Evidence for Induction of Oxidative Stress Caused by Chronic Exposure of Chinese Residents to Arsenic Contained in Drinking Water

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Exposure of experimental animals or cultured cells to arsenic induces oxidative stress, but, to date, no examination of this phenomenon in humans has been reported. In this study we conducted a cross-sectional study in Wuyuan, Inner Mongolia, China, to explore the relationship between chronic arsenic exposure from drinking water and oxidative stress in humans. Thirty-three inhabitants who had been drinking tube-well water with high concentrations of inorganic arsenic (mean value = 0.41 mg/L) for about 18 years constituted the high-exposure group, and 10 residents who lived nearby but were exposed to much lower concentrations of arsenic in their drinking water (mean value = 0.02 mg/L) were selected as the low-exposure comparison group. Results of the present study indicated that although the activity for superoxide dismutase (SOD) in blood did not differ significantly between the two groups, the mean serum level of lipid peroxides (LPO) was significantly higher among the high-exposed compared with the low-exposed group. Elevated serum LPO concentrations were correlated with blood levels of inorganic arsenic and its methylated metabolites. In addition, they showed an inverse correlation with nonprotein sulfhydryl (NPSH) levels in whole blood. The subjects in the high–arsenic-exposure group had mean blood NPSH levels 57.6% lower than those in the low-exposure group. Blood NPSH levels were inversely correlated with the concentrations of inorganic arsenic and its methylated metabolites in blood and with the ratio of monomethylarsenic to inorganic arsenic. These results provide evidence that chronic exposure to arsenic from drinking water in humans results in induction of oxidative stress, as indicated by the reduction in NPSH and the increase in LPO. Some possible mechanisms for the arsenic-induced oxidative stress are discussed. *Key words*: arsenic, chronic arsenic poisoning, drinking water, glutathione, human, lipid peroxidation, methylation, nonprotein sulfhydryl, oxidative stress. *Environ Health Perspect* 110:331–336 (2002). [Online 28 February 2002]
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Arsenic is widely distributed in nature and is mainly transported in the environment by water (*1*). Chronic exposure of humans to high concentrations of arsenic in drinking water is associated with skin lesions (*2,3*), peripheral vascular disease (*4*), hypertension (*5*), blackfoot disease (*2,6*), and a high risk of cancers (*2,7*). Inorganic arsenic (iAs) predominantly exists in two oxidation states naturally: trivalent arsenite [iAs(III)] and pentavalent arsenate [iAs(V)]. It is generally recognized that the toxicity of iAs(III) is a result of its ability to bind with sulfhydryl (SH) groups, especially vicinal thiols in proteins, resulting in the dysfunction of enzyme activity (*8*), and the toxicity of iAs(V) is a result of its ability to substitute phosphate in enzyme-catalyzed reactions, where it can disrupt normal functions of the enzymes (*9,10*). It is well known that animal models are not a good predictor of human carcinogenesis for arsenic, suggesting that the mechanisms for metabolism and toxicity of arsenic in humans differ from those in animals. Recent studies suggest that arsenic also exerts its toxicity through the generation of reactive oxygen species (ROS) (*11–14*), which include hydrogen peroxide and other chemical forms known as free radicals (e.g., superoxide $\cdot O_2^-$, hydroxyl OH·, and peroxyl ROO· radicals) (*15*). iAs(III) has been shown to enhance the production of heme oxygenase, an indicator of oxidative stress (*11*), and to increase intracellular peroxide levels *in vitro* (*12,16*). Acute and chronic intake of arsenic increased lipid peroxides (LPO) in blood, liver, kidney, and other organs of rats (*13,14*). However, no information has been available on the relationship between oxidative stress and chronic exposure of humans to arsenic contained in drinking water.

In biological systems, a number of antioxidant defense systems, including enzymatic and nonezymatic routes, operate to control excessive levels of ROS (*15*). Superoxide dismutase (SOD), an antioxidase catalyzing the dismutation of $\cdot O_2^-$ into hydrogen peroxide and O_2, is thought to be essential for the protection of cells against ROS and is used experimentally and clinically as an antioxidant drug (*17*). SOD has been shown to effectively reduce the frequency of arsenite-induced sister chromatid exchanges in human peripheral lymphocytes (*18*) and XRS-5 X-ray sensitive cells (*19*). Glutathione (GSH), the most abundant nonprotein sulfhydryl (NPSH) in most cells, acts as a nucleophilic scavenger of numerous compounds and their metabolites via enzymatic and chemical mechanisms and plays important roles in the protection against oxidative damage caused by ROS (*20–22*). GSH can react nonenzymatically with ROS or can act as a substrate in the GSH peroxidase-mediated destruction of hydroperoxides (*22*). GSH depletion can impair a cell's defense against the toxic actions of many compounds and may lead to cell injury and death (*21,22*). Cellular toxicity of arsenic was found to be inversely related to intracellular GSH levels and thus may be enhanced by GSH depletion (*23*). Acute administration of arsenic to rats produced a significant reduction in hepatic GSH (*24*). Chronic exposure of rats or mice to arsenic via injections caused up to 35% depletion in hepatic GSH, along with liver injury (*13,25*).

The purpose of this study was to investigate whether prolonged ingestion of arsenic contained in drinking water can cause oxidative stress in humans by comparing serum levels of LPO between the high– and

low–arsenic-exposed subjects. We also sought to elucidate the possible underlying mechanisms of the oxidative stress induced by arsenic.

Materials and Methods

Study participants. The present study was carried out in three villages of Wuyuan, a town in the southwestern area of Inner Mongolia, China, where the drinking water supply system has been dependent on tube wells with a depth of about 15 m. Thirty-three volunteer residents (mean age, 42.4 ± 13 years; range, 13–73 years; 21 male and 12 female) from 11 families who lived in the villages of Yindingtu and Shiba, Wuyuan, were selected as high–arsenic-exposed subjects. The tube-well water consumed by this group contained high concentrations of arsenic (0.41 ± 0.11 mg/L, mean ± SD). Ten residents from five families who lived in the village of Shahe, Wuyuan, located 35 kilometers from Yindingtu and Shiba and with drinking water containing lower arsenic levels (0.02 ± 0.01 mg/L, mean ± SD), were recruited as control subjects (mean age, 37.3 ± 16.4 years; range, 23–68 years; 5 male and 5 female). All of the subjects who were selected in the high– and low–arsenic-exposed groups are of the Han nationality. Mean duration of tube-well water consumption for the high– and low–arsenic-exposed groups was similar (about 18 years). Two trained doctors administered a questionnaire during an in-person interview and obtained information about lifetime drinking sources, medical history, alcohol consumption, cigarette smoking, and dietary habits. Two trained dermatologists blinded with respect to arsenic exposure status conducted detailed physical examinations. All the subjects who participated in the study provided informed consent.

Fasting peripheral venous blood was drawn from the study participants, and the levels of NPSH and SOD activity in blood were determined immediately. Serum was separated immediately from 1.5 mL of blood and transferred on dry ice to China Medical University for the measurement of LPO. In addition, 1.5 mL of each whole blood sample was transferred on dry ice to St. Marianna University School of Medicine in Japan for arsenic measurements. The blood samples of all study subjects were tested simultaneously. Water samples were taken directly from the tube wells and preserved at room temperature before determination of arsenic concentrations.

Arsenic analysis. Quantitation of total arsenic (tAs) in water and iAs, monomethylarsenic (MMeAs), and dimethylarsenic (DMeAs) in blood, urine, and hair was performed as reported previously (*26*). Briefly, 0.25 g of hair or 0.5 mL of each other sample was transferred into a 10 mL polymethylpentene test tube, and after the addition of 2 mL 2M sodium hydroxide to the sample, the mixture was heated at 95°C for 3 hr. The assay sample was stirred with a magnetic stirrer once every 30 min. The treated sample was diluted to make 10 mL, and an aliquot sample was used for each assay. iAs and methylated arsenic compounds do not undergo changes in chemical species (e.g., distribution of methyl groups) even when heated in 2M NaOH (*26*). iAs, MMeAs, and DMeAs were determined by atomic absorption spectrophotometry (Shimadzu model AA-6103; Shimadzu, Kyoto, Japan). Using this method, the detection limit of each of the three chemical species of arsenic was 1 ng, and the coefficient of variation was < 5%. The standard reference material used was oyster tissue (No. 1566) from the National Bureau of Standards (NBS, Washington, DC, USA). Because of limited seafood ingestion, no trimethylarsenic was detected in the blood or urine samples of any subjects.

Assay of serum LPO. Serum LPO was determined by measuring the thiobarbiturate-reactive substances (TBA-RS) by the method of Yagi et al (*27*). Briefly, serum (300 μL) was mixed with 20% trichloroacetic acid (2,700 μL) and 0.67% thiobarbituric acid (1,000 μL) and then incubated at 100°C for 30 min. After cooling with tap water, the resulting chromogen was extracted with *n*-butyl alcohol (4,000 μL) by vigorous shaking. The mixtures were then centrifuged at 3,000 rpm for 10 min, and the supernatants obtained were monitored at a wavelength of 535 nm by a Shimazu spectrophotometer (UV-1600; Shimadzu). 1,1,3,3-Tetraethoxypropane was used as the external standard, and the concentration of LPO was expressed as nanomoles of malondialdehyde equivalents.

Determination of NPSH in whole blood. We determined whole-blood NPSH levels using 5,5′-dithiobis-2-nitrobenzoic acid and measured the absorbance at 412 nm according to the method of Ellman (*28*). Briefly, whole blood (100 μL) was diluted with 0.4 mL of cold double-distilled water (DDW), and 0.5 mL of 10% trichloroacetic acid was added to precipitate the protein. The sample was mixed well and centrifuged at 15,000 rpm for 5 min. The supernatant was used for the determination.

Determination of SOD activity in whole blood. Whole blood (50 μL) was washed with 5 mL saline and centrifuged at 2,000 rpm for 3 min. The rinsed cells were diluted with cold DDW to make a final volume of 0.2 mL. Ethanol (0.1 mL) and chloroform (0.1 mL) were mixed with the erythrocyte lysis to precipitate the hemoglobin. The tubes were shaken vigorously for 15 min and centrifuged at 4,000 rpm for 3 min. The water-ethanol layer was used to determine SOD activity according to the method of Elstner and Heupel (*29*).

Data analysis. We used SPSS software (version 6.0; SPSS Inc., Chicago, IL, USA) to determine statistical significance for the differences of LPO, NPSH, SOD, and arsenic levels between the high– and low–arsenic-exposed groups by independent-sample *t*-test. All *p* values are two tailed.

Results

The concentrations of arsenic in well water used by the study subjects and the prevalence of the main clinical manifestations were described previously (*30,31*). Table 1 shows that the mean blood concentrations of iAs, MMeAs, and DMeAs of the high–arsenic-exposed group were about 3.0, 9.9, and 5.1 times higher, respectively, than those of the low–arsenic-exposed group, and the urinary

Table 2. Comparison of serum LPO concentration, whole blood NPSH level, and SOD activity between the high– and low–arsenic-exposed groups.

Group (*n*)	LPO (μM)[a]	NPSH (μmol/g Hb)[a,b]	SOD (NU/mL blood)
High (43)	8.8 ± 1.8*	4.3 ± 1.2**	2,654 ± 400
Low (10)	7.1 ± 1.0	7.5 ± 0.5	2,830 ± 99

Abbreviations: Hb, hemoglobin; NU, nitrite unit. Values shown are mean ± SD.
[a]Serum level of LPO in five samples and whole blood NPSH in one sample could not be determined because of limited sample volume. [b]Data from Pi et al. (*30*). Significantly different from the low–arsenic-exposure group, *$p < 0.05$; ** $p < 0.01$

Table 1. Levels (mean ± SD) of iAs, MMeAs, and DMeAs in blood and urine and total arsenic in hair of the high– and low–arsenic-exposed subjects.

Group (*n*)	Blood (μg/L)			Urine (μg/L)			Hair (μg/g)
	iAs	MMeAs	DMeAs	iAs	MMeAs	DMeAs	
High (43)	8.2 ± 3.4*	20.7 ± 7.9*	13.2 ± 5.5*	360 ± 173*	468 ± 240*	1,559 ± 529*	1.8 ± 0.8*
Low (10)	2.7 ± 0.9	2.1 ± 0.9	2.6 ± 0.8	71 ± 13	40 ± 22	152 ± 53	0.4 ± 0.2

*Significantly different from the low–arsenic-exposure group, $p < 0.001$.

levels of iAs, MMeAs, and DMeAs of the high–arsenic-exposed group were about 5.1, 11.7, and 10.3 times higher than those of the low–arsenic-exposed controls. Although our analytic method did not separate MMeAs(V) and MMeAs(III), the total MMeAs in blood (49.2%) was markedly higher than iAs (19.4%) and DMeAs (31.4%). In contrast, DMeAs was the most abundant metabolite in urine (65.3%). The total arsenic in hair of the high–arsenic-exposed group was 4.5 times higher than that of the low–arsenic-exposed group.

Table 2 shows that the mean serum LPO level of the high–arsenic-exposed group was significantly higher than that of low–arsenic-exposed controls; conversely, the mean NPSH level in whole blood of the high–arsenic-exposed subjects was only 57.3% of low–arsenic-exposed controls. However, the SOD activity in whole blood showed no significant difference between the high– and low–arsenic-exposed subjects.

As shown in Figure 1, the serum LPO levels were significantly correlated with the concentrations of iAs, MMeAs, DMeAs, and total arsenic in blood. In addition, an inverse correlation of serum LPO concentrations with NPSH levels in whole blood was observed, but no correlation was found between serum LPO concentrations and the SOD activities in whole blood (Figure 2). Figure 3 shows that the NPSH levels in whole blood were correlated with blood concentrations of iAs, MMeAs, DMeAs, and total arsenic. In addition, the concentrations of whole-blood NPSH were significantly correlated with the ratio of MMeAs to iAs in blood, which reflects the extent of the first methylation of iAs in the body (Figure 4), but were not correlated with the ratio of DMeAs to MMeAs in urine, which reflects the extent of the second methylation of MMeAs in the body ($p = 0.69$; data not shown).

Discussion

Recently, some areas with chronic arsenic poisoning were found in Inner Mongolia, China, where underground water was the only source of water supply and was commonly obtained from tube wells of about 15–30 m in depth. The water sources in some villages in our study area of Wuyuan were contaminated with naturally occurring arsenic. The mean arsenic concentration in tube-well water from Yindingtu and Shiba, two of the seriously contaminated villages in Wuyuan, was 0.41 mg/L, which is about 8.2 and 41 times higher than the limit set for drinking water in China (0.05 mg/L) and the present World Health Organization drinking water guideline value (0.01 mg/L) (32), respectively. The mean level in the control village of Shahe was 0.02 mg/L.

Because the groundwater was not used for irrigation and the surface soil contained minimal levels of arsenic, arsenic concentrations in wheat, the main farm crop, grown in both the high-and low-exposure areas were similar (0.029 vs. 0.024 µg/g, respectively; $n = 5$). Hence, we can conclude that the ingestion of tube-well water was the main exposure source of arsenic in our study subjects. The main clinical manifestations of the exposed subjects were similar to those found in other arsenic poisoning areas of the world (2,3,33), mainly including cutaneous

depigmentation, hyperpigmentation, and hyperkeratosis and increased incidence of peripheral vascular disorder, peripheral neuropathy, and liver swelling (30,31). Both blood and urine levels of iAs, MMeAs, and DMeAs were significantly higher in the high-exposure group compared with the low-exposure controls. The proportions of the three metabolites in blood and urine were different: MMeAs was the main metabolite in blood (49.2%), whereas DMeAs was the major metabolite in urine (65.3%). This difference suggests that part
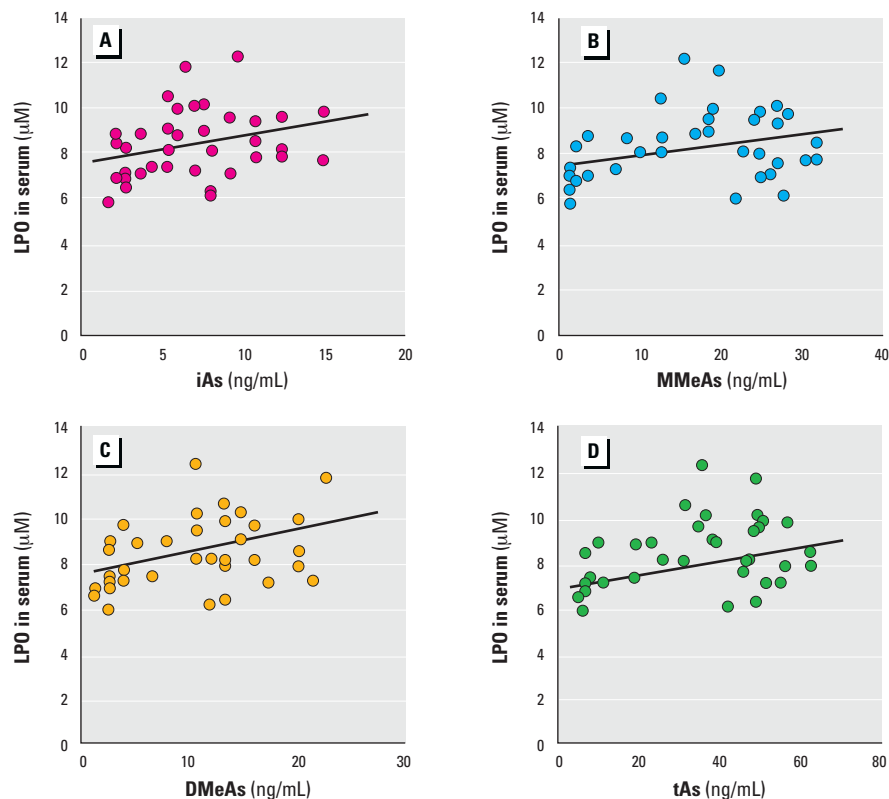

Figure 1. Correlation of serum levels of LPO with (*A*) iAs ($r = 0.34$; $p < 0.05$), (*B*) MMeAs ($r = 0.34$; $p < 0.05$), (*C*) DMeAs ($r = 0.43$; $p < 0.01$), and (*D*) tAs ($r = 0.40$; $p < 0.05$) in whole blood of subjects ($n = 38$). Serum LPO in five samples could not be determined because of limited sample volume.
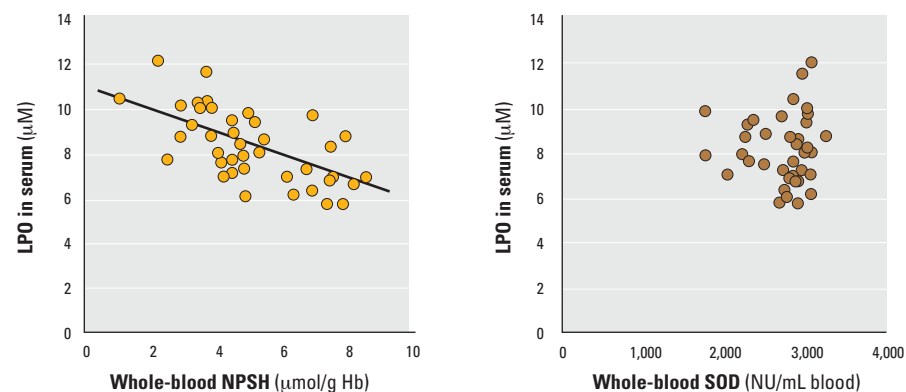

Figure 2. Correlation of serum levels of LPO with whole-blood levels of NPSH ($r = 0.60$; $p < 0.001$) and SOD ($r = 0.002$; $p < 0.99$) activity in subjects ($n = 38$). Abbreviations: Hb, hemoglobin; NU, nitrite unit. Serum LPO in five samples and whole-blood NPSH in one sample could not be determined because of limited sample volume.

of the MMeAs in blood was methylated into DMeAs before being excreted in the urine.

The present study showed that subjects chronically exposed to arsenic-contaminated drinking water in Wuyuan, Inner Mongolia, had increased serum levels of LPO. This may be a result of either overproduction of ROS or accumulation of ROS resulting from dysfunction of antioxidases/antioxidants during chronic arsenic exposure. Previous works suggested that the toxicity of arsenic compounds may be mediated by ROS generated during the metabolism of arsenic compounds (16,34). Genotoxicity of arsenite (35) and arsenic-induced apoptosis in NIH3T3 cells (36), NB4 cells (37), and Chinese hamster ovary cells (16) were triggered by the generation of H_2O_2. $\cdot O_2^-$ and H_2O_2 were produced by endothelial cells exposed to low levels of arsenite (38). DMeAs, a metabolite of iAs, induced DNA damage via formation of $\cdot O_2^-$ and dimethylarsenic peroxyl radical (39,40). 8-Hydroxy-2′-deoxyguanosine (8-OhdG), one of the major ROS-induced DNA base-modified products, was detected in the skin of patients with arsenic-related Bowen's disease (41) and in the liver of rats administered DMeAs (42). Our recent data (43) from animal experiments revealed that $\cdot O_2^-$, which

was identified as a Tiron-reactive signal (Dojindo Lab., Kumamoto, Japan), was generated in intact aortic rings from rabbits chronically exposed to 5 mg/L of arsenate in drinking water for 18 weeks. Also, the levels of H_2O_2 in urine were significantly higher in the arsenate-exposed rabbits than in controls. In the present study, serum LPO concentrations were significantly correlated with blood levels of arsenic and its metabolites, indicating that the overproduction of ROS induced by chronic arsenic exposure was one of the main causes of the elevation of serum LPO observed in the high–arsenic-exposed subjects.

Arsenic has been reported to produce $\cdot O_2^-$ and H_2O_2 in endothelial cells (38,43). It is well recognized that SOD can be up-regulated by overproduction of ROS, and the lack of expression in SOD knock-out experimental animals results in oxidative stress (44). SOD can effectively reduce the frequency of arsenite-induced sister chromatid exchanges in cells (18,19), suggesting that this antioxidant enzyme contributes to the oxidative stress-dependent toxicity caused by arsenic. In the present study, however, the SOD activity was not significantly altered by chronic arsenic exposure. The serum LPO levels were not correlated with the SOD activities in

whole blood. This suggests that the increased LPO level observed in the high–arsenic-exposed group was not associated with the SOD activity in the subjects. The strong inverse correlation between NPSH and LPO levels suggests that the depletion of NPSH was another possible cause of the lipid peroxidation observed in the arsenic-exposed group. Conversely, the overproduction of ROS could cause the depletion of NPSH.

One of the mechanisms by which arsenicals produce toxic effects is through their interaction with cellular sulfhydryl groups in proteins or elsewhere (45). iAs(III) and iAs(V) have been shown to react with some NPSH to form As–SH complexes in vitro at high concentrations (46,47). However, our preliminary examination showed that sulfhydryl groups of dithiols were readily oxidized by incubation with iAs(III) but not iAs(V), MMeAs(V), or DMeAs(V), whereas monothiols were unaffected by all the arsenicals (48). In addition, the concentrations of arsenic in blood of the high–arsenic-exposed subjects were at the micromolar level, which is low compared with the level of NPSH in blood, suggesting that direct binding was not the main cause of NPSH depletion in blood. The depletion was significantly correlated with the ratio of MMeAs to iAs in blood, indicating that the first methylation of iAs would tend to deplete available NPSH. GSH or other NPSHs, such as L-cysteine, plays critical roles in the reduction of As(V) to As(III) in blood and in the methylation reaction of iAs in liver (49,50).

Present results suggest that the two main causes of NPSH depletion were oxidative reaction with overproduced ROS and the arsenic methylation process. However, because GSH is almost always present in all cells at high concentrations (generally millimolar) (22), the two mechanisms alone do not seem to explain the striking depletion of
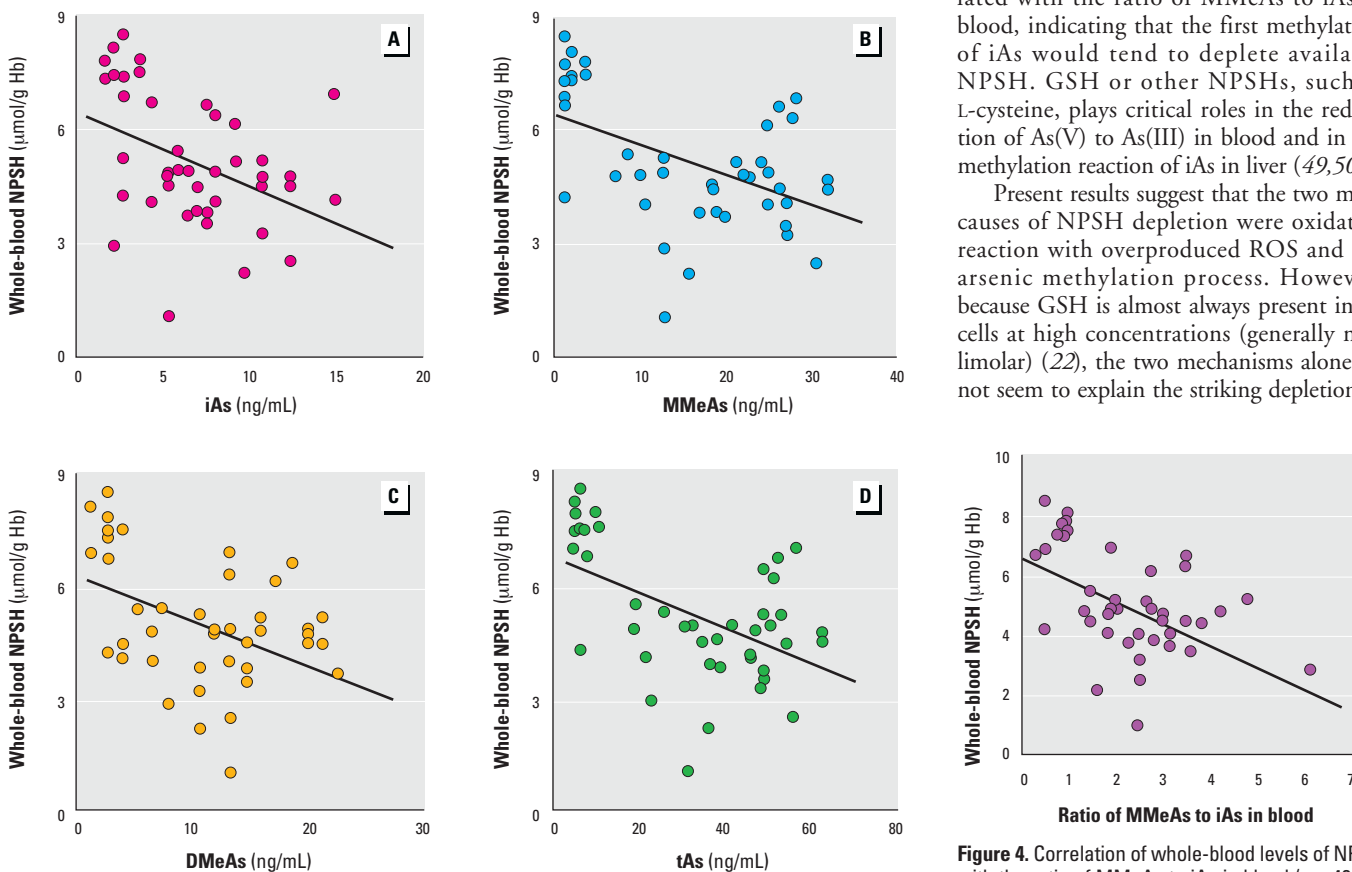

Figure 3. Correlation of levels of NPSH with (A) iAs ($r = 0.41$; $p < 0.01$), (B) MMeAs ($r = 0.50$; $p < 0.001$), (C) DMeAs ($r = 0.46$; $p < 0.001$), and tAs ($r = 0.51$; $p < 0.001$) in whole blood of subjects ($n = 42$). Whole-blood NPSH in one sample could not be determined because of limited sample volume.

Figure 4. Correlation of whole-blood levels of NPSH with the ratio of MMeAs to iAs in blood ($n = 42$; $r = 0.50$; $p < 0.001$). Whole-blood NPSH in one sample could not be determined because of limited sample volume.
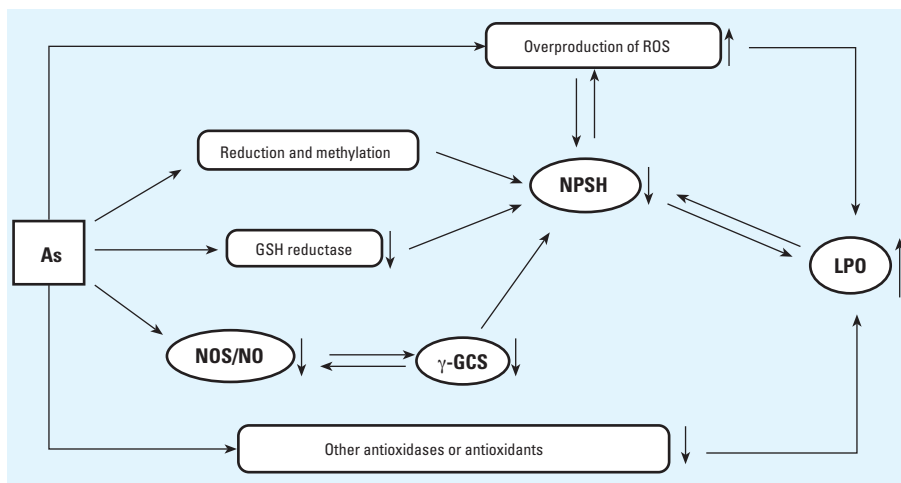
Figure 5. Putative mechanism of oxidative stress induced by chronic arsenic exposure in humans. NOS, nitric oxide synthase.

NPSH in blood. We have reported previously that chronic exposure to arsenic in drinking water results in decreased nitric oxide production in humans (*30*), and inhibition of NO synthesis leads to a marked decrease in GSH synthesis through down-regulation of the rate-limiting enzyme γ-glutamylcysteine synthetase (γ-GCS) *in vitro* and *in vivo* (*51,52*). NO itself up-regulates γ-GCS and increases GSH concentration (*53*). Our recent work showed that levels of serum NO metabolites correlated positively with whole-blood NPSH levels (*30*). Therefore, the observed GSH depletion may be at least in part due to the decreased NO synthesis caused by chronic arsenic exposure through down-regulation of γ-GCS.

Furthermore, the oxidation-reduction cycling of GSH is also central to the cellular response to oxidative stress. The balance between the oxidation of GSH to glutathione disulfide (GSSG) and the rapid reduction of GSSG by GSH reductase contributes to the maintenance of a cellular GSH:GSSG ratio of about 300:1 (*54,55*). Inhibition of GSH reductase diminishes the intracellular level of GSH. Styblo et al. (*56,57*) demonstrated that arsenicals and arsenothiols, especially methylated trivalent arsenicals and arsenothiols, were potent inhibitors of the reduction of GSSG by GSH reductase. The inhibition constants for the di(γ-glutamylcyteinylglycinyl)methyl-dithioarsonite and dicysteinylmethyl-dithioarsenite, two possible intermediates of iAs metabolism *in vivo*, were 0.009 and 0.018 mM, respectively (*56,57*). Either the trivalent organoarsenicals generated during biomethylation of arsenic or the arsenothiols formed in the cell by reaction with NPSH could alter the cellular GSH:GSSG ratio, decreasing the GSH level by the inhibition of GSH reductase. Recently, the concentrations

of MMeAs(III) and MMeAs(V) in hamster liver after a single intraperitoneal dose of iAs(V) (2 mg As/kg of body weight) were 38.5 and 31.4 ng/g, respectively (MMeAs(III)/total MMeAs = 55.1%) (*58*). Although the method we used for determining blood arsenic in the present study could not separate MMeAs(III) and MMeAs(V), high levels of total MMeAs (49.2%) were observed in blood, suggesting the possibility that high levels of MMeAs(III) existed in other organs. Interestingly, MMeAs(III) has been detected in urine after the administration of sodium 2,3-dimercapto-1-propane sulfonate to humans chronically exposed to inorganic arsenic in the drinking water in Inner Mongolia (*59*).

In summary (Figure 5), our data provide evidence that chronic exposure to arsenic from drinking water in humans results in induction of oxidative stress, as indicated by the increase of serum LPO. The oxidative stress induced by chronic arsenic exposure was associated with high levels of iAs and its methylated metabolites in blood, as well as with NPSH depletion caused by iAs and its methylated metabolites via different mechanisms.

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