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Sodium Arsenite in Drinking Water is a Continuous Threat to Maturing Reproductive System: a Study in Prepubertal Male Mice

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Abstract

Human exposure to arsenic in countries known for heavy arsenic load, where ground water arsenic level exceeds the WHO limit, can be detrimental for developing gonads. Developmental and reproductive toxicity of arsenic in rodents after in utero exposure is although known but information on the toxic effects of arsenic on postnatal gonadal development is scant. This aspect was the focus of the present investigation. Postnatal day 25 prepubertal male mice were challenged with sub-chronic and chronic (up to PND 53 & PND 114 respectively) oral exposure (drinking water) to low and high doses (0.01, 5, and 10 $mg.L^{-1}$) of sodium arsenite. Data were compared statistically at $P<0.05$. Sub-chronic exposure to high arsenic doses led to significant increase in the production of reactive oxygen species and lipid peroxidation, while simultaneous significant reduction occurred in the activity of antioxidant enzymes catalase, superoxide dismutase and peroxidase, hormone concentrations (follicle-stimulating hormone, luteinizing hormone, testosterone) and sperm parameters. Testicular cell damage and sperm DNA damage, as revealed by comet assay, were evident at 10 $mg.L^{-1}$arsenic dose. Chronic exposure further aggravated the adverse effects. Severe testicular oxidative stress, reduction in hormones and pronounced histological alterations in testis and epididymis, and sperm parameters together with excessive sperm DNA damage were noticeable even at the lowest test dose of 0.01 $mg.L^{-1}$. The study concludes that arsenic exposure considerably affects the prepubertal gonad, may cause irreversible damage to the developing reproductive system of males and that minute quantity of arsenic in drinking water can be a serious health hazard.

Keywords: Sodium arsenite; Prepubertal mice; Reproductive toxicity; Reproductive hormones; Oxidative stress; Sperm parameters; Genotoxicity

Introduction

The heavy metal arsenic, which has a ubiquitous occurrence in soil, water, and atmosphere, is considered a major toxic metal element due to its carcinogenic, mutagenic, and teratogenic properties [1, 2]. Although 90% of arsenic poisoning is believed to be geogenic, anthropogenic sources like use of pesticides and dumping of tons of quantities of industrial waste have considerably increased its occurrence in the environment [3]. Contamination of natural water reservoirs with the arsenic in West Bengal, China, India, Bangladesh, Pakistan, Afghanistan, Mongolia, Myanmar, Cambodia, DPR Korea, and Nepal is of special concern [1,4,5]. In these countries, arsenic concentration in the drinking water has been found to be well above the permissible limit of 10 ppb or 10$\mu g.L^{-1}$ as outlined by the WHO [6,7]. It has

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been estimated that more than 230 million people worldwide, which include 180 million from Asia, are at risk of arsenic poisoning [8].

In humans, chronic arsenic exposure is known to cause numerous health related problems. For instance, lung cancer, skin cancer, bladder cancer, coronary artery disease, hyperkeratosis, peripheral vascular disease, melanosis, gangrene, and lung diseases are associated with arsenic exposure [9, 10]. Arsenic poisoning has also been linked with infant mortality, impaired intellect and motor dysfunction in children [10, 11]. Depending on the route [12], arsenic adversely affects the male reproduction [13,14]. Its exposure leads to a decrease in the testes and accessory sex organs weight and reduction in testosterone concentrations. This eventually causes spermatotoxicity and general reproductive toxicity in experimental animals [15-17]. Studies on animal models have suggested that arsenic induces necrosis, apoptosis and, through diminished testosterone production, may cause permanent gonadal dysfunction [18-20].

At tissue and cell level, arsenic induces malformations in the male reproductive system through increased production of reactive oxygen species (ROS) [21]. Developmental toxicity caused upon exposure to arsenic includes retardation of fetal growth, gross tissue abnormality and mortality [22]. Numerous studies have reported in vivo toxic outcomes of arsenic on the adult male rat reproductive system [19, 23-25].

However, pre- and post-natal developmental time periods are even more critical, since exposure to a toxicant metal element, chemical agent or a drug in the early days of gonadal maturation can lead to adverse reproductive health effects immediately or in later life. In this context, prenatal exposure of mice to high arsenic concentrations is known to produce cell damaging effects [16, 26,27]. For instance, a low dose of 50 ppb arsenic was shown to induce learning impairments [28] and liver dysfunctions in mice [29]. Adverse outcomes of low doses of arsenic on pre-and post-natal development of mice have been reported [30]. Although it is known that sexual maturation in prepubertal female rats is delayed upon exposure to arsenic [31], similar data on prepubertal male mice are lacking.

The present study therefore aimed to investigate the impact of arsenic exposure on postnatal male mouse reproductive system following exposure to sub-chronic and chronic oral doses of $NaAsO_2$ beginning from PND 25 to PND 53 and PND 25 to PND 114 respectively.

Material and Methods

Animals and maintenance

Eighty Swiss albino male mice (age 21-25 days old) were obtained from the National Institute of Health Islamabad and maintained in the Animal House Facility of Quaid-i-Azam University, Islamabad. Before experiments, mice were kept in rodent cages under standard laboratory conditions: room temperature (24 ± 1 °C), photoperiod (14:10 light:dark hours) and 40% relative humidity. Mice were maintained on standard rodent feed and had free access to drinking water *ad libitum*. Animal handling and the experimental design were approved by the "Bioethical committee of the Faculty of Biological Sciences", Quaid-i-Azam University, Islamabad. Animal handling also strictly complied with the European Union Guidelines on Animals and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and further guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

The mice were divided into four groups, each containing ten mice. $NaAsO_2$ was administered orally in drinking water at 0.01, 5.0, and 10.0 $mg.L^{-1}$concentrations for 28 days (PND 25 to PND 53, sub-chronic) and 90 days (PND 25 to PND 114, chronic).

Procurement of blood plasma and tissue samples

On PND 54 and PND 115, animals were weighed, and venous blood was collected immediately from the heart and centrifuged at 926 g for 10 min to prepare plasma samples. Plasma was stored at −20 °C for determination of hormone concentrations. Mice were then quickly decapitated, and following the dissection, reproductive organs were weighed and separated out. One testis and epididymis of each mouse were rinsed with saline and stored at −20 °C for biochemical analyses. For sperm collection, cauda epididymis tissue was sliced into pieces in 5 ml Human Tubal Fluid (HTF) medium (Merck, UK) and sperms were allowed to swim out in petri dish. These were left for 30 min in an incubator at 37 °C with 5% CO_2 in air. For histology, the other testis and epididymis of each animal were quickly rinsed with saline and fixed in 10% formalin.

Hormone determination

Determination of gonadotropins (LH and FSH)

Luteinizing hormone (LH): Plasma LH was quantitatively determined through competitive enzyme linked immunosorbent assay (ELISA) using a commercial kit (Mybiosource, USA) following the manufacturer's procedure provided for this bioassay. Fifty microliters of standard, blank, and samples were added into wells coated with a biotinylated LH antibody. To each coated well, 50 µl of reagent A was added. The plate was then slightly shaken to clear the cloudiness, then covered and incubated for 60 min. Washing was done three times with 350 µl of washing solution. Wells were decanted and allowed to dry. Reagent B (100 µl) was then added to each well and incubation was carried out for 60 min. After 1 h, wells were washed five times with the washing solution, 90 µl of horseradish peroxidase (HRP) enzyme was then added to each well and incubated

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again for 15-25 min at 37 °C until blue color developed. Fifty microliters of stop solution were then added into each well and mixed thoroughly till blue color turned into yellow. Absorbance was recorded at 450 nm in triplicate within 1 min on an ELISA plate reader (Via Medical Pro reader-96, Germany). The detection range of the kit was 50 mIU/mL to 0.78 mIU/mL, while the lowest detection limit was 0.08 mIU/mL. Inter- and intra-assay coefficient of variations were 8.0% and 12.0% respectively.

Follicle stimulating hormone (FSH): Plasma FSH was quantitatively measured using a commercial kit and following the manufacturer's protocol (Mybiosource, USA). To each of the wells, 100 µL of standard and samples were added. Wells were incubated for 90 min at 37 °C. Subsequently, washing was done twice and 100 µl of biotinylated FSH antibody was added and samples were incubated for 1 h at 37 °C. Washing of the plate was done three times. Leaving out the blank wells, 100 µl of enzyme conjugate was added and incubated for 30 min. After 30 min, five additional washes were given, followed by addition of 100 µl of color reagent and incubation was carried out at 37 °C in dark. After appearance of dark color, 100 µl of color reagent C was added to stop the reaction. The contents were mixed thoroughly, and absorbance was recorded at 450 nm using an ELISA plate reader as above. Three readings were taken within 10 min. Detection range of the kit was 0.25 ng/mL to 50 ng/mL, sensitivity was 0.125 ng/mL, while the intra- and intra assay coefficient of variations were <15%. Concentration is given in mIU/mL.

Testicular homogenate preparation

Testes were homogenized in 1 mL phosphate buffered saline (PBS) and centrifuged at 926 *g* for 15 min. Supernatant was collected for antioxidant bioassays.

Plasma and testicular testosterone

Intra-testicular and blood plasma testosterone: Testosterone concentrations were determined in whole blood plasma and testicular homogenates through quantitative ELISA as mentioned above following the method provided by the manufacturer (Amgenix, USA). Determination of testosterone was based on competitive binding of sample testosterone and testosterone conjugated enzyme (Horseradish peroxidase), whereby, 50 µL of control, standard, and testicular homogenate or blood plasma were added into streptavidin coated wells. To each of the wells, 100 µL of testosterone-enzyme conjugate reagent and 50 µL of biotin reagent were then added. After gently mixing for 30 sec, the ELISA plate was incubated for 1 h at room temperature. Wells were washed three times after incubation with the washing buffer for the removal of any unbound enzyme conjugate. Following this, TMB substrate reagent (100 µL) was added to the wells and another incubation was done for 30 min. Stop solution (50 µL) was added to each of each the wells and mixed for 20 sec. Absorbance was read 450 nm on an ELISA plate reader as above. Sensitivity of the kit was 0.05ng/mL, while the inter- and intraassay coefficient of variations were 5.2% and 7.4%. respectively.

Oxidative parameters

Reactive oxygen species (ROS): ROS production in the testicular homogenates was determined according to [32]. One milligram of N.N-Diethyl para phenylene diamine sulfate (DEPPD) was dissolved in 10 ml distilled water for the preparation of reagent 1. $FeSO_4$ stock solution contained 50 mg ferrous sulfate dissolved in 10 ml sodium acetate buffer (pH 4.8). Fifty microliters of $FeSO_4$ stock solution were mixed with 100 ml of sodium acetate buffer to prepare reagent 2. Both reagents were mixed in a 1:25 ratio and placed in dark for 2 min. Sodium acetate buffer (1200 µl), above mixed reagents (1680 µl), and 60 µl of testis homogenate were mixed in a cuvette. Absorbance was read on UV-Visible Spectrophotometer (Agilent 8453, USA) at 505 nm. For each sample, three readings were taken at an interval of 15 sec intervals and then averaged.

Thiobarbituric acid reactive substance (TBARS) assay: TBARS is an indirect measurement of oxidative stress produced due to lipid peroxidation, whereby malondialdehyde formation in testicular homogenates through its reaction with thiobarbituric acid (TBA) was determined according to the method as described [33]. The reaction mixture was prepared in a test tube by adding 0.1 mL of 1.5 mM ascorbic acid, 0.1 mL of 50 mM Tris -HCL, 0.1 mL of 1mM $FeSO_4$, 0.6 mL of distilled water, and 0.1mL of testis homogenate. After vigorous mixing, the contents were incubated for 15 min at 37 °C. After adding 1 mL of thiobarbituric acid and tricholoroacetic acid (0.375% and 10% respectively), the contents were thoroughly mixed and boiled in a water bath for 15 min at 100 °C and centrifuged at 2500 rpm for 10 min. Absorbance of the supernatant was recorded at 535 nm. For each sample, three readings were quickly taken and then averaged.

Antioxidant enzymes

Catalase (CAT) assay: CAT activity in the samples was determined as described [34] with some modifications. Reaction mixture was prepared by adding 1.99 ml of potassium phosphate buffer (pH 7.0), 1000 µl H_2O_2 (5.9 mM), and 100 µl testicular homogenate. Three absorbance readings were recorded after 1min interval at 240 nm. One unit CAT activity was 0.01 absorbance change unit/min.

Superoxide dismutase (SOD) assay: The enzyme activity was determined according to the method as described [35]. Briefly, reaction mixture was prepared by adding 1.5 mL of L-Mehionine, 0.75 of Triton X-100, and 1mL of nitroblue tetrazolium (NBT). The final volume was brought to 30 ml by adding 50 mM PBS (pH 7.8). One milliliter of this mixture was added to test tubes and 20 µl of sample was added into each one of them. Sample tubes were illuminated with a

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fluorescent lamp for 7 min and the contents were incubated at 37 °C for 5 min. For initiation of reaction, 10 µl of riboflavin were added and the contents were incubated again at 40 °C for 8 min. Absorbance was recorded in triplicate during 1 min at 560 nm.

Peroxidase (POD) assay: Activity of POD in the testis homogenate was determined according to the method as described [34]. The reaction mixture was prepared by mixing 75 µl hydrogen peroxide (40 mM), 625 µl of 50 mM potassium phosphate buffer (50 mM pH 5.0), 25 µl guaiacol (20 mM), and 25 µl of testis homogenate. Absorbance was recorded spectrophotometrically after 1 min at 470 nm. One unit of POD activity was reflected as 0.01absorbance change as unit/min.

Reduced glutathione (GSH) assay: Non-enzymatic reduced glutathione was determined in testicular homogenates according to a previous method [36]. The reaction mixture was prepared by adding 1ml of sodium phosphate buffer (0.4 M), 0.5 ml DTNB, and 0.1 mL of sample in a cuvette. After the development of yellow color, the absorbance was taken at 412 nm. Values were recorded in triplicate and then averaged.

Sperm parameters

Epididymal sperm count: For sperm count, 10 µl of sperm mixture was mixed with 190 µl of distilled water to prepare 1:20 dilution. Ten microliters were then taken and suspended into an improved Neubauer's chamber. Sperms were counted under a photomicroscope (BH2 Nikon, Japan) at 40x magnification as described by [37].

Sperm motility: For the evaluation of sperm motility, a preheated slide was taken and 10 µl of sperm suspension were placed and covered with a cover slip. Epididymal sperm motility was evaluated at 200x magnification.

Sperm viability: Sperm viability was assessed according to a previous protocol [38]. Sperm suspension (20 µl) was combined with the same volume of Eosin-nigrosin stain (0.05%). Following 2 min of incubation, the slides were observed at 40x magnification on a light microscope. Alive sperms carried no color while the dead sperms in the suspension turned pink. Percentage of sperm viability was counted after observing at least 200 sperms in each sample.

Sperm DNA damage: Comet assay was carried out on mice epididymal sperm suspension [39]. Glass microscopic slides were coated with 1% normal melting agarose, covered, and left to solidify at 4 °C for 20 min. Covers were removed gently, and 85 mL of low-melting agarose was spread over the first layer containing 20 µl of sperm suspension and 65 µl of 1% low-melting agarose. After solidification, cell lysis was allowed to complete by plunging slides into freshly prepared chilled buffer and keeping there for 2 h. Electrophoresis was performed at 24 V for 30 min. Slides were stained with 80 µl ethidium bromide and observed on a fluorescent microscope at 40 x magnification (Nikon AFX-1 Optiphot, Japan). The Casplab software was used for scoring comets (version 1,2.3b2, Poland). Around 50 to 100 cells were observed in each sample. Percent tail and head DNA (%) and tail length of (µm) sperm DNA comet were determined.

Histology and light microscopy

Standard procedure of Hematoxylin and Eosin (H&E) was followed for histology of testes and epididymis. Prepared slides were observed using a photomicroscope (Leica, Germany). Images were captured using a digital camera (Canon, Japan) attached to the microscope. Image analysis was done on Image J software (Ver. 1.53t Microsoft Inc. USA). Area was measured in μm^2 for seminiferous tubules, interstitial space, and epididymal tubules. The percent area was then calculated by using the following formula: %A(st)= (A (st)×100)/T

While, A(st) is the area of seminiferous tubules, and T is the total area of the section.

Different types of cells (spermatogonia, spermatocytes, and spermatids) were counted by observing 50 seminiferous tubules for each animal and average values per seminiferous tubule were calculated.

Statistical analyses

Data are shown as mean ± S.E. Mean values of treatment groups were compared with the control values through one-way ANOVA followed by Dunnet's post-hoc test using the GraphPad Prism (5.01, CA USA). Probability was $P < 0.01$.

Results

Body and testis weight

In the sub-chronic exposure to $NaAsO_2$, no statistically significant difference was noticeable at any dose in the body weight or testicular weight between control and treated groups of mice on PND 54 (Table 1). In contrast, chronic exposure to $NaAsO_2$ had caused significant reductions ($P<0.001$) in the body weight and as well testis weight at all doses when determined on PND 114 (Table 1).

Hormone concentrations

Sub-chronic exposure to $NaAsO_2$

Table 2 represents hormonal changes. In the sub-chronic $NaAsO_2$ exposure, plasma LH and FSH concentrations were significantly reduced at 5.0 ($P<0.01$) and 10.0 $mg.L^{-1}$ ($P<0.001$) doses of $NaAsO_2$. Low dose $NaAsO_2$ (0.01 $mg.L^{-1}$) treatment did not cause any change in plasma LH or FSH concentrations. Similarly, significant reduction ($P<0.001$) occurred in both the plasma testosterone and intratesticular testosterone concentrations at 5 and 10 $mg.L^{-1}$doses of $NaAsO_2$, while low dose of 0.01 $mg.L^{-1}$did not cause any statistically significant difference when compared with the control mice (Table 2).

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Chronic exposure to $NaAsO_2$

In the chronic set up, $NaAsO_2$ treatment led to significantly reduction (P<0.001) in plasma LH and FSH concentrations (Table 2). Similarly, both the plasma testosterone and intra-testicular testosterone concentrations were reduced in all treatment groups as compared to control animals (Table 2). Treatment with 5 and 10 $mg.L^{-1}$ $NaAsO_2$ led to a greater reduction (P<0.001) in testosterone concentration as compared to 0.01 $mg.L^{-1}$ dose (P<0.01).

Testicular biochemical parameters

Sub-chronic exposure to $NaAsO_2$

Following the sub-chronic arsenic exposure, ROS and TBARS increased significantly (P<0.001) at 5 and 10 $mg.L^{-1}$ $NaAsO_2$ doses, while significant reduction occurred in the levels of antioxidant enzymes, the CAT, SOD and POD. Similar trend was noticeable in GSH concentrations (Table 3). Sight increases (P<0.05) in ROS levels and decrease (P<0.01) in CAT, SOD and POD were also noticeable at 0.01 $mg.L^{-1}$concentration of $NaAsO_2$ (Table 3).

Table1: Body weight and testis weight of postnatal prepubertal mice following sub-chronic and chronic exposure to different doses of $NaAsO_2$. Values are expressed as mean ± S.E.

	Sub-chronic exposure		Chronic exposure	
Treatments	Body weight (g)	Testis weight (mg)	Body weight (g)	Testis weight (mg)
Control	27.5±0.44	98.7±1.33	37.7±0.32	100.7±0.60
0.01 $mg.L^{-1}$	26.1±0.56	97.4±0.76	34.2±0.40**	96.4±0.90**
5 $mg.L^{-1}$	26.5±0.51	97.1±0.62	33.5± 0.90**	93.0±0.70***
10 $mg.L^{-1}$	26.2±0.83	96.2±0.70	32.3±0.90***	91.2± 1.00***

, * show significant difference at P < 0.01 and P < 0.001 respectively as compared to control

Table 2: Hormone concentrations in postnatal prepubertal mice following exposure to $NaAsO_2$ in drinking water. Data are expressed as mean ± S.E

Groups	LH (mIU/mL)	FSH (mIU/mL)	Plasma Testosterone (ng/ml)	Intratesticular Testosterone (ng/g)
Sub-Chronic				
Control	2.89±0.14	2.80±0.07	54.96±1.48	3.33±0.10
0.01 mg. L^{-1}	2.76±0.13	2.68±0.06	52.92±0.80	3.10±0.06
5.0 mg. L^{-1}	2.20±0.07**	2.52±0.02**	49.17±0.94**	2.86±0.05**
10.0 mg. L^{-1}	2.33±0.11**	2.37±0.07***	47.15±0.79***	2.76±0.10***
Chronic				
Control	3.08±0.03	2.85±0.05	55.2±1.15	3.33±0.13
0.01 mg. L^{-1}	2.72±0.09**	2.53±0.07**	48.6±0.57***	2.92±0.07**
5.0 mg. L^{-1}	2.35±0.07***	2.19±0.06***	45.93±0.69***	2.39±0.10***
10.0 mg. L^{-1}	2.47±0.02***	1.90±0.08***	43.77±1.00***	2.49±0.09***

Table3: Oxidative stress parameters and levels of antioxidant enzymes in testicular homogenates of postnatal prepubertal mice after sub-chronic and chronic oral exposure to $NaAsO_2$ in drinking water. Values are mean ± S.E.

Groups	ROS (Absorbance)	TBARS (nM.min/mg protein) (unit/min)	CAT (unit/mg)	SOD (unit/min)	POD (µM/g)	GSH (µM/g)
Sub-Chronic	(PND 25 – 53)					
Control	3.27±0.25	1.40±0.13	17.7±0.70	80.8±1.33	15.8±0.42	18.6±0.66
0.01 $mg.L^{-1}$	4.00±0.18*	1.87±0.16*	14.8±0.49**	73.5±1.28**	13.5±0.56*	17.9±0.60
5.0 $mg.L^{-1}$	4.16±0.11**	2.72±0.17***	14.6±0.47**	48.7±2.07***	10.6±0.53***	14.3±0.73**
10.0 $mg.L^{-1}$	4.5±0.12***	2.99±0.02***	13.7±0.57***	45.0±0.66***	8.4±0.60***	14.4±1.03**
Chronic	(PND 25 – 114)					
Control	2.97±0.21	1.13±0.02	18.5±0.52	83.8±1.2	15.5±0.6	19.2±0.5
0.01 $mg.L^{-1}$	4.11±0.18***	2.40±0.11***	12.3±9.9***	38.7±1.98**	9.9±0.7***	13.8±0.6**
5.0 $mg.L^{-1}$	4.48±0.12***	3.62±0.21***	11.0±7.1***	34.1±0.98***	7.1±0.2***	11.1±0.4**
10.0 $mg.L^{-1}$	5.07±0.21***	4.21±0.17***	8.2±0.55***	31.2±1.0***	6.4±0.5***	9.91±0.6**

*, **, *** indicate significant difference at P < 0.05, P < 0.01 and P < 0.001 compared to control.

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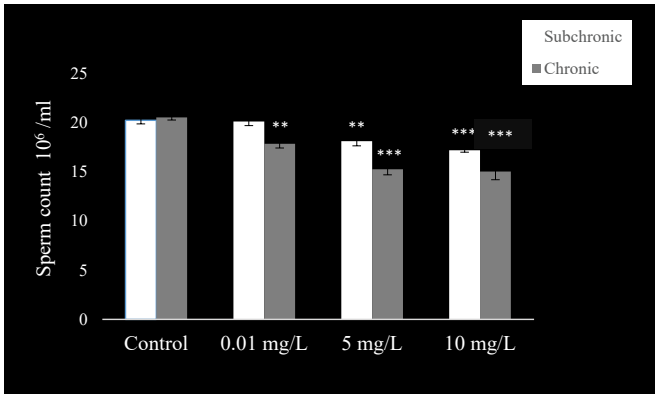

Figure 1: Sperm count decreased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

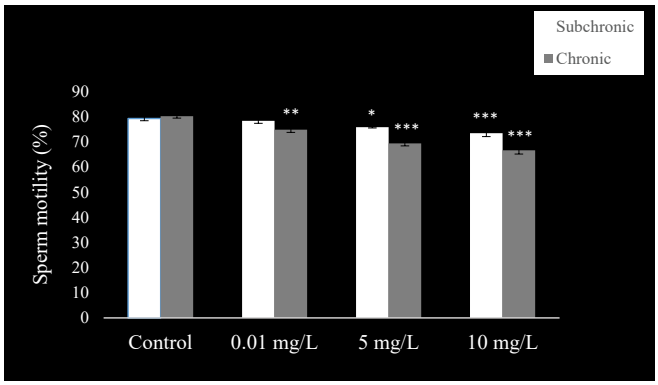

Figure 2: Sperm motility decreased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

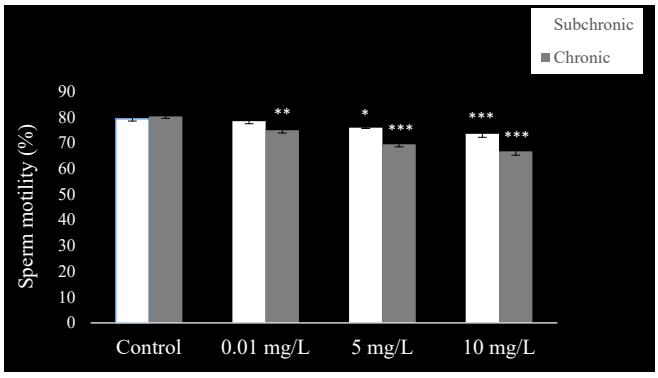

Figure 3: Sperm viability decreased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

Chronic exposure to $NaAsO_2$

In the chronic exposure to $NaAsO_2$, ROS and TBARS increased significantly (P<0.001) at all doses of $NaAsO_2$. Levels of antioxidant enzymes CAT, SOD, POD and non-enzymatic GSH were, in contrast, decreased significantly (P<0.001) at all $NaAsO_2$ doses (Table 3).

Sperm parameters

Sub-chronic exposure to $NaAsO_2$

Sperm count (Fig. 1), motility (Fig. 2), and viability (Fig. 3) were reduced (P<0.001) at 5 and 10 mg.L^{-1} doses of $NaAsO_2$ when compared with the control group of mice. Low dose (0.01 mg.L^{-1}) treatment with $NaAsO_2$ did not induce any alteration in these parameters.

Chronic exposure to $NaAsO_2$

Compared to control mice, significant decline (P<0.001) was observed in sperm count (Fig. 1) with all doses of $NaAsO_2$. Sperm motility (Fig. 2) decreased significantly in all treatment groups (P<0.001), whereas sperm viability (Fig. 3) also decreased significantly (P<0.001) in the 5 and 10 mg.L^{-1}treatment groups as compared to the control group.

Histology and morphometry of testes

Sub-chronic $NaAsO_2$ exposure

Figure 4 shows the testicular morphology following sub-chronic $NaAsO_2$ exposure. In the control group, seminiferous tubules were compactly arranged. Type A and type spermatogonia, spermatids and spermatozoa were readily visible. The tubular lumen contained many spermatozoa while germinal epithelium was normal and thick. Leydig cells showed a normal appearance (Fig. 4A). In the 0.01 mg.L^{-1} $NaAsO_2$ treatment group, slight degeneration of germinal epithelium was noticeable. Cells showed fragmentation and detachment from the germinal epithelium and gathered in the lumen (Fig. 4B). At higher doses of 5 and 10 mg.$L^{-1}$$NaAsO_2$, greater cell damage was noticeable in the germinal epithelium. Cells showed degeneration, detachment from

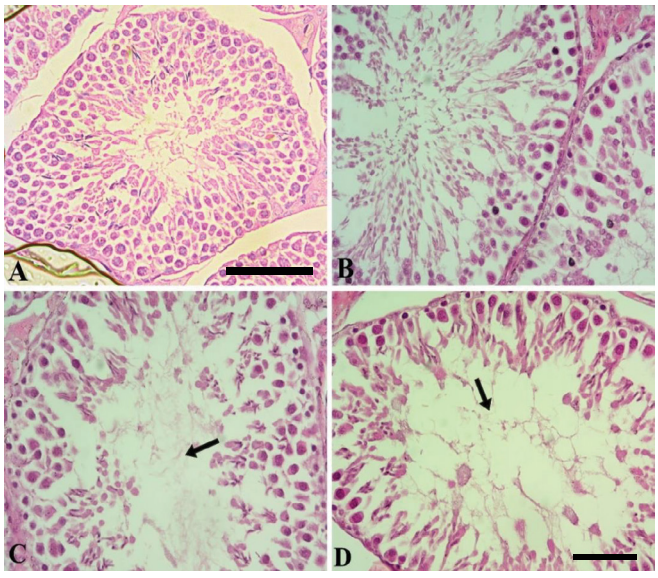

Figure 4: Photomicrograph of postnatal prepubertal mice testes after sub-chronic (PND 25 to PND 54) exposure to $NaAsO_2$ in drinking water. (A) control testis (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Arrows show degenerated spermatozoa. Scale bar= 20 µm.

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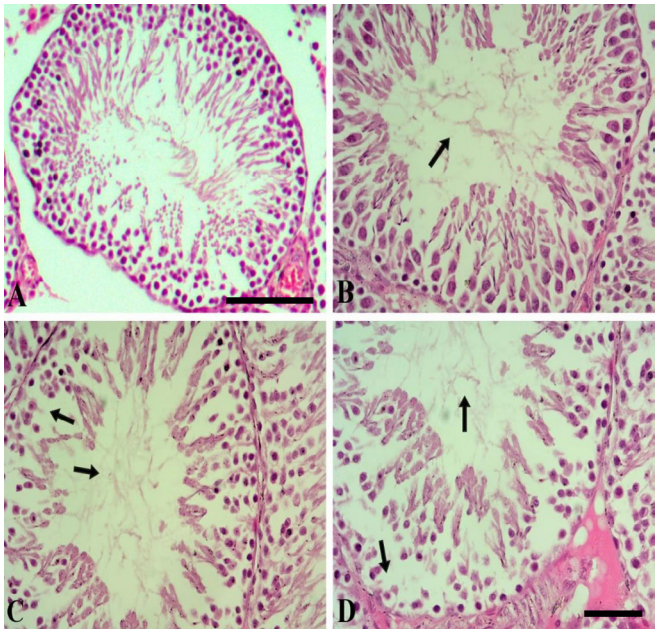

Figure 5: Photomicrograph of postnatal prepubertal mouse testes after chronic (PND 25 to PND 115) exposure to $NaAsO_2$ in drinking water. (A) control testis (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Arrows show degenerated germinal epithelia and degenerated spermatozoa. Scale bar=20 µm.

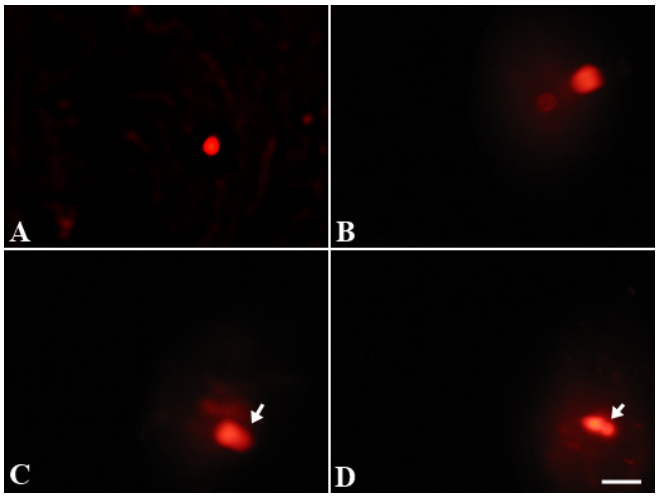

Figure 6: Sperm DNA damage (A) in postnatal prepubertal mice upon sub-chronic (PND 25 to PND 54) exposure to $NaAsO_2$ in drinking water. Control (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L. Arrows show increased tail length with 10 mg/L $NaAsO_2$ treatment. Scale bar=20 µm.

the germinal epithelium, degeneration of type A and Type B spermatogonia and loss of spermatocyte population. Loss of spermatids and spermatozoa were also evident (Fig. 4C & D respectively). Leydig cells also showed degeneration.

Table 4 represents morphometry of mice testis following the sub-chronic and chronic exposure to $NaAsO_2$. At low dose, area of seminiferous tubules and interstitial space although demonstrated statistically non-significant alterations but these were noticeable. The diameter of seminiferous tubules however remained unaffected. Epithelial cell height reduced significantly at 5 $mg.L^{-1}$ ($P<0.01$) and 10 mg.L-^{1} ($P<0.001$) doses of $NaAsO_2$. Diameter of seminiferous tubular lumen showed significant increase ($P<0.01$) only with 10 $mg.L^{-1}$ treatment. Number of spermatocytes decreased significantly in the testes at 10 $mg.L^{-1}NaAsO_2$ dose ($P<0.01$), while spermatid numbers also decreased ($P<0.001$) in the testis at highest test dose of $NaAsO_2$ (10 $mg.L^{-1}$).

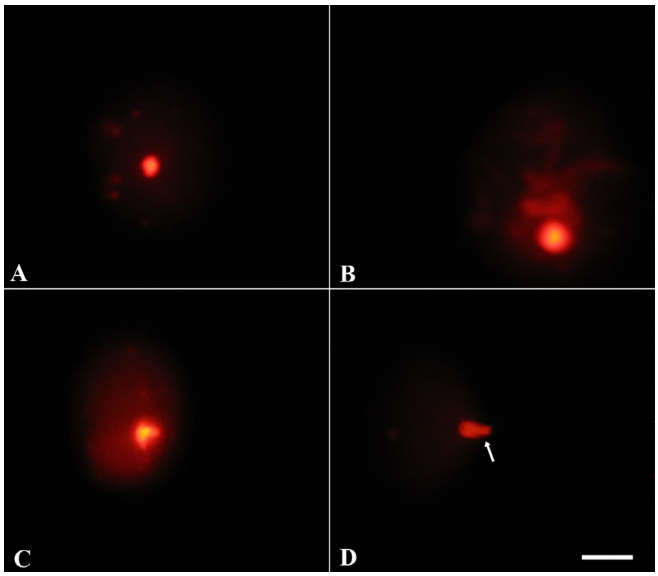

Figure 7: Sperm DNA damage (A) in postnatal prepubertal mice upon chronic (PND 25 to PND 115) exposure to $NaAsO_2$ in drinking water. (A) Control (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L. Arrow shows increased tail length after 5 and 10 mg/L $NaAsO_2$ treatments. Scale bar= 20 µm.

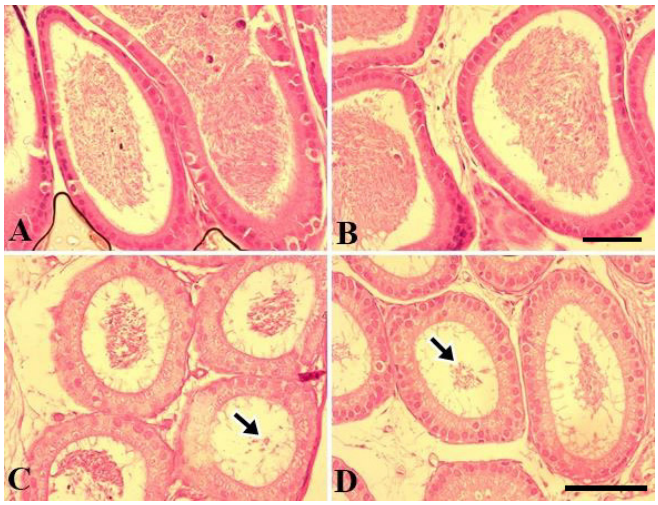

Figure 8: Photomicrographs of postnatal prepubertal mouse epididymal tissue sub-chronic (PND 25 to PND 54) exposure to $NaAsO_2$ in drinking water. (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Arrows show disrupted epithelia and reduction in spermatozoa. Scale bar=20 µm.

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Table 4: Morphometric parameters of postnatal prepubertal mice testes show significant alterations after sub-chronic and chronic oral exposure to $NaAsO_2$ in drinking water. Values are mean ± S.E.

Groups	Area of Seminiferous tubules (%)	Area of Interstitium (%)	Seminiferous Tubule Diameter (µm)	Epithelial Height (µm)	Lumen Diameter (µm)
Sub-Chronic					
Control	89.76±1.56	10.23±1.56	201.78±1.85	56.91±0.92	31.57±0.76
0.01 mg. L^{-1}	89.69±0.95	10.30±0.95	199.45±0.95	56.57±0.82	31.38±0.83
5.0 mg. L^{-1}	88.44±2.75	11.55±2.75	197.78±0.65	53.62±0.57*	33.23±0.67
10.0 mg. L^{-1}	85.14±2.04	14.85±2.04	197.87±0.86	51.83±0.80***	34.64±0.80*
Chronic					
Control	88.66±1.03	11.33±1.03	201.6±1.62	59.19±0.96	29.93±0.32
0.01 mg. L^{-1}	84.97±2.06	15.02±2.06	190.1±1.25***	45.45±1.84***	36.55±0.56***
5.0 mg. L^{-1}	79.48±2.53*	20.51±2.53*	185.9±1.89***	40.73±1.42***	40.00±0.52***
10.0 mg. L^{-1}	76.26±3.49**	23.7±3.49**	183.6±1.29***	39.08±1.26***	40.56±0.54***

*, **, *** indicate significant difference at P < 0.05, P < 0.01 and P < 0.001 respectively compared to control

Table 5: Sperm DNA damage in postnatal prepubertal mice after sub-chronic and chronic oral exposure to $NaAsO_2$ in drinking water. Values are mean ± S.E.

Groups	% Tail DNA	Tail length (µm)	% Head DNA
Sub-Chronic			
Control	5.14 ± 2.29	3.26 ± 0.37	94.84 ± 2.29
0.01 mg. L^{-1}	7.06 ± 0.74	3.66 ± 0.33	92.92 ± 0.75
5.0 mg. L^{-1}	8.70 ± 1.72	4.30 ± 1.35	91.2 ± 1.72
10.0 mg. L^{-1}	15.6 ± 2.37*	10.6 ± 1.67*	84.36 ± 2.38*
Chronic			
Control	6.79 ± 0.95	3.06 ± 0.06	93.1 ± 0.96
0.01 mg. L^{-1}	16.10 ± 2.75	9.66 ± 2.66	83.8 ± 2.75
5.0 mg. L^{-1}	41.27 ± 6.24**	19.0 ± 3.21*	58.8 ± 6.25**
10.0 mg. L^{-1}	48.01 ± 8.8**	20.6 ± 3.93**	51.98 ± 8.8**

*, ** indicate significant difference at P < 0.01, P<0.001 and P < 0.001 respectively compared to control.

Chronic exposure to $NaAsO_2$

Figure 5 shows testicular morphology in the chronic exposure to of mice to $NaAsO_2$. Control testes showed compact seminiferous tubules, normal germinal epithelium, compact presence of type A and type B spermatogonia and spermatocytes. Lumen contained spermatozoa, while the Leydig cells showed normal appearance (Fig. 5A). Conversely, at 0.01 mg.L^{-1}dose of $NaAsO_2$, although the germinal epithelium was near normal, the luminal space increased and a reduction in spermatozoa was readily noticeable (Fig. 5B). At high dose concentrations of $NaAsO_2$ (5 and 10 mg.L-1), excessive degeneration of germinal epithelium and germ cell loss was observed. Type A, type B

Morphometry of mice testis demonstrated significant alterations following chronic exposure to $NaAsO_2$ (Table 4), whereby percent area of seminiferous tubules and interstitium were significantly altered but these changes were non-significant at low dose of 0.01 mg.L^{-1}, while seminiferous tubular diameter and epithelial height decreased significantly (P<0.001), and a significant increase was noticeable in the lumen diameter at low $NaAsO_2$ dose. In contrast, at high $NaAsO_2$ doses, significant decrease occurred in percent seminiferous tubular area (P<0.05 at 5 mg.$L^{-1}$and P<0.01 at 10 mg.L^{-1}doses). Interstitial area on the other increased significantly (P<0.05 at 5 mg/L and P<0.01 at 10 mg.L^{-1}doses). Seminiferous tubular diameter and epithelial height decreased significantly at both low and high arsenic doses (P<0.001) but lumen diameter of seminiferous tubules was significantly increased (P<0.001), in comparison to the control group (Table 4). Compared to control testes, spermatocyte

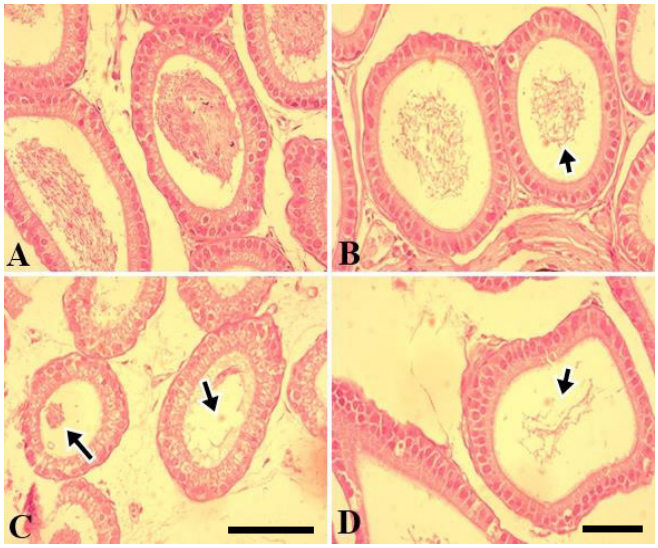

Figure 9: Photomicrographs of postnatal prepubertal mouse epididymal tissue following chronic (PND 25 to PND 54) exposure to $NaAsO_2$ in drinking water. (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Arrows show highly reduced spermatozoa and wider lumen. Scale bar=20 µm.

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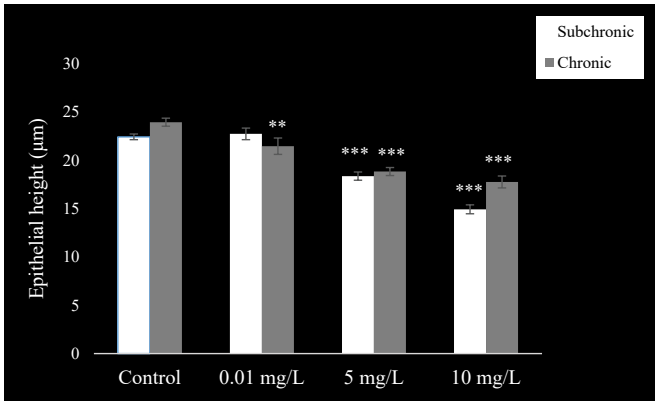

Figure 10: Epididymal epithelial height (µm) decreased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

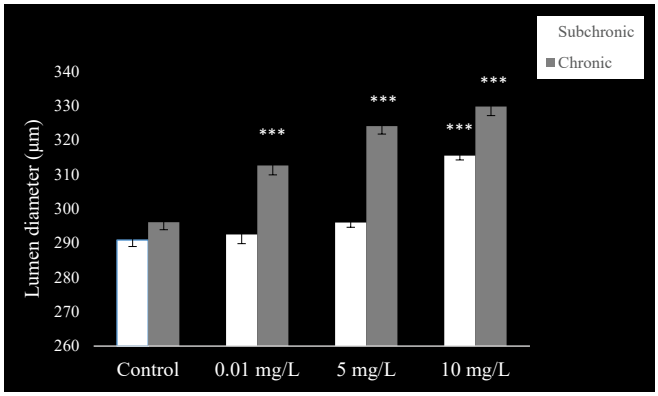

Figure 11: Epididymal luminal diameter (µm) increased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

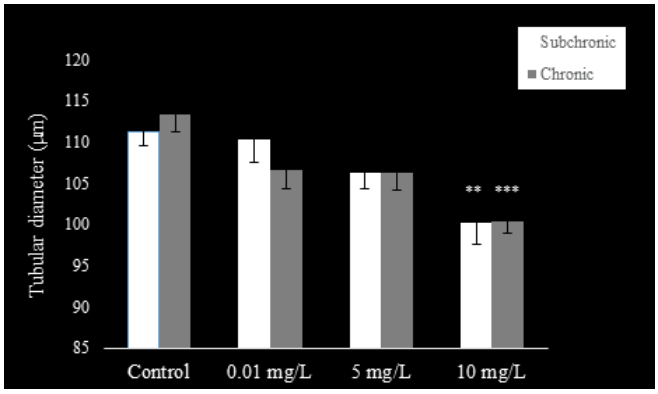

Figure 12: Epididymal tubular diameter (µm) decreased in PND 54 and PND 115 mice after sub-chronic and chronic exposure to oral doses of $NaAsO_2$. Data are mean ± S.E. ** P<0.01 *** P<0.001

and spermatid numbers showed noticeable dose dependent decrease (P<0.001) at low, moderate and high $NaAsO_2$ doses.

Sperm DNA damage

Sub-chronic $NaAsO_2$ exposure

Exposure of postnatal mice to sub chronic $NaAsO_2$ doses revealed significant sperm DNA damage at high dose concentrations (5 and 10.0 $mg.L^{-1}$) (Fig. 6B-D) as compared to control (Fig. 6A) and low dose treatment (Fig. 6B). Sperm tail DNA and tail length were significantly increased (P<0.05), while percent head DNA decreased significantly (P<0.05) after 10 $mg.L^{-1}NaAsO_2$ treatment as compared to control mice (Table 5).

Chronic exposure to $NaAsO_2$

Upon chronic exposure of postnatal mice to $NaAsO_2$, a significant DNA damage was noticeable at all $NaAsO_2$ doses (Fig. 7B-D) when compared with the control (Fig. 7A). Percent tail DNA and tail length increased significantly (P<0.01) at 5 and 10 mg.L-1 doses of $NaAsO_2$, while percent head DNA decreased significantly (P<0.01) at these doses (Table 5).

Histology and morphometry of epididymes

Sub-chronic NaAsO2 exposure

The epithelium of the cauda epididymis showed no morphological alterations in $NaAsO_2$ treated groups (Fig. 8B-D) as compared to the control group (Fig. 8A). Epididymal sperms however demonstrated significant decrease in the lumen at 5 and 10 mg.L-1concentrations of $NaAsO_2$ (Fig. 8C &D). Epithelial cell height of epididymal cells decreased significantly (P<0.001) at 5 and 10 mg/L $NaAsO_2$ doses in the sub-chronic set up (Fig. 10). In contrast, luminal diameter of epididymis increased significantly only at 10 $mg.L^{-1}$ dose of $NaAsO_2$ (Fig. 11). whereas tubular diameter of the epididymis decreased significantly at high dose (Fig. 12).

In the chronic setup, epididymal sperms decreased slightly at 0.01 $mg.L^{-1}$dose of $NaAsO_2$ (Fig. 6B) as compared to the control (Fig. 9A). In contrast, highly significant reduction was noticeable in the epididymal sperms at 5 $mg.L^{-1}$and 10 $mg.L^{-1}$doses of $NaAsO_2$ (Fig. 9C &D). Moreover, epididymal epithelia also showed noticeable structural abnormalities (Fig. 9C &D), whereby a significant decrease (P<0.001) was noticeable in the epididymal epithelial cell height at all arsenic concentrations (Fig. 10). In contrast, luminal diameter of cauda epididymis increased significantly (P<0.001) at all doses (Fig. 11), while the tubular diameter of cauda epididymis decreased significantly (P<0.001) in all treatment groups in comparison to control (Fig. 12).

Discussion

In the present study, postnatal mice (PND 25) were exposed orally to $NaAsO_2$ in drinking water sub-chronically up to PND 54 and chronically to PND 114 at three different dose concentrations, 0.01 $mg.L^{-1}$, 5.0 $mg.L^{-1}$and 10.0 $mg.L^{-1}$. Dose selection criteria were: the lowest dose of 0.01 $mg.L^{-1}$equals 10 ppb or 10 $\mu g.L^{-1}$, a concentration that has been declared by the WHO as safest permissible limit of arsenic

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in drinking water [7,9], whereas the highest dose used in the present experiments (10 mg.L^{-1}) is equivalent to 10,000 ppb, taking into consideration accidental exposure of humans to high arsenic concentration, and also because in several countries of the world, arsenic levels in the ground water exceed well above the safe level up to 50 µg.L^{-1} and exceeding well above in few other countries to 4500 µg.L^{-1} [7,9]. Currently, oral exposure of prepubertal mice to $NaAsO_2$ caused significant reductions in body weight and testicular weight, plasma LH, FSH and testosterone and as well as intratesticular testosterone were decreased, whilst causing significant elevations in ROS and TBARS levels and reductions in the levels of antioxidant enzymes the SOD, POD and CAT and as well as non-enzymatic glutathione reductase (GSH). Histologically, degeneration of seminiferous tubular germinal epithelium occurred. Disintegration of primary and secondary spermatogonia, spermatocytes, spermatids and spermatozoa were readily noticeable, which indicated severe germ cell loss. The percentage area of seminiferous tubules, percent interstitial area, seminiferous tubular diameter and epithelial cell height all decreased, while lumen diameter increased. Sperm count, motility and viability decreased as well. Comet assay further demonstrated significant damage to sperm DNA whereby percent tail DNA and tail length increased, while the percent head DNA was decreased. More severe effects were observed in the chronic set up. Of note, in the chronic set up, 0.01 mg.L^{-1} dose, which was lowest, also caused severe alterations in all above parameters. This showed that chronic exposure of developing male gonad to even lower doses of $NaAsO_2$ can indeed be very harmful to the prepubertal reproductive system which may lead to sever consequences later in the adult life. In the sub-chronic set up, high arsenic doses of 5 mg.L^{-1}and 10.0 mg.L^{-1}were found to be highly damaging.

While some studies demonstrate decline in the body weight of young rats upon chronic exposure to arsenic [40] but others show no effects of arsenic on body weight in adult animals [15, 41,42]. However, present dose dependent decline in the body weight and testis weight upon exposure to $NaAsO_2$ exposure is similar to as reported in adult rats [43,44].

Besides causing weakness, tiredness, inflammation of colon and stomach linings chronic exposure to arsenic induces heart diseases, disruption of the nervous system, and several renal and hepatic diseases [45], weight loss, malabsorption of food and anorexia which is reported to appear after arsenic exposure [46]. It can therefore be safely predicted that indirect systemic and metabolic factors could have played a contributing role in causing a decrease in the body weights and testicular weight in the current in vivo experiments in prepubertal mice.

Decrease in the pituitary gonadotropins, the LH and FSH and gonadal sex hormone testosterone following exposure to $NaAsO_2$ indicates direct suppression of the HPG-axis, most likely via inhibition of the central neuronal pathway for release of gonadotropins. Alternatively, inhibition of steroid biosynthesis by the gonads might also be one of the reasons. In the sub-chronic arsenic exposure, testosterone (intratesticular and plasma), LH, and FSH concentrations were found significantly reduced in the highest dose groups (5 and 10 mg.L^{-1}), but in the chronic exposure to $NaAsO_2$ from PND 25-114, low dose of 0.01 mg.L^{-1}also caused considerable decrease in the hormonal profile. Similar reductions in the plasma testosterone and LH after arsenic exposure have been reported in rats [15, 41]. A significant reduction has been reported in plasma testosterone levels after 1-month exposure to 20 and 40 mg.L^{-1}$NaAsO_2$ in adult mice, but the LH concentrations remained unaffected [14]. Since the testosterone levels are critical for sustaining the normal functioning of the seminiferous tubules and consequently, any reduction in its concentration may cause disintegration of the germinal epithelium [47]. Under normal circumstances, germinal cells are protected from degeneration by FSH, and hence any decline in its concentration might promote the disruption of germinal epithelium of the seminiferous tubules [15,48]. Presently, excessive degeneration of the germinal epithelium, primary and secondary spermatogonia, spermatocytes, spermatids and spermatozoa that was observed in the seminiferous tubules following the exposure of mice to sub-chronic and chronic $NaAsO_2$ treatment was quite possibly due to significantly lower levels of gonadotropins and testosterone.

Moreover, the sperm parameters like the sperm count, motility and viability were found to be significantly reduced with $NaAsO_2$ treatment. Sub-chronic exposure to a low concentration of $NaAsO_2$ (0.01 mg.L^{-1}) did not induce any alterations in the sperm parameters, whereas chronic exposure led to significant toxic effects of arsenic at all concentrations of $NaAsO_2$. Furthermore, alterations in sperm parameters can be attributed to low levels of testosterone but alterations in the levels of other seminal plasma factors may also account for, revealing that the toxicity of arsenic was time and dose dependent. Corroborating with the present results in postnatal mice, exposure of postnatal rats to arsenic has been shown to cause significant reduction in the sperm count [48]. Similar results have been demonstrated in adult mice, whereby arsenic exposure induced alterations in sperm morphology and number [17]. As is evident from present histology also, a decline in sperm count was most likely due to germ cell loss. This decrease in sperm count is also related to a reduced production in the seminiferous tubules, and any blockage or phagocytosis occurring in the ducts [49].

Present $NaAsO_2$ treatment in both the sub-chronic and chronic treatments induced severe morphological alterations in mouse testis and as well as epididymis. In general, arsenic

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treatment induced shrinking of seminiferous tubule diameter, decrease in epithelial cell height, loss of germ cells and mature sperms. Luminal space increased considerably with respect to the arsenic dose. A substantial reduction in the number of sperms stored in the epididymis was also evident in both set of experiments. Low dose arsenic treatment (0.01 $mg.L^{-1}$) although did not induce morphological changes in the testis and epididymis after sub-chronic exposure but chronic treatment with this dose initiated damaging effects on the reproductive organ's histology. Numerous studies have also demonstrated parallel findings in rats [15, 50] and male goats [51] with arsenic treatment. Similar germ cells diminution and reduction in seminiferous tubule diameter has been indicated in other studies [52,53]. Presently, epididymal tissue also demonstrated significant histological alterations, severe reduction in spermatozoa and increased lumen.

Increased ROS and TBARS with an accompanying reduction in antioxidant enzymes were observed in both sub-chronic and chronic treatments. It is already known that ROS formation due to arsenic exposure can damage proteins, lipids, and interstitial tissues [14]. It can improve collagen synthesis and in response the testis interstitial tissue becomes fibroid [54]. Glutathione plays a fundamental role in the mammalian antioxidant defense system. Marked increase in lipid peroxidation, ROS generation, and GSH depletion in rat brain, liver, and RBCs after 12 weeks arsenic exposure has been reported [55]. Concomitant decline that was observed presently in the antioxidant enzyme levels, the SOD, POD, CAT and non-enzymatic reduced glutathione, and as well as gross degenerations and alterations that occurred in the structure of seminiferous tubules indicate that destructive effects to developing gonads quite possibly occurred due to overproduction of ROS and excessive lipid peroxidation inflicted by $NaAsO_2$.

Generalized DNA damage in rats has been strongly linked with the free radicals generated by arsenic [56]. Presently also, chronic exposure of postnatal mice to $NaAsO_2$ revealed considerable DNA damage to developing male mice testes as demonstrated by the Comet assay. This was most likely due to the generation of ROS and a disruption of cellular membranes due to lipid peroxidation. The study overall demonstrated that sub-chronic or chronic exposure to $NaAsO_2$ is detrimental to postnatal male mice reproductive system.

Conclusion

In conclusion, the present study indicated that $NaAsO_2$ induces severe toxic effects in postnatal day 25mice exposed to sub-chronic and chronic doses. Arsenic intake is capable of inducing considerable morphological, biochemical, hormonal, and DNA disruptions during the postnatal reproductive development. The most significant finding of the present study is that even a low dose of 0.01 $mg.L^{-1}$ (10 ppb) of arsenic, which did not cause any significant damage in the sub-chronic set up, the same dose given to mice in the chronic exposure was capable of inducing considerable physiological and cellular damage to developing reproductive organs. Thus, long-term human exposure to even lower concentrations of arsenic is not safe for male reproduction especially in the childhood.

Declaration of interest

The authors declare no conflict of interest.

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