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## Original Contributions

Chronic vitamin C insufficiency aggravated thioacetamide-induced liver fibrosis in *gulo*-knockout mice

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## ABSTRACT

Given the involvement of oxidative stress in liver-disease- or hepato-toxicant-induced hepatic damage and fibrosis, antioxidants are an effective preventive and therapeutic tool. The beneficial results of vitamin C, one of the physiological antioxidants, have been observed both in experimental animals and in humans. However, most of these studies have been concerned with supplementary vitamin C; the effects of under vitamin C insufficiency, which humans sometimes confront, have not been substantially investigated. In the present study, we established a vitamin C-insufficient animal model (half-to-normal serum vitamin C concentration) with *gulo*<sup>-/-</sup> mice that cannot synthesize vitamin C, and induced hepatotoxicity by means of thioacetamide (TAA) injections twice a week for 18 weeks. Additionally, we explored the direct effects of vitamin C both on immortalized human hepatic stellate LX-2 cells and on rat primary hepatic stellate cells. Vitamin C insufficiency resulted in a decreased survival rate and increased serum markers for hepatocyte damage, such as alanine aminotransferase and aspartate aminotransferase. Concomitantly, the levels of reactive oxygen species (ROS) and lipid peroxides in the liver were increased. Histological examinations of the vitamin C-insufficient liver revealed increases in collagen fiber deposition and activated-hepatic-stellate-cell number. Vitamin C, when directly applied to the LX-2 cells as well as the rat primary hepatic stellate cells, suppressed not only proliferation but hydrogen peroxide-induced collagen expression as well. In conclusion, vitamin C insufficiency exacerbated TAA-induced hepatotoxicity. These effects seem to be mainly from insufficient scavenging of ROS in the liver, and possibly in part, by directly affecting hepatic stellate cells.

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## Introduction

Hepatic fibrosis is a reversible physiological wound-healing process. When damage is sustained, however, this process becomes exacerbated and irreversible, leading to cirrhosis [1]. In various liver diseases, such as viral hepatitis, alcoholic liver disease, and others, hepatic damage has been associated with oxidative stress [2], which plays a pathogenic role in hepatotoxicity induced by various drugs and reagents including but not limited to alcohol [3], cisplatin [4], and thioacetamide (TAA) [5]. Based on this evidence, antioxidants have been extensively

investigated as a therapeutic and preventive tool for acute and chronic liver damage [6].

Vitamin C, a water-soluble micronutrient essential for various biological functions, most importantly collagen fiber synthesis [7], is a physiological antioxidant [8] that has been reported to lower oxidative stress, both in smoking and nonsmoking individuals [9].

In animal models, the protective effects of vitamin C against the hepatotoxicity induced by cadmium [10], ethanol [11], cisplatin [4], carbon tetrachloride [12], among others, have been widely studied under either the acute or the chronic condition or both. All these studies reported beneficial anti-hepatic-damage results. However, the results have limited implications. Humans differ from these animals in that they are unable to synthesize vitamin C by themselves, due to the lack of functional L-gulono- $\gamma$ -lactone oxidase, which is essential in vitamin C biosynthesis [13]. Thus, the results obtained in these animals reflect additional vitamin C supplementation, not vitamin C insufficiency, which human sometimes confront. Furthermore, most of these reports did not show any results with respect to hepatic fibrosis. Recently, an experiment involving guinea pigs [14], which do not synthesize vitamin C and thus, like human, are dependent on exogenous supply, was

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; CYP2E1, cytochrome P450 2E1; Gulo, L-gulono- $\gamma$ -lactone oxidase; HD, high dose; HSCs, hepatic stellate cells; LD, low dose; MDA, malondialdehyde; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction;  $\alpha$ -SMA, alpha-smooth muscle actin; TAA, thioacetamide; TGF- $\beta$ 1, transforming growth factor beta 1; WT, wild-type

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conducted. In the results, vitamin C administered during an abstinence period after 90 days of alcohol ingestion facilitated recovery of hepatic damage, including decreased hepatic collagen synthesis. However, this also was an effect of supplementary vitamin C. A study concerning liver fibrosis under vitamin C deficiency was conducted on senescence marker protein 30 knock-out mice [15]. Unexpectedly, vitamin C deficiency attenuated liver fibrosis on chronic carbon tetrachloride challenge. Thus, the effects of vitamin C deficiency/insufficiency on hepatic fibrosis remain unclear.

In the present study, we reevaluated the effect of vitamin C insufficiency on liver damage and subsequent fibrosis in *gulo*<sup>-/-</sup> mice [16] with low serum vitamin C concentration.

## Materials and methods

### Animals

*Gulo*<sup>-/-</sup> mice were obtained from the Mutant Mouse Regional Resource Center (University of California, Davis, CA). Wild-type littermates were used as a control. Serum vitamin C levels were set within the normal or insufficient range by administration of 3.3 and 0.33 g/L (Sigma-Aldrich, St. Louis, MO) in drinking water, respectively, throughout the experimental period. All of the mice were maintained under a specific pathogen-free condition at our Institute's animal facility. Thioacetamide (Sigma-Aldrich) was injected intraperitoneally, twice a week for 18 weeks (10 mg/kg of body weight for 10 weeks; 20 mg/kg of body weight for 8 weeks). Two days after the final injection, the mice were sacrificed. Male Sprague-Dawley (SD) rats were used to isolate hepatic stellate cells. The experiment had been approved by the Institutional Animal Care and Use Committee (permission No.: SNU-111031-1), and all the procedures were performed according to our Institute's SOP.

### Histological analysis

The liver was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 5 μm thicknesses, and subsequently hematoxylin-and-eosin- and Sirius-red-stained. The fibrotic area was assessed by analysis of three sections per mouse under ×100 magnification using the Quantity One computerized morphometry system (Bio-Rad, Hercules, CA).

Preparatory to immunohistochemical staining of α-smooth muscle actin (α-SMA), the liver sections were incubated in 3% hydrogen peroxide in methanol for 30 min and 2% normal goat serum for 1 h prior to primary antibody application. Anti-mouse

α-SMA antibody (clone 1A4, 1: 500, Sigma-Aldrich) was applied as a primary antibody at 4°C overnight. Then, biotinylated anti-mouse IgG antibody (provided in the Vector Mouse on Mouse kit; Vector Laboratories, Burlingame, CA) and avidin-peroxidase conjugate (Vector Laboratories) were sequentially applied at room temperature. A color reaction was performed with diaminobenzidine-hydrogen peroxide (Sigma-Aldrich). The sections were counterstained with Mayer's hematoxylin.

### Biochemical analysis

The serum levels of activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and TGF-β1 concentration were measured using commercial kits (Biotron Diagnostic Inc., Hemet, CA, and Invitrogen, Carlsbad, CA, respectively). For measurement of the ROS level, the liver was homogenized, and supernatants were obtained and incubated with dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) at 36°C for 1 h, after which the fluorescence intensity was measured in a 96-well polystyrene plate (NUNC, Copenhagen, Denmark) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using the SOFTmax PRO 4.0 program (Molecular Devices, Sunnyvale, CA). Lipid peroxidation in the liver was determined by assessment of malondialdehyde (MDA) production, which was assayed according to the level of thiobarbituric acid-reactive substances using a commercial kit (Cell Biolabs, San Diego, CA). The serum and hepatic vitamin C concentrations were measured by the dinitrophenylhydrazine method, modified as previously described [17]. The hepatic collagen concentration was determined using the Sircol collagen assay (Biodye Science, Biocolor Ltd, Carrickfergus, Northern Ireland, UK). Liver protein was quantified with the bicinchoninic acid (BCA) assay. All of the commercial kit procedures were performed according to the respective manufacturers' instructions.

### RNA extraction and RT-PCR

Total RNA was purified from snap-frozen mouse liver or from LX-2 cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA was generated from RNA as per normal, and amplified using the primer pairs listed in Table 1. Each cycle consisted of denaturation at 95°C for 40 s, annealing at 50 to 60°C for 40 s, and amplification at 72°C for 40 s. After 40 cycles, the PCR products were analyzed by 2% agarose gel electrophoresis and subjected to densitometric analysis using Quantity One software (Bio-Rad).

**Table 1**  
List of primers used in this experiment.

Gene	Forward primer	Reverse primer
<i>mTGFβ-1</i>	5'-CGCATCTATGAGAAAACC-3'	5'-GTAACGCCAGGAATTGT-3'
<i>mSMAD2</i>	5'-TCACAGTCATCATGAGCTCAAGG-3'	5'-TGTGACGCATGGAAGTCTCTC-3'
<i>mSMAD3</i>	5'-GTAGAGACGCCAGTCTTACC-3'	5'-GGTTTGAGAACCTCGTCCAT-3'
<i>mSMAD7</i>	5'-GCACAAAGTGTTCCTGGT-3'	5'-TGACGAGAAGGAGGAGGAA-3'
<i>mCOL1A1</i>	5'-GAGCGGAGAGTACTGGATCG-3'	5'-GTTCCGGCTGATGTACCACT-3'
<i>mα-SMA</i>	5'-GCATCCACGAAACCACCTA-3'	5'-CACGAGTAACAATCAAAGC-3'
<i>mPPARγ</i>	5'-CCCTGGCAAAGCATTTGTA-3'	5'-AATCCTTGGCCCTCTGAGAT-3'
<i>mCYP2E1</i>	5'-CCCTGGCAAAGCATTTGTA-3'	5'-TACATGGGTTCTTGCTGTGT-3'
<i>mGAPDH</i>	5'-GTGGAGTCTACTGGCGTCTT-3'	5'-GCCTGCTTACCACCTTCTT-3'
<i>rCOL1A1</i>	5'-GTGTTGCTGAAAGACTACCTCGT-3'	5'-GGTACAGAGTCTTTGCTTCTC-3'
<i>rβ-actin</i>	5'-GTACAACCTTCTTGAGCTCCT-3'	5'-ACAGGATTCCATACCAGGAA-3'
<i>hCOL1A1</i>	5'-GCCATGTATGGCTATTCA-3'	5'-TGACAGGAGCTTGTAGCAT-3'
<i>hGAPDH</i>	5'-GTGGAGTCTACTGGCGTCTT-3'	5'-GCCTGCTTACCACCTTCTT-3'

### LX-2 cell culture

LX-2 cells, a spontaneously immortalized human hepatic stellate cell line [18], were maintained in DMEM (Welgene, Seoul, Korea) with 10% (v/v) FBS (Gibco), 100 U/ml penicillin (Gibco), and 100 mg/ml streptomycin (Gibco). All of the cultures were grown at 37°C under a 5% CO<sub>2</sub> atmosphere. To induce *col1a1* and  $\alpha$ -SMA expression, cells were serum-starved with 0.2% BSA supplementation for 48 h prior to TGF- $\beta$ 1 (2.5 ng/ml) or H<sub>2</sub>O<sub>2</sub> (1 mM/ml) treatment.

### Isolation and culture of the rat primary hepatic stellate cells (HSCs)

Primary HSCs were isolated from male SD rats by sequential digestion of the liver with pronase from *Streptomyces griseus* (Calbiochem), collagenase type IV from *Clostridium histolyticum* (Sigma-Aldrich), and deoxyribonuclease I from bovine pancreas (Sigma-Aldrich), followed by density gradient centrifugation over 11.5 % Nycodenz (Axis-Shield). After obtaining primary HSCs, all cells were seeded in the same media condition of LX-2 cells in a tissue culture flask. After 20 h, cells are washed with PBS and the medium was changed, which was replenished every 2 days afterward. When the culture become confluent, the cells were gathered and reseeded for each experiment.

### Apoptosis and cell-cycle assay

Apoptosis of LX-2 cells was assessed by staining with fluorescein isothiocyanate (FITC)-labeled annexin V (AV, 1  $\mu$ g/ml) and propidium iodide (PI, 0.5  $\mu$ g/ml) (BD Pharmingen, San Diego, CA). PI/Rnase A staining was performed for the purpose of analysis of the cell cycle of LX-2 cells. Cells were obtained and resuspended in 5 ml of 80% cold absolute ethanol/PBS overnight at –20 °C. They were then centrifuged, washed twice with cold PBS, suspended in 500 ml of PI-staining solution (0.1% Triton X-100 in PBS), and incubated with RNase (25 mg/ml) at room temperature for 30 min. Finally, they were stained with PI (50  $\mu$ g/ml) for 15 min and subjected to flow-cytometric analysis (BD FACSCalibur, USA), after which the cell-cycle distribution was analyzed by ModFit LT software (Version 3.3, Verity Software House).

### Statistical analysis

A statistical analysis was performed, with the Mann-Whitney *U* test within-group and the Kruskal Wallis test between-groups, using SPSS II software (SPSS, Chicago, IL). The cumulative survival rate was calculated using the Kaplan-Meier technique, and statistical significance was tested with the log-rank and Wilcoxon tests. A *p* value of 0.05 was set as the determinant of statistical significance.

## Results

### Vitamin C insufficiency exacerbated hepatic damage induced by TAA administration

*Gulo*<sup>-/-</sup> mice were administered with high-dose (3.3 g/L; hereafter, HD group) or low-dose (0.33 g/L; hereafter, LD group) vitamin C in their drinking water, immediately after weaning. Three weeks later, the serum vitamin C level in the HD mice was 96.3  $\pm$  17.2  $\mu$ M, similar to that in their wild-type littermates (91.3  $\pm$  13.2  $\mu$ M; hereafter, WT group) (Fig. 1A, left panel), whereas that in the LD mice was 31.9  $\pm$  16.9  $\mu$ M, less than half the normal level (*P* < 0.05). The hepatic vitamin C concentrations at this point

revealed a pattern similar to that shown by the serum (Fig. 1A, right panel). However, the average body weight did not show any differences among the groups (Fig. 1B). The vitamin C concentrations both in the serum and in the liver were maintained until the end of the experiment (Fig. 1C), while the average body weight in the LD group fell below those in the other two groups (*P* < 0.05, Fig. 1D).

To determine the TAA-elicited hepatotoxicity, AST and ALT levels were measured in the serum (Fig. 1E). In each group, the enzyme levels were elevated compared with those before TAA injection. The elevation was remarkable, especially in the LD group, being significantly higher than in the other groups. Additionally, the survival rate in the LD group was low (Fig. 1F, *P* = 0.028). All of these results suggested an increased vulnerability to TAA-induced hepatotoxicity in vitamin C-insufficient mice.

### Oxidative stress was increased in vitamin C-insufficient mice with TAA administration

The mechanism by which TAA exerts hepatotoxicity is twofold by the metabolite, thioacetamide-S,S-dioxide [19], and by ROS [5], both of which are produced by CYP2E1 during TAA metabolism. Since vitamin C is a well-known antioxidant [8], we anticipated increased oxidative stress as a cause of increased hepatotoxicity in the LD mice. To verify this, we determined the ROS levels in the liver. Prior to TAA treatment, the ROS levels were only minimally detected in all of the groups (data not shown); after TAA treatment, they were elevated in all groups, among which the LD group showed a higher level than the other two (Fig. 2A, *P* < 0.001). Concomitantly, the level of lipid peroxide in the liver, manifested as the concentration of malondialdehyde, was much higher in the LD group (Fig. 2B, *P* < 0.001). This elevated oxidative stress explains, at least in part, the exaggerated hepatotoxicity in the LD group, discussed above.

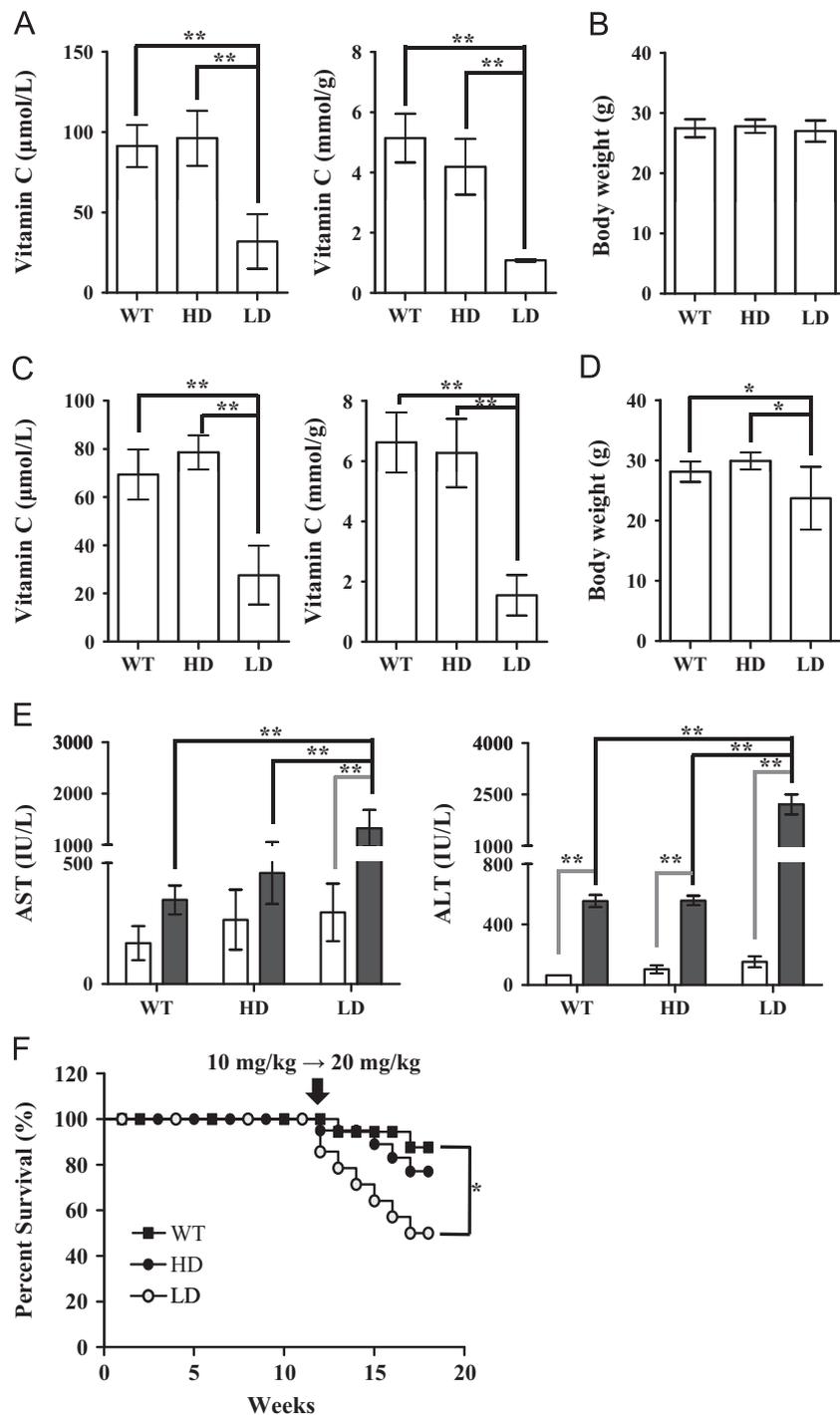
Next, we considered whether the increased ROS level in the LD group could have resulted from increased synthesis, in addition to decreased quenching. If it had, the expression of ROS-producing enzyme also would be elevated. In fact, by RT-PCR analysis, we found that the expression of CYP2E1 was higher in the LD group after TAA treatment (Fig. 2C, Supplementary Fig. 1A, *P* < 0.01), whereas before TAA treatment, it was similar in all of the groups (data not shown).

### Hepatic fibrosis was exaggerated in vitamin C-insufficient mice

Next, we determined whether the increased hepatic damage in the LD group was reflected in the severity of fibrosis. Prior to TAA treatment, there was no fibrosis observed in the liver sections stained with Sirius red (data not shown). After TAA administration, however, centrilobular hepatic damage and accumulation of fibroblast-like cells in the periportal area were observed. Once again, these pathologic changes were more substantial in the LD group (Fig. 3A). By Sirius red staining, heavy deposition of collagen fibers was observed, most prominently in the LD group (Fig. 3B), covering a significantly broader area when morphometrically analyzed (Fig. 3C, *P* < 0.01). Consistent with the morphological findings, the amount of collagen was highest in the LD group (Fig. 3D, *P* < 0.01). These results also were substantiated by RT-PCR analysis results, this time for type I collagen expression (Fig. 3E, Supplementary Fig. 1B).

### Number of activated hepatic stellate cells was increased in vitamin C-insufficient mice

The principal actor in hepatic fibrosis is activated hepatic stellate cells, which secrete collagen fibers [20]. With immuno-

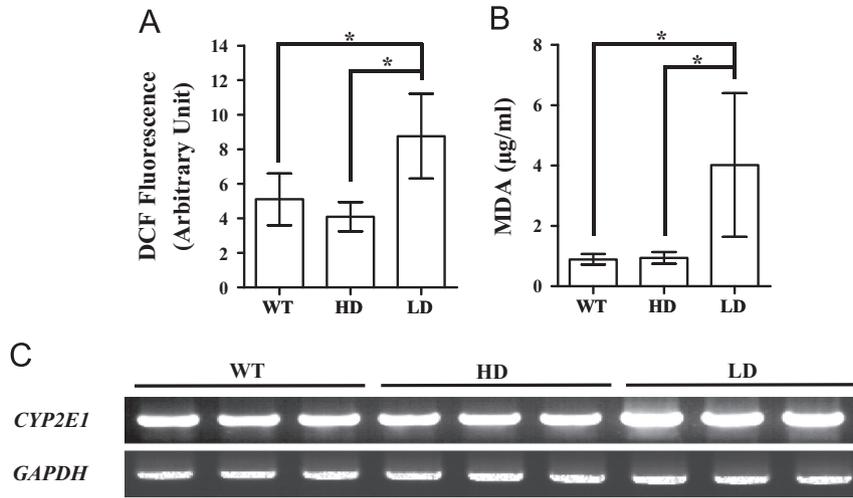


**Fig. 1.** Vitamin C insufficiency exacerbated TAA-induced hepatotoxicity. Experimental animals with vitamin C insufficiency were prepared by administration of low-dose vitamin C in drinking water (the LD group). At the beginning of the experiment, the serum and hepatic vitamin C concentration (A) was low in the LD group compared with the others. However, the average body weight in all of the groups was the same (B). After 18-week TAA treatment, the serum and hepatic vitamin C concentration (C) in the LD group was maintained at a similar level to that prior to TAA treatment, with a modest decrease of body weight (D). Hepatotoxicity, as indicated by the serum levels of AST and ALT activities (E) before (white bars) and after (black bars) TAA treatments, and survival rates (F) were exacerbated in the LD group. The data are shown as the mean  $\pm$  SD.  $n = 7-8$  per group. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HD, high-dose group; LD, low-dose group; WT, wild-type group.

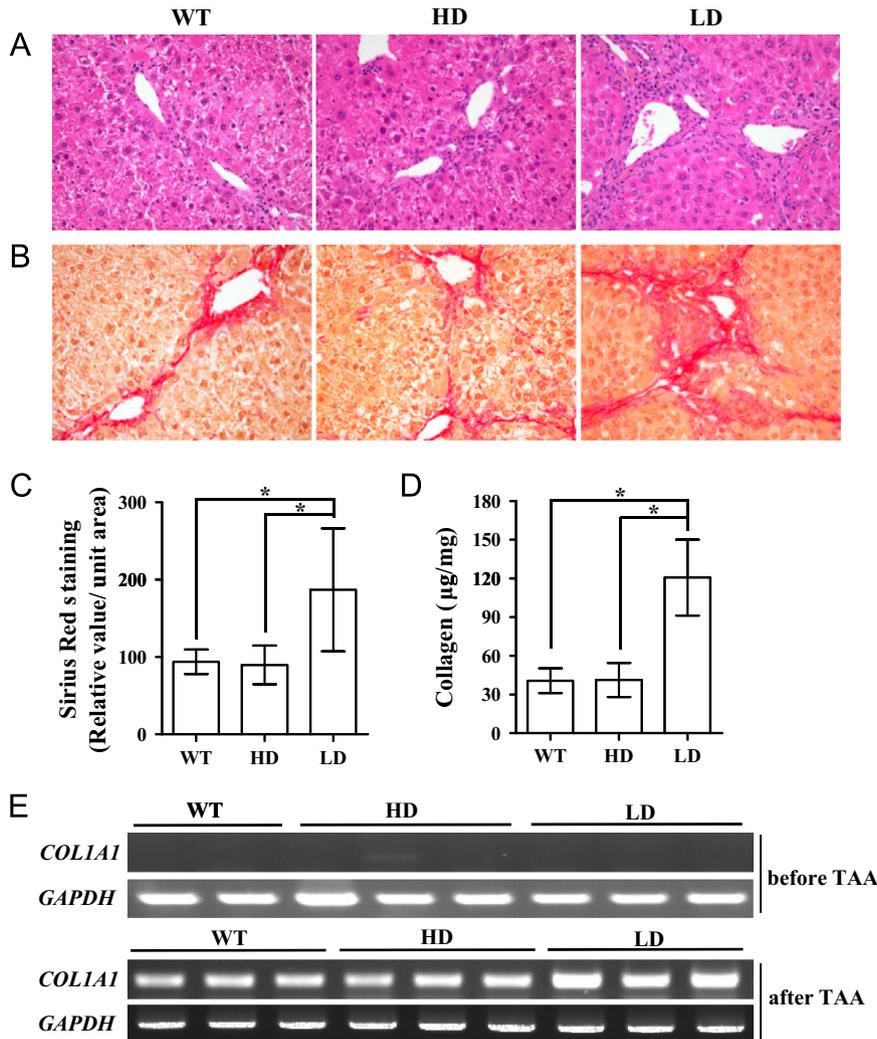
staining for  $\alpha$ -SMA, a specific marker of activated HSCs, no positive cells were observed at all in the normal liver tissue prior to TAA treatment (data not shown); after TAA treatment, immunostained cells were observed in the periportal regions, again most prominently in the LD group (Fig. 4A). An RT-PCR analysis revealed the same results as those observed in the stained tissues (Fig. 4B, Supplementary Fig. 1C,  $P < 0.01$ ).

#### TGF- $\beta$ 1 secretion was increased in vitamin C-insufficient mice

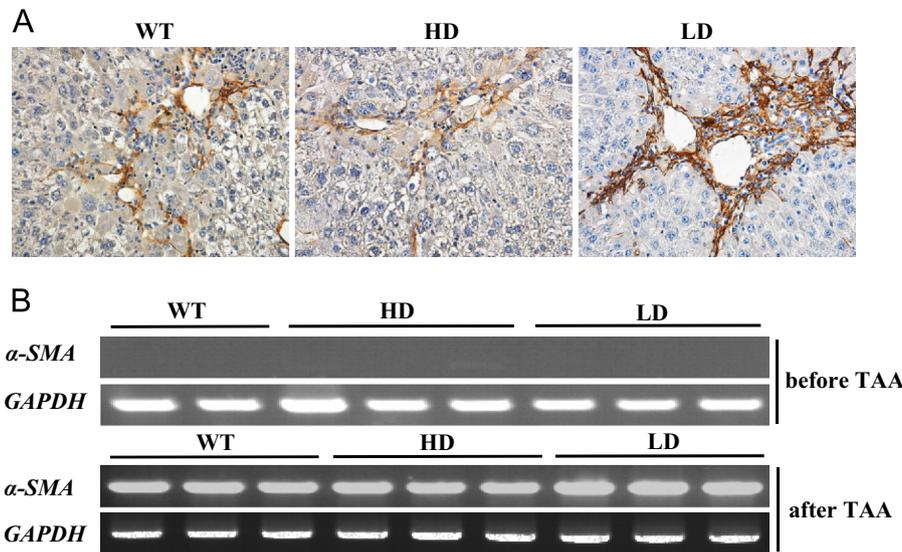
In the activation of quiescent HSCs and the subsequent acquisition of the fibrogenic function, TGF- $\beta$ 1 plays a critical role [21]. Thus, we measured the serum concentration of this cytokine by ELISA. After TAA administration, the serum concentration was increased in each group relative to before (Fig. 5A, \*  $P < 0.05$ , \*\*  $P < 0.01$ ). Among the



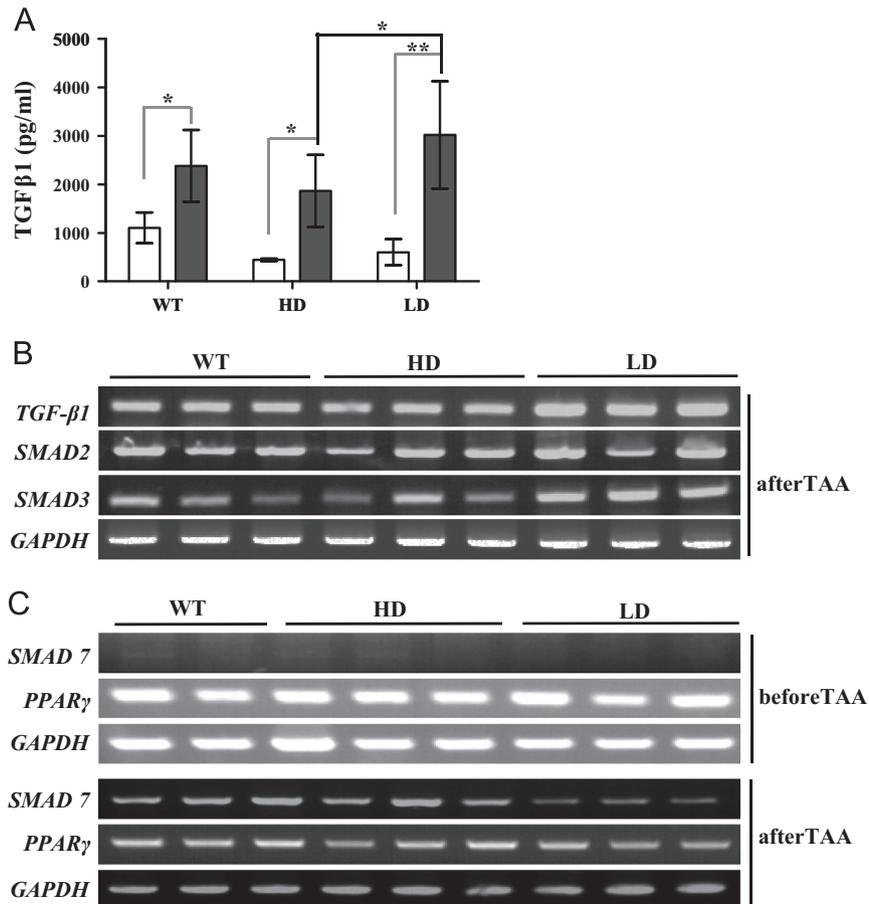
**Fig. 2.** Oxidative stress was increased in vitamin C-insufficient mice. At the end of the experiment, the levels of ROS (A) and MDA, the lipid peroxide (B), were quantified in the liver, both of which were highest in the LD group. The data are shown as the mean  $\pm$  SD ( $*P < 0.001$ ),  $n = 7-8$  per group. (C) The transcriptional levels of CYP2E1 in the liver were similar in all groups prior to TAA treatment. DCFDA, dichlorofluorescein diacetate; MDA, malondialdehyde; TAA, thioacetamide; HD, high-dose group; LD, low-dose group; WT, wild-type group.



**Fig. 3.** Liver fibrosis was exacerbated in vitamin C-insufficient mice. In hematoxylin and eosin staining of the TAA-treated liver tissue (A; magnification =  $\times 400$ ), fibrous septa around the periportal areas were observed, most prominently in the LD liver. Under Sirius red staining (B; magnification =  $\times 400$ ), deposition of collagen fibers was marked. The area occupied by collagen fibers, as morphometrically measured, was larger in the LD group (C). Concomitantly, the biochemically measured amount of collagen (D) was much higher in the LD liver. The data are shown as the mean  $\pm$  SD ( $*P < 0.01$ ),  $n = 7-8$  per group. RT-PCR analyses revealed induction of collagen expression by TAA treatment (E). HD, high-dose group; LD, low-dose group; WT, wild-type group; TAA, thioacetamide.



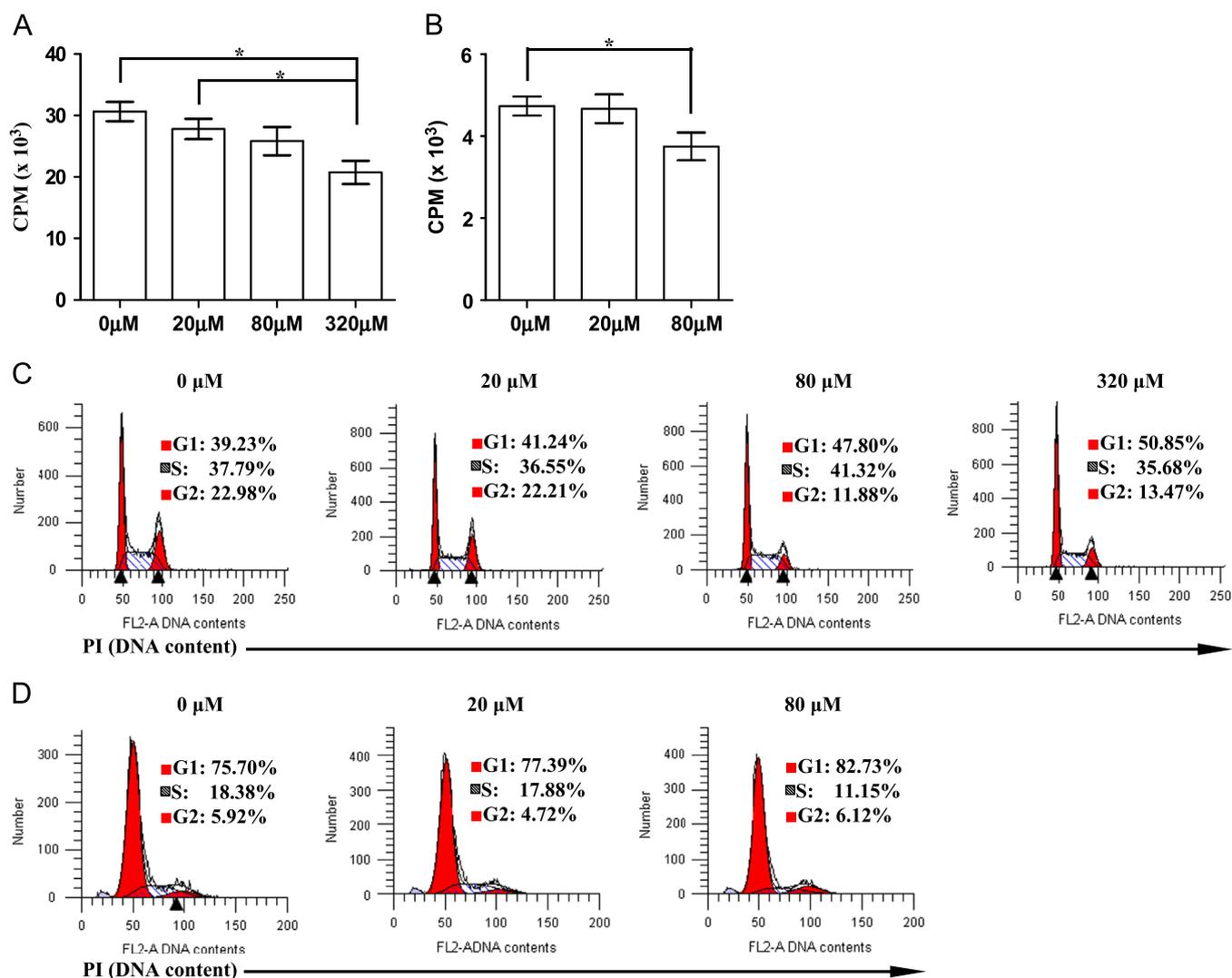
**Fig. 4.** Number of activated HSCs was increased in vitamin C-insufficient mice. Immuno-staining for  $\alpha$ -SMA (A; magnification =  $\times 400$ ), a marker for activated HSCs, in the liver after TAA treatment revealed positive cells in the periportal area, most abundantly in the LD liver. With RT-PCR analyses (B), expression of  $\alpha$ -SMA was hardly observed prior to TAA treatment, but was induced after TAA treatment, most prominently in the LD group. HD, high-dose group; LD, low-dose group; WT, wild-type group; TAA, thioacetamide;  $\alpha$ -SMA, alpha-smooth muscle actin.



**Fig. 5.** TGF- $\beta$  signaling was augmented in vitamin C-insufficient mice. (A) After TAA treatment, the serum level of TGF- $\beta$ 1 was elevated in all of the groups. The level in the LD group was higher than that in the HD group, with statistical significance. The white and black bars represent values before and after TAA treatment, respectively. The data are shown as the mean  $\pm$  SD (\* $P < 0.05$ , \*\* $P < 0.01$ ).  $n = 7$ –8 per group. Accordingly, the mRNA levels of TGF- $\beta$ 1 and its subsequent signaling molecules, Smad2 and 3, in the liver (B) were most prominently elevated in the LD group. By contrast, the transcriptional level of the antifibrotic molecules, SMAD7 and PPAR $\gamma$ , was decreased in the LD group. HD, high-dose group; LD, low-dose group; WT, wild-type group; TAA, thioacetamide.

post-TAA groups, the LD group showed a modestly higher titer than the others. However, in this case, no statistically significant difference was found between the LD and the WT groups.

We also determined the hepatic expression of TGF- $\beta$ 1 and its signaling molecules, Smad2 and Smad3. Before TAA treatment, the mRNA of these molecules was hardly detected by RT-PCR (data not



**Fig. 6.** Vitamin C suppressed HSC proliferation by cell-cycle arrest. LX-2 cells and the rat primary HSCs were cultured in the presence of vitamin C at concentrations indicated in the figures. In vitro application of vitamin C reduced the measured thymidine uptake of LX-2 cells (A) and the rat primary HSCs (B) dose dependently. The data are shown as the mean  $\pm$  SD (\* $P < 0.05$ ).  $n = 7$ –8 per group. Vitamin C treatment increased the frequency of LX-2 cells (C) as well as the rat primary HSCs (D) in the G1 phase, indicating cell-cycle arrest. All of the figures are representative of two or three independent experiments. PI, propidium iodide.

shown); but after TAA treatment, the expression of these molecules was considerable, and was more substantial in the LD group than in the others with statistical significance (Fig. 5B, Supplementary Figs. 1D–F,  $P < 0.05$ ).

Some molecules are known to be antifibrotic. For example, Smad7 is antagonistic against TGF- $\beta$ 1 signaling [22], and PPAR $\gamma$  is a negative regulator of HSC activation [23]. When we evaluated the expression of these molecules, we found that they were less expressed in the LD group (Fig. 5C, Supplementary Figs. 1G and H).

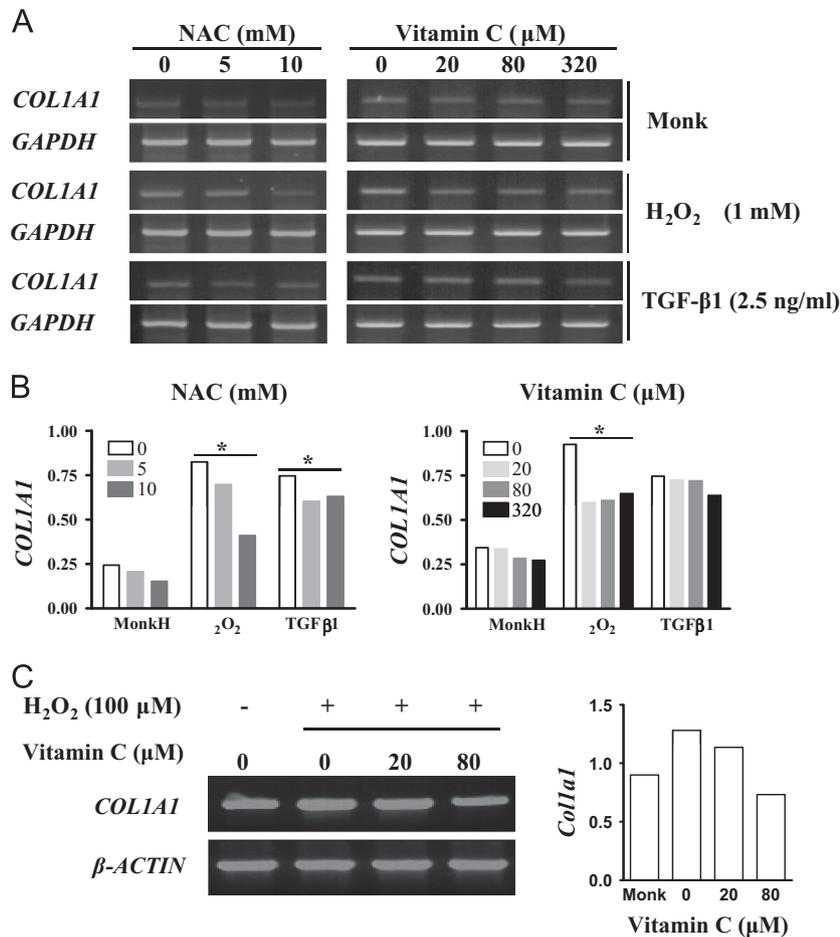
#### Vitamin C arrested proliferation of LX-2 cells in vitro

In light of our finding that vitamin C insufficiency was associated with increased activation of HSCs in the LD liver (Fig. 4), we speculated whether vitamin C did directly affect HSC activation and/or proliferation. To this end, we cultured both LX-2 cells and the rat primary HSCs in the presence of various concentrations of vitamin C. It is known that when plated in a standard culture dish, HSCs are spontaneously activated and begin to proliferate [24]. Our cell count after 48-h culturing (Supplementary Fig. 2A) showed decreased numbers of LX-2 cells with an increased amount of vitamin C. Concomitantly, a thymidine uptake assay (Fig. 6A and B) indicated suppression of cell proliferation by

vitamin C, dose dependently, which seemed to have been due to cell-cycle arrest in the G1 phase (Fig. 6C and D). Meanwhile, apoptosis was not observed with vitamin C in culture media (Supplementary Fig. 2B), indicating that vitamin C decreased the LX-2 cell numbers by arresting cell proliferation, not by inducing cell death.

#### Vitamin C suppressed H<sub>2</sub>O<sub>2</sub>-induced *col1a1* expression

Next, we tested the direct effects of vitamin C on the expression of *col1a1*. After serum starvation, LX-2 cells were stimulated with H<sub>2</sub>O<sub>2</sub> or TGF- $\beta$ 1 for 20 h in the presence or absence of various concentrations of vitamin C or N-acetylcysteine (NAC). In the absence of stimulation, *col1a1* expression was minimal, and was not affected by concentrations of NAC or vitamin C (Fig. 7A and B). With H<sub>2</sub>O<sub>2</sub> or TGF- $\beta$ 1 treatment, LX-2 cells increased the transcriptional level of *col1a1*. Whereas NAC suppressed both H<sub>2</sub>O<sub>2</sub> and TGF- $\beta$ 1-induced *col1a1* expression, vitamin C suppressed only the expression induced by H<sub>2</sub>O<sub>2</sub>, not that by TGF- $\beta$ 1. The same effect was observed in the rat primary HSCs with H<sub>2</sub>O<sub>2</sub> for 20 h in the presence or absence of various concentrations of vitamin C (Fig. 7C).



**Fig. 7.** Vitamin C suppressed  $H_2O_2$ -induced *colla1* expression. LX-2 cells were serum-starved for 48 h and then treated with  $H_2O_2$  or TGF- $\beta$ 1 to induce collagen expression, in the presence of NAC or vitamin C at various concentrations. Twenty-four hours after treatment, the cells were subjected to RT-PCR analysis (A), and the results were quantified by densitometric analysis (B).  $H_2O_2$  or TGF- $\beta$ 1 treatment induced collagen expression, both of which were suppressed by NAC, while vitamin C suppressed only that by  $H_2O_2$ . When the rat primary HSCs were treated with  $H_2O_2$ , collagen expression was induced, which was decreased in the presence of vitamin C dose dependently (C). The data are representative of two independent experiments. NAC, N-acetylcysteine.

## Discussion

The results of the study demonstrated that vitamin C insufficiency, at a subservic level, exacerbated hepatotoxicity induced by long-term TAA injection, as revealed by the elevated markers for hepatocyte damage (serum AST, ALT) and hepatic oxidative stress (ROS, MDA), increased number of activated HSCs, and resultant augmentation of liver fibrosis. Correspondingly, causative factors for these changes, CYP2E1 and TGF- $\beta$ 1, were increased in their expression, while expression of antifibrotic factors such as SMAD7 and PPAR $\gamma$  was decreased.

These results are generally in line with the data previously reported on the beneficial effects of supplementary vitamin C, both in experimental animals [4,10–12] and in humans [9,25]; however, they are quite contrary to the vitamin C “deficiency” findings of Park et al. for SMP30 $^{-/-}$  mice [15]. Specifically, they reported lowered ROS and lipid peroxidation, attenuated fibrosis with a smaller number of activated HSCs, and increased expression of PPAR $\gamma$  in vitamin C-deficient mice relative to WT mice after CCl $_4$  treatment. We have no explanation for these absolutely contradictory results. One remarkable difference between our and their experiments is the serum vitamin C concentration. Whereas that in their mice was undetectable, we maintained it, in the LD group, at a level of around 30  $\mu$ M during the experimental period (18 weeks). Considering that vitamin C is essential for collagen synthesis [7], lower collagen contents and decreased fibrosis in

the SMP30 $^{-/-}$  mice made sense. However, we have no explanations for the other results. We only assume that factors other than vitamin C were operative.

Based on our results and those of others, we can suppose a few mechanisms by which vitamin C insufficiency exacerbates TAA-induced liver damage and fibrosis. TAA is primarily metabolized by CYP2E1 [26], during which ROS is produced. Subsequently, ROS damages hepatocytes by lipid peroxidation of cell membranes. And, Kupffer cells, stimulated by damaged cells, begin to secrete TGF- $\beta$ 1 as well as additional ROS. All of these products—ROS, lipid peroxide, damaged cells, and TGF- $\beta$ 1—activate HSCs and induce them to secrete collagen fibers [27]. We can assume that decreased quenching of ROS due to vitamin C insufficiency allowed the ROS level to elevate, which in turn augmented subsequent events, leading to exacerbated hepatotoxicity.

In addition to oxidative stress, reactive metabolite of TAA also induces hepatotoxicity by covalently binding to macromolecules in the cell [19]. This metabolite is produced via CYP2E1; thus induction of this enzyme increases TAA-induced hepatotoxicity [28,29], while minimal liver injury is induced with TAA in the absence of this enzyme [26]. In the present study, CYP2E1 expression, as revealed by RT-PCR, was much higher in the LD group. Considering that the mRNA level of this enzyme accurately reflects its activity [30], we can assume that the elevated CYP2E1 level under vitamin C insufficiency contributed to the exaggerated hepatotoxicity by increasing the reactive

metabolite, as well as ROS production. However, the exact mechanism for the elevated expression of CYP2E1 remains to be elucidated.

Decreased expression of antifibrotic factors such as Smad7 and PPAR $\gamma$  also explains intense fibrosis under vitamin C insufficiency. PPAR $\gamma$  is crucial for inhibition of HSC activation [23]. Its lowered expression is to be expected, as it has been reported to be inhibited by TGF- $\beta$  signaling, for example, via the  $\beta$ -catenin pathway [31].

Contrary to PPAR $\gamma$ , the expression of Smad7 is induced by TGF- $\beta$  signaling via Smad3 phosphorylation [32]. However, this happens only in the acute phase of liver damage. Under the chronic condition, phosphorylation of Smad3 in HSCs occurs predominantly in the linker region by JNK, yielding pSmad3L, which does not induce Smad7, but is still fibrogenic [33]. Thus, we believe that shifting of Smad3 phosphorylation to pSmad3L occurred more prominently in the LD group. This might be attributable to increased phosphorylation of JNK due to the higher oxidative stress in the LD group [34].

We raised a question about a possible direct effect of vitamin C on HSCs either as an antioxidant, or as a chemical. Thus, we performed in vitro experiments using the human HSC cell line, LX-2 [18]. The growth of LX-2 cells was inhibited, via cell-cycle arrest, dose dependently in the presence of vitamin C. In fact, antioxidants, such as selenium [35], propolis [36], and polyphenols [37], have been reported to elicit cell-cycle arrest in various cell lines, through various mechanisms. Vitamin C also has been reported to arrest the cell cycle in B16F10 murine melanoma cells, specifically by increasing the expression of a cyclin-dependent kinase inhibitor, p21<sup>Waf1/Cip1</sup> [38]. In rat HSCs, another antioxidant, NAC, induces cell-cycle arrest via the same mechanism [39]. Interestingly, both of these authors reported G1 arrest in their cells, as was the case in our results.

Meanwhile, vitamin C directly suppressed H<sub>2</sub>O<sub>2</sub>-induced *col1a1* expression in LX-2 cells. Given its antioxidant property, this result is exactly what was expected, even though dose dependency was not observed. Meanwhile, vitamin C failed to suppress TGF- $\beta$ 1-induced *col1a1* expression, while NAC suppressed it. TGF- $\beta$ 1 has been reported to induce ROS accumulation, specifically H<sub>2</sub>O<sub>2</sub>, which in turn up-regulates *col1a1* expression in rat HSCs, and this effect is mimicked by H<sub>2</sub>O<sub>2</sub> in culture media [40]. This TGF- $\beta$ 1-mediated ROS generation has been actuated via enhanced expression of Nox4 in human HSCs, and NAC has suppressed TGF- $\beta$ 1-induced collagen synthesis in these cells [41], as in our results. If ROS is involved, at least in part, in the process of *col1a1* expression by TGF- $\beta$ 1, we would expect that vitamin C could suppress this process. However, this was not the case. A possible explanation could be the limited ROS-scavenging capacity of vitamin C, which has been demonstrated once in mouse bone-marrow-derived dendritic cells [42]. Or, perhaps vitamin C additionally phosphorylated p38 and ERK1/2 [42], which are involved in the induction of *col1a1* expression by TGF- $\beta$ 1 [43], counterbalancing the suppressive effect through ROS quenching. Further experiments are needed in order to resolve this contradiction.

In summary, we can conclude that vitamin C insufficiency exacerbated liver damage and subsequent fibrosis induced by chronic TAA treatment, principally because of lowered quenching of ROS. Additionally, we posit the possibility of vitamin C's direct inhibitory effects on HSCs with respect to proliferation and collagen synthesis.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2013.10.813>.

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