ASSESSMENT OF LIPID PEROXIDATION MARKERS AND ROINFLAMMATORY CYTOKINES IN ARSENITE-EXPOSED RATS

O. K Afolabi[1], E. B Oyewo[2], A. S. Adekunle[3], O. T. Adedosu[4], A. L. Adedeji[5]

Department of Biochemistry,Faculty of Basic Medical Sciences, College of Medicine, Ladoke Akintola University of Technology, Ogbomoso, NIGERIA.

[1] segunll@yahoo.com

ABSTRACT

Arsenic exposure has been associated with diverse diseased states, including cancer and atherosclerosis. Changes linked with these states include heightened oxidative stress and immunological responses promoted by this intoxication. This study aimed to find the relationships between lipid peroxidation products and proinflammatory cytokines on exposure to arsenic. Method: Rats were exposed to sodium arsenite in their drinking water at doses of 50 and 100 ppm for 7 weeks. At the end of the exposure period, lipid peroxidation products and paraoxonase activity were measured spectrophotometrically while inflammatory cytokines were assayed using ELISA kits. Results: Plasma hydroperoxide levels were increased by more than 150%, while hepatic MDA concentration was raised by 2 fold at high dose of exposure. Arsenite resulted in depression of paraoxonase activity. Concentrations of proinflammatory cytokines: IL-2, IL-6 nd TNF-α were also higher a control group. Rats exposed to arsenite showed positive correlation between lipid peroxidation products and proinflammatory cytokines. Conclusion: Arsenic-induced toxicity may be related to interplay of the peroxidative processes with the process of inflammatory/immunological reactions.

Keywords: Arsenic, lipid peroxidation, proinflammatory cytokines, PON1

INTRODUCTION

Arsenic is an element that is ubiquitous in the environment where it exists in both organic and inorganic forms in different valence (Cullen and Reimer, 1989). It is a natural contaminant of groundwater in certain parts of the world (Kwok, 2006) as a result of its leaching into aquifers from surrounding arsenic-rich geological formation (WHO, 2001) Anthropogenically, it finds its way into the environment marjorly, as a result of metal smelting operations (Ng et al, 2003). Exposure to this metalloid has been associated with a variety of diseases resulting from both its cancer and non-cancer effects. It has been known to cause oxidative stress (Pi et al., 2002) which is one of the underlying causes of diseases associated with arsenic, including cancer and cardiovascular disorders (Bošnjak et al., 2008; Chen et al., 1992, 1995, 1996).

Studies have shown that exposure to arsenic at high levels can lead to development of skin, bladder, liver and lung cancers (Chiou et al., 2001; Steinmaus et al., 2003). Severe metabolic disorders such as diabetes and cardiovascular disorders have also been associated with arsenic exposure (Jana et al., 2006; Sarkar et al., 2003). Arsenic-induced toxicity has been known to be associated with some changes in immune system (Achaya et al, 2010), but there is paucity of studies of these changes with relationship to changes in lipid peroxidation level, especially in atherosclerosis.

In spite of the numerous studies that arsenic induces oxidative stress in effecting its toxicity and the promotion of immunological responses by the metal, no study has been carried out to

investigate the possible correlation existing btween these two processes, to the best of our knowledge. This study, therefore, aimed to find out if there were any dependence between the concentrations of lipid peroxidation products and proinflammatory cytokines during arsenic exposure.

MATERIALS AND METHODS

Animals and Treatment

Twenty four male albino Wistar rats (bred in the Animal House of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria) with a mean body weight of 120g were used for the experiment. The animals were housed in clean plastic cages and were allowed to acclimatize for 2 weeks before the start of the experiment. They were maintained on a standard pellet diet. At the end of the acclimatization period, the animals were weighed and randomly divided into three groups of eight animals each. While one group served as the control group, the remaining two groups were exposed to arsenic in the form of sodium arsenite through their drinking water for 7 weeks. The arsenic doses were 50 and 100 ppm respectively.

At the end of arsenic exposure, blood was collected from the animals into heparinized tubes by cardiac puncture under light ether anesthesia after an overnight fast. The blood samples were centrifuged to obtain the plasma. All samples were stored at -20°C until analyzed.

Biochemical Analyses

Determination of thiobarbituric acid-reactive substances (TBARS)

Level of Lipid Peroxidation was measured by the method of Ohkawa et al. (1979). Briefly, reaction mixture consist of 0.2 ml 8% SDS, 1.5 ml 20% acetic acid and 0.6 ml distilled water. 0.2 ml of tissue homogenate was added to the reaction mixture. Reaction was initiated by adding 1.5 ml of 1%TBA and terminated by 10% TCA. It was the centrifuged and absorbance was read at 532 nm. LPO was expressed in terms of nmoles MDA formed/mg tissue using an extinction coefficient of $1.56 \times 10^5 \, M^{-1} \, cm^{-1}$.

Plasma lipid Hydroperoxides

Lipid hydroperoxides in plasma were assayed by the method of Naurooz- Zadeh et al. (1994). Samples (90 μl) of plasma were mixed with either 10 μl of 10 mM triphenylphosphine (TPP) in methanol or with 10 μl of methanol and incubated for 30 min at a room temperature. Then, 900 μl of FOX2 reagent (250 μM ammonium ferrous sulfate, 100 μM. xylenol orange, 25 mM H_2SO_4 and 4 mM butylated hydroxytoluene in 90% methanol) was added and the sample was incubated for another 30 min. The mixture was centrifuged at 12 000 × g for 10 min to remove flocculated material and the absorbance was read at 560 nm. The absorbance of the sample with TPP was subtracted from the sample without TPP and hydroperoxides concentration was calculated from the standard curve prepared using different concentrations (1–20 μM.) of H_2O_2.

Plasma Paraoxonase

The Paraoxonase activity of PON1 was determined using paraoxon (O, O-diethyl-o-p-nitrophenylphosphate) as the substrate. The increase in absorbance at 405 nm due to the formation of 4-nitrophenol following the hydrolysis of paraoxon was measured as described by Furlong and Richter (2001). The molar extinction co-efficient of 18050 $M^{-1}cm^{-1}$ was used to calculate enzyme activity. PON1 activity is expressed in U/l serum. 1 unit of Paraoxonase

activity was defined as the enzyme quantity that disintegrated 1 micromole of paraoxon substrate in 1 minute.

Determination of Plasma Interleukin 2, 6 and Tumour Necrosis Factor Alpha Concentrations

Plasma concentrations of interleukin 2 (IL-2), 6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α) were determined by enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Inc. USA).

Statistical Evaluation

Results are expressed as mean± S.E. One-way analysis of variance (ANOVA) followed by Tukey's test was used to analyze the results with $p < 0.05$ considered significant. Associations among the parameters and their magnitudes were tested for by using Pearson Correlation analysis.

RESULTS

As shown in Figure 1, plasma hydroperoxide concentrations in the animals were drastically increased by exposure to sodium arsenite by mre than 150% at the doses investigated. Plasma LOOH at both 50 and 100 ppm arsenic doses were 23.96 μmol/L and 26.07 μmol/L respectively compared to the control at 8.88 μmol/L. The hepatic concentrations of MDA in rats after exposure to arsenite exposure are depicted in Figure 2. The concentrations of the lipid peroxidation product increased significantly in the animals ($p < 0.05$) as the arsenic dose increased. The MDA concentration was increased by 2 fold at the high dose of arsenic exposure from 1.23 nmol/g tissue to 2.57 nmol/g tissues. Paraoxonase activity in the plasma was also affected by exposure to the toxicant (Figure 3). Arsenite doses at 50 ppm and 100 ppm reduced the enzyme activity by 30% and 27% respectively.

The concentrations of both TNF-α and IL-2 in the exposed rats were significantly increased ($p < 0.05$) at all doses tested, compared to the controls (Figure 4). At 50 and 100 ppm arsenite doses, IL-2 concentrations were increased to 49.85 nd 51.16 pg/ml respectively from 37.95 pg/ml in control, while TNF-α levels ere raised to 176.76 and 308.46 pg/ml respectively from 95.11 pg/ml in the control. With IL-6, the level was only increased at high dose by 1.62 fold by arsenite.

The correlations between the oxidative stress indices and the proinflammatory cytokines were presented in Table 1. Strong positive correlations were found between the lipid peroxidation products (hepatic MDA and plasma LOOH) and the proinflammatory cytokine (TNF-α, IL-2 and IL-6). While paraoxonase activity, another oxidative stress marker,gave negative correlation with both IL-2 and TNF-α.

DISCUSSION

Inflammation has been implicated in atherogenic process through its adverse effect on lipoprotein metabolism and arterial wall biology making it a major contributor to atherogenesis (Stentz et al. 2004). In this study, arsenic exposure resulted in increased expressions of the proinflammatory cytokines, TNF-α, IL-2 and IL-6. Similar results have been obtained and reported with other metals like cadmium (Villanueva et al., 2000; Afolabi et al., 2012). The inflammatory response could be a consequence of increased oxidative stress, resulting from elevated level of lipid peroxidation (Janabi et al., 2000) as lipid peroxidation products have reportedly induced a pattern of inflammatory genes (Rajavashisth et al., 1990). The increase in the expressions of these proinflammatory cytokines could

increase the atherogenic potential in the system. The proinflammatory cytokines, IL-6 and TNF-α correlated significantly with development of atherosclerosis and have been implicated as atherosclerotic indicators (Haddy et al., 2003). TNF-α is associated with an elevated risk of recurrent myocardial infarction and cardiovascular death (Ridker et al., 2000) and its level correlates with risk of atherosclerosis (Bruunsgaard et al., 1999). Its disruption of the cell-cell adhesion process prevents the formation of F-actin stress fibers leading to loss of endothelial permeability, favoring leukocyte transmigration (Wojciak-Stothard et al., 1998).

From several studies, IL-6 is now recognized as an independent risk factor for coronary artery disease (Elhage et al., 2001; Schieffer et al., 2004). It reportedly correlates with both metabolic and inflammatory factors considered to be significant to atherosclerosis progression (Hardy et al., 2003). IL-6 can act as both pro-inflammatory as well as anti-inflammatory cytokine, depending on its level of expression (Murtaugh et al., 1996). At high concentrations, it has been reported to perform a pro-inflammatory role (Thong-Ngam et al., 2006) and has been implicated in various pathological conditions such as, increased risk of atherosclerosis, heart attack and stroke (Dubiński and Zdrojewicz, 2007). In this study, exposure to high dose of arsenite (100 ppm) provoked a significant increase in plasma IL-6 levels ($p < 0.05$), suggesting a proinflammatory response to this toxicant. IL-6 promotes cell adhesion molecule (CAM) expression by endothelial and smooth muscle cells. It enhances the production of acute phase reactants such as C-reactive protein and TNF-α by the hepatocytes. In addition, it has also been associated with progression of smooth muscle cells into foam cells (von der Thusen et al., 2003).

IL-2 is a pro-inflammatory cytokine produced by T_H1 lymphocytes and is considered atherogenic (Upadhya et al., 2004). It plays a vital role in vascular inflammatory processes and is expressed in atherosclerotic plaques (Upadhya et al., 2004). Helper T lymphocytes expressed this cytokine to regulate immune response towards foreign agents gaining access into the body (Kleeman et al., 2008). Following arsenic intoxication, the expression of IL-2 was increased in the rats. The increases in IL-2 imply a systemic and/ or localized inflammation resulting in the stimulation of the T helper cells.

To investigate the effect of arsenic intoxication on lipid peroxidation in the rats, hepatic MDA and plasma hydroperoxide concentrations were estimated. The MDA concentration was significantly increased at high dose while formation of hydroperoxides in the plasma was elevated at both doses of arsenic investigated. The increases may be caused by increased oxidative stress, resulting from the depletion of the animals' antioxidant scavenger system. Arsenic toxicity has already been related to the production of excess reactive oxygen species (ROS) and hydrogen peoxides (Hughes, 2002; Kitchin and Ahmed, 2003). Our findings further buttressed the reports that arsenic compounds induce lipid peroxidation and oxidative stress in tissues, leading to biochemical injuries (Hughes et al., 2002).

Studies have shown that cytokines are able to induce reactive oxygen species (ROS) production. For example, TNF-a and IL-1 increased the production of hydroxyl radicals and lipid peroxidation mouse tumorigenic fibroblast cells and mesangal cells (Bohler et al., 2000). Other inflammatory cytokines have been reported to stimulate the expression of inducible nitric oxide synthase (NO) in various cells (Hukkanen et al., 1995). The increased level of oxidative stress seen in this study may thus be partly due to the action of the arsenic-induced proinflammatory cytokines. This is highly plausible considering the high level of correlation existing between these proinflammatory cytokines and markers of oxidative stress in ourstudy.

Paraoxonase (PON1), an HDL-associated enzyme, plays a critical role in the metabolism of lipid hydroperoxides and thereby, the retardation of atherogenesis (Mackness et al., 1988). There also exists an inverse relationship between the enzyme activity and the risk of cardiovascular diseases as it prevents the formation of oxidized HDL and low density lipoprotein (Mackness et al., 2003). In this study, plasma paraoxonase activity was decreased following arsenic exposure. PON1 activity is generally considered to vary in response to the consumption of PON1 for the prevention of oxidation (Aviram *et al*., 1999). The decrease may, therefore, be a result of increased generation of reactive oxygen species leading to the inactivation of the enzyme. The increased lipid peroxidation, evidenced from the heightened generation of hepatic MDA concentrations and elevated plasma lipid hydroperoxides (LOOH) in this study, is suggestive of an increased oxidative stress in the exposed rats. Inflammation conditions have been reported to decrease PON1 gene expression in vitro. Proinflammatory cytokines like TNF- α have been linked with the down-regulation of mRNA expression of PON1 in HepG2 cells (Kumon et al., 2002). The increased expression of proinflammatory cytokines elicited by arsenic exposure may, therefore, play a role in the reduced PON1 activity seen. PON1 contributes immensely to the anti-oxidant function of HDL and is responsible for inhibiting the oxidation of both LDL-C and HDL-C (Gatica *et al*., 2006). The reduction in PON1 activity may lead to the oxidation of HDL, compromising its functions (Deakin et al., 2007).

Immunological activation with the attendant inflammatory responses coupled with heightened lipid peroxidation has been suggested to participate in the initiation and progression of atherosclerosis (Donica, 2001). In this study, positive correlations between hepatic MDA concentrations and plasma LOOH (as markers of oxidative stress) on one hand, and the proinflammatory cytokines TNF-a, IL, IL-6, on the other hand, suggest that immunological stimulation along with increased lipid peroxidation processes may be means by which arsenic-induced toxicity intensifies atherosclerotic process.

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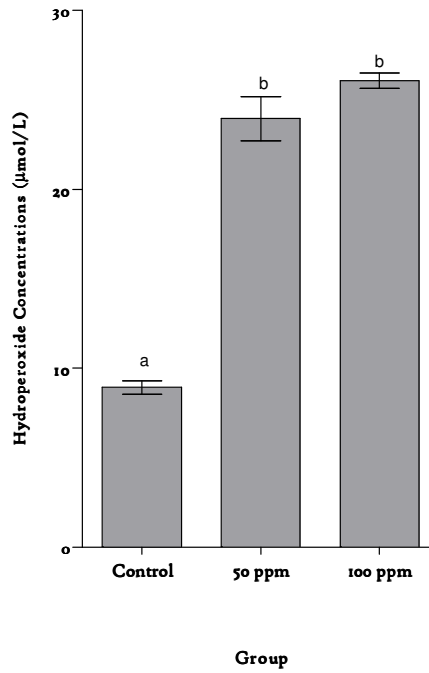
Figure 1: Lipid Hydroperoxide concentrations in plasma of rats exposed to sodium arsenite. Bars carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).
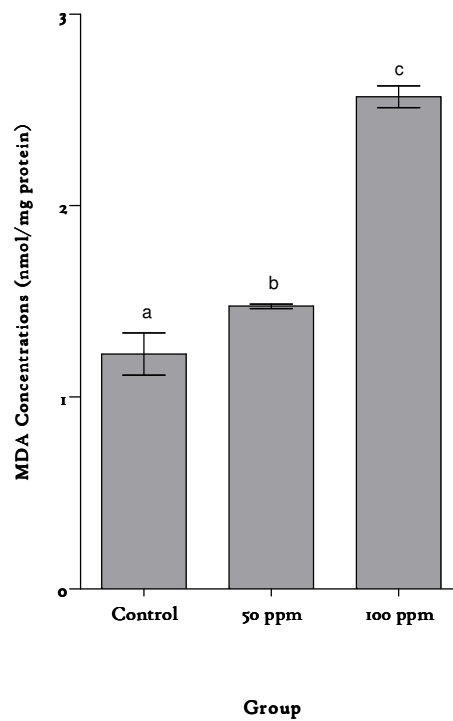

Figure 2: Hepatic MDA concentrations in rats exposed to sodium arsenite for 7 weeks Bars carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).
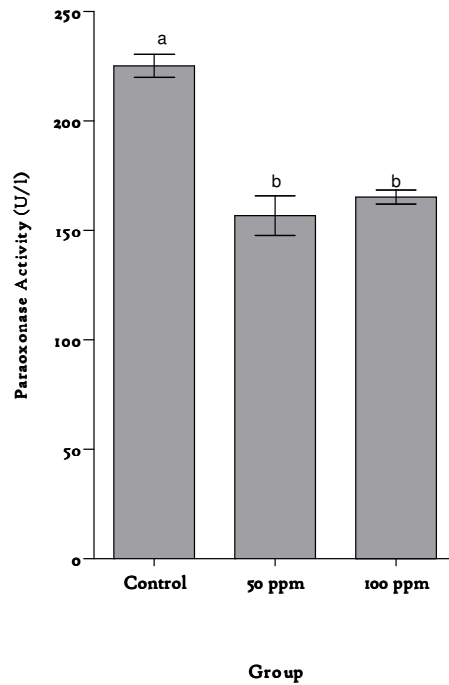
Figure 3: Paraoxonase activity in plasma of rats exposed to sodium arsenite for 7 weeks. Bars carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).
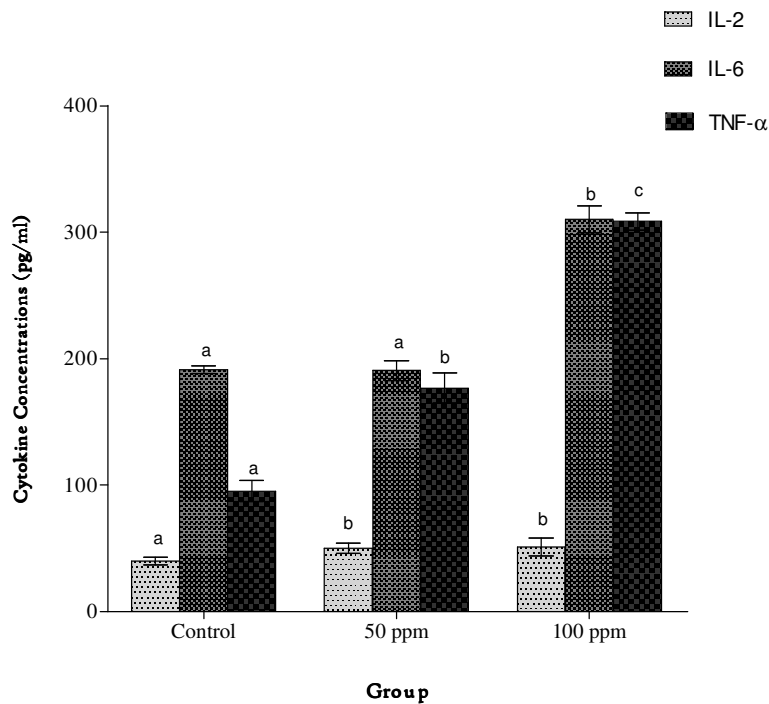

Figure 4: IL-2, IL-6 and TNF-α levels in the plasma of the animals. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).

Table 1. The correlation coefficents for PON1 activity, oxidative stress parameters and proinflammatory cytokines of rats exposed to arsenite in their drinking water

Parameter	Correlation Coefficient (r)	p-Value
Hepatic MDA vs. IL-2	0.580	≤ 0.01
Hepatic MDA vs. IL-6	0.946	≤ 0.01
Hepatic MDA vs. TNF-α	0.973	≤ 0.01
LOOH vs. IL-2	0.686	≤ 0.01
LOOH vs. IL-6	0.589	≤ 0.01
LOOH vs. TNF-α	0.840	≤ 0.01
PON1 vs. IL-2	-0.539	≤ 0.05
PON1 vs. TNF-α	-0.628	≤ 0.01