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Hepatic Damage Caused by Chronic Arsenic Toxicity in Experimental Animals

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ABSTRACT

Objective: Noncirrhotic fibrosis of the liver is common in subjects chronically consuming ground water geologically contaminated with arsenic, but the mechanism of the hepatic fibrosis is not known. Because lipid peroxidation has been implicated in the development of several other forms of hepatic fibrosis, including iron and copper overload, we have explored the roles of oxidative stress and lipid peroxidation in the causation of hepatic fibrosis in a murine model of chronic arsenic toxicity. **Methods:** Male BALB/c mice were given drinking water contaminated with arsenic (3.2 mg/L) or arsenic-free (<0.01 mg/L, control) ad libitum. Mice were sacrificed at 3, 6, 9, 12, and 15 months for examination of hepatic histology and assays of hepatic reduced glutathione content, lipid peroxidation, enzymes of the antioxidant defense system, and membrane-bound sodium/potassium ATPase (Na^+/K^+ ATPase). **Results:** After 12 months of arsenic feeding, the liver weights increased significantly as did serum aspartate aminotransferase and alanine aminotransferase. After 6 months of arsenic feeding, hepatic glutathione and the enzymes glucose-6-phosphate dehydrogenase and glutathione peroxidase were significantly lower than those of the control group. Hepatic catalase activity was significantly reduced at 9 months in the arsenic-fed group, while glutathione-S-transferase and glutathione reductase activities were also significantly reduced at 12 and 15 months. Plasma membrane Na^+/K^+ ATPase activity was reduced after 6 months while lipid peroxidation increased significantly after 6 months of arsenic feeding. Liver histology remained normal for the first 9 months, but showed fatty infiltration after 12

months of arsenic feeding. Histologic evidence of fibrosis was observed after 15 months. **Conclusion:** We have demonstrated hepatic fibrosis due to long-term arsenic toxicity in an animal model. Initial biochemical evidence of hepatic membrane damage, probably due to reduction of glutathione and antioxidant enzymes, may be seen by 6 months. Continued arsenic feeding resulted in fatty liver with serum aminotransferase and alanine aminotransferase elevated at 12 months and hepatic fibrosis at 15 months. The murine model is proposed as relevant to epidemic human toxicity in areas of arsenic contamination.

INTRODUCTION

Cutaneous and hepatic manifestations of arsenic toxicity due to chronic consumption of arsenic-contaminated ground water are seen commonly among a large number of inhabitants of various districts of West Bengal.¹ An important feature of chronic arsenic toxicity in West Bengal is a form of hepatic fibrosis that causes portal hypertension, but does not progress to cirrhosis.²⁻⁴ Prolonged consumption of arsenic-contaminated liquids, such as Fowler's solution, has been known to cause similar hepatic lesions.⁵ Although the association between arsenic toxicity and hepatic fibrosis has been firmly established, the mechanism of this type of liver damage remains unelucidated.

Oxidative damage is thought to underlie several chronic liver diseases that are associated with fibrosis. Oxidative stress, which is based on activated molecular species of oxygen, is a complex process that can result in the peroxidative damage of the major cellular components including amino acids, carbohydrates, lipids, proteins, and nucleic acids.^{6,7} Damage to membrane lipids and the associated alterations in bulk properties of membranes frequently are, however, considered to be the primary basis for chemical-induced hepatocellular injury and a loss of cell viability. Lipid peroxidation has been found to be important in carbon tetrachloride (CCl₄)-induced liver damage, as well as in acetaldehyde and free-radical induced fibrosis in ethanol-fed animals.^{8,9} Similarly, lipid peroxidation has been proposed as the mechanism of hepatic fibrosis due to excess iron^{10,11} or copper deposition¹² in experimental animals. Based on these studies, we hypothesized that lipid peroxidation-mediated membrane damage, presumptively due to oxidative stress and reduced enzymatic antioxidant defense, may be an important mechanism of the hepatic fibrosis that results from chronic arsenic toxicity. To test this hypothesis, we utilized a murine model in which hepatic fibrosis was induced by feeding arsenic-contaminated water for 15 months. Alteration of the enzymes involved in the antioxidant defense system and membrane damage

due to lipid peroxidation precede the pathomorphological lesions of arsenic-induced hepatic fibrosis in the murine model.

MATERIAL AND METHODS

Collection of Arsenic Contaminated Water

Arsenic contaminated subsoil drinking water was collected from a well that had been used as a drinking water source for many years by a family, several members of which manifested clinical features of arsenic toxicity. Total arsenic content of the water was measured spectrophotometrically using silver diethyldithiocarbamate (Ag-DDTC) in chloroform (CHCl₃) with hexamethylene tetramine¹³ and the proportion of the arsenite (As III) and arsenate (As V) were determined.¹⁴ The water sample was preserved at -20° C for the study.

Animals

Inbred male BALB/c mice (7-8 weeks old, weighing 22-25 g) were maintained on standard laboratory chow at a constant temperature and humidity environment with a 12-hour light:dark cycle. All animal experiments were approved by and were performed in accordance with local institutional guidelines for the care and use of laboratory animals.

Experimental Protocol

The mice were divided into 2 groups. The experimental group was provided with the arsenic-contaminated water (3.2 mg/L) ad libitum. The control group drank arsenic-free water (<0.01 mg/L). The mice were killed in batches under ether anesthesia after 3, 6, 9, 12, and 15 months. Blood was collected by cardiac puncture before sacrifice for the estimation of serum proteins, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) by commercially

available kits. The livers were removed and weighed. A small liver fragment from each mouse was fixed in 10% buffered formalin for histologic examination. The remaining liver was perfused with 20 mL of ice-cold isotonic saline through the portal vein, removed, blotted, and divided into 2 portions.

Assay of Enzymes Involved in the Antioxidant Defense System

A portion of the liver was minced and a 10% homogenate was prepared in ice-cold, potassium phosphate buffer (10 mM, pH 7.4) containing 0.15 M KCl, using a glass homogenizer fitted with a Teflon pestle. The homogenate was divided into 3 parts which were processed as follows. One part was used for the assay of reduced glutathione (GSH) and total thiol (-SH).¹⁵ The second part was centrifuged at $2000 \times g$ for 15 minutes at 4°C and the postnuclear supernatant fraction was used for lipid peroxidation analysis.¹⁶ The third part was used for the preparation of cytosolic proteins. The homogenate was centrifuged at $3000 \times g$ for 15 minutes at 4°C and the supernatant was centrifuged again at $105,000 \times g$ for 60 minutes. The supernatant was used for the assay of the activities of glucose-6-phosphate dehydrogenase (G6PD),¹⁷ catalase,¹⁸ glutathione-S-transferase (GST),¹⁹ glutathione peroxidase (Gpx),²⁰ and glutathione reductase (GR).²¹

Plasma Membrane Isolation and Na^+/K^+ ATPase Assay

The second portion of liver tissue was used for the preparation of plasma membrane-enriched fractions as follows. A 20% liver homogenate was prepared in 1 mM NaHCO_3 , containing 0.5 mM CaCl_2 (pH 7.4). The homogenate was centrifuged at $1500 \times g$ for 30 minutes. The plasma membrane enriched fraction was prepared as described by Pascale *et al.*²² In brief, the sediment, after centrifugation at $1500 \times g$ for 30 minutes, was washed twice in $\text{NaHCO}_3/\text{CaCl}_2$ solution and the final sediment containing crude liver plasma membranes (LPM) was suspended in 1.5 mL of 48% sucrose. The suspension was transferred to 30 mL polycarbonate tubes and carefully overlaid with 8 mL of 45% sucrose, 10 mL of 41% sucrose, and 10.5 mL of 37% sucrose. All sucrose solutions contained 2mM Tris-HCl buffer (pH 7.4). After centrifugation at $100,000 \times g$ for 70 minutes, partially purified LPM were found in a band between 37 and 41% sucrose. The LPM suspension was diluted 10 times with

0.1 mM NaHCO_3 , sedimented for 10 minutes at $14,000 \times g$, and finally suspended at about 2 mg of protein/mL in the same buffer. Na^+/K^+ ATPase activities were determined at 37°C according to the method of Ismail-Beigi and Edelman²³ as modified by Gonzalez-Calvin *et al.*²⁴ NADPH-cytochrome c reductase and succinate dehydrogenase activities were determined according to the methods of Feo *et al.*^{25,26} For evaluation of the extent of contamination by microsomes and mitochondria, NADPH, cytochrome c reductase and succinate dehydrogenase activities, respectively, were determined.

Protein Determination

The protein content of the liver homogenates and the subcellular fractions was determined by the method of Lowry *et al.*²⁷

Arsenic Estimation in Liver Tissues

The arsenic content of liver tissue of 5 experimental animals and 4 control animals obtained after 6 months of drinking arsenic-contaminated water and arsenic-free water, respectively, was estimated as described by Das *et al.*²⁸

Liver Morphology

Hematoxylin-eosin, reticulin, and Masson-stained paraffin sections of formalin-fixed liver tissues were evaluated by an examiner who was unaware of the experimental group.

Statistical Analysis

Data were expressed as mean \pm SD. Statistical analysis of the difference between sample means was done by Student's *t*-test.

RESULTS

Long-Term Effect of Arsenic on Body Weight

The ground water fed to the experimental group contained arsenic 3.2 mg/L with an arsenite (As III) to arsenate (As V) ratio of 1:1. Based on the daily water consumption, the average daily arsenic ingestion was calculated to be 70.4 $\mu\text{g}/100 \text{ g}$ body weight. Consumption of the arsenic contaminated water for 15 months caused significant reductions in body weight at 12

Table 1

Liver Function Tests in Control and Arsenic-Exposed Mice at the End of Different Study Periods

Month	Group	T. Protein (g/dL)	Albumin (g/dL)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
3	Control (n = 5)	6.46 ± 0.34	3.24 ± 0.35	22.80 ± 2.77	23.40 ± 4.44	107.6 ± 5.02
	Experimental (n = 6)	6.23 ± 0.46	3.08 ± 0.29	23.83 ± 2.99	24.10 ± 4.57	109.8 ± 6.99
5	Control (n = 10)	6.58 ± 0.58	3.42 ± 0.45	24.30 ± 3.80	23.50 ± 2.27	108.6 ± 8.03
	Experimental (n = 10)	6.50 ± 0.73	3.17 ± 0.48	26.30 ± 4.49	26.00 ± 5.29	111.7 ± 5.83
9	Control (n = 5)	6.52 ± 0.54	3.40 ± 0.60	22.80 ± 2.78	22.60 ± 1.67	113.4 ± 8.64
	Experimental (n = 5)	6.40 ± 1.32	3.08 ± 0.68	31.20 ± 9.65	26.80 ± 4.29	116.8 ± 11.62
12	Control (n = 9)	6.36 ± 0.91	3.06 ± 0.45	25.00 ± 4.27	23.50 ± 2.78	121.5 ± 6.8
	Experimental (n = 14)	6.66 ± 0.86	3.03 ± 0.61	34.60 ± 6.93**	32.80 ± 8.21*	128.4 ± 12.02
15	Control (n = 8)	6.05 ± 0.26	3.11 ± 0.12	27.50 ± 4.10	25.50 ± 6.63	123.7 ± 5.02
	Experimental (n = 6)	5.95 ± 0.32	2.71 ± 0.24*	40.80 ± 5.60**	38.16 ± 6.79*	164.6 ± 10.94**

p < 0.01, **p < 0.001.

months (35.14 ± 8.52 g in the arsenic-fed mice compared to 40.50 ± 3.16 g in the control group [$p < 0.05$]) and at 15 months (31.83 ± 14.58 g in arsenic-fed mice compared to 38.37 ± 2.94 g in the control group [$p < 0.01$]). There was no significant difference in the amount of food or water consumption between the 2 groups.

Liver Weights and Serum Markers of Liver Injury

A significant increase in liver weight was observed after 12 months of arsenic-contaminated water consumption (4.65 ± 1.55 g per 100 g of body weight in arsenic-fed mice vs 3.77 ± 0.29 g in the control groups at 12 months [$p < 0.05$] and 5.9 ± 1.09 g in arsenic-fed mice compared to 4.24 ± 0.29 g in the control groups at 15 months [$p < 0.01$]). Serum ALT and AST levels were significantly increased in the arsenic-fed group after 12 months while an increase in serum ALP levels and a re-

duction of albumin concentration occurred after 15 months of arsenic consumption (Table 1).

Effect of Arsenic Consumption on Hepatic GSH Content and Enzymes of the Antioxidant Defense System

As shown in Figure 1, a time-related reduction in hepatic GSH content occurred in the arsenic-fed mice. Hepatic activities of enzymes related to GSH homeostasis, namely, G6PD, GST, GR, Gpx, and catalase, were also reduced in a time-related manner (Table 2).

Hepatic Lipid Peroxidation

Lipid peroxidation was enhanced in the arsenic-fed mice, as evidenced by increased production of malondialdehyde. As shown in Figure 1, the lipid peroxidation in-

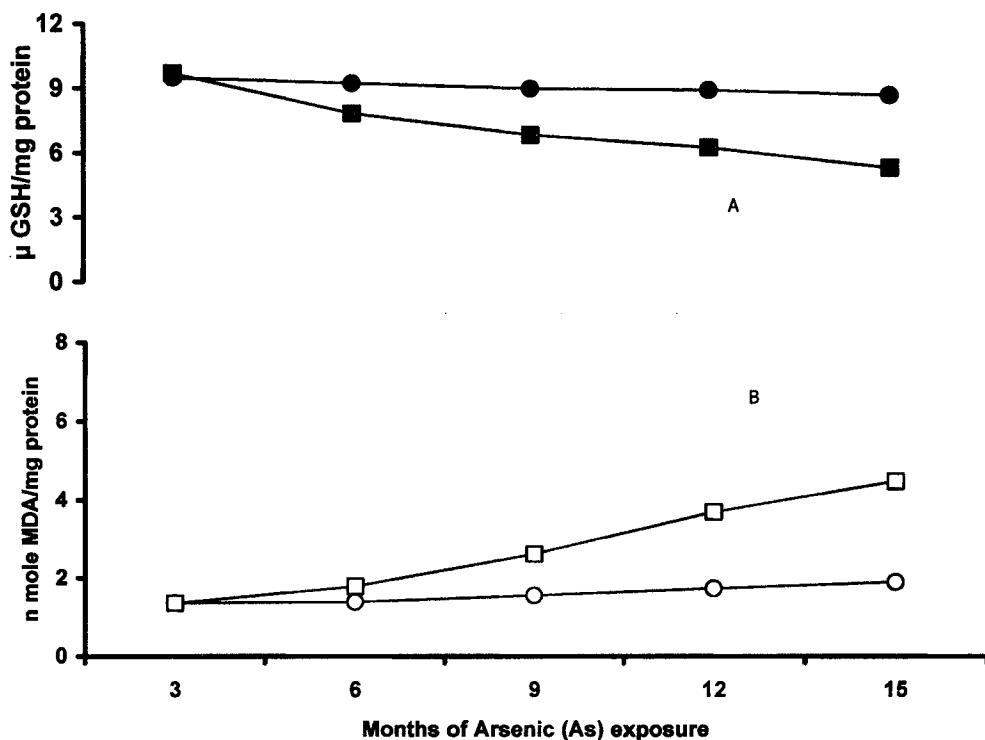


Figure 1. Relationship between hepatic GSH content and MDA concentration, a marker of lipid peroxidation, in mice exposed to chronic arsenic toxicity. A: Shows the time-related reduction of hepatic GSH content in the arsenic-fed mice (■) compared to control (●). B: Shows hepatic MDA levels with time in arsenic-fed mice (□) compared to control (○).

Table 2

Hepatic Glutathione and Other Enzymes of the Antioxidant Defense System in Control and Arsenic-Exposed Mice at the End of Different Study Periods

Month	Group	G6PDH (nmole NADP reduced/min/ mg protein)	GR (μmole of NADPH oxidation/ min/mg protein)	GST (nmole produced/ min/mg protein)	Catalase (μmole H ₂ O ₂ reduced/min/ mg protein)	GSH-Px (μmole NADPH oxidation/ min/mg protein)
3	Control (n = 5)	10.32 ± 0.23	24.38 ± 0.89	118.02 ± 1.12	6.78 ± 0.22	8.28 ± 0.38
	Experimental (n = 6)	11.04 ± 0.56	27.39 ± 2.66*	130.05 ± 11.04*	7.26 ± 0.59	9.19 ± 0.67†
6	Control (n = 10)	10.46 ± 0.84	24.92 ± 0.37	118.42 ± 2.04	6.68 ± 0.33	8.27 ± 0.28
	Experimental (n = 10)	8.39 ± 0.62#	25.43 ± 1.31	121.85 ± 7.30	6.34 ± 1.05	7.49 ± 0.94†
9	Control (n = 5)	10.05 ± 0.41	24.99 ± 0.83	117.41 ± 1.87	6.78 ± 0.27	8.29 ± 0.20
	Experimental (n = 5)	7.02 ± 0.29**	24.24 ± 2.21	113.92 ± 4.70	6.06 ± 0.48*	7.01 ± 0.61#
12	Control (n = 9)	9.80 ± 0.25	25.04 ± 0.44	117.73 ± 2.24	6.74 ± 0.29	8.03 ± 0.24
	Experimental (n = 14)	6.39 ± 0.64**	20.86 ± 1.88**	102.68 ± 6.90**	5.45 ± 0.50**	5.95 ± 1.01**
15	Control (n = 8)	9.94 ± 0.26	24.93 ± 0.79	122.28 ± 4.22	6.47 ± 0.26	8.02 ± 0.55
	Experimental (n = 6)	5.02 ± 0.87**	18.43 ± 2.02**	88.72 ± 18.0**	4.55 ± 0.85**	5.20 ± 1.12**

*p < 0.05, †p < 0.02, #p < 0.01, **p < 0.001.

creased with time and was inversely correlated with hepatic GSH content.

Na⁺/K⁺ ATPase Activity in Plasma Membrane-Enriched Fractions

Hepatocellular plasma membrane preparations contained only minimal contamination of microsomal or mitochondrial membranes as shown by the depletion of NADPH-cytochrome c reductase and succinate dehydrogenase activities, respectively (relative specific activities > 0.05). There was a time-related reduction in hepatic plasma membrane Na⁺/K⁺ ATPase activity in the arsenic-fed mice, compared with the control groups (Figure 2). The reduction of Na⁺/K⁺ ATPase activity was inversely related to the increase in lipid peroxidation.

Liver Arsenic Content

After 6 months of arsenic-contaminated water, the hepatic arsenic of experimental animals ($0.66 \pm 0.35 \mu\text{g}/$

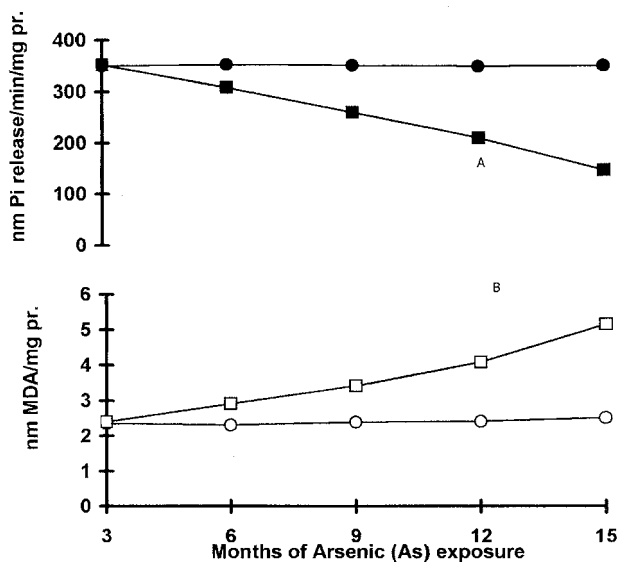


Figure 2. Relationship between hepatic MDA level, a marker of lipid peroxidation and membrane-bound Na⁺/K⁺ ATPase activity in mice exposed to chronic arsenic toxicity. A: Shows a time-related reduction of hepatic membrane-bound Na⁺/K⁺ ATPase activity as expressed in m mole (nm) Pi release/min/mg protein in the arsenic-fed mice (■) compared to control (●). B: Shows increased hepatic MDA levels with time in arsenic-fed mice (□) compared to control (○).

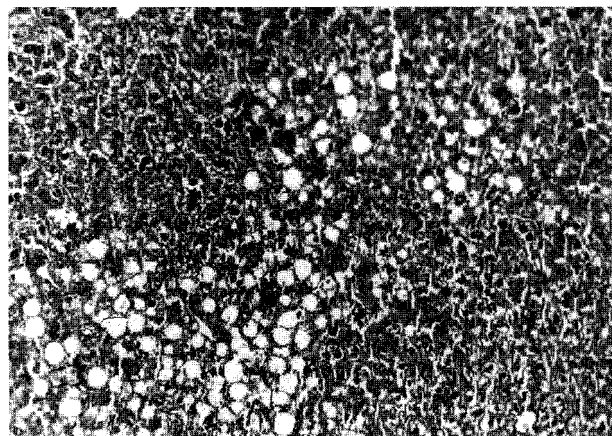


Figure 3. Liver histology of mice exposed to arsenic for 12 months. The histology shows fatty changes in the liver (hematoxylin-eosin, magnification $\times 100$).

g) was significantly elevated above control ($0.029 \pm 0.005 \mu\text{g/g}$) ($p < 0.001$).

Pathomorphology of the Liver

No abnormal hepatic morphology was observed by light microscopy during the first 9 months of arsenic feeding. After 12 months, 11 of 14 mice in the experimental group exhibited histological abnormalities charac-

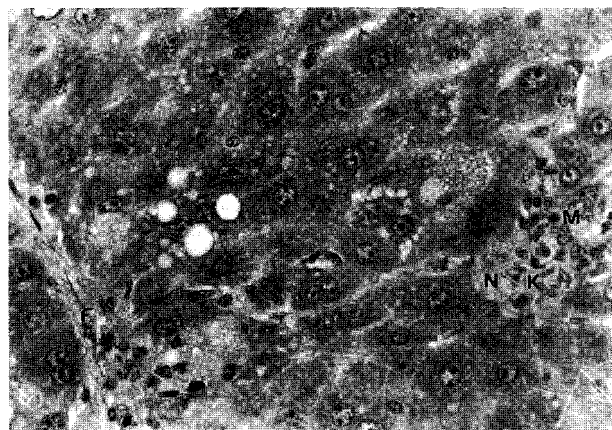


Figure 4. Liver histology of mice exposed to arsenic for 15 months. The histology shows hepatocellular degeneration and necrosis characterized by collections of mononuclear cells and Kupffer cells associated with injured hepatocytes. Streaky fibrosis is seen at one end of the liver lobule (hematoxylin-eosin, magnification $\times 400$).

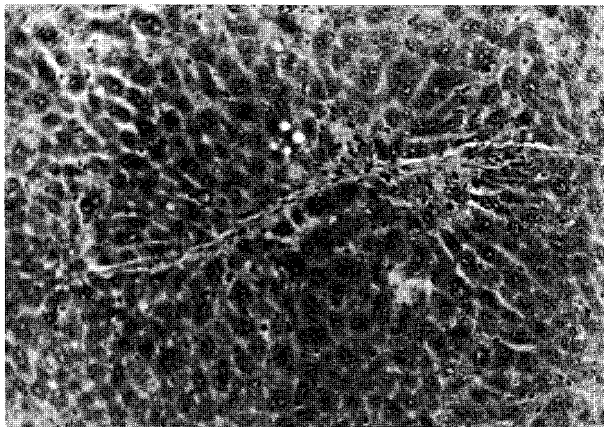


Figure 5. Liver histology of mice exposed to arsenic for 15 months. The histology shows streaky fibrosis in the liver (hematoxylin-eosin, original magnification $\times 100$).

terized by hepatocellular degeneration and focal mononuclear cell collection. Fatty degeneration in the liver (macrovesicular steatosis) was seen in 5 mice (Figure 3). After 15 months of arsenic-contaminated drinking water, hepatocellular necrosis associated with intralobular mononuclear cell infiltration and Kupffer cell proliferation and hepatic fibrosis were evident (Figure 4). A few hepatocytes still contained macrovesicular fat and the nuclei of some hepatocytes showed vacuolation (Figure 4). Stellate scars were seen spreading from portal zones in 4 of 6 mice (Figure 5). Mature fibrous tracts contained only a small number of inflammatory cells (Figure 6). The he-

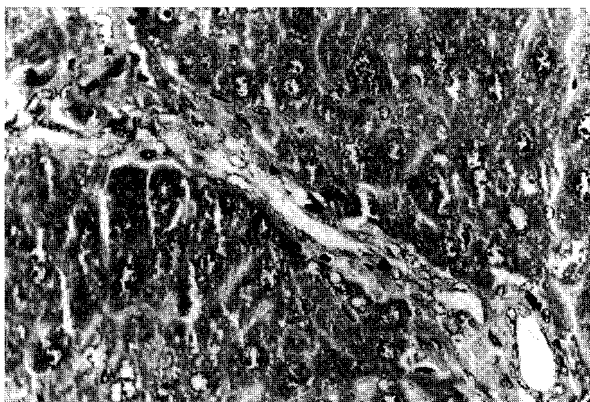


Figure 6. Liver histology of mice exposed to arsenic for 15 months. The histology shows mature collagen deposition spreading from the portal tracts through the liver lobules (Masson Trichrome $\times 400$).

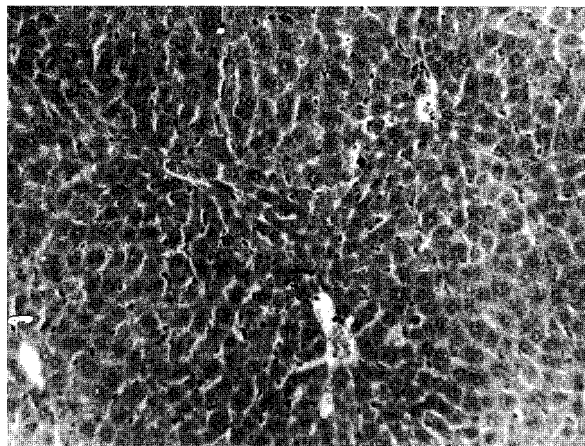


Figure 7. Normal liver histology of control mice at the end of 15 months of feeding As-free water (hematoxylin-eosin, magnification $\times 100$).

patic fibrosis was not associated with nodular regeneration. The liver morphology was normal in all control mice (Figure 7).

DISCUSSION

Noncirrhotic portal fibrosis is a relatively common clinical problem in India, Japan, and some other countries. Our group^{2,3} and other investigators^{5,29,30} have reported a significant incidence of this disorder in people drinking arsenic-contaminated water for long periods of time. Despite the establishment of an association of non-cirrhotic portal fibrosis with drinking of arsenic-contaminated water in several districts of West Bengal, India, elucidation of the mechanism of this disorder has remained conjectural. Our present study is the first to describe arsenic-induced portal fibrosis in an animal model. As in the human subjects, long-term consumption of arsenic-contaminated water was needed for generation of hepatic fibrosis. Although hepatocellular necrosis and a mild degree of mononuclear infiltration was present, there was no evidence of cirrhotic nodules, which is another similarity to the human disease. The elevation of hepatic arsenic after 6 months of arsenic consumption confirmed arsenic accumulation in the liver.

Inorganic arsenic increases the rate of formation of active oxygen species including superoxide anion radical (O_2^-) and hydroxyl (OH) radical through a chain reaction.³¹⁻³³ The mechanism of arsenic toxicity to individual cell type has historically centered around the inhibitory

effects on cellular respiration at the level of mitochondria.^{34,35} Disruption of oxidative phosphorylation and concomitant decrease in the cellular levels of ATP³⁶ are thought to be important central events of arsenic-induced toxicity evoking increased production of hydrogen peroxide. These effects could cause formation of reactive oxygen species resulting in oxidative stress.³⁷ Arsenite-induced oxidative stress in mammalian cells is supported indirectly by the finding that adding superoxide dismutase to the culture medium reduces the frequency of arsenic-induced sister chromatid exchange in human lymphocytes.³⁸

In many animals, including mammals, inorganic arsenic is metabolized in the liver.³⁹ The liver possesses an antioxidant defense system that removes peroxides, free radicals, and superoxide anion generated within the cell. GSH is a critical component of this defense system. GSH is required for reduction of arsenic (V) to arsenic (III) species in preparation for enzymatically catalyzed oxidative methylation. It also promotes arsenic methylation by stabilizing the redox state of the cell.⁴⁰ Methylation of inorganic arsenic protects the cell against some effects of the more toxic inorganic form.⁴¹ The cumulative depletion of hepatic GSH that occurred after 12 months of arsenic consumption may have exacerbated the toxic effects of arsenic, which became histologically obvious at this time.

The glutathione cycle is mediated by several enzymes functioning in a concerted manner. Our study found the decreased enzymes of the GSH regeneration and scavenger systems in the liver of the arsenic-fed animals coincided with the reduction of hepatic GSH level.

The increased lipid peroxidation and plasma membrane damage, as shown by a progressive reduction of Na⁺/K⁺ ATPase activity in the mice drinking arsenic-contaminated water paralleled GSH depletion of the liver. GSH depletion may result in the accumulation of free radicals that initiate lipid peroxidation and initiate biochemical damage by covalent binding to macromolecules. GSH depletion and enhanced lipid peroxidation in rat liver have also been reported in rats treated with a large dose of sodium arsenite (18.2 mg/kg body weight).⁴² Similarly, oxidative damage and peroxidation of lipid membranes have been observed with excessive hepatic iron^{10,11} and copper¹² deposition in experimental animals. Peroxidative breakdown of polyunsaturated fatty acids and membrane phospholipids leads to biochemical changes culminating in the inactivation of membrane enzymes, such as Na⁺/K⁺ ATPase, that may lead to alteration of ion transport and cellular water content, eventually leading to cell death. Several studies have

also implicated lipid peroxidation in the pathogenesis of hepatic fibrogenesis, which was seen as a relatively late outcome of chronic consumption in our study. Considered together, these observations suggest that weakening of the antioxidant defense system of the liver and consequent peroxidative damage of the lipid membranes may play a central role in the generation of liver pathology in chronic arsenic toxicity.

Oxidative stress in the tissue not only causes peroxidative damage of the membrane lipids, but also causes oxidative damage of the cellular proteins. Some evidence of oxidative damage of proteins resulting in hepatocellular damage are available in CCl₄-treated⁴³ as well as ethanol-intoxicated rats.⁴⁴ Although evidence of oxidative protein damage in chronic arsenic intoxication is not yet available, the role of protein oxidation in arsenic-induced liver injury cannot be ruled out. Further studies on protein oxidation are needed to understand the complete sequences of biochemical changes in arsenic-induced liver damage.

In summary, we have demonstrated an animal model of arsenic-induced noncirrhotic fibrosis of the liver. The drinking water used in our experiments was identical to the water consumed by many people living in regions where the ground water is geologically contaminated with arsenic. The prolonged and continuous exposure to arsenic required for the initiation of hepatic fibrogenesis is consistent with the clinical situation. The temporal sequence of the biochemical abnormalities of liver function in our murine model suggests that an initial perturbation of the enzymes involved in the antioxidant defense system results in reduction of hepatic GSH content and hepatocellular membrane damage caused by lipid peroxidation. Whether hepatocellular injury and subsequent hepatic fibrosis, as observed in our study, is caused solely by membrane damage or by additional oxystress-induced cellular protein degradation needs further study. This murine model can be utilized to test the basic mechanisms of hepatic fibrosis induced by environmental arsenic intoxication and other metallotoxins.

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