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Effect of arsenic acid withdrawal on hepatotoxicity and disruption of erythrocyte antioxidant defense system

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ABSTRACT

We investigated the effects of withdrawal from Sodium arsenite ($NaAsO_2$) on the hepatic and antioxidant defense system in male Wistar rats using a before and after toxicant design. Rats were orally gavaged daily with varying doses of $NaAsO_2$ for a period of 4 weeks. One half of the population was sacrificed and the remaining half had the toxicant withdrawn for another further 4 weeks. Biochemical and immunohistochemical techniques were used to assess the impact of withdrawal on the erythrocyte and hepatic systems. Exposure of Wistar rats to $NaASO_2$ led to a significant ($p < 0.05$) increase in hepatic and erythrocyte markers of oxidative stress (malondialdehyde, thiol contents and hydrogen peroxide generation). Concurrently, there was a significant ($p < 0.05$) increase in hepatic and erythrocyte antioxidant enzymes (glutathione-S-transferase, glutathione peroxidase and superoxide dismutase) following exposure. Withdrawal from $NaAsO_2$ exposure led to a decline in both erythrocyte and hepatic markers of oxidative stress and together with a significant improvement in antioxidant defense system. Histopathology and immunohistochemistry revealed varying degrees of recovery in hepatocyte ultrastructure alongside increased expression of the pro-survival protein Kinase B (Akt/PKB) after 4 weeks of $NaAsO_2$ withdrawal. Conclusively, withdrawal from exposure led to a partial recovery from oxidative stress-mediated hepatotoxicity and derangements in erythrocyte antioxidant system through Akt/PKB pathway.

1. Introduction

Of the many naturally occurring elements found abundantly distributed in the earth's crust, arsenic has found its way into prominence as a toxicant of significant public health risk [1]. A heavy metal, specifically classed as a metalloid [2], it is naturally produced during processes such as volcanic eruptions and the biodegradation of other organic minerals and rocks [3]. Increased dependency on arsenic among other heavy metals for anthropogenic causes has resulted in widespread release of arsenic by-products into the environment [4]. Human exposure is then unavoidable, not just from occupational causes, but also from atmospheric pollution, ingestion of contaminated food and water sources and from contact with certain finished industrial products [5].

Of the routes to exposure, the most important source, by far appears to be dietary. Tchounwou et al. [1] report that each individual has an

average intake of 50 μg per day. Recent studies show a rise in the levels of inorganic arsenic in food items, especially rice, a staple of third world and underdeveloped countries [6–8]. Arsenic was found to accumulate more in the liver than other tissues after one month of exposure, when administered orally and subcutaneously. However, after 3 months of exposure, was found to accumulate more in the kidney than in the liver or other tissues. [9]. With a high correlation established between exposure and increased health risks, and an uptick in the incidence of health-related conditions among affected populations, it is no wonder arsenic is listed as one of the high malignancy causing elements today [10,11].

Certain heavy metals as cobalt and copper have been classified as essentials due to their absolute requirements in trace quantities for normal biochemical and physiological functions in the human body [12]. Arsenic, on the other hand, has been found to have no beneficial physiologic role in the human body and is classified as a non-essential

metal [13]. Even at low exposure levels, it has been shown to cause multiple organ damage by generating reactive oxygen species (ROS) and promoting oxidative stress: a redox deficiency or imbalance that can cause bimolecular damage [2,14], disrupting cellular structures and components [15], affecting detoxification and repair enzymes [16], causing DNA damage, disrupting normal cell cycle regulation and in extreme cases, cancer and cell death [17].

Free radicals are produced in living organisms during normal metabolism (e.g., the reactions of mitochondrial respiratory chain and cytochrome P450), inflammation, phagocytosis and other physiological processes. The most important category of free radicals is constituted by ROS, such as superoxide radical anion ($O_2 \cdot -$), hydroxyl radical ($OH\cdot$), peroxyl radical ($RO\cdot_2$) and hydrogen peroxide (H_2O_2) [18]. The mechanisms of liver toxicity and damage in chronic arsenic toxicity are primarily a consequence of increased free radical activity and oxidative stress [19].

From this point, two possible routes to hepatic damage have been reported: The activation of kinase signaling molecules such as C-Jun N-terminal kinases (JNK), p38 Mitogen activated protein kinase (p38 MAPK) and cytochrome P450, leading to cell lysis, apoptosis and accumulation of bile acids [20,21], or increased peroxidation of lipids which also cause hepatocyte damage [22]. It has been reported that in erythrocytes, $NaAsO_2$ can absorb into blood circulation and bind to hemoglobin. This might lead to the oxidation of sulfhydryl groups, which are critical for heme synthesis, with a concomitant reduction in oxygen uptake capabilities [19]. Also, significant decreases are triggered in erythrocyte reduced glutathione levels, coupled with increases in malondialdehyde and protein carbonyl levels, all indicative of oxidative damage to erythrocyte membranes. Grossly, this is evident as distorted, misshapen erythrocytes [23].

In poor resource regions of the world where government regulatory mechanisms are weakened or virtually non-existent, and where indiscriminate dumping of heavy metals and their metabolites occur on a daily basis, the dangers to health from arsenic exposure persist. We explore to see the effects of withdrawal from exposure on the known disease states arising from the multi-organ damage associated with this toxicant.

2. Materials and methods

2.1. Chemicals

Epinephrine, $NaAsO_2$, Hydrogen peroxide (H_2O_2), hydrochloric acid, sulphuric acid, xylenol orange, sodium hydroxide, potassium iodide, reduced glutathione (GSH), potassium dichromate, O-dianisidine, sodium potassium tartarate, copper sulphate, glacial acetic acid, ethanol, sodium azide, 2-dichloro-4-nitrobenzene (CDNB), thiobarbituric acid (TBA), Trichloroacetic acid, Ellman's reagent (DTNB), ammonium ferrous sulphate, sorbitol was purchased from Sigma (St Louis, MO, USA). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System was purchased from (KPL, Inc., Gaithersburg, Maryland, USA). Akt/PKB antibody was purchased from (Bioss Inc. Woburn, Massachusetts, USA) while 3, 3′-Diaminobenzidine (DAB) tablets were purchased from (AMRESCO LLC. Ohio, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

2.2. Experiment and design

80 healthy adult male Wistar rats (200–250 g) obtained from the Experimental animal unit of the Faculty of Veterinary Medicine, University of Ibadan were used for this study. The animals were handled humanely according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science and published by the National Institute of Health. Ethical regulations were followed in accordance with national and institutional

guidelines for the protection of animal welfare during experiments [24].

All animals were kept in wire mesh cages under controlled light cycle (12 h light/12 h dark) and temperature of $32 \pm 3\,°C$. The rats were fed with commercial rat chow (Ladokun Feeds Nigeria Limited) *ad libitum* and liberally supplied with clean tap water. Randomly, they were assigned into four groups comprising twenty animals per group. Group A was provided tap water with no test substance added. Group B was treated with tap water into which $NaAsO_2$ was dissolved at 10 mg/kg/day; Group C was treated with tap water into which $NaAsO_2$ was dissolved at 20 mg/kg/day; Group D was treated with tap water into which $NaAsO_2$ was dissolved at 40 mg/kg/day.

One half of the population was sacrificed at the end of 4 weeks of $NaAsO_2$ exposure. $NaAsO_2$ was withdrawn from the remaining rats and they were administered clean tap water only for another 4 weeks. The rats were monitored weekly for clinical signs of toxicity. After four weeks, the remaining animals were sacrificed. For each phase of sacrifice, the rats were starved overnight and the following procedure was observed. Blood was drawn from the retro-orbital venous plexus of the animals into vials containing heparin as an anticoagulant. The animals were then sacrificed by cervical dislocation. The liver was removed, rinsed in 1.15% KCl and homogenized in aqueous potassium phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at $10,000g$ for 20 min to obtain the supernatant fraction stored at $4\,°C$ till use.

2.3. Preparation of erythrocytes for biochemical assays

The preparation of erythrocytes was described according to the method of Steck and Kant [25]. The erythrocytes were washed three times with ice-cold phosphate buffer saline at pH 7.4, and centrifuged. The erythrocyte membrane was lysed and the pellets were resuspended in PBS at 1:109 dilutions until the time of use. The pellets thus obtained were washed repeatedly in the same buffer to obtain haemoglobin-free white membranes.

2.4. Biochemical assays and hematological parameters

Protein concentration was carried out as described by Gornall et al. [26]. Hydrogen peroxide generation was evaluated as described by Wolff [27]. The malondialdehyde concentration was determined according to Farombi et al. [28]. Nitric oxide was quantified as described by Olaleye et al. [29], while the thiol contents were estimated by the method of Ellman [30].

The erythrocyte and hepatic reduced glutathione (GSH) concentration was determined using the method of Jollow et al. [31]. The superoxide dismutase (SOD) activity was evaluated by the method of Misra and Fridovich [32] with slight modification [33]. Glutathione peroxidase (GPx) and Glutathione-S-transferase (GST) activities were measured by the method of Rotruck et al. [34] and Habig et al. [35], respectively. The packed cell volume (PCV) was determined by microhaematocrit method. The haemoglobin (Hb) concentration was determined by Cyanmethaemoglobin method while red blood cell (RBC) and white blood cell (WBC) counts were determined using an haemocytometer.

2.5. Histopathology

Small pieces of liver tissues were collected in 10% buffered formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5–6 μm in thickness were made and stained with haematoxylin and eosin for histopathological examination [36].

2.6. Immunohistochemistry of protein kinase B

Immunohistochemistry of paraffin embedded tissue of the liver was performed after the tissues were obtained from buffered formalin

perfused rats. The paraffin sections were melted at 60 °C in the oven. Dewaxing of the samples in xylene was followed by passage through ethanol of decreasing concentration (100-80%). Peroxidase quenching in 3% H_2O_2/methanol was carried out with subsequent antigen retrieval performed by microwave heating in 0.01 M citrate buffer (pH 6.0) to boil. All the sections were blocked in normal goat serum (10%, HistoMarkR, KPL, Gaithersburg MD, USA) and probed with PKB antibody (AbclonalR, Baltimore, MD, USA), 1:200 overnight at 4 °C in a refrigerator. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0 μg/ml) secondary antibody and subsequently, streptavidin peroxidase (Horse Radish Peroxidase-streptavidin system) according to manufacturer's protocol (HistoMarkR, KPL, Gaithersburg MD, USA).

Reaction product was enhanced with diaminobenzidine (DAB, AmrescoR, USA) for 6–10 min and counterstained with high definition hematoxylin (EnzoR, NY − USA), with subsequent dehydration in ethanol. The slides were covered with coverslips and sealed with DPx mountant. The immunoreactive positive expression of intensive regions were viewed starting from low magnification on each slice then with $100 \times$ magnifications using a photo microscope (Olympus) and a digital camera (ToupcamR, Touptek Photonics, Zhejiang, China).

2.7. Statistical analysis

All values are expressed in mean ± S.D. Parameters obtained from the experimental groups were compared with the control. The data obtained were analyzed using repeated measures one-way analysis of variance (ANOVA) with the Tukey post-hoc analysis for the analysis of scientific data using GraphPad Prism® 5.01 Values were considered statistically significant at p < 0.05.

3. Results

3.1. Hepatic antioxidant status and markers of oxidative stress following $NaAsO_2$ exposure and withdrawal

Rats exposed to $NaAsO_2$ showed significant (p < 0.05) reduction of hepatic total protein (Table 1). Concurrently, there were significant (p < 0.05) increases in thiol, malondialdehyde (MDA), hydrogen peroxide (H_2O_2) generated and nitric oxide levels following exposure at 10 mg/kg, 20 mg/kg and 40 mg/kg of $NaAsO_2$ (Table 1). These markers of oxidative levels were normalized after the withdrawal of $NaAsO_2$ (Table 1). On the other hand, the activities of anti-oxidant enzymes including glutathione peroxidase (GPx), glutathione-S-transferase (GST) and superoxide dismutase (SOD) were observed to increase significantly (p < 0.05) following exposure to $NaAsO_2$ at 10 mg/kg, 20 mg/kg and 40 mg/kg, respectively (Table 2). Furthermore, upon

$NaAsO_2$ withdrawal, there was a significant increase in the activity of hepatic GPx might be due to adaptive response (Table 2).

3.2. Erythrocyte antioxidant profile and indicators of oxidative stress following $NaAsO_2$ exposure and withdrawal

As observed in the liver, there was significant (p < 0.05) declines in erythrocyte total protein levels following 4 weeks' exposure to $NaAsO_2$ (Table 3). The markers of oxidative stress followed the similar pattern as observed in the liver. A significant (p < 0.05) increase in MDA, H_2O_2 and nitric oxide levels was observed following exposure to $NaAsO_2$ (Table 3).

Upon withdrawal of the toxicant, values for total protein, and the other markers of oxidative stress appeared to return to pre-exposure levels (Table 3). Further, significant increases were observed in the erythrocyte GSH content and the activities of SOD, GPx and GST activity both at exposure and withdrawal phases. However, there was no significant changes in the values of erythrocyte GSH contents during the withdrawal period of $NaAsO_2$ (Table 4).

3.3. Hematological parameters following exposure and withdrawal of $NaAsO_2$

Following four weeks of exposure to $NaAsO_2$, anemia was observed with a significant (p < 0.05) reductions in PCV, WBC and RBC indices across all treatment groups compared to the control (Table 5). On the other hand, platelets showed dose dependent increases of 20.7%, 28.8% and 45.2% in the order of 10 mg/kg, 20 mg/kg and 40 mg/kg treatment $NaAsO_2$, respectively (Table 5). Upon withdrawal of $NaAsO_2$ however, a reverse picture of the hematological parameters was observed. Across the treatment groups, upon withdrawal of increases $NaAsO_2$, in PCV, Hb, RBC and WBC values were obtained when compared to the control (Table 5). This demonstrated that the anemia observed upon exposure was responsive, and regenerative.

3.4. Histopathology

After four weeks' exposure to $NaAsO_2$, the liver ultrastructure of the control group showed no visible lesions. Conversely, across all treated groups, peri-portal inflammation was observed around the hepatic portal veins alongside congestion of the hepatic blood vessels (Plate 1). Following withdrawal of $NaAsO_2$, the hepatocytes showed varying degrees of macrovesicular steatosis, indicative of long-term fat deposition within the hepatocytes together with different degrees of congestion in the hepatic vessels (Plate 2).

Table 1
Liver total protein and markers of oxidative stress.

Experimental groups		Group A	Group B	Group C	Group D
Total Protein	Exposure	2.64 ± 0.51	2.23 ± 0.24	2.09 ± 0.19*	2.09 ± 0.16*
	Withdrawal	2.56 ± 0.24	2.53 ± 0.31	2.47 ± 0.33	2.43 ± 0.26
Total Thiol	Exposure	73.76 ± 4.81	95.83 ± 4.88*	86.12 ± 5.48*	85.51 ± 4.92*
	Withdrawal	64.62 ± 8.13	45.21 ± 5.32*	63.90 ± 4.12	61.62 ± 8.02
Nitric Oxide	Exposure	3.41 ± 0.23	4.21 ± 0.24*	4.29 ± 0.30*	4.65 ± 0.25*
	Withdrawal	2.88 ± 0.51	1.82 ± 0.26*	2.92 ± 0.25	2.45 ± 0.23
MDA	Exposure	0.17 ± 0.02	0.28 ± 0.03*	0.44 ± 0.04*	0.45 ± 0.02*
	Withdrawal	0.09 ± 0.02	0.11 ± 0.01	0.10 ± 0.02	0.10 ± 0.02
H_2O_2 generated	Exposure	21.54 ± 0.38	23.53 ± 0.86*	24.89 ± 1.46*	25.29 ± 1.43*
	Withdrawal	26.38 ± 3.44	27.03 ± 3.74	26.23 ± 2.13	27.08 ± 2.75

Values are presented as mean ± standard deviation.
Group A (Control), Group B (Sodium arsenic (10 mg/kg; p.o.), Group C (Sodium arsenic (20 mg/kg; p.o.) Group D (Sodium arsenic (40 mg/kg; p.o.).
Asterisk (*) Indicates significant difference from control at p < 0.05 in each row. Total protein (Mg/dl), H_2O_2 (hydrogen peroxide generation; μmol/mg protein), MDA (malondialdehyde; μmol of MDA formed/mg protein), Nitric oxide (μmol/mg protein) Total thiol (μmol/L).

Table 2
Liver antioxidant enzyme profile following exposure and withdrawal of Sodium Arsenite.

Experimental groups		Group A	Group B	Group C	Group D
GSH	Exposure	61.71 ± 3.75	60.60 ± 2.75	61.79 ± 2.27	59.96 ± 1.31
	Withdrawal	52.30 ± 2.00	50.91 ± 1.64	53.33 ± 3.36	52.04 ± 1.32
SOD	Exposure	16.10 ± 2.27	18.54 ± 1.90	19.75 ± 1.82*	19.79 ± 1.49*
	Withdrawal	**19.80 ± 0.18**	**20.60 ± 0.15**	**20.70 ± 0.27**	**20.80 ± 0.20**
GST	Exposure	1.74 ± 0.28	2.02 ± 0.21	2.14 ± 0.20*	2.12 ± 0.16*
	Withdrawal	1.76 ± 0.16	1.83 ± 0.13	1.85 ± 0.27	1.85 ± 0.17
GPx	Exposure	89.08 ± 13.88	103.54 ± 10.57	110.14 ± 10.20*	109.42 ± 7.52*
	Withdrawal	83.32 ± 7.73	86.68 ± 6.15*	80.05 ± 5.48	90.31 ± 5.97*

Values are presented as mean ± standard deviation.
Group A (Control), Group B (Sodium arsenic (10 mg/kg; p.o.), Group C (Sodium arsenic (20 mg/kg; p.o.) Group D (Sodium arsenic (40 mg/kg; p.o.).
Asterisk (*) Indicates significant difference from control at $p < 0.05$ in each row. GSH (reduced glutathione; μmol/mg protein), SOD (Superoxide dismutase; Units/mg protein), GST (Glutathione S-Transferase; μmol/min/mg protein), GPx (Glutathione peroxidase; μmol/mg protein).

Table 3
Erythrocyte total protein and markers of oxidative stress.

Experimental groups		Group A	Group B	Group C	Group D
Total Protein	Exposure	1.15 ± 0.19	0.99 ± 0.02*	0.98 ± 0.06*	1.26 ± 0.27
	Withdrawal	1.14 ± 0.04	1.08 ± 0.05*	1.09 ± 0.05	0.99 ± 0.04*
Total Thiol	Exposure	54.33 ± 8.74	55.06 ± 10.41	52.39 ± 10.44	47.61 ± 10.39
	Withdrawal	99.21 ± 13.56	82.30 ± 15.82	71.31 ± 15.66*	40.13 ± 8.85*
Nitric Oxide	Exposure	0.47 ± 0.05	0.49 ± 0.04	0.44 ± 0.07	0.97 ± 0.03*
	Withdrawal	0.83 ± 0.09	0.59 ± 0.05*	0.51 ± 0.11*	0.37 ± 0.10*
MDA	Exposure	0.28 ± 0.01	0.31 ± 0.01*	0.35 ± 0.02*	0.24 ± 0.01*
	Withdrawal	0.16 ± 0.02	0.16 ± 0.03	0.17 ± 0.02	0.17 ± 0.02
H_2O_2 generated	Exposure	13.98 ± 0.25	14.31 ± 0.58	14.23 ± 1.39	14.64 ± 0.63
	Withdrawal	15.73 ± 0.74	15.73 ± 0.79	15.77 ± 1.05	14.73 ± 0.84

Values are presented as mean ± standard deviation.
Group A (Control), Group B (Sodium arsenic (10 mg/kg; p.o.), Group C (Sodium arsenic (20 mg/kg; p.o.) Group D (Sodium arsenic (40 mg/kg; p.o.).
Asterisk (*) Indicates significant difference from control at $p < 0.05$ in each row. Total protein (Mg/dl), H_2O_2 (hydrogen peroxide generation; μmol/mg protein), MDA (malondialdehyde; μmol of MDA formed/mg protein), Nitric oxide (μmol/mg protein) Total thiol (μmol/L).

Table 4
Erythrocyte antioxidant enzyme profile following exposure and withdrawal of Sodium arsenite.

Experimental groups		Group A	Group B	Group C	Group D
GSH	Exposure	51.41 ± 0.70	52.26 ± 0.37*	51.98 ± 0.83	53.11 ± 1.31*
	Withdrawal	53.33 ± 0.53	53.40 ± 0.42	54.11 ± 0.72	53.61 ± 0.26
SOD	Exposure	41.33 ± 7.04	47.16 ± 1.31*	47.33 ± 3.29*	40.25 ± 5.68
	Withdrawal	**42.90 ± 0.16**	**46.10 ± 0.18***	**45.20 ± 0.18***	**49.70 ± 0.19***
GST	Exposure	3.39 ± 0.49	3.89 ± 0.18*	3.95 ± 0.25*	3.60 ± 0.33
	Withdrawal	3.31 ± 0.11	3.49 ± 0.16*	3.43 ± 0.14	3.74 ± 0.15*
GPx	Exposure	208.51 ± 33.39	238.49 ± 4.79*	239.59 ± 14.81*	193.48 ± 37.74
	Withdrawal	188.85 ± 7.52	200.72 ± 9.19*	199.27 ± 7.58*	217.17 ± 8.12*

Values are presented as mean ± standard deviation.
Group A (Control), Group B (Sodium arsenic (10 mg/kg; p.o.), Group C (Sodium arsenic (20 mg/kg; p.o.) Group D (Sodium arsenic (40 mg/kg; p.o.).
Asterisk (*) Indicates significant difference from control at $p < 0.05$ in each row. GSH (reduced glutathione; μmol/mg protein), SOD (Superoxide dismutase; Units/mg protein), GST (Glutathione S-Transferase; μmol/min/mg protein), GPx (Glutathione peroxidase; μmol/mg protein).

3.5. Immunohistochemistry

Following four weeks' exposure to NaAsO$_2$, the 10 mg/kg, 20 mg/kg and 40 mg/kg treated groups showed lower PKB expressions when compared with the control group (Plate 3). Interestingly, upon withdrawal of the NaAsO$_2$, the exposed rats showed even higher expressions of PKB when compared with the control group (Plate 4).

4. Discussion

In this study, we investigated the effects of long term exposure and withdrawal of NaAsO$_2$ on the liver and erythrocyte. It was well established that arsenic causes toxicity by the process of oxidative stress [37–40]. Also, reactive oxygen and nitrogen species (ROS, RNS) are generated and are due to the presence of unpaired electrons in their outer orbit that ultimately make them extremely reactive [41]. Such free radicals are needed in trace quantities for normal physiologic reactions, including defense mechanisms against infectious agents and in maintaining redox homeostasis [42]. When in excess, these oxidants initiate a chain reaction, culminating in the oxidation of lipids (lipid peroxidation), proteins and DNA, creating imbalances, disrupting redox signaling and causing molecular damage while incapacitating the antioxidant defense systems [43].

Our results showed significant arsenic acid (10 mg/kg, 20 mg/kg and 40 mg/kg) induced increases in MDA content, H$_2$O$_2$ generated and nitric oxide content in the liver and erythrocytes following exposure to arsenic with the non-significant increase in the values of H$_2$O$_2$ generated in the erythrocytes. This is an indication of oxidative stress

Table 5
Hematological parameters following sodium arsenite intoxication.

Experimental groups		Group A	Group B	Group C	Group D
PCV (%)	Exposure	46.30 ± 1.50	42.30 ± 1.20*	43.30 ± 1.50*	42.80 ± 1.64*
	Withdrawal	47.00 ± 1.90	50.40 ± 1.50*	50.00 ± 4.20	46.70 ± 1.53
Hb (g/dl)	Exposure	14.50 ± 0.70	14.50 ± 0.90	14.30 ± 0.50	14.10 ± 0.75
	Withdrawal	14.60 ± 0.50	15.40 ± 0.40*	15.90 ± 1.50	14.40 ± 0.44
PLATELETS (x10^3/μL)	Exposure	590.20 ± 190.00	712.40 ± 199.10	760.70 ± 166.40	857.50 ± 75.74*
	Withdrawal	675.80 ± 97.90	577.00 ± 156.00	696.20 ± 98.00	544.70 ± 17.47*
RBC(x1012/L)	Exposure	8.10 ± 0.20	7.50 ± 0.50*	7.80 ± 0.20*	7.60 ± 0.28*
	Withdrawal	8.10 ± 0.20	8.60 ± 0.20*	8.80 ± 0.80*	8.20 ± 0.20
WBC (x109/L)	Exposure	17.8 ± 5.80	15.9 ± 4.00	15.1 ± 2.50	7.3 ± 5.82
	Withdrawal	11.60 ± 3.50	15.10 ± 4.90	16.90 ± 8.10	13.40 ± 2.50

Values are presented as mean ± standard deviation.
Group A (Control), Group B (Sodium arsenic (10 mg/kg; p.o.), Group C (Sodium arsenic (20 mg/kg; p.o.) Group D (Sodium arsenic (40 mg/kg; p.o.).
Asterisk (*) Indicates significant difference from control at p < 0.05 in each row.

precipitated by NaAsO$_2$ exposure. However, upon withdrawal of the NaAsO$_2$ from the exposed rats, the markers of oxidative stress were found to return to near normal values suggesting that withdrawal of NaAsO$_2$ following exposure might show some degree of amelioration.

On the one hand, the hepatic GSH, (which provides the reduction of hydroperoxides in membrane phospholipids via glutathione-S-transferases and glutathione peroxidase, and thereby the activation of glutathione antioxidant system to interrupt lipid peroxidation chain reaction) was largely unaffected following exposure to NaAsO$_2$. On the other hand, significant increases in erythrocyte and hepatic GPx, GST and SOD activity was observed. We propose two possible explanations for this development, both involving the up-regulation of antioxidant enzyme activity by Nrf2 [44,45].

First, is the cysteine-thiol modification of the Keap 1 arm of the Nrf2-Keap1 pathway [44]. In the absence of stressors, Nrf2 is kept at low levels by Keap 1 [45], but in the presence of ROS, the sulfhydryl groups present on the Keap 1 arm oxidizes cysteine residues, especially Cys 151, changing the critical conformation of Keap 1 and inhibiting it from binding to Nrf2 [46,47].

Subsequently, Nrf2 is released within the cytosol, moves into the nucleus to heterodimerize with small Maf proteins and then, bind to regulatory gene regions known as the antioxidant response elements (ARE) [48]. The substances which elicit a response at the ARE include quercetin, environmental contaminants, metals and certain oxidants like nitric oxide, and hydrogen peroxide [49]. The Nrf2-Maf-ARE complex subsequently activates a whole array of antioxidant and
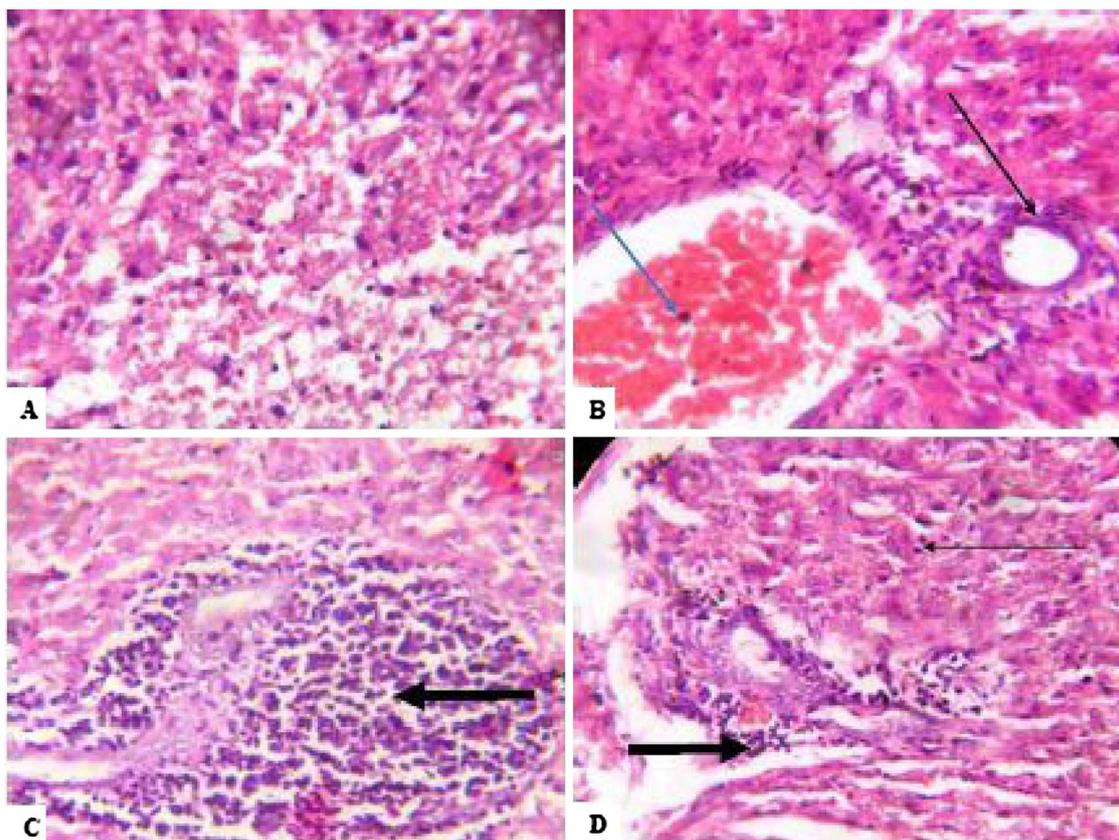
Plate 1. Photomicrograph showing Liver of rats. (A) − Control: Plate shows no significant lesion (B) −10 mg/Kg: Plate shows congestion of vessels (light blue arrow) and mild periportal inflammation (light black arrow). (C) −20 mg/Kg: Plate show severe disseminated periportal inflammation (black arrows) (D) −40 mg/Kg: Plate shows mild periportal inflammation (black arrow). All plates are after 4 weeks exposure to Sodium Arsenite. Magnification: 100× with Haematoxylin and Eosin stains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
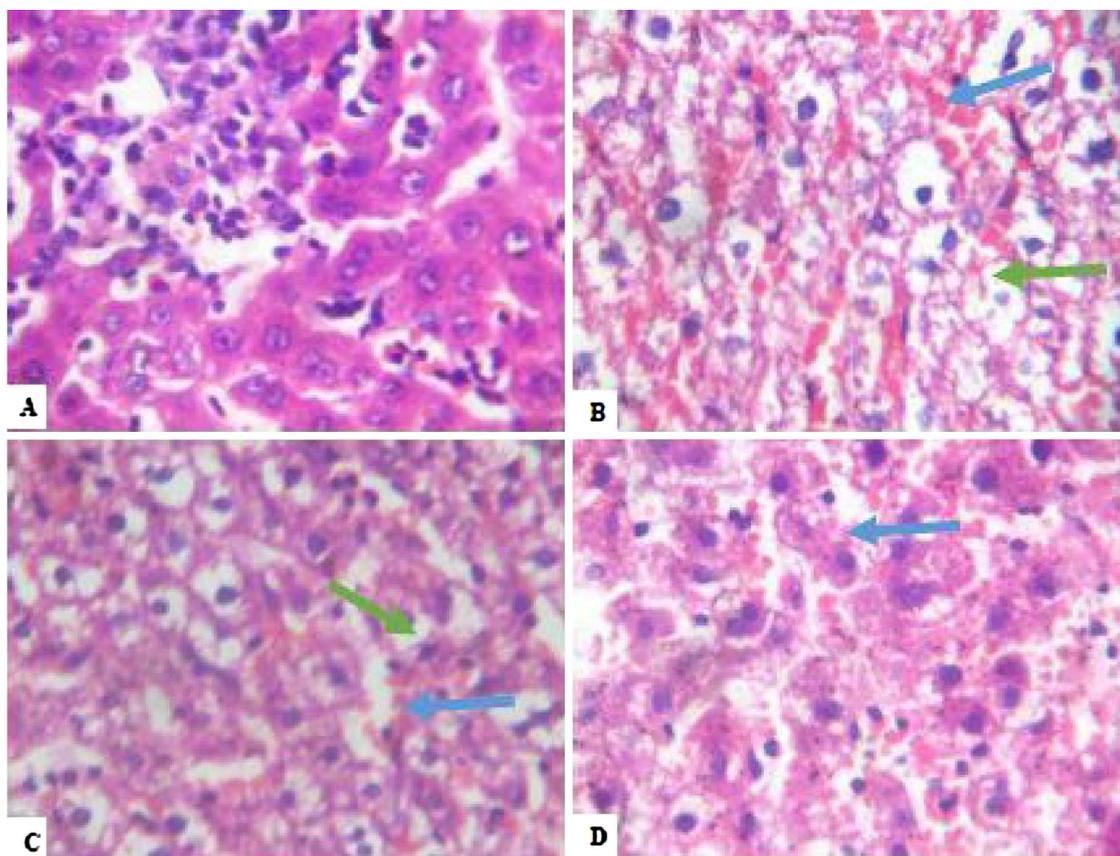
Plate 2. Photomicrograph showing Liver of rats. (A) – Control: Plate shows no significant lesion. Groups B-D show disseminated macrovesicular steatosis (green arrows) as well as congestion of vessels and sinusoid (blue arrow). All plates are after 4 weeks exposure to Sodium Arsenite. Magnification: 100× with Haematoxylin and Eosin stains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detoxification genes including those of glutathione-S-transferase and many other enzymes involved in antioxidant defense, thus increasing antioxidant activity and detoxifying free radicals [42].

Secondly, arsenic itself has been discovered to activate the Nrf2-Keap1 antioxidant pathway and prolong Nrf2 activation [50–52]. But, unlike ROS and other free radicals, it works independent of the Keap 1 and Cys 151 mechanism. Studies suggest that it involves the autophagy protein p62 binding to Keap 1, thereby disrupting the Nrf2-Keap1 complex and releasing Nrf2, which then initiates the activation of antioxidant enzymes [53,54].

Following withdrawal of the animals from exposure, there was a stepwise reduction in the hepatic and erythrocyte markers of oxidative stress. Due to the apparent diminishing of the toxicant in systemic circulation, a negative feedback mechanism could be responsible for this, possibly through Keap 1 signaling to auto-regulate Nrf2 concentrations back to minimum levels as reported by [45]. Interestingly, the hepatic and erythrocyte antioxidant enzyme activities improved significantly both at exposure and withdrawal of NaAsO$_2$. During oxidative stress, the activities of antioxidant enzymes should decline, but the reverse was recorded in the present study. We therefore hypothesize that the observable increase in the acidities of SOD, CAT, GST and GPx might be due to adaptive response to the NaAsO$_2$ toxicity through Nrf2 activation and (Akt/PKB) signaling.

Our results showed sections of mild to severe periportal inflammation, steatosis and congestion of portal vessels, but this was still mild when compared to established histo-pathological manifestations of arsenic toxicity [55,56]. We speculate that the lesions seen were initial signs of toxicity and that the up-regulation of hepatic and erythrocyte antioxidant enzyme activity, secondary to Nrf2 activation, prevented further fibrotic damage to the liver ultrastructure. This agrees with similar reports where Nrf2 activity was shown to protect against liver damage [57–59].

Following exposure to NaAsO$_2$, our results showed a progressive, dose-dependent pancytopenia across all treatment groups. This corroborates established literature demonstrating arsenic to have a wide-ranging suppressive effect on the hematopoietic system, spanning the bone marrow, spleen and erythrocytes [60]. Increased cytosolic calcium levels, ceramide formation, and disruption in erythrocyte membrane integrity alongside decreased ATP availability all contribute to cell death. The short life span of arsenic laden RBCs has been shown to impact a direct negative effect on erythrocyte and hemoglobin values, leading to anemia [19,61].

An exception to the pancytopenia observed in this study was the thrombocytosis seen in response to exposure and the concomitant thrombocytopenia in response to withdrawal. The exact cause is unclear as this differs from established platelet responses in arsenic toxicity [60,62], but a possible explanation is reported by [63] who linked iron deficiency with secondary thrombocytosis and platelet activation. The oxidation of the sulfhydryl groups on hemoglobin by arsenic and the subsequent reduction in heme synthesis could have led to depleted iron stores, possibly triggering a secondary thrombocytosis. Withdrawal of the inciting toxicant should have the reverse effect, freeing up the iron stores and reducing platelet levels as was seen in this study. Overall, withdrawal from NaAsO$_2$ appeared to alleviate and reverse arsenic induced anemia, and improve blood count across all groups.

Protein kinase B, synonym Akt is a serine/threonine kinase that is expressed to varying degrees in response to stimuli which impact cell homeostasis or destruction Nicholson and Anderson [64]. A marker of cell survival, it is central to homeostasis, replication and existence [65]. There is thus a positive association to be expected between its expression and cell survival. In this study, we observed a decrease in the expression of Akt/PKB in the exposed groups. This could be interpreted
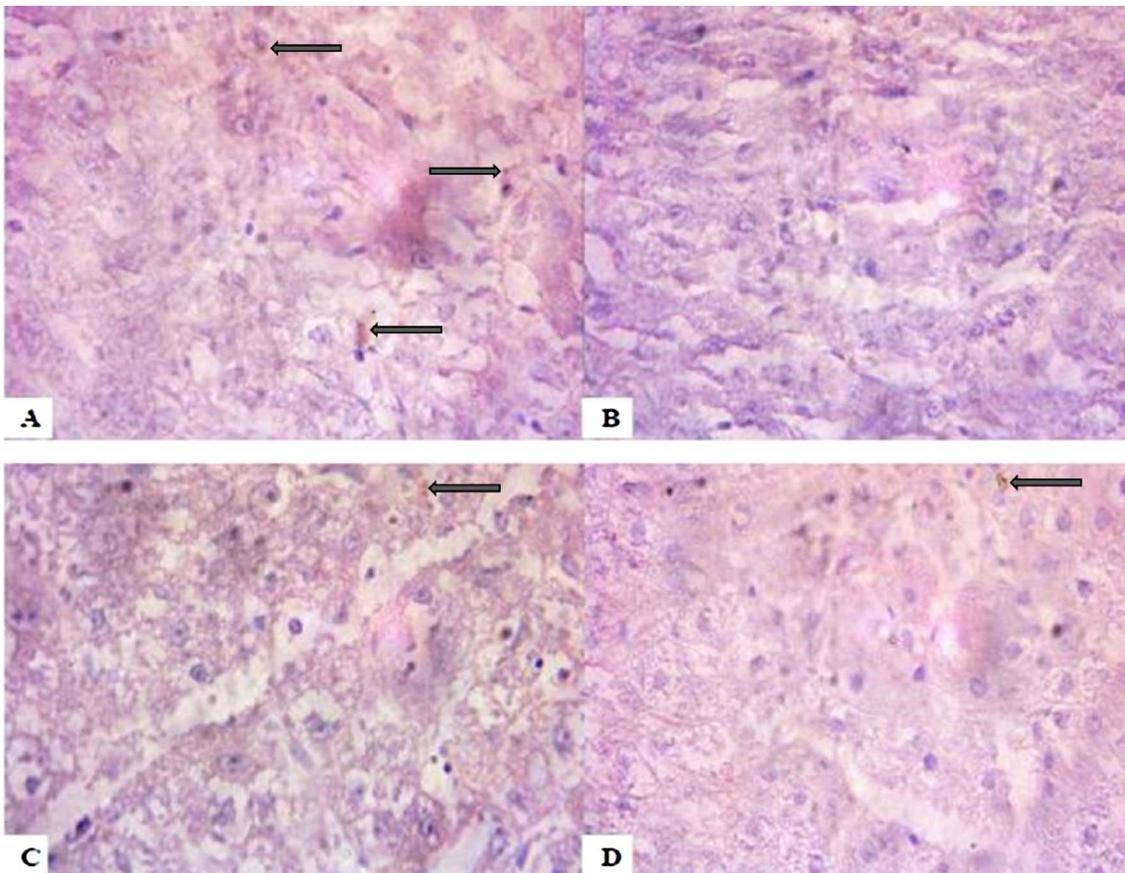
Plate 3. Control group (A) showed higher expression of the survival protein PKB. Groups B–D treated with Sodium arsenite showed lower PKB expression 4 weeks after exposure. (Immunohistology slides were prepared with $100\times$ magnification using a digital microscope.).

Plate 4. Control group (A) showed lower PKB expressions. Groups B–D treated with Sodium arsenite showed higher PKB expression 4 weeks after withdrawal. (Immunohistochemistry slides were prepared with $100\times$ magnification using a digital microscope.).

that exposure to NaAsO$_2$ had deleterious effects on cell survivability. On withdrawal from the toxicant however, higher expressions of Akt/PKB were observed in NaAsO$_2$ treated rats. This suggests that Akt/PKB could be involved in promoting cell survival after withdrawal from NaAsO$_2$. We surmise that withdrawal from NaAsO$_2$ reduced the impact of toxicant on cell ultrastructure, reversed the progression of cell destruction and improved the survival of hepatic tissue.

5. Conclusion

Taking all into account, NaAsO$_2$ toxicity even at low doses is still of grave public health concern. NaAsO$_2$ induced oxidative stress, causing significant damage to the liver and hematopoietic systems. However, withdrawal of the exposed animals seemed to reverse the aforementioned NaAsO$_2$ toxicity by increasing the antioxidant defense system through Akt/PKB signaling as speculatively demonstrated by immunohistochemistry. It is possible that Akt/PKB signaling might play a protective role in human populations from NaAsO$_2$ contamination following withdrawal. A future research direction is to focus on the molecular analysis of Akt/PKB signaling pathway.

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