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ORIGINAL ARTICLE

Effectiveness of zinc supplementation in containing arsenic induced acute toxicity in rat erythrocytes by modulating antioxidant defense system and hematological parameters

Payal BHARDWAJ, Devinder Kumar DHAWAN
Department of Biophysics, Panjab University, Chandigarh, India

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ABSTRACT
Arsenic is a toxic metalloid that can cause adverse effects on physiological systems of animals and human beings. It is one of the major health concerns owing to its exposure through contaminated drinking water. A growing body of evidence also suggests that trace elements, in particular zinc, do exert many positive effects on a number of physiological functions. So, the current study was designed to evaluate the protective effects of zinc supplementation on rat erythrocytes during arsenic induced acute toxicity. Male Wistar rats, weighing 200–250 g were randomly segregated into four groups: Control, Arsenic treated (400 ppm), Zinc treated (227 mg/l $ZnSO_4.7H_2O$) and Arsenic+Zinc treated. Various investigations were undertaken to evaluate the role of zinc in conditions of arsenic induced toxicity using parameters, which included antioxidant enzymes and hematological indices. Antioxidant enzyme activities and lipid peroxidation were altered in the lysates of rat erythrocytes following acute arsenic toxicity. The results clearly showed significant improvement in the activities of anti-oxidative enzymes upon zinc supplementation. Further, hematological parameters showed significant reduction in the levels of total leukocytes, neutrophils and lymphocytes following arsenic treatment, which were normalized upon zinc supplementation. The study highlights the potential role of zinc supplementation in augmenting the key indices of antioxidant defense system of erythrocytes in conditions of arsenic induced toxicity.

KEY WORDS: zinc; arsenic; erythrocytes; antioxidant enzymes; acute toxicity

Introduction

The existence of various pollutants in the soil, air and water, as a function of time and concentration, may adversely affect the environment producing cascading effects on human health. Sources of pollution comprise of different elements in different proportions (Patterson & Settle, 1987; Flora, 2011). Arsenic is one such pollutant that occurs naturally and its exposure is generally through the consumption of food and water (Rodriguez *et al.,* 2003; Ahmada *et al.,* 2013). Various studies have shown that the toxicity caused by arsenic mainly depends on the type of arsenic. The pentavalent inorganic arsenate [As(V)] is the most prevalent form present in the environment and is also considered less toxic than the trivalent inorganic arsenite [As(III)] (Aposhian, 1989). Arsenate ions can substitute for phosphate ions thereby interfering with ATP synthesis mechanism, whereas arsenite interacts with the thiol groups and affects many functional proteins like metallothioneins and transcription factors (Aposhian, 1989). Arsenic may also exert epigenetic effects probably by interfering with the DNA methylation process, which has a bearing in fetal developmental origin of disease processes (Vahter, 2007). Arsenic first encounters the blood or the erythrocytes as soon as it enters the body. This interaction may cause toxic effects on the erythropoietic cells of bone marrow and probably result in anemic conditions (Lerman *et al.,* 1983; Selvaraj &

Correspondence address:
Dr. Devinder Kumar Dhawan
Department of Biophysics
Panjab University
Chandigarh, 160014, India
TEL.: 09878253746 • E-MAIL: dhawan@pu.ac.in

Naorem 2014). Oxidative stress has been identified as an important mechanism in arsenic induced toxicity. In particular, arsenic has been shown to induce oxidative DNA damage and also enhance generation of malondialdehydes (Mondal *et al.,* 2013; Ganger *et al.,* 2016).

Furthermore, there is growing evidence that intake of micronutrients exerts a positive effect in containing the toxicity and carcinogenesis as a result of exposure from a number of xenobiotics. Micronutrients can modify the hysiological response as a consequence of exposure to toxic metals by modulating their metabolism and transport processes. Various aspects of cellular mechanisms are zinc-dependent (Cunningham *et al.,* 1990; Yang *et al.,* 2017). Zinc plays important role in the development, growth, immune response, reproduction and toxicity. Over 300 different enzymes with vital roles in the physiological function rely on zinc that acts as a cofactor for proper functioning. Zinc is known to have an antioxidant potential through the non-enzymatic stabilization of iomembranes integrity (Malhotra & Dhawan 2008; Goel *et al.,* 2006). Zinc loss from the biological membranes increases their susceptibility to oxidative damage and micro-architectural deterioration. Zinc is also a necessary structural component of DNA-binding proteins that affect gene expressions. Further, zinc deficiency has been shown to have adverse effects on growth rate, hematological parameters and essential elements (Hendy *et al.,* 2001; Hamid *et al.,* 2018).

In this study, we have attempted to evaluate the role of zinc to provide protection, if any, to erythrocytes during arsenic induced toxicity in rats. Furthermore, the present study may provide new projection for zinc to act as a nutritional supplement to protect from the adverse effects of acute arsenic toxicity.

Materials and methods

Experimental design and treatment schedule

Male Wistar rats in the weight range of 200–250g were procured from the central animal house, Panjab University, Chandigarh. The animals were housed in polypropylene cages in the departmental animal house under hygienic conditions. The animals were maintained on standard laboratory pelleted feed and water and were acclimatized for one week to laboratory conditions prior to the start of the experimental procedure. The animals were randomly segregated into four groups. Control: The animals in this group were given a standard diet as well as drinking water, Arsenic treated: The animals in this group were given arsenic at a dose level of 400 ppm, Zinc treated: Zinc in the form of $ZnSO_4.7H_2O$ was given to the animals at a dose level of 227 mg/l in drinking water (Goel & Dhawan 2001), Arsenic+Zinc treated: The animals in this group were given arsenic and zinc at a similar dose level as mentioned above. The experimental design and procedures were approved by the Ethical Committee on Animal Experiments of the Central Animal House, Panjab University. All the treatments were continued for a total duration of four weeks. Thereafter, the following investigations were undertaken.

Blood collection and erythrocytes lysate preparation

The blood samples were taken from the animals belonging to control and all the treated groups at the termination of treatment period by puncturing the retro-orbital plexus of animals with the fine sterilized glass capillary. Blood samples were collected in stopper vacutainer tubes containing sodium citrate as an anticoagulant. Erythrocyte lysates were prepared by following the method of Ceballos-Picot *et al.* (1996). Briefly, erythrocyte pellets were obtained from the obtained blood samples by centrifugation at 500× g for 15 min at room temperature. The plasma and buffy coat were then removed and the erythrocytes were washed twice in the normal saline and stored at -20°C until used. The lysates were prepared by freezing and thawing the erythrocytes two times and by addition of three volumes of ice-cold distilled water. The cell membranes were removed by centrifugation at 1000×g for 20 min and the supernatants were considered as the erythrocyte lysates.

Lipid Peroxidation and antioxidant enzymes

The following biochemical estimations were carried out in the blood lysates taken from the control and the treated groups.

Lipid peroxidation (LPO)

Lipid peroxidation in erythrocyte lysates was determined by measuring malondialdehyde (MDA) levels on the basis of the reaction between MDA present in the sample and thiobarbituric acid at 532 nm (Ohkawa *et al.,* 1979). The values were expressed as nmol mg^{-1} protein for MDA in lysates.

Catalase

The activity of the Catalase enzyme was estimated by following the method of Luck (1971). The enzyme activity was expressed as mM of H_2O_2 decomposed min^{-1} mg^{-1} protein.

Glutathione-s-transferase (GST)

The enzyme activity of GST was measured by using the method of Habig *et al.* (1974). The activity of the enzyme was measured as µmol GSH conjugate formed min^{-1} mg^{-1} protein. The protein contents in the erythrocyte lysates were estimated by following the standard method of Lowry *et al.* (1951).

Differential leukocyte count (DLC) and total leukocyte count (TLC)

Differential leukocyte count

Differential leukocyte counts were recorded according to the method of Dacie & Lewis (1969). A fresh drop of blood was placed on the slide and smear was made. The slide was finally fixed in methanol for 10 min and then was stained in a freshly diluted Giemsa stain for 30 minutes. Then, the slide was washed thoroughly in running water and air dried. Further, the percentages of different leukocytes i.e., lymphocytes, neutrophils, monocytes, eosinophils, basophils were calculated by observing the slides under the light microscope at 100×.

Total leukocyte count

Total lymphocytes were measured by using a routine method with the help of Heamocytometer following routine laboratory method (Lampasso, 1968).

Statistical Analysis

The data were expressed as the mean ± the standard deviation (S.D.). For the data analysis, one way analysis of variance (one-way ANOVA) was used to evaluate the differences among the groups. Newman–Keul's *post hoc* test was then used for the multiple comparisons between different groups. Differences were considered statistically significant at $p<0.05$. Statistical analysis software, SPSS (14 version) was adopted for processing the data of the present study.

Results

Body weights

The animals from control, zinc treated and combined arsenic plus zinc treated groups showed a steady increase in their body weights with time. In the arsenic treated animals, a gradual decrease in body weights was observed at different time intervals as compared to the controls but the decrease was not statistically significant (Table 1).

Antioxidant defense system

The erythrocyte catalase activity was found to be significantly decreased in arsenic treated animals. However, zinc treatment to arsenic treated animals showed a statistically significant increase in catalase activity ($p<0.001$) when compared to arsenic treated animals. Further, zinc treatment alone to control animals also showed a marked increase in catalase activity ($p<0.01$), when compared with the control animals. Further, a statistically significant increase in the levels of lipid peroxidation was observed in arsenic treated ($p<0.001$) groups. Interestingly, zinc treatment to arsenic treated rats resulted in lowering the process of lipid peroxidation ($p<0.001$) to a significant extent as compared to the arsenic treated animals. However, zinc treatment alone to control rats did not indicate any significant change in LPO when compared to control rats. Further, a significant ($p<0.005$) reduction in the enzyme activity of GST was observed in arsenic treated group as compared to control animals, which was statistically increased upon zinc supplementation and the values were normalized (Table. 2).

Differential leukocyte count (DLC) and Total leukocyte count (TLC)

A significant decrease in lymphocytes and neutrophils counts was observed in arsenic treated animals as compared to controls. However, no significant change was observed in the counts of monocytes, eosinophils and basophils in the all treated groups. Zinc treatment to arsenic treated animals resulted in a significant increase in the levels of neutrophils ($p<0.01$) in comparison to arsenic treated animals. Further, zinc treatment to arsenic treated animals also resulted in an increase in the counts of lymphocytes, but the increase was not statistically significant.

A statistically significant decrease was observed in the TLC following arsenic treatment, which was restored to normal levels upon zinc supplementation. Zinc treatment to control animals did not show any significant change in TLC when compared to control group (Table 3).

Table 1. The body weights of the animals subjected to different treatments were recorded at weekly intervals. The body weights are expressed in grams

	Control	Arsenic Treated	Zinc Treated	Arsenic +Zinc Treated
Zero Day	220.0±7.07	219.0±7.41	222.0±13.04	221.0±8.94
One week	226.6±5.03	212.0±12.55	230.4±13.90	220.0±7.90
Two weeks	234.0±4.18	201.0±16.73	242.4±15.70	212.0±7.58
Three Weeks	243.0±4.47	190.0±16.96	252.8±15.80	205.0±7.90
Four weeks	253.2±4.43	181.0±15.57	264.0±15.57	198.0±6.70

Table 2. Effect of zinc supplementation on Catalase, LPO and GST in erythrocytes of arsenic treated rats

Group	Catalase	LPO	GST
Control	1.67±0.15	50±14.19	2.44±0.6
Arsenic	1.14±0.09 [a]	169.21±24.34 [c]	0.57±0.07 [a]
Zinc	2.58±0.89 [b]	47.22±7.53	3.89±0.04
Arsenic+Zinc	2.02±0.32 [f]	65.31±7.61 [f]	2.18±0.47 [d]

The values are mean±SD (n=6). The values with a superscript are significantly different from the control group (a, $p<0.05$; b, $p<0.01$; c, $p<0.001$). The values of arsenic+zinc treated group are compared with arsenic group (d, $p<0.05$; e, $p<0.01$; f, $p<0.001$), Catalase (μmoles H_2O_2 decomposed $min^{-1}mg^{-1}$ protein; LPO (μ moles of MDA min^{-1} mg^{-1} protein; GST (μ moles of CDNB conjugated min^{-1} mg^{-1} protein)

Table 3. Differential leukocyte counts and total leukocyte counts (expressed as per mm^3) of the control animals and the animals given different treatments.

Group	Lymphocyte	Neutrophils	Monocytes	Eosinophils	Basophils	TLC
Control	1464±49	2468±202	351±10	55±5	200±7	5000±200
Arsenic Treated	1318±33 [b]	1199±277 [c]	316±21	52±5	181±1	2600±530 [c]
Zinc treated	1502±55	2296±65	350±14	54±5	210±14	4380±574
Arsenic+Zinc	1370±50	2324±134 [e]	355±6	51±3	201±3	5100±100 [f]

Each value is the mean±SD (n=6).Values with a superscript are significantly different from the control group (a, $p<0.05$; b, $p<0.01$; c, $p<0.001$) or values of arsenic+zinc treated group compared with arsenic group (d, $p<0.05$; e, $p<0.01$; f, $p<0.001$)

Discussion

Earlier reports suggested that arsenic causes induction of reactive oxygen and nitrogen species and thereby affects the antioxidant defense system of the body (Ramos *et al.,* 1995; Flora *et al.,* 2007; Shen *et al.,* 2013). Arsenic has been shown to inhibit a number of antioxidant enzymes and proteins involved in defense mechanism of the body such as glutathione, glutathione-s-transferase, thioredoxin reductase, and superoxide dismutase (Mazumder, 2005; Clare *et al.,* 2017). It causes oxidative stress and changes the redox status of the cell. Toxicity may also result during zinc deficient conditions, thereby adversely affecting the zinc dependent enzymes of the physiological systems. Also, the cysteine rich biomolecules, having zinc as their component, may also get affected causing rapid dissociation of zinc. This might result in zinc deficient condition. Therefore, we can say that zinc supplementation in arsenic toxicity may restore activity of a number of zinc dependent enzymes (Ganger *et al.,* 2016).

Erythrocytes are the first to interact with increased reactive oxygen species and in the process exhaust zhey their enzymatic potential (Karatas *et al.,* 2003). In the current study, acute arsenic toxicity in rats has caused a considerable increase in malondialdehyde levels in erythrocyte lysates, which is the result of enhanced oxidation of membrane lipids in response to toxicity. Earlier studies have also shown similar findings (Kader *et al.,* 2002; Kumar, 2010; Messarah *et al.,* 2013). Increase in the production of reactive oxygen species in response to toxicity have apparently caused impairment in the membranes of erythrocytes thereby resulting in the alteration of their defense system. The normalization of malondialdehyde levels upon zinc supplementation would be due to the antioxidative property of zinc as shown in earlier studies from this lab (Pathak *et al.,* 2002). Zinc has understandably provided protection to fatty acids of the erythrocyte membranes from peroxidation by inhibiting the process of reactive species formation. Earlier studies also showed that zinc modulated the metal toxicity by causing inhibition of both endogenous as well as lipid peroxidation to stabilize the biomembranes (Sidhu *et al.,* 2004; Braga *et al.,* 2015). Furthermore, zinc is considered to regulate the levels of metallothioneins that serve as hydroxyl radical scavenger. Thus, the inhibition of production of malondialdehyde would provide protection to body against arsenic-induced toxicity. Also, a number of studies that have been carried out earlier unraveled the role of many other antioxidants in decreasing toxicity caused by arsenic, which included supplementation of ascorbic acid, alpha-tocopherol, plant extracts, flavonoids, polyphenols, or selenium (Chattopadhyay *et al.,* 2002, Bongiovanni *et al.,* 2007).

A significant decrease in the catalase and glutathione-s-transferase activities has been observed in arsenic treated rats. The decrease in the activities of these enzymes suggested their increased utilization in response to increased reactive oxygen species and enhanced incorporation of thiol groups to the substrate. Modulation of the activities of the enzymes upon zinc supplementation indicates the antioxidative property of zinc as it acts at the transcription level and stimulates the synthesis of metallothioneins. Earlier reports also showed the role of zinc in regulating the catalase activity in conditions of metal toxicity (Kowaltowski *et al.,* 2001; Sidhu *et al.,* 2005; Cambray Guerra, 2014).

A significant decrease in total leukocyte counts and differential leukocyte counts has been observed in the arsenic treated animals in comparison to the control animals (Table 3). Neutrophils were found to be significantly decreased in the arsenic treated group. Anemia, leucopenia and thrombocytopenia are the common effects of arsenic poisoning in humans following acute and chronic exposures (Correia *et al.,* 2009). Neutrophils are considered as the first line of defense against number of infectious agents, parasites, tissue injury and inflammatory material. They remove foreign materials by phagocytosis and play a role in coagulation, fibrinolysis and in preventing cytotoxicity of other body cells. Therefore, in case of arsenic induced toxicity, neutrophils might get activated and participate in protecting the cell from foreign substances. At the same time, lymphocytes are functional in antibody production in any diseased condition and therefore their decrease is understandable in such a situation. It has been observed that zinc supplementation to arsenic intoxicated rats normalized the levels of leukocyte count, which is arguably due to reduction of oxidative stress following zinc supplementation. Someya *et al.,* (2009) also explained that decreased leukocyte count could be due to zinc deficient conditions.

The mechanism, by which zinc provides protection to lipid bilayer membranes is that it prevents lipid peroxidation by scavenging free radicals. Also, arsenic toxicity alters the redox status of the cell and may cause zinc deficient condition. So, zinc supplementation in condition of arsenic toxicity increases its labile concentration and restores the function of a number of enzymes involved in the defense mechanism.

Hence, it can be concluded from the study that zinc can play a key role in containing arsenic induced toxicity by improving the antioxidant defense system.

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