Differential oxidative stress and DNA damage in rat brain regions and blood following chronic arsenic exposure

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Chronic arsenic poisoning caused by contaminated drinking water is a wide spread and worldwide problem particularly in India and Bangladesh. One of the possible mechanisms suggested for arsenic toxicity is the generation of reactive oxygen species (ROS). The present study was planned 1) to evaluate if chronic exposure to arsenic leads to oxidative stress in blood and brain – parts of male Wistar rats and 2) to evaluate which brain region of the exposed animals was more sensitive to oxidative injury. Male Wistar rats were exposed to arsenic (50 ppm sodium arsenite in drinking water) for 10 months. The brain was dissected into five major parts, pons medulla, corpus striatum, cortex, hippocampus, and cerebellum. A number of biochemical variables indicative of oxidative stress were studied in blood and different brain regions. Single-strand DNA damage using comet assay was also assessed in lymphocytes. We observed a significant increase in blood and brain ROS levels accompanied by the depletion of GSH/GSSG ratio and glucose-6-phosphate dehydrogenase (G6PD) activity in different brain regions of arsenic-exposed rats. Chronic arsenic exposure also caused significant single-strand DNA damage in lymphocytes as depicted by comet with a tail in arsenic-exposed cells compared with the control cells. On the basis of results, we concluded that the cortex region of the brain was more sensitive to oxidative injury compared with the other regions studied. The present study, thus, leads us to suggest that arsenic induces differential oxidative stress in brain regions with cortex followed by hippocampus and causes single-strand DNA damage in lymphocytes. Toxicology and Industrial Health 2008; 24: 247–256.

Key words: arsenic; chronic exposure; comet assay; DNA damage; oxidative stress

Introduction

Arsenic is a widespread environmental and industrial toxicant that may cause neuropathy, skin lesions, and even cancer upon prolonged exposure (Goering, et al., 1999; Hindmarsh, 2000; Nemeti and Gregus, 2002). Arsenic exists in both inorganic and organic forms in different oxidation states (−3, 0, +3, +5). However, the trivalent and the pentavalent forms of arsenic are of major concern to the toxicologists in case of an environmental exposure. It is well known that exposure to arsenic compounds may cause acute, as well as chronic effects, not only in humans but also in animals.

Inorganic arsenic compounds, which are found throughout the environment, are now classified as human carcinogens (Yamamoto, et al., 1995; Shi, et al., 2004). Arsenic compounds find a wide range of applications like clarifier in glass industry, wood preservative (copper arsenite), semiconductor (gallium arsenide) industry, desiccant and defoliants in agriculture, and as a byproduct of smelting of...
non-ferrous metals, particularly gold and copper (Flora, et al., 2007; Ratnaike, 2003). Thus, arsenic can be classified as an environmental and occupational hazards. Chronic arsenic toxicity caused by drinking arsenic-contaminated water has been reported from many countries (Lewis, et al., 1999; Chowdhury, et al., 2000; Nickson, et al., 1998). Recently, a large population in West Bengal in India and Bangladesh have reported to be affected with arsenic (Smith, et al., 2000; Guha Mazumder, et al., 1998). Exposure to arsenic via drinking water is correlated with a significantly elevated risk of skin and bladder cancer (Rossman, 1998). It is assumed that an estimated six million people in an area of 38,865 km² in West Bengal of India are presently drinking arsenic contaminated water (>50–3000 μg/L; Chowdhury, et al., 2001). This level is well above the World Health Organization (WHO) recommended permissible limit of 10 μg/L (WHO, 1993). Chronic exposure to high concentration of arsenic (>50 μg/L) is of prime importance to people particularly in India and Bangladesh. Skin manifestation and depigmentation and keratosis are the two most common clinical manifestations, whereas neurological disorders appear after long-term exposure to arsenic. We have observed in experimental animal models (in rats and guinea pigs) that a number of clinical and biochemical manifestations start appearing after exposure 4–8 months of exposure to high dose of arsenic (i.e., 10 or 25 ppm in drinking water; Kannan, et al., 2001). In the earlier reported study, rats exposed to sodium arsenite (5 mg/kg) for 60 days showed changes in neurotransmitters level (Rodriguez, et al., 2003). Itoh, et al. (1990) also reported increased 5-hydroxytryptamine and noradrenaline in brain regions of mice exposed to 10 mg/kg arsenic as arsenic trioxide. Similar results were reported by Rodriguez, et al. (2001) in rats orally exposed to 10 or 20 mg/kg arsenic as arsenic trioxide. However, data from long-term chronic exposure is lacking particularly on oxidative stress in brain regions.

Arsenic may exert its toxicity through reaction with various sulfhydryls that exist in the cells (Aposhian, 1989). Recent studies suggest that arsenic compounds during their metabolism in cells generate reactive oxygen species (ROS) like superoxide, hydroxyl radical, and hydrogen peroxide, which are responsible for some of the toxic effects of arsenic (Mishra, et al., 2008; Kalia, et al., 2007; Flora, 1999; Liu, et al., 2001). Arsenic exposure has been reported to suppress the functions of antioxidant defense system leading to oxidative damage to cellular macromolecules including DNA, proteins, and lipids. Antioxidants protect the cellular machinery from peroxidative injury inflicted by ROS (Halliwell and Arouma, 1993). Neuropathy caused by arsenic toxicity is well known (Calderon, et al., 2001; Tripathi, et al., 1997). It is known that most of the neurotoxic effects of arsenic are mediated through generation of ROS (Garcia-Chavez, et al., 2007). However, brain being a dynamic system is known to show differential response to arsenic toxicity. However, till date no study is reported that describes the susceptibility of a particular brain region towards arsenic.

In the present study, we studied the toxic effect of arsenic on whole blood, red blood cells (RBCs), and different brain regions (pons medulla, cerebellum, cortex, corpus striatum, and hippocampus) in terms of oxidative stress. We also studied the single-strand DNA breaks by comet assay in the lymphocytes after chronic exposure of arsenic. The study aimed at determining if there is a differential effect of arsenic on different brain regions.

**Materials and methods**

**Chemicals and reagents**

Sodium arsenite was obtained from Merck (Darmstadt, Germany). All other analytical laboratory chemicals and reagents were purchased from Merck (Darmstadt, Germany), Sigma (St Louis, USA) or BDH Chemicals (Mumbai, India). Ultra-pure water prepared by Millipore (New Delhi, India) was used throughout the experiment to avoid metal contamination and for the preparation of reagents/buffers that are used for various biochemical assays in our study.

**Animals and treatment**

All the experiments were performed on male rats weighing 200 ± 20 g. Animals were obtained from animal house facility of Defence Research and Development Establishment (DRDE), Gwalior.
Animal ethical committee of DRDE, Gwalior, India, approved the protocols for experiments. Before dosing, they were acclimatized for 7 days to light from 6 a.m. to 6 p.m. alternating with 12 h darkness. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2 °C. The rats were allowed standard animal’s chow diet (Lipton’s, Kolkata, India) throughout the experiment. Forty animals were randomized into two groups of twenty rats each and were treated as below for the period of 10 months.

Group I → No treatment (drinking water) (Control)
Group II → 50 ppm sodium arsenite (in drinking water)

We selected only one dose of sodium arsenite for the present study as we have already reported significant changes in biochemical variables in RBCs and brain and alterations in neurochemical variables with comparatively lower doses (10, 20, 25 ppm in drinking water; Bhadauria and Flora, 2007; Gupta and Flora, 2006; Kannan and Flora, 2004). There are also reports that show significant changes in brain oxidative stress and damage to DNA following exposure to 100 ppm arsenic (Waalkes, et al., 2003; Flora, et al., 2005; Tripathi, et al., 1997). However, to the best of our knowledge, no studies are available in the literature reporting arsenic-induced oxidative stress in different brain regions. On the basis of the above-mentioned studies and doses that have been tried in experimental animals, we selected a median dose to investigate if there is a differential effect of arsenic on brain regions.

Animals were weighed every month, and they were killed under light ether anesthesia after 10 months. Five animals were used for each parameter. All extra animals used in the study were used to dissect out the brain parts to perform the biochemical analysis. Blood was collected in heparinized vials through intra-cardiac puncture. Brain tissue samples were removed washed with normal saline, and all the extraneous materials were removed. Tissues were kept in ice-cooled conditions throughout the experiment. Brain of each animal was removed and dissected into different regions viz cerebral cortex, cerebellum, pons medulla, hippocampus, and corpus striatum for various biochemical studies. All biochemical analyses were performed within 48 h, and samples were stored at −70 °C for all other experiments.

Biochemical assays

**ROS level in brain and RBCs**

The amount of ROS in RBCs was measured using 2′,7′-dichlorofluorescin diacetate (DCFDA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). Dichlorofluorescein fluorescence was used to determine both the location and amount of electrophilic lipid-dependent ROS formation. It can still be used to effectively detect ROS/Reactive Nitrogen Species RNS such as hydrogen peroxide and peroxynitrite in cells. The assay was performed as described by Socci, et al. (1999). Briefly, 5% RBC hemolysate was prepared and diluted to 1.5% with ice-cold 40 mM tris–HCl buffer (pH 7.4) and further diluted to 0.25% with the same buffer and placed on ice. The samples were divided into two equal fractions. In one fraction, 40 μL of 1.25 mM DCFDA in methanol was added for ROS estimation. Another fraction in which 40 μL of methanol was added served as a control for tissue/hemolysate auto fluorescence. All samples were incubated for 15 min in a water bath at 37 °C. Fluorescence was determined at 488 nm excitation and 525 nm emission wavelength using a fluorescence plate reader (Perkin Elmer model LS-55, Buckinghamshire, UK).

**Brain reduced (GSH) and oxidized glutathione (GSSG) level**

Brain GSH and GSSG levels were measured following the method described by Hissin and Hilf (1973). Briefly, 0.25 g of tissue sample was homogenized on ice with 3.75 mL of 0.1 M phosphate–0.005 M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) and 1 mL of 25% HPO₃, which was used as a protein precipitant. The homogenate (4.7 mL) was centrifuged at 1,00,000 × g for 30 min at 4 °C. For the GSH assay, 0.5 mL supernatant and 4.5 mL phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 mL) contained 100 μL supernatant, 1.8 mL phosphate–EDTA buffer, and 100 μL O-phthaldehyde (1000 μL/mL in absolute methanol, prepared fresh). After mixing, fluorescence was determined at 420 nm with an excitation wavelength of 350 nm using a spectrofluorometer (Model RF 5000 Shimadzu, Tokyo, Japan).
For the GSSG assay, 0.5 mL of supernatant was incubated at room temperature with 200 μL of 0.04 mol/L N-ethylmaleimide solution for 30 min. To this mixture, 4.3 mL of 0.1 mol/L NaOH was added. A 100-μL sample of this mixture was taken for the measurement of GSSG using the procedure described above for GSH assay, except that 0.1 mol/L NaOH was used as the diluents instead of phosphate buffer.

Whole blood glutathione (GSH) and δ-aminolevulinic acid dehydratase (ALAD) activity

Analysis of blood GSH concentration was performed as described in detail by Flora, et al. (2005). Briefly, 0.2 mL of whole blood was added to 1.8 mL of distilled water and incubated for 10 min at 37 °C for complete hemolysis. After hemolysis, 3 mL of 4% sulphosalicylic acid was added, and tubes were centrifuged at 2500 × g for 15 min. Supernatant (0.2 mL) was mixed with 0.4 mL of 10 mM 5, 5′-dithiobis-(2-nitrobenzoic acid) and 1 mL phosphate buffer (0.1 M, pH 7.4). At the end, absorbance at 412 nm was recorded.

Activity of blood ALAD was assayed according to the procedure of Berlin and Schaller (1974). In total, 0.2 mL of heparinized blood was mixed with 1.3 mL of distilled water and incubated for 10 min at 37 °C for complete hemolysis. After adding 1 mL of standard δ-aminolevulinic acid, the tubes were incubated for 60 min at 37 °C. Enzyme activity was stopped after 1 h by adding 1 mL of 10% trichloroacetic acid. After centrifugation (1500 × g) of reaction mixture, equal volume of Ehrlich reagent was added to the supernatant, and the absorbance was recorded at 555 nm after 5 min. Pink color chromogen was extracted in butanol–pyridine solution (15:1) and read at 532 nm.

Glucose-6-phosphate dehydrogenase (G6PD) in different brain regions

Glucose-6-phosphate dehydrogenase activity was measured in 10% tissue homogenate, and the tissue homogenate was centrifuged at 10,000 rpm for 10 min at −1 °C. The supernatant was tested for G6PD on the same day. Polyacrylamide gel electrophoresis was carried out using the method of Ornstein (1964) and stained as described by Sholl and Anders (1973). The staining mixture contained α-glucose-6-phosphate (G6P) (0.02 g), nicotinamide adenine dinucleotide phosphate (NADP) (0.02 g), phenazine methosulfate (PMS) (0.001 g), nitro blue tetrazolium (NBT) (0.01 g) dissolved in 40 mL of 0.1 M Tris–HCl buffer (pH 8.0). Gel was incubated at 37 °C overnight in dark. Acetic acid (7%) was used as the storing solution for gels. G6PD staining is based on the principle that the substrate G6P combines with NADP, which acts as an electron acceptor and gets converted to reduced state NADPH and 6-phosphogluconate. The latter when combines with the dyes PMS and NBT gives the characteristic violet-colored bands. PMS acts as catalyst in binding of NBT to the phosphogluconate, NADP being the cofactor.

Determination of single-strand breaks by comet assay

Single cell gel electrophoresis or comet assay was done as per the protocol of Singh, et al. (1988) with some modifications by Rao, et al. (1999). Briefly, 20 μL of whole blood was mixed with 200 μL of 0.75% low-melting agarose in phosphate buffered saline at 37 °C and quickly put onto a microscope slide, which already had a dried layer of 0.1% agarose. A cover glass was put slowly on the slide to make a uniform layer of agarose-cell mixture. This slide was put onto ice for 5 min to allow rapid solidification of gel. Then the cover glass was removed, and 200 μL of agarose was placed on the slide to make an additional layer of agarose above the layer containing the cells. The slide was again cooled for 5 min before the cover glass was removed, and slides are immersed into cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris), 1% Triton X-100, and 10% meso 2,3-dimercaptosuccinic acid (DMSA) were added to the lysing solution.
added freshly. After 12 h of lysis, slides were transferred to a horizontal electrophoresis unit containing running buffer (30 mM NaOH and 1 mM EDTA pH 13.0). Slides were allowed for unwinding of DNA for 20 min. Then electrophoresis was conducted for 20 min at 0.4 V/cm. Slides were carefully removed from the unit and washed with neutralizing buffer (0.4 M Tris, pH 7.5). DNA staining was done with ethidium bromide (200 ng/mL), and comets were visualized in a LEICA GMBH, Germany fluorescent microscope.

**Statistical analysis**

Data are expressed as means ± SEM. Data comparisons were carried out using Student’s t-test to compare both the groups Difference between unexposed (without treatment) with a *P* value <0.05 was considered significant.

**Results**

**Effects on animal body weight and general morphology**

Rat body weights were monitored throughout the experiment for a period of 10 months and are depicted in Figure 1. There was a significant and consistent increase in the body weight of normal animals; however, arsenic-exposed animals did not show considerable weight gain when compared with normal animals. Arsenic-exposed animals also showed signs of hair loss after 5–6 months of arsenic exposure.

**Effects on blood and brain ROS**

Figures 2 and 3 show the changes in the level of ROS in blood and different brain regions, respectively. There was a significant increase in blood ROS levels (nearly 2.5-fold) compared with control animals (Figure 2). Similar results were observed in rat brain regions where a significant increase in ROS levels was noted in all the brain regions compared with the corresponding values in normal animals (Figure 3). Cortex showed maximum ROS increase closely followed by cerebellum and hippocampus.

**Effect on brain GSH/GSSG ratio**

Figure 4 shows the changes in GSH/GSSG ratio in different brain regions namely pons medulla, cerebellum, corpus striatum, hippocampus, and cortex. There was significant decrease in GSH/GSSG ratio in all the brain regions of arsenic-exposed rats except an increase in hippocampus compared with normal animals depicting oxidative stress in brain.

**Effects on blood and brain oxidative stress variable**

Table 1 depicts the effects of arsenic exposure on blood ALAD, GSH, and brain TBARS levels.
The results indicate that arsenic exposure caused a significant decrease in ALAD and GSH levels in rats. However, TBARS levels increased significantly in different brain regions with the maximum increase noted in the cortex region followed by hippocampus and corpus striatum. Pons showed the least increase in the TBARS levels (Table 1).

**Effect on brain G6PD activity**

Figure 5 depicts the G6PD electrophoretic pattern in different brain regions of normal and arsenic-exposed rats. A significant decrease in G6PD band intensity was observed in all the brain regions of arsenic-exposed animals compared with normal animals especially in hippocampus and corpus striatum indicating a depleted antioxidant system in arsenic-exposed animals.

**Effect on single-strand breaks in circulating lymphocyte**

Single-strand breaks in circulating lymphocytes of normal and arsenic-exposed animals were deter-
mined through comet assay, and the results are shown in Figure 6. There were no comets single-strand breaks observed in control lymphocytes, whereas chronic arsenic-exposed cells showed typical comets with long tails indicating heavy damage to the DNA.

Discussion

Arsenic is a well-known toxicant. Besides a number of mechanisms that have been proposed, oxidative stress has now become one of the major factors in arsenic-induced toxic effects (Mishra, et al., 2008). Free radicals have been suggested to be the most likely factor responsible for producing various toxic effects in chronic arsenic exposure (Flora, 1999; Flora, et al., 2005). Studies by Ito, et al. (1998) indicated that arsenic may exert its toxic effects by generating radicals like superoxide anion, hydroxyl radicals, and hydrogen peroxides. Liu, et al. (2001) suggested the following sequence of events for arsenic-induced mutagenesis in mammalian cells: arsenic $\rightarrow$ superoxide anions $\rightarrow$ hydrogen peroxide $\rightarrow$ hydroxyl radicals $\rightarrow$ genotoxicity. Thus, arsenic induces oxidative stress by stimulating ROS and diminishing antioxidant enzyme activities leading to the decrease in the concentration of non-enzymatic antioxidant molecule, GSH in arsenic-poisoned animals. Arsenic disturbs antioxidant defense system, and this might lead to the impairment in cell prooxidant/antioxidant balance ultimately to oxidative injury. Superoxide anion, hydroxyl radical, and hydrogen peroxides are the intermediates formed during oxygen metabolism (Flora, et al., 2007).

The present study showed a significant decrease in GSH/GSSG ratio in brain along with a significant increase in TBARS levels. An increase in TBARS level suggests lipid peroxidation although this parameter has its own limitation, and one of the major disadvantages of TBARS measurement is that it provides an indirect evidence of lipid peroxidation. The decrease in GSH/GSSG ratio may further lead to disturbed brain functioning and cause severe neurological complications. Ercal, et al. (1996) suggested that the GSH/GSSG ratio could be a sensitive indicator of oxidative stress. Cellular GSH can reduce the toxicity of a variety of essential and non-essential metals, including arsenic, probably through chelation (Huang, et al., 1995). It is also reported that cellular GSH is a critical cofactor for the enzymatic methylation of arsenite to monomethyl or dimethyl arsenic acid (Zakharyan, et al., 1996). It has been suggested that reactive intermediates might react with GSH either by a direct chemical reaction or via glutathione-S-transferase mediated reactions and can be converted into oxidized glutathione (GSSG) (Zakharyan, et al., 1996). When the ratio of oxidized glutathione exceeds the capacity of glutathione reductase to reduce, the oxidized glutathione (GSSG) is actively transported out of the cell and is thereby lost. This process might explain the reduction of GSH/GSSG ratio after arsenic exposure. To recycle GSSG, cell utilizes the NADPH-dependent GSH reductase (Langdon, 1958). In return, NADPH is supplied by glucose-6-phosphate dehydrogenase (G6PDH) (Grimes, 1980). Glucose-6-phosphate dehydrogenase (G6PD) is the first enzyme of pentose phosphate pathway. It regulates the operation of the hexose monophosphate shunt (HMS; Luzzatto and Battestuzzi, 1984), the pathway that produces NADPH and pentose phosphates. It supplies extra-mitochondrial NADPH to the cells through the oxidation of glucose-6-phosphate to 6-phosphogluconate. This NADPH maintains the GSH at a constant level. In this study, a significant decrease in G6PD activity in brain suggests decrease in NADPH level, which reduces the conversion of oxidized glutathione into reduced glutathione. Arsenic, being a potent cellular toxicant, may affect the synthesis of glucose-6-phosphate dehydrogenase at its DNA level and may alter mRNA regulated enzyme production (Pal and
The fact that arsenic toxicity is mediated via generation of ROS was also supported by Nishigori, et al. (2004). ROS, in turn, is implicated in the development of carcinogenesis (Wang and Huang, 1994) and other cytotoxic effects.

We also observed that arsenic exposure caused significant DNA damage in terms of comet tail in lymphocytes. Andrew, et al. (2006) have reported earlier that comet assay could be used as an indicator of arsenic poisoning. However, most of these studies involving human and animals were reported after acute and sub-acute arsenic exposure, and the present study confirms these results in chronically exposed animals. We noted a loss of DNA integrity because of single-strand breaks (Figure 6). The underlying mechanism for arsenic-induced DNA damage could be attributed to the fact that DNA is organized in large supercoiled structures which, when relaxed locally by strand breaks in the negatively charged DNA, would be stretched out towards anode by electrophoresis (Klaude, et al., 1996). The extent of DNA migration/DNA shearing is directly related to the frequency of DNA strand breaks (McCarthy, et al., 1997). Furthermore, there are reports that suggest that arsenite activates NADH oxidase to produce superoxide ion, which subsequently causes oxidative DNA damage (Bachrovsky, et al., 1999; Lee and Ho, 1995). This process involves Fenton’s reaction in which hydrogen peroxide and metal ions (Fe²⁺ or Cu²⁺) react to produce hydroxyl radicals. However, the exact mechanism by which arsenic causes oxidative DNA damage is still not clear. Recent studies have proposed two mode of action for arsenic-induced DNA damage: 1) inhibition of various enzyme involved in DNA repair and expression, for example, poly ADP-ribose polymerase-I (PARP-I), an important DNA repair enzyme (Yager and Wiencke, 1997) and 2) induction of ROS capable inflicting DNA damage (Schwerdtle, et al., 2006). Apart from this, we also recently observed that cortex was the primary site of action of arsenic closely followed by hippocampus and cerebellum (data not published). This further suggests that there is a differential susceptibility of arsenic in different brain regions. However, one needs to correlate the functional aspects of each region with the biochemical indices to get a better idea of neurotoxic effects of arsenic.

Thus, to conclude, our present report suggests that arsenic causes oxidative damage in brain regions by decreasing GSH/GSSG ratio and shows differential susceptibility in various brain regions based on G6PD and TBARS. Our study also provides insight that chronic exposure to arsenic causes single-strand DNA breaks.

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References


Ercal, N, Treraratphan, P, Hammond, TC, Matthews, RH, Grannemann, N, Spitz, D (1996) In vivo indices of oxidative stress in lead exposed C57BL/6 mice are reduced by...


