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Lactate Dehydrogenase Isoenzymes in Dimethylnitrosamine-Induced Hepatic Fibrosis in Rats

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Summary Alteration of liver function is an inevitable phenomenon in hepatic fibrosis. Since many enzymes are useful in diagnosing liver diseases, the alteration of the lactate dehydrogenase (LDH) isoenzyme pattern was studied in experimentally induced liver fibrosis. The liver injury was induced by intraperitoneal injections of dimethylnitrosamine (DMN) into adult male albino rats belonging to the Wistar strain. The injections were given on the first 3 consecutive days of each week over a period of 3 weeks. The degree of hepatic fibrosis was assessed by histopathological examination as well as by monitoring the collagen content of the liver tissue. The LDH isoenzymes in serum and liver were separated by polyacrylamide gel electrophoresis. The percentage distribution of the isoenzymes was determined, and the M/H ratio was calculated. The total LDH activity was also studied in the liver and serum samples. The results demonstrated many pathological changes including centrilobular necrosis and fibrosis after DMN treatment. The collagen content was increased to about 4 times normal by the 21st day of DMN administration. The total LDH activity was significantly increased in the serum on the 14th and 21st days. But in the liver tissue, a significant increase was noticed only on the 7th day. The LDH isoenzyme patterns indicated a sharp increase in the activities of LDH_4 and LDH_5 in the serum on the 14th and 21st days of DMN treatment. A remarkable increase was noticed in the M/H ratio on the 14th and 21st days in the serum and on all the days in the liver tissue. Increased serum LDH activity suggests deterioration of liver functions during hepatic fibrosis. The present study demonstrated that serum LDH isoenzyme assay is a useful tool in the diagnosis of hepatic fibrosis along with other biochemical tests.

Key Words: lactate dehydrogenase, LDH isoenzymes, dimethylnitrosamine, hepatic fibrosis, liver collagen

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Hepatic fibrosis is characterized by a widespread structural alteration in the liver, which consists primarily of increased deposition of connective tissue components, especially collagen and glycoproteins [1-3]. The alteration of connective tissue metabolism may lead to various changes in normal liver functions. Inflammatory cells, which increase in number in hepatic fibrosis are also responsible for the alteration of certain liver activities. In most forms of liver diseases, the pathophysiological processes of hepatic necrosis, inflammation, and fibrogenesis usually occur concurrently. Even though liver biopsy is a precise tool to assess the extent of liver damage, blood analysis and liver function tests are also important for a better interpretation of the disease.

It was reported that some of the serum enzymes are useful in diagnosing liver diseases [4, 5]. Lactate dehydrogenase (LDH, EC 1.1.1.37) is a cytoplasmic enzyme that catalyzes the oxidation of lactate to pyruvate and *vice versa*. LDH is present in almost all tissues of the body. Classically, five LDH isoenzymes are present in the serum, of which the electrophoretically slowest moving band (LDH_5) corresponds to the liver isozyme. The appearance of LDH_5 isoenzyme in the serum indicates hepatocellular damage. Since the LDH isoenzyme patterns are different in various organs and tissues, its serum analysis is useful in establishing the location of the cell injury.

It has been shown 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 alcoholic cirrhosis of human beings [6, 7]. Deterioration of liver functions with hypoproteinemia and jaundice were reported in DMN-induced liver damage [6]. Histopathological examination of DMN-treated liver tissue revealed extreme centrilobular necrosis accompanied by pericellular fibrosis and sinusoidal capillarization [8, 9]. In addition, inflammation, neutrophilic infiltration, and proliferating Ito cells were also demonstrated in this experimental model of hepatic fibrosis [10-12]. But the changes in the LDH isoenzyme patterns either in the serum or liver were not studied in hepatic fibrosis. So, the present investigation was aimed at studying the alteration of LDH isoenzyme patterns in the serum and liver samples during the progression of DMN-induced hepatic fibrosis in adult male albino rats. The H and M subunits of LDH were determined, and the M/H ratio was calculated. The total LDH levels were also monitored in serum and liver samples.

MATERIALS AND METHODS

Chemicals. Dimethylnitrosamine, L-hydroxyproline, chloramine-T, bovine serum albumin, lithium lactate, nicotinamide adenine dinucleotide (NAD), sodium pyruvate, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), acrylamide, N,N′-methylene-bisacrylamide, ammonium persulfate, Tris, N,N,N′, N′-tetramethyl-ethylenediamine (TEMED), and bromophenol blue were purchased from Sigma Chemical Company, St. Louis, MO. Ethylene glycol mono-

methyl ether (methyl cellosolve) was procured from Fluka AG, Switzerland; and *p*-dimethylaminobenzaldehyde, from E. Merck, Darmstadt, West Germany. Glycine, 2,4-dinitrophenylhydrazine, sodium chloride, sodium potassium tartarate, and perchloric acid were obtained from Loba Chemie, Bombay, India. All other chemicals used were of analytical grade.

Animals. Adult male albino rats of the Wistar strain, about 3 months in age and weighing between 180–200 g, were used for the experiment. The animals were bred and maintained in special polypropylene cages with a wire mesh top and a hygienic bed of husk. They were provided with commercial rat feed pellets (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*.

Induction of hepatic fibrosis. Hepatic fibrosis was induced by intraperitoneal injections of dimethylnitrosamine (DMN) in a dose of 1 μl (diluted to 1 : 100 with 0.15 M sterile NaCl)/100 g body weight. The injections were given on the first three consecutive days of each week over a period of 3 weeks. Control animals ($n=6$) also received an equal volume of 0.15 M NaCl without DMN. The injections were given without anesthesia. Treated animals ($n=6$ in each group) 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 mean value was used as the control. All the animals were anesthetized with diethyl ether before sacrifice. Blood was collected from a deep cut made in the right jugular vein of the neck with a scalpel blade. It was allowed to clot at 30°C for 1 h and the serum was separated by centrifugation at 2,000 $\times g$ for 10 min. The serum was not stored, and the enzyme assays were carried out immediately after the collection.

Preparation of liver extract. The livers were rapidly removed, rinsed in ice-cold 0.15 M NaCl to remove blood-borne contaminants, and blotted. Each liver specimen was chopped after having been weighed on an electronic balance, and homogenized separately in a Polytron homogenizer (Kinematica AG, Switzerland) with ice-cold 0.15 M NaCl solution at 4°C. The final concentration of the homogenate was adjusted to 100 mg tissue/ml and used for enzyme assays within 2 h. The total protein present in the liver extract was determined by the method of Lowry [13].

Assessment of the degree of hepatic fibrosis. The degree of hepatic fibrosis was evaluated both histopathologically and biochemically. In addition, the liver weight and body weight of the animals were also monitored during DMN administration. For histopathological evaluation, the liver sections were stained with hematoxylin and eosin and examined with a Nikon labophot microscope.

The biochemical evaluation of hepatic fibrosis was carried out by determining the collagen content in the liver tissue. The total collagen content in the liver was determined by the estimation of hydroxyproline, a characteristic imino acid in collagen. For the determination of hydroxyproline, all samples were hydrolyzed in 6 N 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 then 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 [14]. 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 M 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 determined with a spectrophotometer at 560 nm.

The total collagen content in the liver tissue was calculated by multiplying the hydroxyproline content by the factor 7.46, as postulated by Neuman and Logan [15].

Assay of total LDH activity in serum and liver. The total LDH activity in serum and liver homogenate was determined according to the method of King [16]. The spectrophotometric assay of LDH involves the use of lactate as substrate and NAD as coenzyme. In brief, 1 ml of buffered substrate was mixed with 0.1 ml of 1 : 10 diluted serum/liver homogenate and treated as the test sample. For the control, 1 ml of buffered substrate was mixed with 0.2 ml water. Both tubes were warmed at 37°C for 5 min. To each test sample, 0.2 ml of coenzyme (0.5% NAD in water) was added, and the tubes were incubated for exactly 15 min. The reaction was stopped by the addition of 1 ml of 2,4-dinitrophenylhydrazine (20 mg in 100 ml of 1 N HCl) to both test and control tubes followed by diluted enzyme to the controls. The tubes were incubated at 37°C for another 15 min, and 5 ml of 0.4 N NaOH were then added to each tube and mixed well. The intensity of the color was measured at 440 nm with a Shimadzu UV-160 spectrophotometer.

A standard curve was prepared with 50–300 nmol of sodium pyruvate. It was prepared along with 1 mM NADH in buffered substrate solution and 0.5% NAD in distilled water. The enzyme activity was expressed as μmol of pyruvate formed/h/ml serum and μmol of pyruvate formed/min/100 mg protein, for serum and liver, respectively.

Determination of LDH isoenzymes in serum and liver by polyacrylamide gel electrophoresis. The LDH isoenzymes present in serum and liver samples were determined by the disc electrophoretic method of Dietz and Lubrano [17]. The isoenzymes were separated on polyacrylamide gel since it has the advantage of high resolving power and excellent separation of proteins into sharp bands. The isoenzymes were identified by numbers according to their electrophoretic mobility on the gel. The fastest moving component is LDH_1; and the slowest moving, LDH_5.

The isoenzymes were separated on a 5% polyacrylamide gel without sodium dodecyl sulfate. The gel tubes, fixed on a special stand, were filled with gel mixture and allowed to polymerize by preventing contact with the atmosphere by layering a drop of water gently on top of the mixture. After polymerization at 20°C, the water on the top of the gel was blotted out, and 10 μl of diluted serum/liver

homogenate supernatant (1 : 1 dilution with 40% sucrose) was added. Then 30 μl of 40% sucrose was layered over the sample and the tubes were filled with running buffer. The tanks were filled with diluted glycine buffer [6 g Tris and 28.8 g glycine in 1 liter water (pH 8.3), diluted 10 times before use] and 0.2 ml bromophenol blue (10 mg/100 ml) was added to the cathode chamber. Electrophoresis was performed at 4°C with a constant current of 3 mA/tube till the marker dye reached the bottom of the tube. After the run, the gels were removed and stained. The staining solution was prepared freshly by mixing 1 ml of 1 M lithium lactate, 1 ml of NAD (10 mg/ml), 1 ml of 0.1 M NaCl, 1 ml of 5 mM $MgCl_2$, 2.5 ml of 0.5 M phosphate buffer (pH 7.4), 2.5 ml of nitroblue tetrazolium (1 mg/ml), and 0.25 ml of phenazine methosulfate (1 mg/ml). All solutions, with the exception of NAD, were prepared and stored at 4°C in the dark. NAD was prepared freshly. The staining was performed at 37°C in the dark for about 1 h.

The stained bands were scanned in a LKB Laser Densitometer, model 2202 Ultroscan (LKB-Produkter AB, S-16126, Bromma, Sweden) connected to a LKB 2220 computing integrator. The percentage of H and M subunits was calculated according to the method of Markert [18]. This was based on the tetramer structure of LDH with bands 1 to 5 containing 0, 25, 50, 75 and 100% of the M-LDH, respectively ($LDH_1 = H_4M_0$, $LDH_2 = H_3M_1$, $LDH_3 = H_2M_2$, $LDH_4 = H_1M_3$ and $LDH_5 = H_0M_4$). The M/H ratio was calculated for both control and treated animals.

Statistical analysis. The results were statistically evaluated by analysis of variance (ANOVA). Arithmetic mean and standard error were calculated for the data. The control values were compared with the treated values on different days by the least significant difference method. A value of $p < 0.05$ was considered as significant. Correlation analysis was used to assess the linear curve fitting of the LDH standard curve, and the correlation coefficient was found to be > 0.999.

RESULTS

The alteration in the body weight and liver weight of the animals monitored during the progression of hepatic fibrosis is demonstrated in Fig. 1. The DMN-administered animals did not gain body weight during the course of treatment. A significant decrease ($p < 0.001$) was noticed in the mean body weight of the animals after the 14th and 21st days of DMN administration. Similarly, liver weight was also reduced significantly ($p < 0.001$) on the 14th and 21st days of DMN treatment (Fig. 1).

The histopathological examination of the liver tissue demonstrated severe centrilobular congestion and marked dilatation of central veins and sinusoids on the 7th day. On the 14th day, there were massive centrilobular necrosis and intense neutrophilic infiltration. On the 21st day of DMN treatment, the liver showed many changes common to those seen in alcoholic fibrosis. There was diffuse centrilobular necrosis in all cases. Hydropic and focal fatty changes were also seen.

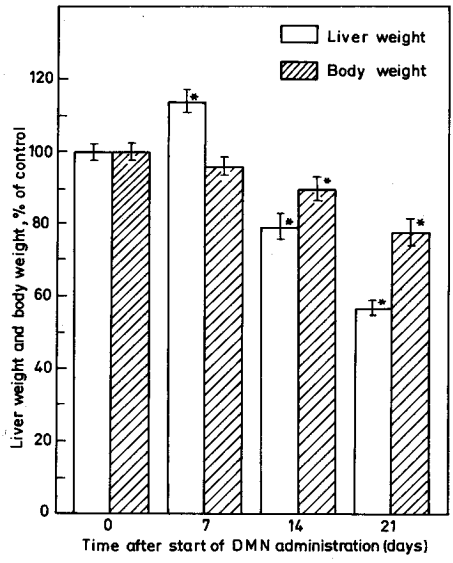

Fig. 1. Changes in liver weight and body weight of the animals during DMN-induced hepatic fibrosis in rats. The liver weight is expressed as mg/g body weight. Values given are the mean and standard deviation (*$p<0.001$ by ANOVA).

Increased fibrosis with bile duct hyperplasia was noticed consistently, and regeneration of hepatocytes was seen in many cases. The hepatocytes showed Mallory's hyaline within the cytoplasm. Apoptosis and dysplasia were frequent. There was bridging necrosis and early fibrosis between the portal tract and central vein. All the 21st day liver sections showed well developed fibrosis around central veins.

The total collagen content in the liver during the pathogenesis of DMN-induced hepatic fibrosis is shown in Fig. 2. By the 21st day of DMN treatment, fibrosis was well developed with a 4 fold increase in the total collagen content in the liver.

The total LDH activity in the serum during the progression of DMN-induced hepatic fibrosis is demonstrated in Fig. 3. A significant increase ($p<0.001$) was noticed in the total LDH activity on the 14th and 21st days of DMN administration. The difference was not significant on the 7th day. The maximum increase observed on the 21st day, was about 3 fold greater than the control. The total LDH activity in the liver tissue is shown in Fig. 4. A significant increase ($p<0.001$) was noticed in the hepatic LDH activity only on the 7th day after the start of DMN administration. On the 14th and 21st days, the total LDH activity in the liver tended to decrease with the greatest depletion on the 21st day. But the difference was not significant when this value was compared with the control one.

Figure 5 demonstrates LDH isoenzyme patterns in the rat serum during DMN administration. Polyacrylamide gel electrophoretic separation of isoenzymes revealed a sharp increase in the activities of LDH_4 and LDH_5 isoenzymes on the 14th and 21st days of DMN treatment (lanes C and D in Fig. 5). No significant

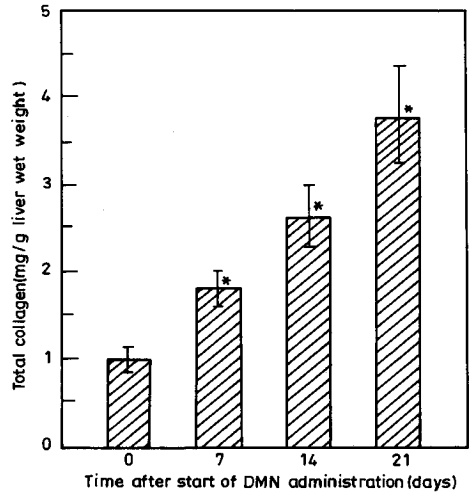

Fig. 2. Total collagen content in the liver during DMN-induced hepatic fibrosis in rats. The values given are the mean and standard deviation (*$p<0.001$ by ANOVA). The collagen content in the liver tissue was determined by estimating hydroxyproline, a characteristic imino acid present in collagen.

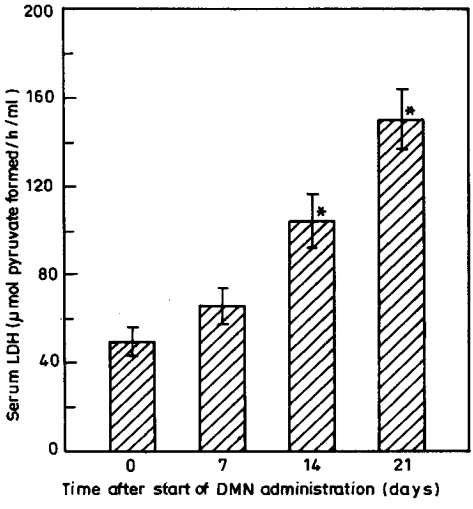

Fig. 3. Total LDH activity in the serum during DMN-induced hepatic fibrosis in rats. The values represent mean and standard deviation (*$p<0.001$ by ANOVA).

alteration was noticed in the serum LDH isoenzyme patterns on the 7th day of DMN administration. Figure 6 represents LDH isoenzymes in the liver during DMN-induced hepatic fibrosis. A significant increase was noticed in the LDH_5 isoenzyme activity in the liver tissue on all days of DMN treatment (Fig. 6). The maximum increase was on day 21.

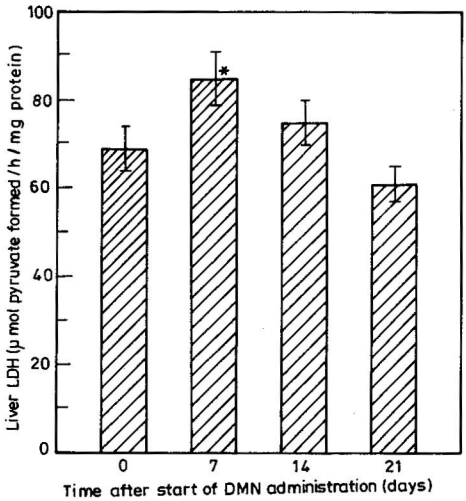

Fig. 4. Total LDH activity in the liver tissue during DMN-induced hepatic fibrosis in rats. The values are mean and standard deviation ($*p<0.001$ by ANOVA).

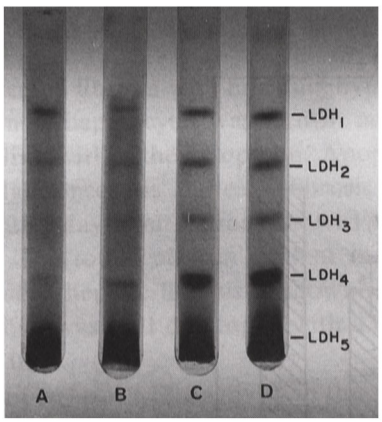

Fig. 5

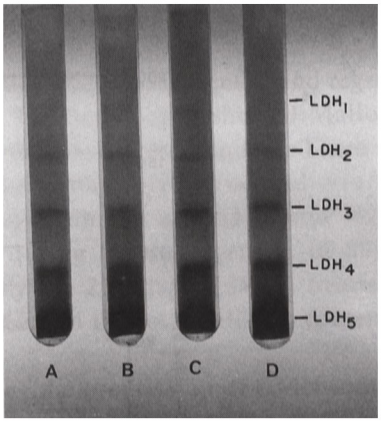

Fig. 6

Fig. 5. LDH isoenzymes in rat serum during the progression of DMN-induced hepatic fibrosis. The isoenzymes were separated by polyacrylamide gel electrophoresis. Lane A, control; lane B, day 7 of treatment period; lane C, day 14; lane D, day 21.

Fig. 6. Polyacrylamide gel electrophoretic pattern of LDH isoenzymes in rat liver tissue during DMN administration. Lane A, control; lane B, day 7 of treatment period; lane C, day 14; lane D, day 21.

Table 1 depicts the percentage distribution of LDH isoenzymes in the rat serum after laser densitometry of the stained bands. The total M subunits in the serum were increased on the 21st day of DMN administration, and the total H subunits were significantly reduced on the 14th and 21st days. As a result, the M/H ratio was remarkably increased on the 14th and 21st days of DMN treatment

Table 1. Percent distribution of serum LDH isoenzymes, M and H subunits and M/H ratio in DMN-induced hepatic fibrosis in rats.

Isoenzymes	Control	Day 7	Day 14	Day 21
LDH_1	18.84±1.56	16.34±1.08	10.87±0.96	9.14±1.06
LDH_2	14.13±0.90	12.38±0.65	8.01±1.11	7.63±0.92
LDH_3	7.34±0.38	9.62±0.61	5.13±0.61	3.19±0.31
LDH_4	9.02±0.28	10.56±0.86	20.63±1.62	22.96±1.86
LDH_5	50.67±2.65	51.10±3.11	55.36±3.12	57.08±3.12
Total M subunits	64.65±3.37	66.92±4.21	75.40±4.27	77.81±4.46*
Total H subunits	35.35±2.07	33.08±2.18	24.60±1.98**	22.19±1.81***
M/H ratio	1.83±0.12	2.02±0.15	3.06±0.23***	3.51±0.28***

Values are the mean±standard error vs. control ($n=6$). *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Table 2. Percent distribution of liver LDH isoenzymes, M and H subunits and M/H ratio in DMN-induced hepatic fibrosis in rats.

Isoenzyme	Control	Day 7	Day 14	Day 21
LDH_1	1.60±0.12	1.08±0.11	0.80±0.06	1.04±0.08
LDH_2	8.74±0.71	5.21±0.71	5.21±0.41	3.38±0.37
LDH_3	16.54±1.08	11.34±0.96	15.70±1.06	13.07±1.06
LDH_4	25.22±1.68	28.74±2.18	21.50±2.08	20.26±1.86
LDH_5	47.90±3.11	53.63±3.16	56.79±3.17	62.25±4.12
Total M subunits	77.26±4.12	82.15±5.06	82.06±5.56	84.82±5.31
Total H subunits	22.74±2.01	17.85±1.42	17.94±1.36	15.18±0.97**
M/H ratio	3.40±0.18	4.60±0.34*	4.57±0.32**	5.58±0.43***

Values are the mean±standard error vs. control ($n=6$). *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

($p<0.001$). No significant alteration was noticed in the isoenzyme pattern on the 7th day.

The percentage distribution of LDH isoenzymes in the rat liver is demonstrated in Table 2. The total M subunits in the liver showed only a plateau on all the days of DMN administration. But the H subunits were considerably decreased on the 21st day ($p<0.01$). The M/H ratio was significantly increased on all the days of DMN treatment, with a maximum on day 21.

DISCUSSION

Hepatic fibrosis is always accompanied by impaired hepatocyte metabolism and deposition of connective tissue components in the liver [19, 20]. A significant decrease in liver weight [21] and decreased liver weight to body weight ratio [22] were reported in DMN-induced hepatic fibrosis. In the present study, an increase was noticed in the liver weight to body weight ratio on the 7th day of DMN administration. This could have been due to the induction of liver microsomal enzyme systems by DMN, which in turn, induces increased metabolic activity and a corresponding increase in liver weight. But in the later stages, the liver weight

was reduced due to decreased protein synthesis, cell necrosis, and collapse of the liver parenchyma.

The histopathological changes observed in the present investigation were on a par with the pathophysiological alteration reported in human hepatic fibrosis. The 21st day course of controlled DMN administration in rats produced massive centrilobular necrosis and well developed fibrosis around the central veins. The approximately 4 fold increase in total liver collagen observed in the present study coincides with the previous investigations on DMN-induced hepatic fibrosis in rats [2, 22].

Reports are not available regarding LDH isoenzyme patterns either in experimental or human hepatic fibrosis. A significantly increased total LDH activity in the serum was reported in DMN-induced chronic liver injury in dogs [23]. Elevated serum total LDH activity was also reported in human liver cirrhosis [24]. In the present investigation also, significantly increased total LDH activity was observed in the serum on the 14th and 21st days after administration of DMN. The increased enzyme activity in the serum is due to the extreme necrosis of the liver tissue during fibrosis and simultaneous leakage of the enzyme into the blood stream.

Data are not available with regard to total LDH activity in the liver tissue during experimentally induced hepatic fibrosis. A normal LDH activity was reported in the liver tissue in hepatic cirrhosis of alcoholic origin [25]. An increased release of LDH from cultured mouse hepatocytes treated with dimethylnitrosamine was reported [26]. In the present study, a significant increase in the liver LDH activity was noticed only on the 7th day of DMN administration. This indicates an increased synthesis of the enzyme in the liver during the early stages of hepatic fibrosis. But in later stages the enzyme activity is diminished due to a reduction in the number of functional hepatocytes due to extreme necrosis of the liver tissue.

The LDH_5 isoenzyme level in the serum is reportedly a sensitive indicator of hepatic injury in patients with acute liver disorders [27, 28]. A significant increase in the serum level of the fifth LDH fraction was reported in rats treated with carbon tetrachloride [29]. Elevated levels of serum LDH_5 isoenzyme activity was observed in patients with various liver diseases [30]. An increased level of LDH_4 and LDH_5 isoenzymes was also reported in patients with metastatic liver disease [31]. In the present investigation, a sharp increase was noticed in the activities of LDH_4 and LDH_5 isoenzymes on the 14th and 21st days after DMN administration. These LDH isoenzymes, especially LDH_5, are very abundant in liver and are a marker of liver tissue. Dimethylnitrosamine treatment leads to extreme hepatocellular necrosis and simultaneous spillage of LDH_5 isoenzyme into the circulation. An increase in the LDH_5 isoenzyme level in the serum is a characteristic feature of almost all liver diseases.

Data are not available or are scanty regarding the alteration of M and H subunits of LDH isoenzymes either in serum or liver tissue during hepatic dis-

orders. In the present study, a significant increase was noticed in the case of M subunits in the serum on the 21st day of DMN treatment. This led to a dramatic decrease in H subunits, resulting in a significant increase in the M/H ratio on the 14th and 21st days of DMN treatment. This was due to the significant increase in the M subunit-dominant LDH_4 and LDH_5 isoenzymes in the serum. In the liver tissue also, the increase observed in the M/H ratio was due to the alteration of M and H subunits during DMN administration.

The present investigation demonstrated alteration of total LDH and LDH isoenzyme patterns in the serum and liver during DMN-induced hepatic fibrosis. It also revealed changes in the amounts of M and H subunits and an increase in the M/H ratio during DMN administration. The increased LDH activity in the serum indicates deterioration of liver functions during hepatic fibrosis. Since there is a variation in LDH isoenzyme patterns in both serum and liver tissue of rats and humans, caution must be exercised before extrapolating the results to man.

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