Phosphorylated and non-phosphorylated forms of p38 MAPK, JNK and ERK were examined. GAPDH was used as a loading control. COS, chitooligosaccharides; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

whereas no significant inhibitory effect was observed in the control group and cells treated with NC-siRNA (P>0.05). Nrf2 knockdown cells were treated with COS (1.0 mg/ml) for 1 h prior to the challenge with 80 mM ethanol. Cell viability was measured by MTT assay. Compared to ethanol-treated cells (45.1±2.2%), COS pretreatment significantly increased the amount of viable cells to 74.9±1.8% in the NC-siRNA system, whereas the percentage of viable cells in the Nrf2 knockdown system was further decreased to 24.1±2.3% by ethanol. COS, therefore, failed to protect liver cells from ethanol-induced cell death in the Nrf2 knockdown system, as shown by the 36.4±2.4% cell viability observed (Fig. 7C). We found Nrf2 siRNA treatment, not only aggravated the adverse effects of ethanol, but also abrogated the protective effects of COS.

COS treatment attenuates ethanol-induced MAPK phosphorylation in L02 cells. MAPK cascades are key signaling pathways involved in the regulation of normal cell proliferation and survival (12). Therefore, we measured the level of phosphorylated MAPKs in the presence and/or absence of COS. As shown in Fig. 8, p38 MAPK, JNK and ERK were highly phosphorylated in L02 cells when treated with ethanol alone. By contrast, the increase of p38 MAPK, JNK and ERK phosphorylation were suppressed by COS pretreatment. COS reduced MAPK activation in a dose-dependent manner, suggesting that MAPK inhibition by COS can result in increased survival in L02 cells.

Discussion

Ethanol-induced liver disease remains a worldwide health concern without effective therapies. Since oxidative stress is a well-established factor in the progression of liver damage due to alcohol intake, using antioxidants to ameliorate oxidative stress and alleviate ethanol hepatotoxicity is a logical approach. This approach has shown a high rate of success in *in vitro* and *in vivo* models. For example, EGCG is the major and most active polyphenolic antioxidant present in green tea. It has been confirmed that EGCG exerted a protective action against ethanol-induced toxicity in Chang liver cells (16). Diosmin, a naturally occurring flavone glycoside readily obtained by dehydrogenation of hesperidin, alleviated alcoholic liver injury via inhibition of oxidative stress markers in female wistar rats (17). Lucidone, isolated from the fruits of *Lindera lucida*, also protected HepG2 cells against ethanol-induced cytotoxicity (10). In the present study, we have demonstrated that COS effectively inhibited ethanol-induced oxidative damage in human L02 normal liver cells. A similar effect was observed in an *in vitro* system in which ethanol was used to induce oxidative stress in cultured human hepatic (HepG2) cells (6). These results suggest that oxidative stress critically contributes to ethanol hepatotoxicity, and that antioxidant protection is an effective strategy to prevent alcohol liver diseases.

Oxidative stress-induced cell death is associated with an increase in ROS, such as hydrogen peroxide, nitric oxide, superoxide anion, hydroxyl radicals and singlet oxygen (18).

The accumulation of ROS in the liver was found to cause dysfunction of cellular membrane systems, protein and DNA oxidation and eventually hepatocyte injury. This damage if unrepaired irreversibly caused cells to undergo apoptosis (19). Ethanol promoted oxidative stress, by the increased formation of ROS and by the depletion of oxidative defenses in the cell (20). Thus, removal of excess ROS or suppression of their generation by antioxidants may be effective in preventing oxidative cell death induced by ethanol. Studies have been conducted to investigate the antioxidant effects of natural products and their findings showed significant results in ROS production (21,22). In the present study, we have shown from the results obtained that COS is a potential source that can be used in medical- or pharmaceutical-related fields. Ethanol exposure was found to induce the overproduction of ROS in L02 cells, leading to oxidative stress. However, treatment with COS significantly suppressed ethanol-induced ROS generation and increased the survival rate of L02 cells probably through potent antioxidant activity.

Lipid peroxidation is a prominent manifestation of ROS and oxidative stress in biological systems. Exposure to chemical or physical agents triggers membrane-free radical reactions in living cells, which accelerates lipid peroxidation (23). A large number of toxic byproducts are formed during lipid peroxidation such as MDA, 4-hydroxynonenal, conjugated-dienes, lipid hydroperoxides and isoprostanes (24). The concentration of MDA in cells or tissue lysates was considered to be a major lipid peroxidation marker (10). We observed that ethanol exposure sustained MDA levels in L02 cells, whereas the increase of MDA levels was significantly inhibited by COS pretreatment. In addition, GSH can act as a non-enzymatic antioxidant by direct interaction of its sulfhydryl group with ROS or it can be involved in the enzymatic detoxification of ROS, as a cofactor or coenzyme (25). Therefore, reduced GSH content was also used to evaluate oxidative stress in biological systems. In the present study, we found that ethanol exposure decreased GSH levels in L02 cells, which is in agreement with a previous report (26). Notably, results of this study also show that COS pretreatment prevented ethanol-induced GSH depletion in liver cells.

Besides exogenous antioxidant defense, the body depends on several endogenous defense mechanisms to protect against oxidative stress. Among these antioxidant molecules, the phase II enzymes including HO-1, NQO-1 and SOD play important roles in the exclusion of ROS (27). HO-1 is a stress inducible protein. Various stimuli, such as thiol scavengers, ultraviolet radiation and oxidative stress act as inducers of HO-1 (28). Increased HO-1 expression has been reported to reduce cell injury such as oxidative stress, pro-inflammatory cytokine production and the activation of pro-apoptotic inducers (29). NQO-1 is an antioxidant that is upregulated in response to hyperoxia and oxidative stress (30). SOD is the major enzyme for scavenging ROS, which can catalyze the conversion of the superoxide anion into oxygen and hydrogen peroxide (31). In the present study, we found that COS treatment significantly enhanced HO-1, NQO-1 and SOD expression at the mRNA and protein levels. As HO-1, NQO-1 and SOD can be activated by Nrf-2, a major transcription factor regulating ARE-driven phase-II gene expression, the cytosolic and nuclear protein levels of Nrf-2 were then detected by western blotting. The data showed that the nuclear protein levels of Nrf-2 were dose-dependently increased in L02 cells treated with COS, while the cytosolic protein levels of Nrf-2 were decreased, which indicated that COS was able to enhance the nuclear translocation of Nrf-2. Previous studies have shown that increasing Nrf-2 activity in hepatic tissues was highly hepatoprotective during ethanol-induced oxidative stress (32). We also found Nrf2 knockdown decreased the protective effects of COS in L02 cells. These results strongly suggested that COS protects liver cells from oxidative stress and cell death via the Nrf2-dependent pathway. In a previous study, COS was found to increase cellular antioxidant defense through the Nrf2 pathway in rat pheochromocytoma (PC12) cells (12). Thus, it can be concluded that Nrf2 activation by COS is not cell type-dependent and it can be elicited in cell lines of human and rat origin.

MAPKs are serine-threonine protein kinases that play a significant role in signal transduction from the cell surface to the nucleus (12). Diverse cellular functions, ranging from cell survival to cell death, are regulated by MAPK signaling. A number of extracellular and intracellular stimuli that induce ROS production concomitantly can activate MAPK pathways in multiple cell types (33). For example, direct exposure of vascular endothelial cells to lipopolysaccharide (LPS), to mimic oxidative stress, led to the activation of MAPK pathways (34). PC12 cells treated with hydrogen peroxide, a ROS-generating agent, increased the phosphorylation of MAPKs. Ethanol itself and ROS produced by ethanol also modulated MAPKs in hepatocyte-like VL-17A cells (35). Therefore, we detected the phosphorylation of MAPK family proteins including ERK, JNK and p38 MAPK. Results of the western blot analysis revealed that ethanol significantly activated the phosphorylation of these MAPKs. However, COS pretreatment markedly inhibited the phosphorylated levels of MAPK in ethanol-induced L02 cells. It has been reported that COS suppressed p38 MAPK and ERK phosphorylation in LPS-induced N9 microglial cells (36). Based on these results, we conclude that the protective effects of COS on L02 cells may be attributed to the inhibition of phosphorylated levels of MAPKs.

In conclusion, our findings have demonstrated that COS inhibited ethanol-induced oxidative stress in L02 cells through inhibition of ROS generation and upregulation of antioxidant genes including HO-1, NQO1 and SOD at the transcription and translation levels. The fact that COS regulated Nrf2 activation and MAPK phophorylation, along with its established antioxidant effect, makes this compound a candidate to be studied towards the development of a therapeutic strategy for alcoholic liver disease.

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