

Oxidative stress in liver of mice exposed to arsenic-contaminated water

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Background: Oxidative stress has been implicated in the initiation of hepatic damage caused by various agents. Not much data on oxidative stress in liver in chronic arsenic exposure are available in the literature. We therefore studied this aspect in a murine model. **Methods:** BALB/c mice were given arsenic-contaminated (3.2 mg/L) or arsenic-free (<0.01 mg/L, control) drinking water *ad libitum*. Batches of mice were sacrificed after 2 and 4 months, and blood samples and liver tissue were collected. Liver histology was examined and levels of hepatic reduced glutathione (GSH), malondialdehyde, and enzymes of the antioxidant defense system in the liver tissue were determined. Arsenic content in liver tissues obtained at 4 months was estimated. **Results:** Two-month exposure to arsenic caused significant elevation of hepatic GSH (11.4 [0.8] µg/mg protein) compared to control mice (9.3 [0.4]; p<0.01). Levels of enzymes related to GSH homeostasis were also elevated. At 4 months, hepatic GSH was significantly reduced (8.4 [0.5] µg /mg protein) when compared to control mice (9.3 [0.4]; p<0.01). Arsenic content in the liver tissue after 4 months of exposure was significantly higher (0.40 [0.05] µg /g) as compared to control mice (0.04 [0.04]; p<0.01). **Conclusion:** The results suggest that the antioxidant defense system in the liver of mice is activated after exposure to arsenic for 2 months. However, prolonged exposure to arsenic probably causes overuse failure of this system, which might result in initiation of biochemical injury to the liver. [*Indian J Gastroenterol* 2000;19:112-115]

Key words: Antioxidants

Arsenic contamination of subsoil drinking water and consequent chronic arsenic toxicity is a major public health problem in eight districts of West Bengal.¹ Although such an environmental health problem has been reported from various parts of the world,^{2,3,4} the largest has involved vast areas of West Bengal and Bangladesh.^{5,6} The source of such contamination is geological. The arsenic content of most drinking water sources in these areas exceeds the WHO permissible limit of 50 µg /L.

Prolonged arsenic ingestion leads to hepatic fibro-

sis and non-cirrhotic portal hypertension.^{7,8} Although described for nearly a century, the mechanism of arsenic-induced liver injury remains elusive.

In several animal models, free radical-mediated oxidative stress has been shown to be responsible for hepatocellular injury and hepatic fibrosis, in particular those induced by CCl₄⁹ and ethanol.¹⁰ Free radicals have also been proposed to be involved in the liver damage caused by excess deposition of iron¹¹ and copper.¹² Previously, we have shown that feeding arsenic for prolonged periods in mice caused perturbation of the enzymes involved in the antioxidant defense system, resulting in depletion of hepatic GSH and elevation of lipid peroxidation. This, in turn, produced hepatocellular injury and eventually fibrosis.¹³ Based on this information, we hypothesized that oxidative stress may play an important role in the genesis of hepatic damage that results from chronic arsenic exposure.

In the present communication, we present data on the initial biochemical events in relation to oxidative stress following administration of arsenic for short duration.

Methods

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 Ag-DDTC in CHCl₃ with hexamethylene tetramine¹⁴ and the proportions of arsenite (As III) and arsenate (As V) were determined.¹⁵ The water sample was preserved at -20°C for the study.

Inbred BALB/c mice (7-8 weeks old, weighing 20 - 22 g) were maintained on standard laboratory chow at a constant temperature and humidity environment in 12-hour light-dark cycles. All animal experiments were approved by the institution and were performed in accordance with local institutional guidelines for the care and use of laboratory animals.

The mice were divided into two groups. The experimental group was provided with the arsenic-contaminated water (3.2 mg/L) *ad libitum* while the control group was provided arsenic-free water (<0.01 mg/L). The mice

were sacrificed in batches under ether anesthesia after 2 (10 animals in each group) and 4 (5 animals in each group) months. Blood was collected from each mouse by cardiac puncture before sacrifice; total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated by standard laboratory techniques. The livers were removed and weighed; a small liver specimen from each mouse was fixed in 10% buffered formalin for histologic examination; another specimen from each mouse in the 4 month experiment was stored at -20°C for estimation of arsenic content. The remaining liver tissue was perfused with 20 mL of ice-cold isotonic saline through the portal vein, removed and blotted.

The perfused liver tissue 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 three portions, which were processed as follows. One was used for assay of reduced glutathione (GSH).¹⁶ The second part was centrifuged at 2000xg for 15 min at 4°C, and the post-nuclear supernatant was used for assay of malondialdehyde (MDA), a marker of lipid peroxidation.¹⁷ The third part was centrifuged at 3000xg for 15 min in a cold centrifuge at 2°C (Remi C24); the supernatant was centrifuged at 105,000xg for 1 h at 2°C (Spino Model L ultracentrifuge) and the pellet was discarded. The supernatant (cytosol) was used to assay the activities of glucose 6-phosphate dehydrogenase (G6PD),¹⁸ glutathione reductase (GR),¹⁹ glutathione-S-transferase (GST),²⁰ catalase²¹ and glutathione peroxidase (GSH-Px).²²

Protein content of the liver homogenates and sub-cellular fractions was determined by the method of Lowry *et al.*²³ Arsenic content of liver tissue of 5 experimental animals and 4 control animals (in the fifth control animal, liver tissue obtained was inadequate) obtained after 4 months of feeding was estimated as described by Das *et al.*²⁴ Hematoxylin-eosin and reticulin-stained paraffin sections of formalin-fixed liver tissues were evaluated by an examiner who was unaware of the arsenic-exposure status of the animal from which the tissue had been harvested.

Data are expressed as mean (SD). Intergroup differences were analyzed using Wilcoxon's rank sum test at a significance level of 0.05.

Results

The well water fed to the experimental group contained 3.2 mg/L of arsenic with arsenite (As III) to arsenate (As V) ratio of 1:1. Based on average water consumption by the mice, average daily arsenic ingestion was calculated to be 70.4 mg/100 g body weight. Serum levels of ALT,

Table 1: Liver function test of control and experimental mice exposed to arsenic (mean [SD])

	2 months		4 months	
	Control (n=10)	Experimental (n=10)	Control (n=5)	Experimental (n=5)
Total protein (g/dL)	6.2 (0.3)	6.2 (0.3)	6.3 (0.2)	6.1 (0.2)
Albumin (g/dL)	3.4 (0.2)	3.3 (0.3)	3.5 (0.4)	3.6 (0.3)
ALT (IU/L)	21.5 (2.0)	21.6 (3.3)	22.2 (1.3)	22.4 (1.3)
AST (IU/L)	22.2 (2.2)	22.1 (2.6)	22.4 (1.3)	22.7 (1.7)
ALP (IU/L)	111.0 (2.5)	111.6 (4.3)	110.0 (5.7)	109.1 (7.1)

AST and albumin remained unaltered in experimental mice compared to control (Table 1).

Liver arsenic content of arsenic-exposed mice at the end of 4 months (0.40 [0.05] µg/g; $p < 0.01$) was significantly higher as compared to control mice (0.04 [0.04] µg/g).

Various parameters related to glutathione homeostasis after arsenic exposure are presented in Table 2. After 2 months of exposure to arsenic, hepatic GSH level was significantly higher than that in the control mice. Enzymes related to GSH homeostasis, such as GSH-regenerating enzymes G6PD and GR, and scavenging enzymes GST and GSH-Px, too were increased. However, there was no difference in catalase level between experimental and control mice.

After 4 months of arsenic exposure, the rise in hepatic GSH that was observed at 2 months had been reversed (Table 2). Also, hepatic GSH content was depleted as compared to control mice. GSH-regenerating enzyme G6PD was markedly reduced whereas GR activity was still maintained at a high level. Levels of two scavenging enzymes, GST and GSH-Px, which were el-

Table 2: Hepatic glutathione and other enzymes of antioxidant defense system in control and arsenic-exposed mice (mean [SD])

	2 months		4 months	
	Control (n=10)	Exptl. (n=10)	Control (n=5)	Exptl. (n=5)
GSH (µg/mg protein)	9.3 (0.4)	11.4# (0.8)	9.3 (0.4)	8.4# (0.5)
Lipid peroxidation (nmole malondialdehyde formed/mg protein)	1.4 (0.1)	1.3 (0.2)	1.4 (0.2)	1.6 (0.2)
<i>GSH-regenerating enzymes</i>				
G6PD (nmole NADP reduced/min/mg protein)	10.4 (0.3)	13.6# (2.0)	10.3 (0.4)	9.3* (0.5)
GR (µmole NADPH oxidised/min/mg protein)	24.8 (0.9)	28.1* (2.0)	25.0 (0.3)	27.0# (1.1)
<i>Scavenging enzymes</i>				
GST (nmole product/min/mg protein)	118.1 (3.5)	143.2# (17.4)	116.9 (3.3)	124.7 (8.7)
GSH-Px (µmole NADPH oxidised/min/mg protein)	8.2 (0.4)	10.2# (0.8)	8.2 (0.3)	8.9 (0.9)
Catalase (µmole H ₂ O ₂ reduced/min/mg protein)	6.83 (0.4)	7.4 (1.0)	6.9 (0.4)	7.0 (1.2)

* $p < 0.05$, # $p < 0.01$ compared to control animals

evaluated after exposure to arsenic for 2 months, also returned to normal at the end of 4 months of arsenic exposure (Table 2). Catalase level remained unaltered.

MDA level, a marker of lipid peroxidation, in the livers of arsenic-exposed mice at 2 and 4 months was comparable to that in control mice (Table 2).

No abnormal hepatic morphology was observed by light microscopy after 2 and 4 months of feeding arsenic-contaminated water.

Discussion

Exposure to arsenic for 2 months resulted in significant elevation of hepatic GSH content. This elevation may reflect an attempt to counteract the increased oxidative stress induced by chronic arsenic exposure, and was probably mediated by elevation in the activities of G6PD and GR that we observed. Increase in G6PD activity leads to activation of hexose monophosphate (HMP) shunt pathway, thereby increasing the supply of NADPH, which in turn results in increased GR activity and rise in levels of GSH. The observed increases in activities of the scavenging enzymes GST and GSH-Px further suggest activation of the adaptive mechanism of hepatocytes against oxidative stress. The increased activity of GSH-Px may allow scavenging of lethal peroxide radicals and thereby maintain normal lipid peroxidation and hepatocyte integrity.

However, after 4 months of arsenic exposure, a fall in GSH was noted. This may reflect a failure of the antioxidant defense system. A fall in G6PD activity supports this observation. Increased level of hepatic GR, despite the fall in G6PD activity, suggests a tendency of the hepatocytes to regenerate GSH from its oxidized state to combat the oxidative assault. Elevated levels of GST and GSH-Px returned to normal levels at the end of 4 months of arsenic exposure, further suggesting a failure of the antioxidant defense system. The scavenging enzyme GSH-Px helps GSH in the formation of stable products from peroxides. Accumulation of arsenic in the livers of arsenic-exposed mice at 4 months may be due to depletion of hepatic GSH content. For metabolism of inorganic arsenic, GSH is required for its methylation to monomethylarsonic acid and dimethylarsenic acid.²⁵ Once inorganic arsenic is deposited in the liver, it may produce a continuous oxidative stress and initiate biochemical damage in the liver, the main site of its xenobiotic metabolism.

Active oxygen species such as superoxide (O_2^-) and hydroxyl (OH^\cdot) ions are formed during arsenic metabolism through a chain of reactions.²⁶ Moreover, metabolites of arsenic, such as dimethylarsenic acid, also produce active oxygen species and peroxy radicals.²⁷ The liver possesses an antioxidant defense system that removes free radicals, superoxides and peroxides gener-

ated within the hepatocytes. GSH plays the most important role in deactivating the intermediate products of xenobiotics metabolism²⁸ and prevent peroxidation of membrane lipids. Further, GSH is required for reduction of arsenic (V) to arsenic (III) species, methylation of inorganic arsenic and thus protect the cell against some effects of the more toxic inorganic form.²⁹

Exposure to arsenic-contaminated drinking water for a prolonged period is associated with pathological changes in the liver as evidenced by clinical^{7,8} and experimental¹³ studies. Feeding of arsenic-contaminated water to mice for 15 months and sacrifice of these mice at different intervals revealed no histological changes in the liver up to 9 months, hepatocellular steatosis associated with significant reduction in hepatic antioxidant defense system at 12 months, and evidence of liver fibrosis associated with further impairment of antioxidant defense system at 15 months.¹³

In summary, exposure to arsenic for a short period initiates activation of the liver antioxidant defense system by increasing hepatic GSH content to counteract and protect the hepatocytes against free radical-mediated oxidative assault. This antioxidant defense system becomes less effective subsequently due its overactivity. This might initiate hepatic injury.

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Acknowledgement: The authors acknowledge the Indian Council of Medical Research, New Delhi for providing financial assistance for this study

Received July 9, 1999. Received in final revised form December 16, 1999. Accepted January 7, 2000