

Ascorbic acid concentrations in dimethylnitrosamine-induced hepatic fibrosis in rats

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Abstract

Background: Ascorbic acid is a potent antioxidant and is involved in many metabolic activities including collagen biosynthesis. In the present investigation, ascorbic acid and lipid peroxides were monitored in the blood and liver samples during the progression of experimentally induced hepatic fibrosis. **Methods:** Liver injury was induced by intraperitoneal injections of dimethylnitrosamine (DMN) on three consecutive days of every week over a period of 21 days. The progression of fibrosis was assessed by histopathological examination and by monitoring of the collagen content of the liver tissue. Ascorbic acid and lipid peroxides were monitored in both blood and liver samples on days 0, 7, 14, and 21 after the start of DMN administration. The liver total protein was also measured during the investigation. **Results:** Histopathological examination demonstrated centrilobular necrosis, fibrosis, and early cirrhosis during DMN treatment. The collagen content increased four-fold on the 21st day of investigation. Lipid peroxides were elevated significantly in both blood and liver specimens on days 7, 14, and 21. A drastic decrease was observed in the ascorbic acid concentrations in both liver and blood samples on all days after the start of DMN administration. Liver total protein concentrations were significantly reduced during DMN administration. **Conclusions:** The exact mechanism of the decrease of ascorbic acid during DMN-induced hepatic fibrosis is not clear. The most probable reason for the decreased blood and liver ascorbic acid during DMN-induced hepatic fibrosis is the increased utilization of ascorbic acid for free radical scavenging in order to reduce the highly elevated oxidative stress.

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Keywords: Ascorbic acid; Vitamin C; Dimethylnitrosamine; Hepatic fibrosis; Oxidative stress; Lipid peroxidation

1. Introduction

Ascorbic acid (vitamin C) is involved in many metabolic activities and is essential for collagen biosynthesis. It increases blood circulation and plays an

important role as an antioxidant. Ascorbic acid is an essential cofactor for the enzymes prolyl hydroxylase and lysyl hydroxylase, which catalyzes the hydroxylation of proline and lysine residues, respectively, during collagen biosynthesis [1]. Data are not available regarding the role of ascorbic acid in experimentally induced liver injury. Significantly decreased ascorbic acid concentrations were reported in patients with alcoholic liver disease and primary biliary cirrhosis [2,3].

Hepatic fibrosis is a dynamic and highly integrated cellular response to chronic liver injury [4]. Recent

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Table 1
Biochemical parameters during the progression of DMN induced hepatic fibrosis in rats

Parameters	Control (n = 12)	Day 7 (n = 12)	Day 14 (n = 10)	Day 21 (n = 8)
Body weight (g)	192.25 ± 7.21	186.45 ± 8.33	167.36 ± 11.46**	145.33 ± 9.38**
Liver weight (g)	7.71 ± 0.58	7.41 ± 0.62	4.76 ± 0.42**	3.45 ± 0.35**
Total collagen ^a	1.03 ± 0.07	1.96 ± 0.13**	3.02 ± 0.18**	4.16 ± 0.32**
Total protein ^a	150.63 ± 8.77	136.34 ± 10.45*	128.57 ± 12.74**	121.16 ± 10.29**

Values are mean ± S.D.

^a Expressed as milligrams per gram of liver wet weight.

* $p < 0.01$ (by ANOVA).

** $p < 0.001$ (by ANOVA).

investigations suggest that oxidative stress is the mechanism of the development of liver fibrosis in most of the clinical and experimental conditions [5–7] and is generally associated with decreased antioxidant defenses. Increased lipid peroxidation and formation of oxidative stress-related molecules may serve as the mediators to modulate tissue and cellular events responsible for chronic liver injury and associated fibrosis. Ascorbic acid, vitamin E, glutathione, and selenium are the first-line defenders against free radicals and oxidative stress in biological systems.

We showed that dimethylnitrosamine (DMN)-induced hepatic fibrosis in rats is a good and reproducible animal model for studying biochemical and pathophysiological alterations associated with the development of hepatic fibrosis and cirrhosis in humans [8–10]. Since oxidative stress plays a major role in the progression of hepatic fibrosis and cirrhosis, we investigated the role of a major antioxidant, ascorbic acid, in hepatic fibrosis. In order to interpret the results, we monitored the blood and liver ascorbic acid concentrations during the progression of experimentally induced hepatic fibrosis in rats and evaluated with other parameters (see Table 1). The blood and liver lipid peroxidation and liver protein concentrations were also measured.

2. Materials and methods

2.1. Chemicals

Dimethylnitrosamine, L-hydroxyproline, chloramine-T, 2,4-dinitrophenylhydrazine, ascorbic acid, thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, and bovine serum albumin were from Sigma (St. Louis, MO). Ethylene glycol monomethyl ether

(methyl cellosolve) was procured from Fluka (Switzerland), and *p*-dimethylaminobenzaldehyde was purchased from E. Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Animals

Adult male albino rats of the Wistar strain, at about 3 months old and weighing between 180 and 200 g, were used for the induction of liver injury. The animals were bred and maintained under 12-h light/12-h dark cycles in an air-conditioned animal house, with commercial rat feed pellets (Hindustan Lever, Bombay, India) and water available ad libitum. They were housed in polypropylene cages with a wire mesh top and a hygienic bed of husk.

2.3. Induction of hepatic fibrosis

Hepatic fibrosis was induced by intraperitoneal injections of dimethylnitrosamine in doses of 1 mg (10 µl diluted to 1 ml with 0.15 mol/l sterile NaCl)/100 g body weight. The injections were given on three consecutive days of every week over a period of 21 days. Control animals also received an equal volume of 0.15 mol/l NaCl without DMN. The injections were given without anesthesia. Treated animals were sacrificed on days 7, 14, and 21 from the beginning of the exposure by decapitation. Some of the control animals were sacrificed at the beginning of the experiment and some together with the treated animals on days 7, 14, and 21. The pooled mean value was used as control. All the animals were anesthetized with diethyl ether before sacrifice. Blood was collected from the orbital of the eye using a heparinized capillary tube under anesthesia. The control and the seventh day group comprised 12 rats each, while the 14th and 21st day

groups consisted 10 and 8 rats, respectively. The body weight of the experimental animals was monitored throughout the study.

2.4. Preparation of liver extract

The livers were rapidly removed and blotted. The livers were weighed and a portion was chopped with scissors and homogenized separately using a Polytron homogenizer (Kinematica, Switzerland) with ice-cold 0.15 mol/l NaCl. The final concentration of the homogenate was adjusted to 100 mg tissue/ml and used for the determination of ascorbic acid, lipid peroxides, and proteins.

2.5. Assessment of the progression of hepatic fibrosis

The progression of hepatic fibrosis was assessed both histopathologically and biochemically. Tissue blocks were cut from the median lobe and fixed in 10% phosphate-buffered formalin. The paraffin-embedded blocks were cut into 5- μ m sections and stained with hematoxylin and eosin. The stained sections were examined using a Nikon labophot microscope and photographed.

Collagen content in the liver tissue was measured as a biochemical parameter to assess the progression of fibrosis. The total collagen content in the liver tissue was determined by the estimation of hydroxyproline, a characteristic imino acid present in collagen. To estimate hydroxyproline, all samples were hydrolyzed in 6 mol/l HCl in sealed tubes at 110 °C for 16 h. The hydrolyzed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was redissolved in distilled water and made up to a known volume. It was treated with activated charcoal and filtered through Whatman filter paper. The clear filtrate was used for the determination of hydroxyproline according to the method of Woessner [11]. In brief, 1 ml of the filtrate was mixed with 1 ml of freshly prepared chloramine-T solution and allowed to stand for 20 min. It was further mixed with 1 ml of 3.15 mol/l perchloric acid and left for 5 min. Finally, 1 ml of freshly prepared *p*-dimethylaminobenzaldehyde was added and mixed well, and the mixture was incubated in a water bath at 60 °C for 20 min. The absorbance of the solution was measured in a spectrophotometer at 560 nm.

The total collagen content in the liver tissue was calculated by multiplying the hydroxyproline content by a factor of 7.46, as postulated by Neuman and Logan [12].

2.6. Extraction and estimation of total protein in liver

The total protein present in the liver tissue was extracted in 10% TCA. About 100 mg of liver tissue homogenate was mixed with 10 ml of 10% TCA and placed in a water bath at 90 °C for 30 min with intermittent stirring. It was filtered and the filtrate was dissolved in 0.1 mol/l NaOH by gentle warming. The total protein present in the liver extract was determined by the method of Lowry et al. [13] using bovine serum albumin as standard.

2.7. Determination of lipid peroxides in blood and liver tissue

The lipid peroxides present in the blood sample were determined by the spectrofluorometric method of Yagi [14] employing thiobarbituric acid reagent. Fluorometric method eliminates the measurement of nonspecific colored products of TBA reaction while using blood samples. In brief, 50 μ l of heparinized blood was diluted to 1 ml with physiological saline and mixed gently. It was centrifuged at 3000 rpm for 10 min and 0.5 ml of the supernatant was mixed with 4 ml of 3 mol/l sulfuric acid and 0.5 ml of 10% phosphotungstic acid. The tube was allowed to stand for 5 min at room temperature and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the sediment was suspended in 2 ml of distilled water. One milliliter of 0.67% TBA reagent (prepared freshly by dissolving TBA in 50% glacial acetic acid with gentle warming) was added. A standard solution was prepared using 1 nmol of 1,1,3,3-tetramethoxypropane in 2 ml of distilled water and 1 ml of TBA reagent. The reaction mixture is then heated for 60 min at 95 °C in a water bath to generate malondialdehyde (MDA). After cooling in ice-cold water, the TBA–MDA adduct was extracted in 5 ml of spectroscopic grade *n*-butanol. It was centrifuged at 3000 rpm for 10 min and 4 ml of the butanol layer was collected. The fluorescence intensity was measured in a Hitachi spectrofluorometer (model 650-40; Hitachi Koki,

Tokyo, Japan) at an excitation of 515 nm and an emission of 553 nm. The lipid peroxides present in the blood were expressed as nanomoles of malondialdehyde per 100 ml.

Lipid peroxides present in the liver tissue were determined by the thiobarbituric acid reaction described by Ohkawa et al. [15] using tetramethoxypropane as standard. An aliquot of 0.5 ml of liver homogenate was treated with 3.5 ml of ice-cold 10% trichloroacetic acid (TCA) and mixed well. The tubes were centrifuged at 3000 rpm for 10 min and 2 ml of the supernatant was mixed with 2 ml of 0.6% thiobarbituric acid reagent (prepared freshly by dissolving in 0.25 mol/l HCl with gentle warming). It was covered with glass marbles and placed in a boiling water bath for 15 min. The absorbance was measured using a Shimadzu UV-260 spectrophotometer at 532 nm.

2.8. Estimation of ascorbic acid in blood and liver

The ascorbic acid (vitamin C) present in the blood sample and liver tissue was determined by the spectrophotometric method of Omaye et al. [16]. In brief, 1 ml of 10% liver homogenate and 0.6 ml of blood sample were made up to 2 ml with 10% TCA and mixed well. It was centrifuged at $3500 \times g$ for 10 min and 1 ml of the supernatant was mixed with 0.5 ml of 2,4-dinitrophenylhydrazine reagent (3 g of 2,4-dinitrophenylhydrazine, 4 g of thiourea, and 50 mg of copper sulfate in 100 ml of 9 mol/l H_2SO_4) and incubated at $37^\circ C$ for 3 h. The tubes were removed and 2.5 ml of ice-cold 85% H_2SO_4 was added. It was mixed well and kept at room temperature for 30 min, and the absorbance was measured using a Shimadzu UV-160 spectrophotometer at 520 nm. A standard was prepared using 10 μg of pure ascorbic acid.

2.9. Statistical analysis

Arithmetic mean and standard deviation were calculated for the data. The results were statistically evaluated using one-way analysis of variance (ANOVA). The control mean values were compared with the treated mean values on days 7, 14, and 21 of the experiment using the least significant difference method. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Animal body weight and liver weight

Animal body weight and liver weight monitored during the progression of hepatic fibrosis are demonstrated in Table 1. The DMN-administered animals did not gain body weight during the course of treatment. A significant decrease was noticed in the mean body weight of animals on days 14 and 21 after the start of DMN administration. The liver weight was also significantly ($p < 0.001$) reduced on days 14 and 21. About a 55% decrease was observed in the liver weight on day 21 compared with the control value.

3.2. Assessment of the progression of hepatic fibrosis

Fig. 1A–D demonstrates the histopathological changes of rat liver treated with DMN over a period of 21 days. The control liver depicted normal lobular architecture with central vein and radiating hepatic cords (Fig. 1A). There was severe centrilobular congestion and marked dilatation of central vein and sinusoids on the seventh day (Fig. 1B). It also showed hemorrhagic necrosis and mild bile duct hyperplasia. On the 14th day, there were massive hepatocytes necrosis, neutrophilic infiltration, and initiation of fibrosis (Fig. 1C). On the 21st day of DMN treatment, the liver section demonstrated collagen fibers, marked fibrosis, intense neutrophilic infiltration, and regeneration of hepatocytes (Fig. 1D). Bridging necrosis and apoptosis were present in certain cases.

3.3. Liver collagen

The total collagen in the liver, measured by estimating hydroxyproline content, was significantly increased ($p < 0.001$) on all the days after the start of DMN administration (Table 1). The maximum increase was on day 21 and it was about fourfold higher when compared to the total collagen content in the control liver. The fourfold increase of total liver collagen on day 21 coincides with the observation of marked fibrosis and accumulation of collagen bundles in the liver by histopathological examination.

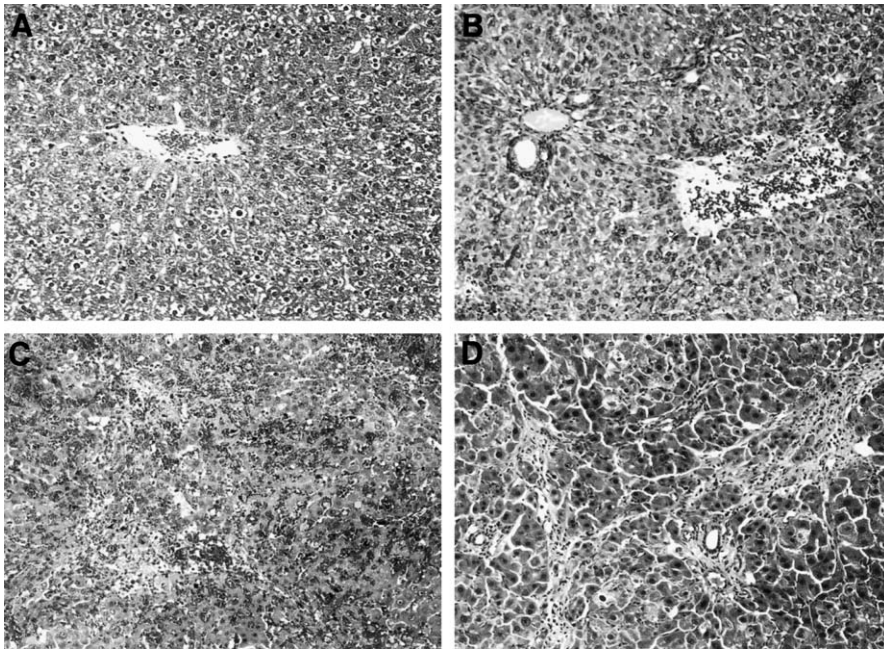


Fig. 1. Hematoxylin and eosin (H&E) staining of rat liver section after DMN administration demonstrating necrosis and fibrosis. (A) Control liver ($\times 100$). (B) DMN day 7 ($\times 100$): centrilobular congestion and marked dilatation of central vein and sinusoids. (C) DMN day 14 ($\times 100$): massive necrosis, neutrophilic infiltration, and initiation of fibrosis. (D) DMN day 21 ($\times 100$): marked fibrosis, intense neutrophilic infiltration, and regeneration of hepatocytes.

3.4. Liver protein

The total protein content in the liver during DMN-induced hepatic fibrosis is demonstrated in Table 1. A significant decrease was noticed in total protein concentration on all the days after the start of DMN administration. The maximum decrease was on day 21. It was observed that on day 21, the DMN-treated liver shrank drastically with a significant decrease of total liver weight.

3.5. Lipid peroxidation

The lipid peroxides present in the blood and liver specimens, measured in terms of malondialdehyde formed, were significantly increased on all days after the start of DMN administration (Figs. 2 and 3, respectively). During the course of study, the elevation of lipid peroxides was gradual from control to the day-21 samples. The maximum increase was observed on day 21 in both liver and blood specimens after the start of DMN administration. A positive correlation

($r=0.998$) was noticed with regard to the rise in lipid peroxides in blood and liver samples. The remarkable increase of lipid peroxides in both blood and liver specimens during DMN administration indicates the formation of reactive oxygen species (ROS), which plays a major role in cell injury and pathogenesis of hepatic fibrosis.

3.6. Ascorbic acid

The alteration of ascorbic acid in the blood during DMN administration is represented in Fig. 4. The blood ascorbic acid was significantly ($p<0.001$) and remarkably decreased on all the days after the start of DMN administration. The decrease was gradual from control to the day-21 samples. The maximum decrease was on day 21 (more than five-fold compared to the control value). The mean blood ascorbic acid concentration in control animals was 51.87 mg/l, while on day 21, it was decreased to 9.67 mg/l.

The ascorbic acid concentrations in the liver during the course of DMN administration are dem-

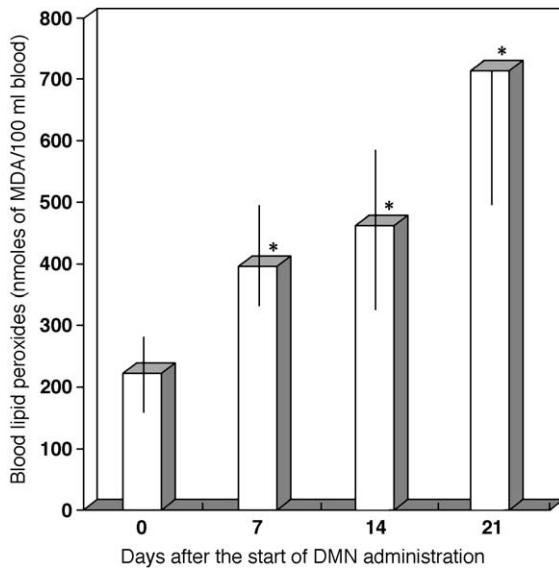


Fig. 2. Lipid peroxides in the blood during DMN-induced hepatic fibrosis in rats. The blood lipid peroxides are expressed as nanomoles of malondialdehyde (MDA) liberated ($*p < 0.001$ by ANOVA when compared with control).

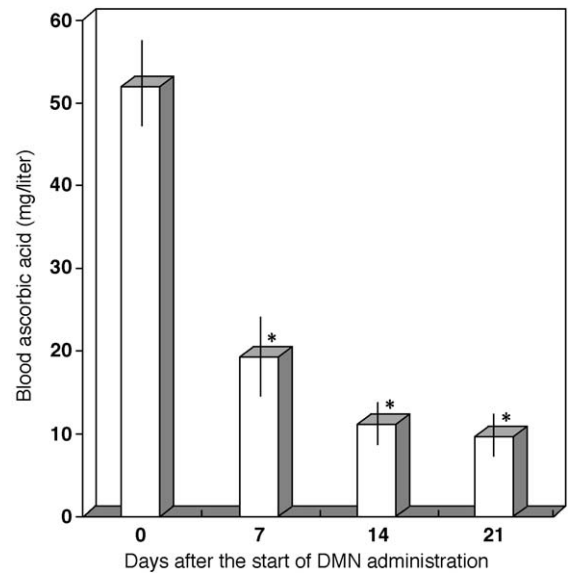


Fig. 4. Ascorbic acid concentrations in the blood during DMN-induced hepatic fibrosis in rats ($*p < 0.001$ by ANOVA when compared with control).

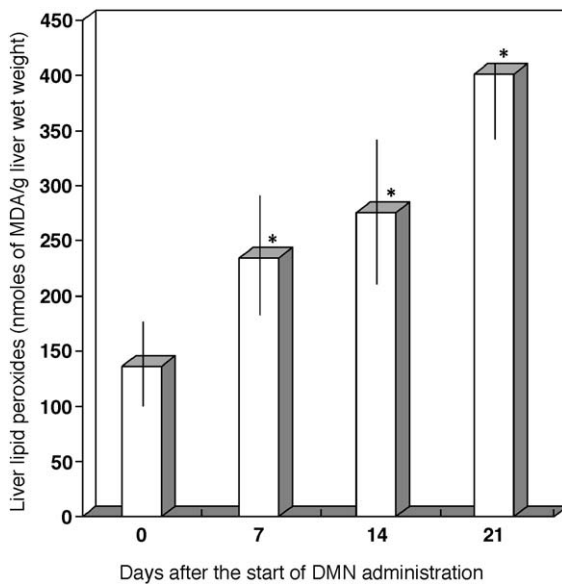


Fig. 3. Lipid peroxides in the liver during DMN-induced hepatic fibrosis in rats. Lipid peroxides are expressed as nanomoles of malondialdehyde liberated ($*p < 0.001$ by ANOVA when compared with control).

onstrated in Fig. 5. As in the case of blood samples, a drastic decrease was observed in the liver ascorbic acid concentrations on all the days after the start of

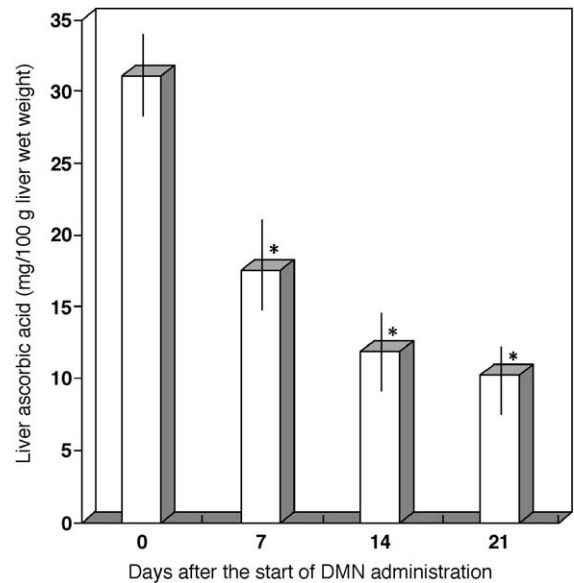


Fig. 5. Ascorbic acid concentrations in the liver during DMN-induced hepatic fibrosis in rats ($*p < 0.001$ by ANOVA when compared with control).

DMN administration. The maximum decrease was on day 21 (about three-fold). A positive correlation ($r=0.992$) was observed with regard to the decrease of ascorbic acid in blood and liver specimens during the course of DMN administration.

4. Discussion

The dimethylnitrosamine-induced liver injury in rats is a reproducible and potentially valuable animal model for studying the pathogenesis of human hepatic fibrosis and cirrhosis. The 21-day course of DMN administration in rats produced centrilobular necrosis and well-developed fibrosis, as present in alcoholic liver diseases. The approximately fourfold increase of total liver collagen observed in the present study coincides with the previous investigations on DMN-induced hepatic fibrosis in rats [10,17].

The pathogenesis of hepatic fibrosis is a dynamic process mediated through oxidative stress and hepatocyte injury [5,18,19]. Lipid peroxidation, the oxidative alteration of polyunsaturated fatty acids, is a mechanism generally recognized as being the most important in the pathogenesis of liver injury by a number of toxic compounds [7,20]. It may lead to a decrease in the fluidity of the lipid phase of the biomembrane, and this can be expected to have important consequences in relation to many of the major metabolic functions dependent on membrane structure and integrity [21]. The generation of reactive oxygen species that can attack biomolecules directly, with the consequent enhancement in membrane lipid peroxidation, is considered as the major factor involved in the mechanism of liver cell injury [22–24].

Increased oxidative stress and lipid peroxidation have been reported in DMN-induced hepatic fibrosis [7] as well as in carbon tetrachloride-induced liver injury in rats [25,26]. In the present investigation also, significantly increased concentrations of lipid peroxides were noticed in the blood and liver specimens (Figs. 2 and 3). This suggests that DMN-induced liver damage generates free radicals, which react with polyunsaturated fatty acids of hepatic microsomal system and cause rearrangement of the double bonds to generate diene conjugated lipids. The positive correlation ($r=0.998$) noticed between increased lipid

peroxidation in blood and liver samples indicates extensive extrahepatic circulation of lipid peroxides generated in the liver.

Reports are not available regarding ascorbic acid concentrations in experimentally induced hepatic fibrosis or cirrhosis. Significantly reduced leukocyte ascorbic acid concentrations were observed in alcoholic liver disease and primary biliary cirrhosis [2]. A significant decrease in plasma ascorbic acid was also reported in patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma [27]. In the present investigation, a remarkable decrease of ascorbic acid has been observed in both blood and liver specimens (Figs. 4 and 5). The continuous decrease of ascorbic acid may indicate increased oxidative stress during the progression of fibrosis.

The exact mechanism of reduced concentrations of ascorbic acid in DMN-induced hepatic fibrosis is not clear. It has been reported that ascorbic acid stimulates the rate of collagen biosynthesis [28,29] through increased transcription of the collagen genes [30,31]. The theory of increased utilization of ascorbic acid for the enhanced biosynthesis of collagen in hepatic fibrosis cannot be applied because vitamin C is not consumed stoichiometrically in the peptidyl proline 4-hydroxylase and peptidyl lysine hydroxylase reactions, and both enzymes can catalyze their reactions for a number of catalytic cycles in the complete absence of this vitamin [32–34]. It was reported that oral contraceptives, corticosteroids, tetracycline, calcitonin, aspirin, and other drugs significantly reduce plasma and tissue ascorbic acid concentrations in humans [35]. The influence of DMN metabolism towards the decrease of blood and liver ascorbic acid is yet to be investigated.

It was reported that oral administration of vitamin C ameliorated the necrotic and fibrotic changes in carbon tetrachloride-induced liver damage [36]. Ascorbic acid is a potent scavenger of reactive oxygen species in plasma and extracellular compartments of the liver [37]. Ascorbic acid scavenges and destroys free radicals in combination with vitamin E and glutathione. The drastic decrease of ascorbic acid in DMN-induced hepatic fibrosis may indicate increased oxidative stress, free radical formation, and simultaneous damage of the liver plasma membrane lipid bilayers. The increased oxidative stress and the formation of reactive oxygen species cause extensive

necrosis of the liver, which finally contributes to the development of fibrosis and cirrhosis.

After scavenging the reactive oxygen species, an amount of ascorbic acid could regenerate either enzymatically using monodehydroascorbate reductase, or nonenzymatically by spontaneous dismutation. The enzymatic regeneration of the ascorbic acid may occur principally intracellularly at the expense of reduced glutathione. The highly reduced concentrations of glutathione in liver diseases [38,39] may affect the enzymatic regeneration of the ascorbic acid, which results in the sacrifice of ascorbic acid during scavenging of reactive oxygen species in liver diseases, including DMN-induced hepatic fibrosis. Under physiologic conditions, the regenerating system of ascorbic acid may function normally, but during extreme necrosis of the liver, it may be impaired, which contributes to a decreased ascorbic acid concentration in both liver and circulating system.

In summary, the present study demonstrated drastic decreases of both blood and liver ascorbic acid concentrations in DMN-induced hepatic fibrosis in rats. It also depicted increased lipid peroxides, oxidative stress, and simultaneous formation of free radicals, which induce extensive liver necrosis and ultimate fibrosis.

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