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Hepatotoxicity Induced by Arsenic Trioxide in Adult Mice and Their Progeny

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***Abstract*—**In this investigation, we have evaluated the effects of arsenic trioxide on hepatic function in pregnant and lactating Swiss albino mice and their suckling pups. Experiments were carried out on female mice given 175 ppm As_2O_3 in their drinking water from the 14th day of pregnancy until day 14 after delivery. Our results showed a significant decrease in plasma levels of total protein and albumin, cholesterol and triglyceride in As_2O_3 treated mice and their pups. The hyperbilirubinemia and the increased plasma total alkaline phosphatase activity suggested the presence of cholestasis. Transaminase activities as well as lactate deshydrogenase activity in plasma, known as biomarkers of hepatocellular injury, were elevated indicating hepatic cells' damage after treatment with As_2O_3. Exposure to arsenic led to an increase of liver thiobarbituric acid reactive substances level along with a concomitant decrease in the activities of superoxide dismutase, catalase and glutathione peroxidase and in glutathione.

***Keywords*—**Antioxidant status, arsenic trioxide, hepatotoxicity, mice, oxidative stress.

I. Introduction

ARSENIC (As) is ubiquitous in the earth's crust and biosphere. It is widely distributed in nature and released into the environment through industrial processes and agricultural usage. Human may be exposed to inorganic arsenic via ingestion through drinking water as a major route, or via inhalation and skin absorption as a minor route. Besides the natural sources of arsenic contamination in drinking water, use of arsenic-contaminating herbicides, insecticides, rodenticides, preservatives and by products of fossil fuels are also the potential sources of toxicity [1].

A chronic exposure through contaminated drinking water became an increasing global problem of public health concern [2]. Epidemiological studies have shown that inorganic arsenic exposure may lead to cancer of the liver, kidney, bladder, prostate, skin, lung, colon and nasal cavity [2]. Other clinical manifestations include blackfoot disease [3], ischemic heart disease, hypertension [4], diabetes mellitus [4] and atherosclerosis [5].

It has been recognized that arsenic exerts its toxic effects through several mechanisms, the most significant of which is the reversible reaction with sulfhydryl group's especially vicinal dithiols. The binding of arsenic to thiol containing amino acid residues in proteins has provided a mechanistic framework for envisioning interactions between proteins and arsenicals and for understanding the inhibition of the activities of enzymes by arsenical. In fact, As_{III} is the main form of arsenic interacting with sulfhydyl groups that exists in the cells, and forms strong complexes with thiols, causing inhibition of many enzymes such as dihydrolipoate, dehydrooxygenase, pyruvate dehydrogenase, monoamine oxidase, succinate oxidase and DNA polymerase [6].

Liver and kidneys are the primary target organs for toxic effects of arsenic as evidenced by clinical manifestations and biochemical alterations. Arsenic is known to produce damage in both liver and kidney tissues by enhancing peroxidation of membrane lipids [7], which is a fatal process exclusively carried out by free radicals [8].

Moreover, there exist concerns about the potential teratogenicity and developmental toxicity of inorganic arsenic. Both inorganic and organic arsenic species cross the placenta in human [9] and rodents [10]. After *in utero* exposure to inorganic arsenic (As^{5+} and As^{3+}), it was reported malformations, particularly neural tube defects, in hamsters [11], mice [12] and rats [13].

On the other hand, arsenic is a transplacental carcinogen in mice. It was shown after *in utero* exposure that arsenic induced tumor in the liver, adrenal, lung, and ovary of C3H mice [14]. More recent studies using CD1 mice showed that transplacental arsenic induced urogenital system tumours, mostly begnin tumours of the ovary and uterus, and adrenal adenoma. When combined with diethylstilbestrol malignant urogenital tumours appeared [15].

Moreover early life exposure to high levels of arsenic in drinking water has been associated with arteriosclerosis and myocardial infarction in one year old infants living in the Antofagasta region of Chile [16].

Numerous studies concerning abnormal function of liver after acute [17] and chronic arsenic toxicity have been carried out on humans and adult rats [13], but reports remain scarce for adult mice and appear to be lacking for suckling mice.

In an effort to better our understanding of the mechanism of As_2O_3 liver toxicity, the present study was designed to investigate the effects of As_2O_3 on liver metabolism changes in adult mice and their pups during the suckling period.

II. Materials and Methods

A. Chemicals

As_2O_3 was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were purchased

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from standard commercial suppliers.

B. Animals and Treatments

All Experiments, performed on female Swiss albino mice and weighing about 40 g were purchased from SIPHAT (Tunisia). After a one-week adaptation period in a room with controlled temperature (22±2◦C) and lighting (12 h-light/12 h dark), female mice were mated with males. A sperm-positive vaginal smear was taken to indicate the first day of pregnancy. They were housed individually in polycarbonate cages and were provided daily with standard pellet diet (SICO, Tunisia) and water *ad libitum*. Twelve pregnant female mice were randomly divided into two groups of six each: group I served as controls and group II received daily 175 ppm of As_2O_3 (equivalent to 54 mg/kg bw) through the drinking water from the 14^{th} day of pregnancy until day 14 after delivery. The arsenic dose used in our experiment represented 1/4 of oral LD_{50} in mice [18]. Before starting the treatment we have tested different doses of arsenic. No toxic effects and no oxidative stress were observed in pups whose mothers were treated by 100 ppm of Arsenic (1/7 of LD_{50}). From 175 ppm of As_2O_3, a moderate oxidative stress was mentioned. At a dose 200 ppm (2/7 of LD_{50}) abortion, general depression, abdominal constriction and lethal effect were observed. The day of parturition was considered as day 0 of lactation and pups born were counted, their sex and weight were recorded and each litter was reduced to eight pups (4 males and 4 females if possible) as it has been shown that this procedure maximized lactation performance [19]. Daily As_2O_3 intake by lactating mice was determined after measuring drinking water consumption. So each lactating mouse, treated with As_2O_3, ingested daily 1.63 ± 0.13 mg of As_2O_3. All animals were observed for signs of treatment-related effects. The experimental procedures were carried out according to the general guidelines on the use of living animals in scientific investigations and approved by the Ethical Committee of our country.

On postnatal day 14, 12 dams and 96 pups were sacrificed after anesthesia with chloral hydrate. Blood samples were collected into heparined tubes by aortic puncture in dams and brachial artery in pups. Plasma samples were drawn from blood after centrifugation at 2200 g and kept at -80°C until biochemical determinations.

Livers were removed and cleaned. Some portions in the median part of each liver were collected, weighed and washed in saline solution, homogenized (10% w/v) in phosphate buffer (pH 7.4) and centrifuged at 8000 g for 20 min at 4°C.

C. Biochemical Assays

Plasma levels of total protein and albumin were determined by colorimetric method using commercial kits (Diasys, Germany, ref: FS 12311021 and FS 10220021 respectively). Glycemia was assayed with a commercial kit (Biomaghreb, Tunisia, ref: 20121) and determined by enzymatic colorimetric method using glucose oxidase enzyme.

Plasma cholesterol and triglyceride levels and total ALP were determined by the enzymatic colorimetric methods using kits from Elitech diagnostics, France (Ref: CHSL 4900 and TGML 0425, PASL-0500 respectively). Direct bilirubin level in plasma was evaluated by colorimetric method using kit from Biomerieux, France (Ref: 61037).

Plasma LDH, AST and ALT activities were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Biomaghreb, Ariana, Tunisia. Ref, 20012; 20043; 20047), respectively.

The concentration of MDA in tissues, an index of lipid peroxidation, was determined spectrophotometrically according to [20]. An aliquot of 0.5 ml of liver extract supernatant was mixed with 1 ml of trichloroacetic acid solution and centrifuged at 2500 g for 10 min. One milliliter of a solution containing 0.67 % thiobarbituric acid (TBA) and 0.5 ml of supernatant were incubated for 15 min at 90◦C and cooled. Absorbance of TBA-MDA complex was determined at 532 nm using a spectrophotometer. Lipid peroxidation was expressed as nmol of thiobarbituric acid reactive substances (TBARS), using 1,1,3,3-tetra-ethoxypropane as standard.

SOD activity was estimated according to [21]. The reaction mixture contained 50 mM of tissue homogenates in potassium phosphate buffer (PH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 mM Nitro-Blue-Tetrazolium (NBT). The developed blue colour in the reaction was measured at 560 nm. Units of SOD activity were expressed, as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expessed as units/mg of protein.

CAT activity was assayed by the method of [22]. Enzymatic reaction was initiated by adding an aliquot of 20 µl of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmole H_2O_2 consumed/min/mg of protein.

GPx activity was measured according to [23]. The enzyme activity was expressed as nmol of GSH oxidized/min/mg protein.

GSH in the liver was determined by the method of Ellman [24] modified by [25] The method is based on the development of a yellow colour when DTNB (5,5-dithiobtis-2 nitro-benzoic acid) was added to compounds containing sulfhydryl groups. 500 µL of tissue homogenate in phosphate buffer were added to 3 ml of 4% sulfosalicylic acid. The mixture was centrifuged at 1600 g for 15 min. Five hundred microliters of supernatants were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as mg/mg of protein in the liver.

D. Statistical Analysis

Results were expressed as arithmetical mean ± SD. Statistical significance among groups was determined by using Student's-t-test. $p<0.05$ was considered as significant.

III. Results

Exposure mothers to 175 ppm As_2O_3 reduced glycemia in

dams and in their suckling pups (Table I). Compared to the controls, the As_2O_3-treated mothers and their pups had a significant decrease in plasma levels of total protein and of albumin. Compared to the controls, plasma levels of cholesterol and triglyceride in As_2O_3-treated dams decreased in mothers and in their pups (Table I).

TABLE I
PLASMA LEVELS OF GLUCOSE, PROTEIN, ALBUMIN AND LIPID PARAMETERS IN ADULT MICE AND THEIR PUPS, CONTROLS AND As_2O_3 TREATED, FROM THE 14TH DAY OF PREGNANCY UNTIL DAY 14 AFTER DELIVERY

Plasma parameters and treatment	Mothers	Offspring
Glucose (mg/dL)		
Controls	125.21 ± 18.64	112.66 ± 17.29
As_2O_3 group	67.91 ± 5.19 ***	74.01 ± 5.63 **
Total protein (g/dL)		
Controls	6.46 ± 0.68	18.42 ± 1.71
As_2O_3 group	4.48 ± 0.33 ***	13.59 ± 1.15 ***
Albumin (g/dL)		
Controls	2.898 ± 0.217	2.779 ± 0.222
As_2O_3 group	2.348 ± 0.102 **	2.214 ± 0.104 **
Cholesterol (mg/dL)		
Controls	126.65 ± 6.34	216.99 ± 9.28
As_2O_3 group	107.23 ± 9.31 ***	179.06 ± 9.55 **
Triglyceride (mg/dL)		
Controls	95.28 ± 5.82	230.77 ± 16.78
As_2O_3 group	40.20 ± 5.39 ***	107.39 ± 15.95 ***

Values represent means ± SD (n = 6 for each group).
As_2O_3 vs. controls. ** p<0.01 ; *** p <0.001.

While, activities of AST, ALT and LDH in plasma increased significantly in As_2O_3-treated dams and in their pups as compared to those of controls (Table II).

Table II showed also direct bilirubin level and ALP activity which was increased in mothers and in their suckling pups when compared to those of control group.

As_2O_3 (175 ppm) administered to mothers by their drinking water from the 14th day of pregnancy until day 14 of parturition, provoked a significant increase of MDA levels in liver of dams and of their offspring (Table III).

In As_2O_3 group, GSH level in liver homogenates were decreased in mothers and in their offspring (Table III).

As_2O_3 treatment led to a significant decrease in SOD, CAT and GPx activities in dams and in their pups (Table III).

IV. DISCUSSION

High levels of arsenic impair gestational development as reported by epidemiological studies. Both inorganic and organic species cross the placenta in humans [9] and rodents [10]. Thus, *in utero* arsenic exposure may play an important role in the environmental arsenic induced disease.

In the present study, we have focused our attention on As_2O_3 potential causing alterations in liver function of adult mice and their pups during the suckling period for two main reasons. First, the developmental effects of exposure to relevant levels of As have scarcely been examined [26], most experimental studies of reproductive and developmental toxicity used high doses of As and examined obvious endpoints, such as mortality or major malformations. Second, in the perinatal period the fetus is vulnerable to changes caused by chemical compounds.

TABLE II
PLASMA AST, ALT AND LDH ACTIVITIES, BILIRUBIN AND ALP LEVELS IN ADULT MICE AND THEIR PUPS, CONTROLS AND As_2O_3 TREATED, FROM THE 14TH DAY OF PREGNANCY UNTIL DAY 14 AFTER DELIVERY

Plasma parameters and treatment	Mothers	Offspring
AST (IU/L)		
Controls	161.59 ± 13.6	154.81 ± 8.25
As_2O_3 group	350.11 ± 15.33 ***	232.82 ± 29.79 ***
ALT (IU/L)		
Controls	30.28 ± 2.33	17.51 ± 2.37
As_2O_3 group	79.03 ± 4.84 ***	41.45 ± 3.93 ***
LDH (IU/L)		
Controls	102.01 ± 8.75	88.09 ± 14.59
As_2O_3 group	150.28 ± 16.97 ***	164.07 ± 19.22 ***
Direct bilirubin (mg/dL)		
Controls	0.623 ± 0.07	0.558 ± 0.057
As_2O_3 group	0.853 ± 0.02 ***	0.828 ± 0.031***
ALP (IU/L)		
Controls	304.01 ± 13.82	912.01 ± 112.66
As_2O_3 group	541.91 ± 83.22 ***	1303.91 ± 105.49 ***

Values represent means ± SD (n = 6 for each group).
As_2O_3 treated vs controls: ***p<0.001.

TABLE III
LIVER MDA AND GSH LEVELS AND SOD, CAT AND GPX ACTIVITIES IN ADULT MICE AND THEIR PUPS, CONTROLS AND As_2O_3 TREATED, FROM THE 14TH DAY OF PREGNANCY UNTIL DAY 14 AFTER DELIVERY

Liver parameters and treatment	Mothers	Offspring
MDA [a]		
Controls	6.68 ± 1.46	13.59 ± 1.15
As_2O_3 group	13.11 ± 1.69 ***	18.42 ± 1.71 ***
GSH [b]		
Controls	87.08 ± 1.86	105.93 ± 4.62
As_2O_3 group	66.59 ± 7.77 ***	90.19 ± 0.52 ***
SOD [c]		
Controls	162,02 ± 2.45	120,51 ± 2.88
As_2O_3 group	63,62 ± 3.56 ***	55,11 ± 1.23 ***
CAT [d]		
Controls	44,25 ± 2.12	15,71 ± 1.98
As_2O_3 group	6,45 ± 1.09 ***	9,3 ± 2.02 **
GPx [e]		
Controls	7,84 ± 0.45	21,175 ± 1.63
As_2O_3 group	5,8 ± 0.21***	10,983 ± 0.67 ***

Values represent means ± SD (n = 6 for each group).
As_2O_3 treated vs controls: ** p<0.01; ***p<0.001.
[a]MDA= nmoles/g tissue
[b]GSH = µg/g tissue
[c]SOD = (U/mg protein)
[d]CAT = (µmoles H_2O_2 degraded/min/mg protein)
[e]GPx = (nmoles of GSH/min/mg protein)

As a very active site of metabolism and an organ of vital importance, the liver is especially sensitive to arsenic intoxication [27]. The current study examined As-induced changes of some biochemical parameters. However a significant decrease of total protein as well as of albumin levels in plasma was recorded. These results were in agreement with other finding in adult rats [28]. The decrease

in the protein concentration of As-treated mice might be due to changes in protein synthesis and/or metabolism [29]. The present investigation revealed also a marked decrease in blood glucose levels of mice. The marked decrease in blood glucose level after arsenic exposure may result from glycosuria caused by impairment of renal tubular reabsorption of glucose [30]. This finding is further supported by the same authors who reported that 24 h urinary excretion of glucose was increased markedly after arsenic intoxication [31]. The basic mechanism of trivalent arsenical toxicity lies in the fact that arsenite has a high affinity for sulfhydryl groups and reacts most avidly with vicinal mechanism thiols to hydroxyls. Consequently, it was suggested that the molecular mechanism of arsenic toxicity probably lies with the ability of arsenite to bind protein thiols [32].

By the virtue of this ability arsenite inhibits puruvate and α-ketoglutarate dehydrogenases and consequently depression of citric acid cycle and generation of NADH, NADPH and energy rich phosphates [33].

The information available about the effects of As_2O_3 on biochemical parameters, particularly on lipid metabolism is scanty. Indeed, As_2O_3 treatment causes changes in lipid profile; cholesterol and triglycerides decreased, suggesting that the abnormal activities of lipase enzymes seem to be one of the chief factors responsible for changes of lipid profile status.

Liver is the target organ of As toxicity, and the leakage of hepatic enzymes such as ALT and AST are commonly used as an indirect biochemical index of hepatocellular damage [27]. Transaminases (AST and ALT) are the most sensitive biomarkers directly implicated in the extent of cellular damage and toxicity because they are cytoplasmic in location and are released into the circulation after cellular damage [34]. In the present study, the activities of AST and ALT significantly increased in plasma of As_2O_3-treated mothers in and their pups. Thus might be due to the leakage of these enzymes from the liver cytosol into the blood stream and/or liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane resulting from hepatocyte membrane damage.

Another biochemical marker employed in this study to evaluate liver function is LDH activity, one of the key enzymes of glucose metabolism, in the liver of mice. LDH is an enzyme present in all human cells catalyzing the pH dependent interconversion of lactate into pyruvate. Changes in the serum LDH pattern have been employed for the detection of physiological changes. LDH levels in the As_2O_3-treated group increased in plasma. It may be attributed to a generalized increase in membrane permeability.

In addition, hepatotoxicity is evidenced in the present study by an increase of direct bilirubin level and ALP activities in the plasma of arsenic exposed mice. Our results were in agreement with previous findings of [35] who suggest that the increase in serum bilirubin is a clear marker of hepatic dysfunction. The increase in plasma bilirubin may also result from a decreased liver uptake, conjugation or an increased bilirubin production from hemolysis [36]. The increased level of bilirubin conjugates in plasma is indicative of an impaired hepatic clearance of conjugated bilirubin. It is also possible that the increase in bilirubin, a heme degradation product, could be explained by the induction of heme oxygenase (HO), the rate-limiting enzyme of heme catabolism, since it has been reported that arsenic induces HO activity in rodents associated with marked elevations in the biliary excretion of bilirubin [37]. The increase in serum ALP activity observed in As-exposed mice, compared with the control group, supported the idea that cholestasic injury was present in these animals. Our results were similar to those of [38] who have shown, in humans chronically exposed to arsenic via drinking water, predominantly conjugated hyperbilirubinemia and increased serum ALP activity, suggesting the presence of cholestasis.

Besides a number of mechanisms that have been proposed, oxidative stress is a relatively new theory for arsenic-induced toxic effects [39]. The measurement of lipid peroxidation by-products and the status of antioxidant enzymes like SOD, CAT and GPx are appropriate indirect ways to assess the prooxidant-antioxidant status in the tissues and the estimation of MDA, a by-product of lipid peroxidation, continues to be a reliable method to assess the degree of peroxidative damage to cell membrane. In the present investigation, lipid peroxidation was enhanced, while levels of GSH and activities of SOD, CAT and GPx were significantly decreased in the liver of adult mice and their pups after treatment with arsenic. Recent studies have clearly demonstrated that arsenic compounds during their metabolism in cells generate reactive oxygen species like superoxide anion, hydroxyl radical and hydrogen peroxide leading to oxidative stress [40]. Enhanced production of free radicals and inhibition of antioxidant enzymes have been suggested as possible mechanisms to explain arsenic induced oxidative damage [40].

Inorganic arsenic increases the rate of active oxygen species formation, including superoxide anion radicals, hydroxyl radical through a chain reaction [41]. The mechanism of arsenic toxicity to individual cell type has historically centered around the inhibitory effects on cellular respiration at the level of mitochondria. Disruption of oxidative phosphorylation and concomitant decrease in the cellular levels of ATP are thought to be the important central events of arsenic-induced toxicity evoking increased production of hydrogen peroxide. These effects could cause formation of reactive oxygen species resulting in oxidative stress.

A remarkable decrease in the levels of GSH has been observed in liver of arsenic exposed dams and their pups. Thiols are thought to play a crucial role in protecting cells against reactive oxygen species. Trivalent arsenicals react in vitro with thiol containing molecules such as GSH, cysteine or hemoglobin forming As-SH complex or (GS)3 As_{III} and this property is considered to be the mechanism by which arsenic exerts its toxic effects [42]. Our results were in accordance with previous studies of Maiti and Chatterjee who have found after acute administration of arsenic to rats, a significant reduction in hepatic GSH level [43].

GSH depletion resulted in the accumulation of free radicals that initiated lipid peroxidation resulting in biochemical

damage by covalent binding to macromolecules. Arsenic-generated ROS also attack SH groups leading to their oxidation, thus damaging proteins and enzymes requiring SH groups [44]. Antioxidant enzymes are considered to be the primary defense that prevents biological macromolecules from oxidative damage. SOD, catalase and GPx are the most important enzymes against toxic effects of oxygen metabolism [45].

SOD accelerates the dismutation of superoxide (O_2^-) to H_2O_2, which can be considered as a primary defense and it prevents further generation of free radicals. The decrease in the levels of SOD activity in arsenic treated rats could be due to the accumulation of superoxide anion radical during arsenic metabolism [41]. Catalase catalyzes the removal of H_2O_2 formed during the reaction catalyzed by SOD. A decrease in the activity of SOD could be attributed to an enhanced superoxide production during arsenic metabolism. The superoxide radical also inhibits the activity of catalase [46].

In the present study a decrease of catalase activity in arsenic treated mice may be explained by the insufficient supply of NADPH, which is required for the activation of catalase for its regeneration from its inactive form [47]. Therefore, it is possible that paucity of NADPH production during arsenic exposure could decrease the catalase activity. The decline of this enzyme, in arsenic exposed mice, may be the causal for the increased accretion of hydrogen peroxide thereby leading to the increased LPO. GPx reduces lipid hydroperoxides into lipid alcohols in the presence of GSH. The decreased level of GSH and the enhanced level of lipid peroxidation may cause a depletion of GPx activity during arsenic exposure. Moreover, a decrease in the activity of GPx may be due to a decrease of selenium which acts as a co-factor of this enzyme [48].

V. Conclusion

Large Overall, data indicate, that a high concentration of As_2O_3 in drinking water, ingested by pregnant and lactating mice, induced an oxidative stress in hepatic tissue of both dams and their suckling pups, leading to lipid peroxidation. Balance between oxidative and antioxidant systems was disturbed in As_2O_3-treated mice. High As_2O_3 intake by lactating mice appeared to have pronounced toxic effects in their suckling pups. These evidences, presented in our experimental data, show for the first time the occurrence of hepatotoxicity after exposure mice to As_2O_3 during pregnant and lactating periods, make clear the need for attention of pregnant women exposed to As_2O_3.

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