

Biochemical Abnormalities during the Progression of Hepatic Fibrosis Induced by Dimethylnitrosamine

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Objectives: The pathogenesis of hepatic fibrosis is accompanied with several biochemical and metabolic abnormalities. To obtain more information about the alteration of biochemical and metabolic parameters during alcoholic liver fibrosis, we have monitored the changes of certain important biochemical compounds in experimentally induced hepatic fibrosis.

Design and methods: The liver injury was induced in adult male albino rats by using dimethylnitrosamine (DMN) in doses of 1 mg/100 g body weight. Total collagen, total protein, cholesterol, lipid peroxides, glucose, urea, and inorganic phosphorus were monitored in liver and blood/serum samples on Days 0, 7, 14, and 21 after the start of DMN administration. Serum insulin levels were assayed by radioimmunoassay. The serum and urinary levels of hydroxyproline, uric acid, and creatinine were also monitored.

Results: The total collagen content in the liver was increased about 4-fold by Day 21 after the start of DMN administration. A significant increase was observed in lipid peroxide levels in both liver and blood samples. Although inorganic phosphorus level decreased in both serum and liver tissue, cholesterol was lowered only in the serum. Increased serum insulin level with impaired glucose tolerance was observed after 21 days. Serum hydroxyproline level increased throughout after the start of DMN administration. The urinary excretion of hydroxyproline was also significantly increased with a striking elevation on Day 7. Elevated uric acid levels were recorded in serum and urine samples during the latter periods of DMN treatment. No alteration was observed in blood urea and creatinine levels.

Conclusions: The results of the present investigation demonstrated important alterations in metabolic parameters and biochemical abnormalities during experimentally induced liver damage. All alterations are compatible with the deterioration of liver functions during the pathogenesis of hepatic fibrosis. Copyright © 2000 The Canadian Society of Clinical Chemists

KEY WORDS: dimethylnitrosamine; hepatic fibrosis; biochemical abnormalities; liver collagen; urinary hydroxyproline.

Introduction

Hepatic fibrosis is due to the imbalance in the synthesis and breakdown of connective tissue components of the liver. The development of hepatic

fibrosis is associated with a number of biochemical changes, which lead to structural and metabolic abnormalities in liver. Hepatic fibrosis induced by dimethylnitrosamine (DMN) in rat appears to be a good and reproducible model accompanied with many decompensating features of human alcoholic fibrosis (1,2). Some of these biochemical changes are characterized by alteration in the levels of many metabolic products, which are released into the blood and finally excreted through urine. The pathobiochemical and cytophysiological factors responsible for many of these metabolic alterations in hepatic fibrosis are not clear.

Stimulation of lipid peroxidation and accumulation of malondialdehyde is an important event in hepatic fibrosis. Biomembranes contain substantial amounts of polyunsaturated fatty acids, which are highly susceptible to peroxidative breakdown. Lipid peroxidation is a free radical mediated chain reaction, which is enhanced as a consequence of oxidative stress and results in an oxidative deterioration of these membranous polyunsaturated fatty acids. The generation of hepatic lipid peroxidation by free radicals has been proposed as a mechanism for ethanol-induced hepatotoxicity (3,4). However, the cytopathological relationship between increased lipid peroxidation and hepatocyte injury in liver fibrosis is not clear. It has been reported that the increased production of reactive oxygen intermediates in fibrotic liver is responsible for the upregulation of transforming growth factor- β (TGF- β) gene expression, which enhances collagen synthesis (5).

Increased plasma insulin concentrations with insulin resistance have been reported in cirrhotic patients (6). Hyperglycemia and impaired glucose tolerance have also been observed in patients with established hepatic cirrhosis (7,8). An abnormal insulin level with impaired glucose tolerance may affect carbohydrate metabolism and protein synthesis. Depletion may occur in both total and esterified serum cholesterol levels in chronic liver diseases due to an impaired lipid metabolism and a reduced cholesterol synthesis by the damaged liver.

Although the connective tissue metabolism has

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been extensively studied during experimentally induced hepatic fibrosis (9–11), very little information is available about the biochemical abnormalities and changes in metabolic parameters. Hence, in the present investigation, several clinical and biochemical parameters associated with the pathogenesis of hepatic fibrosis have been undertaken after the administration of DMN in male albino rats. Total collagen, total protein, lipid peroxides, cholesterol, glucose, urea inorganic phosphorus, and insulin were studied in liver and blood/serum samples. Serum and urinary levels of hydroxyproline, uric acid, and creatinine were also monitored after the start of DMN administration.

Materials and methods

CHEMICALS

Dimethylnitrosamine, *L*-hydroxyproline, chloramine-T, bovine serum albumin, thiobarbituric acid, diacetyl monoxime, cholesterol, creatinine and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma (St. Louis, MO, USA). Ethylene glycol monomethyl ether (methyl cellosolve) was procured from Fluka (Buchs, Switzerland) and *p*-dimethylaminobenzaldehyde from E. Merck (Darmstadt, West Germany). Uranyl acetate and sodium tungstate were obtained from BDH Chemicals (Poole, England, UK) and *p*-methylaminophenol sulfate (metol) from Agfa-Gevaert (D-5090, Leverkusen, Germany). Lithium carbonate, uric acid, *o*-toluidine, trichloroacetic acid, potassium sodium tartarate, thiourea and Folin-Ciocalteu's phenol reagent were bought from Loba Chemie (Bombay, India). The insulin radioimmunoassay kit was procured from Radiopharmaceuticals division, Bhabha Atomic Research Center (Bombay, India). All other chemicals used were of analytical grade.

ANIMALS

Three-month-old adult male albino rats of the Wistar strain, weighing between 180 to 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 the air-conditioned animal house with commercial rat feed (Hindustan Lever, Bombay, India) and water available *ad lib*. They were housed in polypropylene cages with a wire mesh top and a hygienic bed of husk.

INDUCTION OF HEPATIC FIBROSIS

Hepatic fibrosis was induced by intraperitoneal injections of dimethylnitrosamine (DMN) in doses of 1 mg (10 μ L diluted to 1 mL with 0.15 mol/L sterile NaCl)/100 g body weight. The injections were given on the first three consecutive days of each 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. The ani-

mals were transferred to metabolic cages 24 h before sacrifice for urine collection. Urine was collected under a layer of toluene and stored at 4 °C until analyzed.

Treated animals were sacrificed on Days 7, 14, and 21 from the beginning of exposure. 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 and the pooled mean value was used as control. The control and the 7th day group comprised 12 rats each, while the 14th and 21st day group consists 10 and 8 rats, respectively. All animals were anesthetized with diethyl ether before sacrifice. Blood was collected from a deep cut made on the right jugular vein on the neck with a scalpel blade. A portion of the blood was immediately treated with ice cold 10% TCA and used for the estimation of glucose and lipid peroxides. The livers were immediately removed, kept in the ice bath in cold saline, and weighed in the wet state after blotting of the water. The liver was then minced with scissors and homogenized in 9 mL of ice cold 1.15% KCl per g of tissue by using a Polytron homogenizer (Kinematica GmbH, CH-6010 Kriens, Switzerland) at 4 °C. The homogenate was stored for up to 72 h at -70 °C until analyzed.

The serum was separated in the conventional way after incubating the blood at 30 °C for 1 h. Serum with lysis of red blood cells were not included in the study. The serum sample for insulin assay was stored at -70 °C, and for the remaining assays it was stored at 4 °C in a refrigerator.

Histopathology was carried out to evaluate the progression of hepatic fibrosis after administration of DMN. The liver sections were stained with hematoxylin and eosin and the degree of fibrosis was assessed by using a Nikon Labophot microscope (Melville, NY, USA).

EXTRACTION OF TOTAL PROTEIN FROM THE 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 N NaOH by gentle warming.

ESTIMATION OF PROTEINS IN LIVER, SERUM, AND URINE

The total protein present in the liver extract was determined by the method of Lowry *et al.* (12). Proteins present in the serum and urine samples were determined by the modification of biuret method described by Reinhold (13).

ESTIMATION OF GLUCOSE AND UREA IN BLOOD

The glucose present in the blood was estimated by the method of Winckers and Jacobs (14) using *o*-toluidine and urea by the method of Natelson *et al.* (15) using diacetyl monoxime.

DETERMINATION OF LIPID PEROXIDES IN BLOOD AND LIVER TISSUE

The lipid peroxides present in the blood was determined by the spectrofluorometric method of Yagi (16) employing thiobarbituric acid (TBA) reagent and tetramethoxypropane as standard. 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 physiologic saline and mixed gently. It was centrifuged at 3000 rpm for 10 min and 0.5 mL of the supernatant mixed with 4 mL of 3 N 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 suspended in 2 mL of distilled water and added 1 mL of 0.67% TBA reagent (prepared freshly by dissolving TBA in 50% glacial acetic acid with gentle warming). The 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 heated for 60 min at 95 °C in a water bath. After cooling in cold water, 5 mL of spectroscopic grade n-butanol was added and shaken vigorously for 2 min. 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 Co., Tokyo, Japan) at an excitation wavelength 515 nm and emission at 553 nm. The lipid peroxides in the blood was expressed as nmols malondialdehyde/100 mL.

Lipid peroxides present in the liver tissue was determined by the thiobarbituric acid reaction described by Ohkawa *et al.* (17) using tetramethoxypropane as standard. The liver tissue was homogenized in 9 mL of ice cold 1.15% KCl per gram of wet tissue. An aliquot of 0.5 mL 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 N HCl with gentle warming). It was covered with glass marbles and placed in a boiling water bath for 15 min. The absorbance was measured by using a Shimadzu UV-260 spectrophotometer (Chiyoda-Ku, Tokyo, Japan) at 532 nm.

DETERMINATION OF CHOLESTEROL, INORGANIC PHOSPHORUS, URIC ACID, AND CREATININE

The cholesterol present in serum and liver homogenate was determined by the method of Jung (18) and inorganic phosphorus by the method of Gomori (19). The uric acid present in serum and urine was determined by the method of Caraway (20). Serum creatinine was estimated by the method of Owen *et al.* (21) and urine creatinine by the method of Bonsnes and Taussky (22).

DETERMINATION OF HYDROXYPROLINE IN LIVER, SERUM, AND URINE

Hydroxyproline content in liver homogenate, serum, and urine samples was determined by the method of Woessner (23). All the samples were hydrolyzed in 6 N HCl (final conc.) 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 dissolved in distilled water and made up to a known volume. It was decolorized with activated charcoal and filtered through Whatman filter paper (Tewksbury, MA, USA). For the determination of hydroxyproline, 1 mL of the clear filtrate was mixed with 1 mL of freshly prepared chloramine-T solution and allowed to stand for 20 min. The samples were further mixed with 1 mL of 3.15 mol/L perchloric acid and waited for 5 min. Finally, 1 mL of freshly prepared *p*-dimethylaminobenzaldehyde was added, mixed well, and placed in a water bath at 60 °C for 20 min. The absorbance of the solution was determined by using a spectrophotometer at 560 nm.

The total collagen content in the liver was calculated by multiplying the hydroxyproline content by the factor 7.46 as postulated by Neuman and Logan (24).

ASSAY OF INSULIN IN SERUM

The immunoreactive insulin present in serum was determined by the double antibody radioimmunoassay method of Soeldner and Sloane (25) using rat insulin as standard. All the samples and standards were assayed in duplicate. A standard curve was prepared by using 0 to 200 μ units of insulin/mL. To the sample tubes, 0.3 mL of assay buffer was added followed by 0.1 mL serum. The standards and a blank with 0.4 mL assay buffer were mixed with 0.1 mL of insulin-free serum. This was followed by the addition of 0.1 mL of insulin antibody (raised in guinea pig) to all the tubes except the blank. The tubes were mixed gently and incubated at 4 °C overnight in a refrigerator. After incubation, 0.1 mL of radioiodinated insulin (¹²⁵I) was added to all the tubes and 0.1 mL was taken separately for total counts. It was mixed gently and incubated for 3 h at room temperature. At the end of incubation, 0.1 mL of second antibody (raised in rabbit) and 1 mL of PEG solution was added to all the tubes and mixed well. The tubes were incubated at room temperature for exactly 20 min. After incubation, all the tubes were centrifuged together at 3000 rpm for exactly 20 min. The supernatant was decanted carefully, the solution adhered to the rim of the tube was blotted out, and the tube with the precipitate was collected for γ -counting. The radioactivity was measured by using an LKB automatic γ -counter, model 1272 CliniGamma (LKB-Wallac, Turku, Finland). The concentration of insulin in unknown samples was obtained from the logit-log curve created in the computer attached to the instrument. The immuno-

TABLE 1
Total Collagen, Total Protein, Lipid Peroxides, Total Cholesterol, and Inorganic Phosphorus in Liver During DMN-Induced Hepatic Fibrosis in Rats

Parameters Assayed	Control (<i>n</i> = 12)	Day 7 (<i>n</i> = 12)	Day 14 (<i>n</i> = 10)	Day 21 (<i>n</i> = 8)
Total collagen ^a	0.98 ± 0.03	1.75 ± 0.05**	2.72 ± 0.09**	3.76 ± 0.15**
Total protein ^a	151.63 ± 3.77	120.16 ± 5.45**	130.34 ± 6.74**	125.57 ± 6.29**
Lipid peroxides ^b	136.02 ± 9.23	234.75 ± 11.20**	266.16 ± 12.30**	387.88 ± 16.06**
Total cholesterol ^a	6.59 ± 0.48	6.41 ± 0.39	5.46 ± 0.22	6.69 ± 0.40
Inorganic phosphorus ^c	72.15 ± 1.53	64.22 ± 2.25*	55.41 ± 1.94**	44.52 ± 1.45**

Values are mean ± standard error.

^aExpressed as mg/g liver wet weight.

^bExpressed as nanomoles of malondialdehyde/g liver wet weight.

^cExpressed as mg/100 g liver wet weight.

p* < 0.01 and *p* < 0.001 (by ANOVA).

reactive hormone present in the serum was expressed as μ units/mL.

STATISTICAL ANALYSIS

Arithmetic mean and standard error 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. The value of *p* < 0.01 was considered as statistically significant. Student's *t*-test and Pearson and Lee's correlation coefficient were also used in certain cases.

Results

ASSESSMENT OF THE DEGREE OF HEPATIC FIBROSIS

The progression of hepatic fibrosis, evaluated by histopathological examination of the liver tissue demonstrated diffuse centrilobular congestion with dilation of central vein and sinusoids on the 7th day. Intense neutrophilic infiltration and mild bile duct hyperplasia were observed on the 14th day. The centrilobular necrosis initiated on the 7th day became prominent during this period. The 21st day liver specimens demonstrated severe centrilobular necrosis in all cases. Increased fibrosis with focal fatty changes and bile duct hyperplasia was noticed consistently. Enlargement of nucleus and regeneration of hepatocytes were seen in many cases. There was bridging necrosis and early fibrosis between portal tract and central vein. All the 21st day liver sections depicted well-developed fibrosis around central vein.

COLLAGEN AND HYDROXYPROLINE

The total collagen in the liver, measured in terms of hydroxyproline content, was significantly increased (*p* < 0.001) on all the days of DMN treatment (Table 1). The maximum increase was noticed on the 21st day, which was ~4-fold when compared

to the control value. The serum hydroxyproline content was also found to be increased on the 7th, 14th, and 21st days of DMN administration (Table 3). The maximum increase was on the 7th day, indicating a high rate of degradation of newly formed liver collagen. Even although, the difference between control and 14th and 21st days of serum hydroxyproline levels were significant, the latter values tend to be decreased and the differences were significant (*p* < 0.01 and *p* < 0.001, respectively) when compared with the 7th-day value by Student's *t*-test.

The urinary excretion of hydroxyproline during the progression of DMN-induced hepatic fibrosis is demonstrated in Table 4. A highly significant increase (*p* < 0.001) was observed in the urinary excretion of hydroxyproline on the 7th, 14th, and 21st days after the administration of DMN. The increase was striking especially in the early stages of DMN treatment. A maximum of 6-fold raise was recorded on the 7th day, indicating increased metabolic degradation of newly synthesized collagen. But on the 14th and 21st days, the urinary excretion of hydroxyproline tend to be decreased and the differences were highly significant (*p* < 0.001) when compared with 7th-day value. The 21st-day value was also significantly different from the 14th-day value (*p* < 0.001). No correlation was observed between the increased accumulation of collagen in the liver and increased excretion of hydroxyproline in the urine.

PROTEINS

The total protein content in the liver during DMN-induced hepatic fibrosis is demonstrated in Table 1. A significant decrease (*p* < 0.001) was noticed in the levels of total liver protein per g of wet weight of tissue on all the days after administration of DMN. The maximum decrease observed was on the 7th day as per the liver wet weight ratio. No significant alteration was noticed in the case of serum total protein during DMN administration (Table 3). A significant increase (*p* < 0.001) was observed in urine protein levels on the 14th and 21st days of

TABLE 2
Lipid Peroxides, Glucose, and Urea in Blood During DMN-induced Hepatic Fibrosis in Rats

Parameters Assayed	Control (<i>n</i> = 12)	Day 7 (<i>n</i> = 12)	Day 14 (<i>n</i> = 10)	Day 21 (<i>n</i> = 8)
Lipid peroxides ^a	113.27 ± 7.50	198.89 ± 11.87*	231.58 ± 15.65**	365.60 ± 85.08**
Glucose ^b	78.21 ± 2.47	81.56 ± 3.12	84.70 ± 4.04	101.39 ± 5.58**
Urea ^b	22.25 ± 0.85	19.23 ± 1.26	24.47 ± 1.16	26.73 ± 2.52

Values are mean ± standard error.

^aExpressed as nanomoles of malondialdehyde/100 mL blood.

^bExpressed as mg/100 mL blood.

p* < 0.01 and *p* < 0.001 (by ANOVA).

DMN treatment (Table 4). On the 7th day, the urine protein levels showed a plateau only. On the 21st day, the urinary proteins demonstrated a 3- to 4-fold increase. This indicates a great enhancement in the catabolism of proteins in DMN-induced hepatic fibrosis in rats.

LIPID PEROXIDATION

The lipid peroxide levels in liver and blood, measured in terms of the formation of malondialdehyde, were significantly increased on all days of DMN administration (Table 1 and Table 2). Over the period of the 21-day study, the increase in lipid peroxide level was gradual from control to the Day 21 samples. The maximum increase was observed on the 21st day of DMN treatment in both liver and blood specimens. A positive correlation (*r* = 0.998) was noticed between the increase in the lipid peroxidation of liver and blood samples during the course of DMN administration.

TOTAL CHOLESTEROL

The serum total cholesterol was significantly decreased on the 14th and 21st days of DMN administration compared to control values (Table 3). The maximum decrease was on the 21st day and the difference was not significant on the 7th day. No

significant alteration was noticed in the liver total cholesterol levels during the course of DMN treatment (Table 1).

INORGANIC PHOSPHORUS

A significant decrease was observed in the liver inorganic phosphorus level on the 7th, 14th, and 21st days of DMN-induced hepatic fibrosis (Table 1). During the entire period of study, the decrease was gradual and the maximum decrease was on the 21st day when compared to control values. The serum inorganic phosphorus also decreased significantly on the 14th and 21st days of DMN administration, but the decrease was not significant on the 7th day (Table 3).

INSULIN AND BLOOD GLUCOSE

The serum insulin levels measured by double-antibody radioimmunoassay, were significantly increased on the 14th and 21st days of DMN administration (Table 3). The blood glucose was significantly increased (*p* < 0.001) only on the 21st day. It was important to note the linear relationship between the significantly increased serum insulin and blood glucose levels on the 21st day of DMN treatment. Even although, the insulin concentrations were increased in the blood, it was unable to

TABLE 3
Total Cholesterol, Inorganic Phosphorus, Creatinine, Uric acid, and Insulin Levels in Serum During DMN-induced Hepatic Fibrosis in Rats

Parameters Assayed	Control (<i>n</i> = 12)	Day 7 (<i>n</i> = 12)	Day 14 (<i>n</i> = 10)	Day 21 (<i>n</i> = 8)
Hydroxyproline ^a	9.85 ± 0.34	15.30 ± 0.69**	12.29 ± 0.53**	11.15 ± 0.47*
Total proteins ^b	7.16 ± 0.21	7.50 ± 0.23	7.37 ± 0.26	7.06 ± 0.30
Total cholesterol ^c	82.46 ± 4.43	73.54 ± 4.66	58.18 ± 3.11**	56.66 ± 3.66**
Inorganic phosphorus ^c	7.64 ± 0.28	7.44 ± 0.38	6.30 ± 0.35*	4.81 ± 0.29**
Creatinine ^c	1.40 ± 0.06	1.53 ± 0.07	1.47 ± 0.06	1.60 ± 0.08
Uric acid ^c	3.29 ± 0.20	3.12 ± 0.13	4.28 ± 0.27	6.74 ± 0.38**
Insulin ^d	8.61 ± 1.16	12.29 ± 2.04	18.22 ± 2.93*	22.21 ± 3.41**

Values are mean ± standard error.

^aExpressed as μg/mL serum.

^bExpressed as g/100 mL serum.

^cExpressed as mg/100 mL serum.

^dExpressed as μunits/mL serum.

p* < 0.01 and *p* < 0.001 (by ANOVA).

TABLE 4
Effect of DMN-induced Hepatic Fibrosis on the Urinary Excretion of Hydroxyproline, Creatinine, Uric acid, and Proteins

Urinary excretion	Control (n = 12)	Day 7 (n = 12)	Day 14 (n = 10)	Day 21 (n = 8)
Hydroxyproline	26.70 ± 1.20	160.42 ± 10.35**	128.15 ± 8.22**	62.05 ± 4.42**
Creatinine ^b	59.27 ± 3.53	50.83 ± 3.14	51.25 ± 3.18	73.33 ± 4.26
Uric acid ^c	387.82 ± 11.26	423.98 ± 21.58	526.28 ± 24.06*	887.33 ± 59.54**
Proteins ^d	10.70 ± 0.59	13.50 ± 0.65	24.67 ± 1.60**	37.41 ± 2.21**

Values are mean ± standard error.

^aExpressed as µg/mg creatinine in 24-h urine sample.

^bExpressed as mg/100 mL of 24-h urine.

^cExpressed as µg/mg creatinine.

^dExpressed as mg/mg creatinine.

p* < 0.01 and *p* < 0.001 (by ANOVA).

reduce the increased blood glucose level indicating insulin resistance in DMN-induced hepatic fibrosis.

BLOOD UREA AND CREATININE

No significant alteration was noticed in blood urea and serum creatinine levels during DMN-induced hepatic fibrosis in rats (Table 2 and 3). The urinary excretory level of creatinine, which was monitored in 24-h urine sample, also did not show any considerable alteration during the period of DMN administration (Table 4).

URIC ACID

A significant increase (*p* < 0.001) was observed in serum uric acid levels only on the 21st day of DMN-induced hepatic fibrosis in rats (Table 3). The increase was not significant on the other days studied. In urine samples, the increase was significant on the 14th and 21st days after DMN administration (Table 4). A correlation (*r* = 0.959) was noticed between increased serum uric acid levels and enhanced urinary excretion of uric acid indicating a normal kidney function, in relation to raised uric acid clearance from the blood, during DMN-induced experimental liver injury.

Discussion

The histopathological changes observed in the present investigation were on par with the pathologic alterations reported in human hepatic fibrosis. The 21-day course of controlled DMN administration in rats produced massive centrilobular necrosis and well-developed fibrosis around the central veins. The ~4-fold increase in total liver collagen observed in this study coincides with the previous investigations on DMN-induced hepatic fibrosis in rats (9,26).

The alteration of hydroxyproline levels in liver, serum, and urine is considered as an index of collagen metabolism and provides valuable information about the biochemical and pathologic events of hepatic fibrosis. Significantly increased levels of serum

hydroxyproline have been reported in patients with alcoholic liver cirrhosis (27) and chronic liver diseases (28). In the present investigation also, a significant increase in serum hydroxyproline was observed on all days of DMN treatment with a maximum on the 7th day (Table 3). This reflects the extensive degradation of newly synthesized collagen in the liver by collagenolytic enzymes during the early stages of fibrosis. Increased hepatic collagenolytic activity was reported in carbon tetrachloride-induced early fibrosis (29) and patients with chronic liver diseases (30).

The significant decrease of total liver protein observed on all days of DMN administration (Table 1) was analogous to a previous report (26). An increase in protein breakdown with an overall fall in whole-body protein turnover has been reported in patients with alcoholic cirrhosis (7). In the present study, a significant increase was observed in the urinary excretion of total proteins. This indicates an increased catabolism of other proteins, in addition to collagen. This enhanced catabolism may be due to the extreme centrilobular necrosis of the liver tissue during hepatic fibrosis.

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 (31). It may lead to 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 (32). Lipid peroxidation can therefore be of major significance to cell injury produced by free-radical mechanisms (33,34).

It has been reported that ethanol metabolism plays an important role in the pathogenesis of hepatic disturbances leading to lipid peroxidation (35, 36). Furthermore, increased formation of malondialdehydes was observed in the hepatic microsomal fraction of rats treated with carbon tetrachloride (37). In the present investigation also, significantly increased levels of lipid peroxides were noticed in the liver and blood samples (Table 1 and 2). This

suggested that DMN-induced liver damage also 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 liver and blood samples indicates that the formation of lipid peroxides is not restricted to the liver tissue alone, but spreads to the extrahepatic regions also (36,38). The extensive portal circulation and extreme necrosis may be responsible for the corresponding increase of lipid peroxides in the blood.

In all chronic liver diseases, including carbon tetrachloride-induced liver cirrhosis, the total serum cholesterol levels are markedly depressed. In this investigation also, serum total cholesterol levels were significantly reduced on the 14th and 21st days of DMN administration (Table 3). The decreased serum cholesterol may be due to the reduction in the packed cell volume associated with chronic liver diseases. A depression in packed cell volume and anemia were observed in this study. However, decreased serum cholesterol level does not have much clinical significance in the physiologic system.

Reports are not available about inorganic phosphorus levels in experimental liver fibrosis. In the present study, a significantly decreased serum and liver inorganic phosphorus have been observed especially during later periods of DMN treatment. The decreased serum inorganic phosphorus may be due to the increased insulin levels in hepatic fibrosis, which causes phosphate ions to enter the cells along with glucose. An elevated serum insulin level has been noticed in this study (Table 3). Enhanced carbohydrate and nucleic acid metabolism, which is common in cirrhosis, can also cause depletion in phosphorus levels.

Significantly elevated insulin level was reported in carbon tetrachloride-induced experimental cirrhosis in rats (39). In the present investigation, increased serum insulin levels were observed on the 14th and 21st days after the start of DMN administration (Table 3). The fasting blood glucose level was also raised on the 21st day indicating insulin resistance (Table 2). This observation of insulin resistance is a common feature in alcoholic liver cirrhosis (40,41). The exact reason for insulin resistance in hepatic fibrosis is not well understood. It was suggested that hyperinsulinemia and impaired glucose tolerance in liver cirrhosis could be related to reduced peripheral utilization of glucose and insulin due to receptorial and postreceptorial defects and altered feedback inhibition of insulin secretion (41, 42). Because liver is directly involved in the regulation of plasma insulin levels, the extensive destruction of liver parenchyma in hepatic fibrosis might result in decreased hepatic insulin degradation.

An abnormally low serum creatinine concentration (43) and a decreased rate of creatinine production (44) have been observed in patients with severe hepatic disease. Diminished urea synthesis with alteration in protein and amino acid metabolism have also been reported in cirrhotic subjects (45).

But in the present investigation, a normal blood urea, serum, and urine creatinine levels were observed. This indicates that the kidney function is normal in DMN-induced hepatic fibrosis in rats. Normal urea cycle enzyme activity (46) and normal blood urea nitrogen levels (39) were reported in carbon tetrachloride-induced cirrhosis in rats. The low serum creatinine in patients with cirrhosis is due to decreased synthesis of creatinine from diminished muscle mass and inadequate production of creatine, a creatinine precursor, by the liver (44,47).

Increased plasma uric acid level has been reported in patients with liver cirrhosis (48). In this study, significantly elevated uric acid levels were observed in serum and urine samples during the latter periods of DMN administration (Table 3 and 4). Because uric acid is the end product of purine catabolism, an elevated plasma level and urinary excretion of uric acid indicates increased degradation of nucleic acids in DMN-induced hepatic fibrosis.

In conclusion, DMN-induced hepatic fibrosis in rats demonstrated several biochemical abnormalities such as elevated urinary excretion of hydroxyproline, increased lipid peroxidation and decrease in cholesterol levels. It also depicted an impaired glucose tolerance with insulin resistance, a reduction in inorganic phosphorus level and increased protein and nucleic acid catabolism. All these alterations are in connection with the deterioration of liver functions during the pathogenesis of hepatic fibrosis.

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References

- Jenkins SA, Grandison A, Baxter JN, Day DW, Taylor I, Shields R. A dimethylnitrosamine induced model of cirrhosis and portal hypertension in the rat. *J Hepatol* 1985; **1**: 489-99.
- George J, Chandrakasan G. Lactate dehydrogenase isoenzymes in dimethylnitrosamine induced hepatic fibrosis in rats. *J Clin Biochem Nutr* 1997; **22**: 51-62.
- Di Luzio NR, Hartman AD. Role of lipid peroxidation in the pathogenesis of the ethanol induced fatty liver. *Fed Proc* 1967; **26**: 1436-42.
- Situnayake RD, Crump BJ, Thurnham DI, Davies JA, Gearty J, Davis M. Lipid peroxidation and hepatic antioxidants in alcoholic liver disease. *Gut* 1990; **31**: 1311-7.
- Garcia-Trevijano ER, Iraburu MJ, et al. Transforming growth factor β 1 induces the expression of α 1(I) procollagen mRNA by a hydrogen peroxide-C/EBP β -dependent mechanism in rat hepatic stellate cells. *Hepatology* 1999; **29**: 960-70.
- Petrides AS, DeFronzo RA. Glucose and insulin metabolism in cirrhosis. *J Hepatol* 1989; **8**: 107-14.
- Morrison WL, Bouchier IAD, Gibson JNA, Rennie MJ. Skeletal muscle and whole body protein turnover in cirrhosis. *Clin Sci* 1990; **78**: 613-9.

8. Alberti KGMM, Johnston DG, Taylor R. Carbohydrate metabolism in liver disease. In: Wright R, Millward-Sadler GH, Alberti KGMM, Karran S, Eds. *Liver and Biliary Disease*. Pp. 45–64. London: WB Saunders, 1985.
9. George J, Chandrakasan G. Molecular characteristics of dimethylnitrosamine induced fibrotic liver collagen. *Biochim Biophys Acta* 1996; **1292**: 215–22.
10. George J, Chandrakasan G. Glycoprotein metabolism in dimethylnitrosamine induced hepatic fibrosis in rats. *Int J Biochem Cell Biol* 1996; **28**: 353–61.
11. Shiba M, Shimizu I, Yasuda M, Ii K, Ito S. Expression of type I and type III collagens during the course of dimethylnitrosamine induced hepatic fibrosis in rats. *Liver* 1998; **18**: 196–204.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–75.
13. Reinhold JG. Total protein, albumin and globulin. In: Reiner M, Ed. *Standard Methods in Clinical Chemistry, Vol. 1*. Pp. 88–97. New York: Academic Press, 1953.
14. Winckers PLM, Jacobs PH. Simple automated determination of glucose in body fluids using an aqueous *o*-toluidine-acetic acid reagent. *Clin Chim Acta* 1971; **34**: 401–8.
15. Natelson S, Scott ML, Beffa C. A rapid method for the estimation of urea in biological fluids by means of the reaction between biacetyl and urea. *Am J Clin Pathol* 1951; **21**: 275–81.
16. Yagi K. Assay for blood plasma or serum. *Methods Enzymol* 1984; **105**: 328–31.
17. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351–8.
18. Jung DH, Biggs HG, Moorehead WR. Colorimetry of serum cholesterol with use of ferric acetate/uranyl acetate and ferrous sulfate/sulfuric acid reagents. *Clin Chem* 1975; **21**: 1526–30.
19. Gomori G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J Lab Clin Med* 1942; **27**: 955–60.
20. Caraway WT. Determination of uric acid in serum by carbonate method. *Am J Clin Pathol* 1955; **25**: 840–5.
21. Owen JA, Iggo B, Scandrett FJ, Stewart CP. The determination of creatinine in plasma or serum and in urine: a critical examination. *Biochem J* 1954; **58**: 426–37.
22. Bonsnes RW, Taussky HH. The colorimetric determination of creatinine by the Jaffe reaction. *J Biol Chem* 1945; **158**: 581–91.
23. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961; **93**: 440–7.
24. Neuman RE, Logan MA. The determination of collagen and elastin in tissues. *J Biol Chem* 1950; **186**: 549–56.
25. Soeldner JS, Sloane D. Critical variables in the radioimmunoassay of serum insulin using the double antibody technique. *Diabetes* 1965; **14**: 771–9.
26. Ala-Kokko L, Pihlajaniemi T, Myers JC, Kivirikko KI, Savolainen ER. Gene expression of type I, III and IV collagens in hepatic fibrosis induced by dimethylnitrosamine in the rat. *Biochem J* 1987; **244**: 75–9.
27. Mata JM, Kershenobich D, Villarreal E, Rojkind M. Serum free proline and free hydroxyproline in patients with chronic liver disease. *Gastroenterology* 1975; **68**: 1265–9.
28. Yamada S, Hirayama C. Clinical significance of serum hydroxyproline containing peptides with special reference to hyproprotein. *Eur J Clin Invest* 1983; **13**: 129–33.
29. Murawaki Y, Yamada S, Koda M, Hirayama C. Collagenase and collagenolytic cathepsin in normal and fibrotic rat liver. *J Biochem* 1990; **108**: 241–4.
30. Murawaki Y, Hirayama C. Hepatic collagenolytic cathepsin in patients with chronic liver disease. *Clin Chim Acta* 1980; **108**: 121–8.
31. Tribble LD, Aw TW, Jones AP. The pathophysiological significance of lipid peroxidation in oxidative cell injury. *Hepatology* 1987; **7**: 377–87.
32. Slater TF, Cheeseman KH, Davies MJ, Proudfoot K, Xin W. Free radical mechanisms in relation to tissue injury. *Proc Nutr Soc* 1987; **46**: 1–12.
33. Sevanian A, Hochstein P. Mechanisms and consequences of lipid peroxidation in biological systems. *Annu Rev Nutr* 1985; **5**: 365–90.
34. Slater TF. Free radical mechanisms in tissue injury. *Biochem J* 1984; **222**: 1–15.
35. Di Luzio NR, Stege TE. The role of ethanol metabolites in hepatic lipid peroxidation. In: Fisher MM, Rankin JG, Eds. *Alcohol and the Liver*. Pp. 45–62. New York: Plenum Press, 1977.
36. Vendemiale G, Altomare E, Grattagliano I, Albano O. Increased plasma levels of glutathione and malondialdehyde after acute ethanol ingestion in humans. *J Hepatol* 1989; **9**: 359–65.
37. Corongui FP, Lai H, Milia A. Carbon tetrachloride, bromotrichloromethane and ethanol acute intoxication. New chemical evidence for lipid peroxidation in rat tissue microsomes. *Biochem J* 1983; **112**: 625–31.
38. Nordmann R, Ribiere C, Rouach H. Ethanol induced lipid peroxidation and oxidative stress in extrahepatic tissues. *Alcohol Alcohol* 1990; **25**: 231–7.
39. Ariosto F, Riggio R, Cantafora A, et al. Carbon tetrachloride induced experimental cirrhosis in the rat: A reappraisal of the model. *Eur Surg Res* 1989; **21**: 280–6.
40. Riggio O, Merli M, Cangiano C, et al. Glucose intolerance in liver cirrhosis. *Metabolism* 1982; **31**: 627–34.
41. Cavallo-Perin P, Cassader M, Bozoo C, et al. Mechanism of insulin resistance in human liver cirrhosis. *J Clin Invest* 1985; **75**: 1659–65.
42. Cavallo-Perin P, Bruno A, Nuccio P, Gorla M, Pagano G, Lenti G. Feedback inhibition of insulin secretion is altered in cirrhosis. *J Clin Endocrinol Metab* 1985; **63**: 1023–7.
43. Takabatake T, Ohta H, Ishida Y, Hara H, Ushioji Y, Hattori N. Low serum creatinine levels in severe hepatic disease. *Arch Intern Med* 1988; **148**: 1313–5.
44. Cocchetto DM, Tschanz C, Bjornsson TD. Decreased rate of creatinine production in patients with hepatic disease. Implications for estimation of creatinine clearance. *Ther Drug Monit* 1983; **5**: 161–8.
45. Rudman D, DiFulco TJ, Galambos JT, Smith RB, Salam AA, Warren WD. Maximal rates of excretion and synthesis of urea in normal and cirrhotic subjects. *J Clin Invest* 1973; **52**: 2241–9.
46. Snodgrass PJ. Urea cycle enzyme activities are normal and inducible by a high-protein diet in CCl₄ cirrhosis of rats. *Hepatology* 1989; **9**: 373–9.
47. Papadakis MA, Arief AI. Unpredictability of clinical evaluation of renal function in cirrhosis: a prospective study. *Am J Med* 1987; **82**: 945–52.
48. Masuda S, Okano T, Osawa K, Shinjo M, Suematsu T, Kobayashi T. Concentrations of vitamin D-binding protein and vitamin D metabolites in plasma of patients with liver cirrhosis. *J Nutr Sci Vitaminol* 1989; **35**: 225–34.