# *IN VITRO* AND *IN VIVO* TOXICOLOGICAL EFFECTS OF SODIUM ARSENITE ON PREPUBERTAL AND ADULT MALE MICE REPRODUCTIVE SYSTEM

**A Thesis Submitted in Partial Fulfillment of The Requirements for The Degree of Doctor of Philosophy**


**By**

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“In the Name of ALLAH, the most Beneficent, the most Merciful”

*Dedicated to my loving Parents*

*Mr. and Mrs. Muhammad Anwar*

## DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is original work. I have not previously presented any part of this work elsewhere for any other degree.

***Naureen Anwar***

# CERTIFICATE

This dissertation **"*In vitro* and *in vivo* toxicological effects of sodium arsenite on prepubertal and adult male mice reproductive system"** by **Ms. Naureen Anwar** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Doctor of Philosophy in Physiology.

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***Naureen Anwar***

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# List of Abbreviations

| | |
|---|---|
| α-MEM | Alpha- Minimum essential medium |
| BrdU | Bromodeoxyuridine |
| CAT | Catalase |
| CC3 | Cleaved caspase-3 |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DEPPD | N, N-Diethyl-p-phenylenediamine sulphate salt |
| DMA | Dimethyl arsenic acid |
| DMEM | Dulbecco's modified eagle's medium |
| EIA | Enzyme immunoassay |
| ELISA | Enzyme linked immune sorbent assay |
| FSH | Follicle stimulating hormone |
| GSH | Reduced glutathione |
| H & E | Hematoxylin and eosin |
| $H_2O_2$ | Hydrogen peroxide |
| HRP | Horse radish peroxide |
| HSD | Hydroxysteroid dehydrogenase |
| HTF | Human tubal fluid |
| IHC | Immunohistochemistry |

| | |
|---|---|
| KSR | Knockout serum replacement |
| LH | Luteinizing hormone |
| MMA | Monomethylarsonic acid |
| MVH | Mouse Vasa homolog |
| $NaAsO_2$ | Sodium arsenite |
| NBT | Nitroblue tertazolium |
| PBS | Phosphate buffer saline |
| PND | Post-natal day |
| POD | Peroxidase |
| RBC | Red blood cell |
| RNS | Reactive nitrogen specie |
| ROS | Reactive oxygen species |
| RPM | Revolutions per minute |
| SOD | Superoxide dismutase |
| STWS | Scott's tap water substitute |
| TBARs | Thiobarbituric acid reactive substances |
| WHO | World Health Organization |

# Abstract

The metal element namely “Arsenic” is abundantly found on earth. It exists in several trivalent and pentavalent oxidation states like -3, +3 and +5. In both human body and atmosphere, it occurs as inorganic and organic compounds. Sodium arsenite ($NaAsO_2$) is considered more toxic and biologically active than sodium arsenate. Arsenic is a renowned carcinogen an an integral part of around 245 minerals. Its has also been used in industrial, agricultural, and medicinal fields. Arsenic accumulates preferentially in the skin, hair and nails of mammals. Drinking water is a common source through which living organisms are exposed to arsenic. Arsenic levels < 10 µg/L are declared safe for drinking purposes by the World Health Organization (WHO) whereas, the maximum contamination levels are 50 µg/L. In recent years, arsenic contamination through drinking water has immensely increased in the sub-continent, especially in Pakistan with some most populated cities having arsenic concentration up to 200 ug/L. Adult male reproductive toxicity caused by arsenic has been extensively reported in adult males, however no data are present as regards to the *in vitro* gonadotoxicity due to $NaAsO_2$ in prepubertal and adult male mice. Therefore, the present study was designed to (i) evaluate possible *in vitro* toxic effects of $NaAsO_2$ in prepubertal and adult male mice reproductive organs (ii) and to assess the *in vivo* reproductive organs development and function after sub-chronic and chronic exposures of $NaAsO_2$.

Three experiments (six replicates for each) were conducted using post-natal day (PND) 5 Balb-c mice testis in an *in vitro* culture system. Prepubertal (infantile) mice testis pieces approximately 1 $mm^3$ were placed on nucleopore membrane, floating on 1ml of α-MEM and 10% KSR. Testicular cultures were incubated at 34°C in a 5% $CO_2$ incubator. Cultures were left untreated for 24 h (Day1). Media were changed after 24 h to provide fresh nutrients to the growing culture. In the first experiment, cultures were treated with 0.01, 0.05, 0.1, 0.5, and 1 µM $NaAsO_2$ exposure for 1 day. Whereas, in second (0.1, 0.5, and 1 µM) and third experiment (10, 50, and 100 µM) $NaAsO_2$ concentrations were administered for six days. Cultures were fixed on day seven and proceeded for hematoxylin and eosin staining and double immunohistochemistry, Cleaved caspase-3 (CC3) and mouse vasa homologue (MVH) for determining apoptosis and germ cells, respectively. Adult mice testicular fragments were incubated at 37°C, while the caudal part of epididymes were incubated at 33°C, each for 2 and 24 h, with $NaAsO_2$ at 1, 10, 50, and 100 µM concentrations. Levels of Reactive oxygen

species (ROS), TBARS, antioxidant enzymes, testosterone concentrations, and the extent of sperm DNA damage were estimated. Sperms were incubated with Human tubal fluid at 37°C in 5% $CO_2$ incubator, and sperm motility, viability, and morphology were assessed after 2 h.

For evaluating the reproductive toxicity of arsenic *in vivo* following the chronic and sub-chronic exposure, $NaAsO_2$ (0.01, 5, and 10 mg/L) was orally administered in male mice from the PND 25 to PND 53 and 114. Histological, hormonal, sperm parameters, oxidative stress, antioxidant enzymes, and DNA damage were assessed.

Results demonstrated that *in vitro* exposure of prepubertal (infantile) testicular cultures to $NaAsO_2$ for 1 and 6 days with 1µM $NaAsO_2$ as the highest dose in experiment 1 and 2 respectively, did not cause any change in the testicular morphology, germ cell density, or CC3 expression. However, exposure of prepubertal (infantile) testis to arsenic concentrations in the third experiment (10, 50, and 100 µM $NaAsO_2$) induced pronounced changes, whereby germ cell number revealed reduction in a dose-dependent manner, while CC3 expression increased a hundred-fold after 50 µM arsenic exposure.

Two hours incubation of adult mice testicular and epididymal tissues with $NaAsO_2$ demonstrated significant increases in the ROS, lipid peroxidation, and sperm DNA damage. However, decreases occurred in catalase, peroxidase, and superoxide dismutase levels at 50 and 100 µM concentrations. Whereas, following 24 h incubation, groups treated with 10, 50 and 100 µM $NaAsO_2$ concentrations, revealed significantly greater toxic effects by elevating oxidative stress markers and sperm DNA damage while decreasing levels of antioxidant enzymes, As for testosterone concentration, a non-significant decrease occurred at 2 h of incubation while significant reduction was noticeable after 24 h of incubation with $NaAsO_2$. Low dose of 1 µM $NaAsO_2$ remained unable to induce any toxic effects. *In vitro* sperm motility and viability were substantially reduced with increasing concentrations of $NaAsO_2$. Similarly, sperm morphology was affected at higher tested doses (50, and 100 µM $NaAsO_2$). On the other hand, *in vivo* sub-chronic (PND 25-53) $NaAsO_2$ exposure at high doses (5 and 10 mg/L) produced significant decrease in the antioxidant enzymes, sperm parameters (sperm count, viability, and motility), and hormones (LH, FSH, testosterone), while ROS and TBARS levels were substantially increased. Morphological changes in the testis as well

as sperm DNA damage were evident only at 10 mg/L dose. In contrast, *in vivo* chronic exposure (PND 25-114) of $NaAsO_2$ even at low dose (0.01 mg/L) induced severe harmful effects on mouse reproductive organs. Considerable changes in mouse testis, and body weight, as well as morphological changes in testis and epididymes were noticed in arsenite exposed mouse. Testicular oxidative stress was induced along with reduction in hormonal, and sperm parameters. Sperm DNA damage was observed at 5 and 10 mg/L arsenic concentrations. *In vivo* experiment results showed that higher doses of $NaAsO_2$, after both sub-chronic and chronic exposure, initiated adverse effects on mouse reproductive system.

Overall, the present study concludes that *in vitro* and *in vivo* $NaAsO_2$ treatment induces both time and dose-dependent toxic effects on prepubertal and adult male mouse reproductive organs.

# CHAPTER # 1

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

Metals are electropositive elements with the tendency of losing electrons. This property makes them good conductors of electricity, highly malleable and lustrous. Depending on the geographical distribution earth's crust is composed of variable proportions of several metals. Physical and chemical properties of metals along with related environmental cues are used to monitor their atmospheric distribution (Khlifi and Hamza-Chaffai, 2010). Unlike other toxins, metals being natural elements are neither produced nor destroyed by anthropogenic means. However, they have become hazardous for human health through human exploitation in terms of their transport and transformation (Beijer, 1986). Metals having specific density greater than 5 $g/cm^3$ and exerting harmful effects on living organisms are termed as heavy metals (Järup, 2003). These are required in very low concentrations for maintaining the physiological and biochemical stability in different life forms. They can however become toxic if their concentrations exceed beyond safer limits. Despite well-recognised deleterious effects caused by higher residual properties, anthropogenic release of heavy metals is still increasing worldwide on daily basis. Its bioaccumulation poses serious risk to the ecosystems (Nagajyoti et al., 2010; Jaishankar et al., 2014). Metals enter into our environment through both anthropogenic and natural means. Some of which include industrialisation and urbanisation resulting in increased sewage and runoff wastewater. Besides these, agriculture sources are also adding the heavy metals in the surroundings through application of insecticides and pesticides (Morais et al., 2012). Chromium, arsenic, nickel, cadmium, copper, zinc and lead are the hazardous heavy metals released in water (Lambert et al., 2000).

Although essential for maintaining normal biochemical functions in living organisms; however, if escaped from cellular checkpoints, heavy metals get involved in un-required chemical reactions and displace the naturally bound metals at specific binding sites located on proteins. This is how heavy metals can disturb normal homeostasis followed by cellular toxicity. Moreover, it is also well-known that heavy metals induce oxidative stress and deteriorate the structures of biomolecules by directly binding to nuclear proteins and DNA (Flora *et al.,* 2008).

### 1.1. History of metals

Human exposure to metals can be traced back to ancient times, which later on proved to exert toxic effects. Lead (Pb) is one of those heavy metals which had been intensively used since 2000 B.C. due to easy access from smelting of silver ores. Another example is arsenic extracted from copper and tin melting which was used to decorate the tombs in Egypt. Other heavy metals posing health risks have been recently discovered. Such as, in 1817, cadmium was discovered in zinc carbonate ores. Less than thirty out of total eighty metals of the periodic table have been found toxic for humans. Indium and gallium are some of the previously least recognised metals however their exposure is increasing with their increased applications with technological advancements (Goyer and Clarkson, 1996).

### 1.2. Occurrence of metals

Flow of the metal ions in the ecosystem continues through biological and geological cycles. Rocks and ores are gradually eroded through rainwater and resistant debris is transported ultimately to the oceans where it is either precipitated into sediments or moved to rainwater through transpiration. Metal elements then enter into food chain through bioaccumulation in plants and animals. In some cases, natural means of bio-concentration exceed the anthropogenic means for e.g. mercury. However, human activities still remain the primary source of metal release from natural ores resulting in contamination of atmosphere, as well as land and water bodies. The metals are derived in the form of fine particles from the extraction site and are distributed throughout the world (Goyer and Clarkson, 1996).

### 1.3. Mechanism of heavy metal toxicity

Cellular level toxic effects of heavy metals are mediated through alterations in the biochemical pathways and target receptors at cellular membranes. This involves binding of metal ion with receptor or with another ligand for recruitment to their final targets. The toxic potential of metal ions is determined through the dose concentration, valance state of these ions and the specific binding ligand. In addition, some metals make organo-metallic compounds through covalent binding with carbon atoms (Goyer and Clarkson, 1996).

### 1.4. Bioaccumulation sites and detection of heavy metals

Major sites for bioaccumulation of heavy metals include hair, blood and urine which provide an ideal evidence for direct analyses of accumulation level either from natural exposure or experimental doses. Whereas, metabolic models are used to quantitatively evaluate the metals in specific organs through tissue autopsies. Acute exposure is analysed from samples of blood and urine while hair samples are used for evaluation of chronic exposure (Goyer and Clarkson, 1996).

### 1.5. Arsenic

Arsenic is abundantly found almost everywhere on earth which makes it 20$^{th}$ abundant element, while its number is 14$^{th}$ for ocean and 12$^{th}$ in human body (Woolson, 1975). In 1250 A.D. it was isolated for the first time by Albertus Magnus. Since then, it has faced several controversies due to its unique features. In periodic table arsenic is placed at the 33$^{rd}$ number. Commonly, it is found in several oxidation states like -3, +3 and +5. In both the human body and atmosphere, it is found in inorganic and organic compounds. It is also an integral part of around 245 minerals (Bissen and Frimmel, 2003). Its use has been seen not only in medicine but also other industrial and agricultural fields (Nriagu and Azcue, 1994).

Arsenic has multiple binding potential and forms trivalent compounds such as arsenic trichloride, and $NaAsO_2$, whereas the pentavalent compounds, for instance, calcium arsenate, lead arsenate, sodium arsenate, and arsenic pentoxide. Arsenite (III) and arsenate (V) are its inorganic forms which are further modified in-vivo either through methylation converting into monomethylarsonic (MMA) or dimethyl arsenic acids (DMA) (Waalkes et al., 2004b).

#### 1.5.1. Arsenic sources and occurrence

Arsenic has an abundance of 1.5-3 mg/kg on land. It becomes available in the environment through both natural and human sources. In nature, it exists in a variety of forms which are counted to be over 200. Among these, arsenates, sulfides and sulfosalts account for 60%, and 20% respectively while remaining 20% is comprised of oxides, arsenites, elemental arsenic, and silicates. As compared to rocks, soils are major source of terrestrial arsenic (Peterson et al., 1981). Soils become concentrated through rock erosion and anthropogenic means. Other factors which regulate the arsenic levels in soil include climate, soil composition and redox status etc. Different foodstuffs contain variable content of arsenic like seafood is rich in organic compounds of arsenic

including arsenocholine and arsenobetaine which are highly resistant against chemical deterioration (Lauwerys et al., 1979).

Many of the natural activities and volcanic explosions result in the accumulation of a high concentration of arsenic in the natural environment (Bissen and Frimmel, 2003). Besides the natural contribution of arsenic contaminants in environment, the human activities such as (i) industrial discharge to open air (ii) extraction of metals from arsenic bearing ores (iii) burning crude oil and natural gas and use of arsenic-containing chemicals in previous hundred years (Bissen and Frimmel, 2003). Land areas which are barren and devoid of the human activities, air contains only nanograms per cubic meter of arsenic contaminants (Atri, 1987). Atmospheric exposure to humans is very low as its normal range varies from 0.4-30 ng/$m^3$ in air (Mandal and Suzuki, 2002).

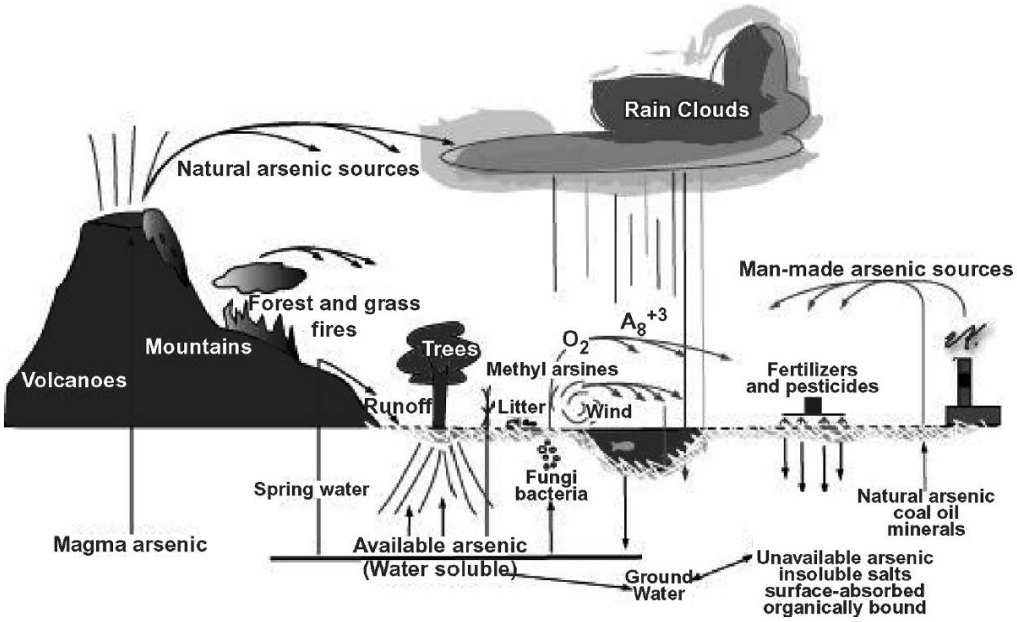


Fig 1.1. Cycling of arsenic and its sources in environment (NRC, 1999)

Trivalent arsenic is more toxic and soluble than the pentavalent form. In sediments, it occurs in concentrations less than 10 mg/kg, dry weight (Crecelius et al., 1975) with variable worldwide distribution. In the atmosphere, airborne particles carry a mixture of arsenite and arsenate (Davidson et al., 1985). Daily dose of exposure through inhalation is about 50 ng in unpolluted regions (WHO, 1981) while in other cases, out of 120 ng of daily dose inhaled, 30 ng is absorbed (Zuane, 1990).

Arsine gas vulnerability is one of the major environmental health-related drawbacks in many vocational related activities for earning and survival. Arsine is tasteless, colourless and odourless gas and its inhalation causes the abrupt damage to RBCs and also results in the renal dysfunctioning which leads to fatal condition without proper treatment. The major causes of arsine toxicity are the exposure to acid and metals ore which constitutes the arsenic as an integral impurity (Fowler and Weissberg, 1974).

Arsenic is accumulated preferentially in skin, hair and nails of mammals. Based on wet weight, humans and domestic animals contain less than 0.3 µg/g of arsenic. With increasing age, the total content of arsenic in the human body tends to be increased from normal range of 3-4 mg. Body tissues except, those originating from ectoderm demonstrate less than 0.3–147 µg/g of arsenic (Smith, 1964).

### 1.5.2. Anthropogenic sources of arsenic

Arsenic is released into the lithosphere, hydrosphere and atmosphere through human activities. This release directly affects both plant and animal life. Soils become contaminated mainly through arsenic-containing pesticides, fertilizers, dust from combustions of fuels and animal and industrial waste disposal (Piver, 1983; Woolson, 1983).

#### 1.5.2.1 Herbicides

Arsenic had been used to eradicate the unwanted weeds since 1890. $NaAsO_2$ is one of those herbicides used for this purpose (Mandal and Suzuki, 2002).

#### 1.5.2.2. Insecticides

Initially, application of arsenic was practiced only in the synthesis of pesticides and insecticides. Overall production of white arsenic was 37,000 tons in 1955 of which only U.S. produced 10,800 tons whereas, its utilization exceeded above the limit of 18,000 tons (Vallee et al., 1960). These pesticides include copper acetoarsenite, lead arsenate, Paris-Green (copper acetoarsenite), arsenic acid, cacodylic acid, monosodium methanearsonate, and disodium methanearsonate are used as pesticides for cotton production.

#### 1.5.2.3. For wood preservation

The process of desiccating cotton piles had been performed by using arsenic acid for years. One such chemical used in 1918 in USA for the first time for wood preservation was Fluor–Chrome–Arsenic–Phenol (FCAP) (Thornton and Farago, 1997).

#### 1.5.2.4. Feed additives

Food preservatives and additives also utilize several formulations containing arsenic compounds like arsenic acid, 3-nitro-4-hydroxyphenylarsonic acid, 4-nitrophenylarsonic acid etc. According to a law passed in 1958, phenyl arsonic acids were allowed to be used as food additives (Mandal and Suzuki, 2002).

#### 1.5.2.5. Medicinal use

Arsenic has been praised for its medicinal properties 2500 years ago. Such as, Austrian peasants used to consume wholesome amount of arsenic for aesthetic purpose i.e. for softening, cleaning and improving the skin complexion along with resolving of breathing problems (Chevallier, 1854; Sollmann, 1957). Some common arsenic formulations used for medicinal purposes are Fowler's solution (potassium arsenite), de Valagin's solution (liquor arsenii chloridi), Donovan's solution (composed of mercuric iodides and arsenic), and Asiatic pills (black pepper and arsenic trioxide) (Vallee et al., 1960).

## 1.6. Drinking water and arsenic

Drinking water is the common source through which living organisms are exposed to arsenic. Such as, normal range of arsenite concentration in the drinking water is 0.01-4 mg/l. The inorganic arsenic is the major contaminant in drinking water and shows much adverse effects as compared to arsenic contaminants in food staffs for example vegetables and grains (Akter et al., 2005). The average arsenic level in sea water is almost 1.5 µg/L while in fresh water it is 1 mg/L (Leonard and Lauwerys, 1980). The WHO claimed that less than 10 µg/L of arsenic concentration in water is considered to be safe from health point of view and suggested that maximum contaminant level (MLC) of arsenic is 50 µg/L (Basu et al., 2001; Brown and Ross, 2002).

## 1.7. Toxicokinetics of arsenic

Absorption through gut is the primary route from where approximately 80-90% of trivalent and pentavalent arsenic compounds are absorbed in both humans and other experimental animals. Moreover, less soluble arsenic compounds including arsenic selenide, gallium and lead arsenides etc. exhibit lower absorption as compared to those with higher solubility. Another route leading to arsenic toxicity is skin especially in individuals with continuous exposure to inorganic arsenic formulations (Hostynek et al., 1993). Although atmospheric arsenic is found in the form of arsenic oxide however, both chemical form and particle size determine the accumulation of arsenic particles in lungs and airways of exposed animals and humans (Morrow et al., 1980). Urine is the

primary source of absorbed arsenic once excreted. Half-life of inorganic arsenic upon ingestion is 10 h with approximately 80% excretion within 3 days while, methylated arsenic exhibits half-life of 30 h. Excretion of organic and trivalent arsenic compounds is faster than that of inorganic and pentavalent forms. Amount of arsenic excretion via urine varies from 5 to 40 µg /day with excess of 100 µg released in case of acute and sub-acute toxicity (Arnold et al., 1990).

In comparison to arsenate, arsenite is biologically two to ten times more vigorously reactive and poisonous (Kosentt, 1994). Among different arsenic compounds, the trivalent arsenic oxides have the potential to assess the dermal route of its entry to body due to its lipid solubility (Winship, 1984).

In the body, arsenic is readily accumulated in skin and is excreted with sloughed off skin cells and sweat. Furthermore, it is also concentrated in nails leading to appearance of white bands in fingernails which is a late symptom of arsenic toxicity. The distance of these lines from base of nail and the overall growth rate of nail are good indicators of exposure time of arsenic. Another determinant of arsenic toxicity is hair which also depicts the previous exposure and absorption of arsenic. Anionic arsenic forms are more soluble and are readily absorbed within the human body as compared to arsenic forms with low solubility. Hair and other tissues enriched with keratin specifically accumulate inorganic arsenic (Smith, 1964). Time of exposure can be determined from arsenic concentration along with the hair length (Pearson and Pounds, 1971). In hair, arsenic concentrations at 1.0 µg/g and exceeding this limit depict the arsenic poisoning (Arnold et al., 1990).

### 1.7.1. Biotransformation of arsenic

Transformation of inorganic arsenic into its methylated derivatives further complicates the arsenic metabolism and toxicity in living body. Both oxidation states of arsenic take part in methylation which is primarily performed in the liver. After absorption, a considerable portion of pentavalent arsenic is readily converted into trivalent form which is further methylated to MMA or DMA. This conversion is considered to involve the two-electron process leading to thiol oxidation. The product of this reaction is trivalent arsenic which is reactive towards S-adenosylmethionine (SAM) thus transferring the methyl group to arsenic from sulfur (Fig. 1.2).

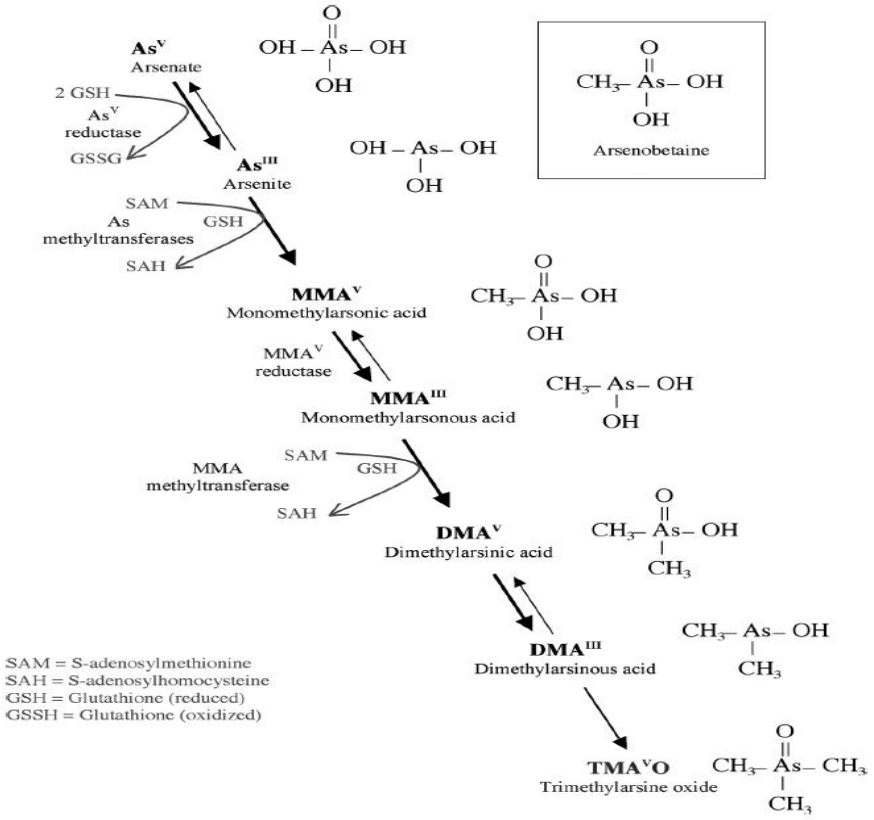


Fig 1.2. *In vivo* biomethylation pathways of arsenic (Wang et al., 2006)

Methylated derivatives of arsenic are considered less toxic due to their lower reactivity for living tissues and rapid excretion through urine. Therefore, this conversion is a kind of detoxifying mechanism which reduces the toxicity of inorganic arsenic compounds and releasing them in the terminal metabolite form i.e. di-methyl arsenic (Goyer and Clarkson, 1996). Previous investigations on human hepatocyte cultures have revealed that increase in exposure level results in increased production of methylated forms. Moreover, increased concentration of trivalent arsenic causes reduction in DMA/MMA ratio (Styblo et al., 1999). Various mammalian species exhibit the differences in biotransformation mechanisms (Vahter, 1994; Vahter et al., 1995), for instance DMA is the primary metabolic product in several animals. In contrast, investigations on chimpanzees and marmoset monkeys revealed no such methylation of inorganic arsenic. Whereas, estimation of arsenic metabolites in urine samples of humans demonstrate the variable percentages of inorganic and methylated arsenic forms as 10-30% inorganic, 10-20% MMA, and 55-76% DMA. These results exhibit both general and occupational exposure. In addition to environmental factors, genetic and physiological cues including polymorphisms, age and sex etc. also regulate the methylation level of arsenic. Exposure of populations in northern Argentina and Chili

of Spanish origin who were exposed through drinking water, revealed the presence of 2-4% MMA in the urine samples (Concha et al., 1998a). However, a similar study conducted on Taiwanese population illustrated the presence of 27% MMA in the urine samples (Chiou et al., 1997).

### 1.8. Chronic arsenic exposure

Severe and long-term poisoning are the outcomes of arsenic exposure. The long-term arsenic exposure includes the non-specific health related signs such as long-term weakness, dropping of reflexes, tiredness, inflammation of stomach linings, inflammation of colon, anorexia, and baldness. Arsenic can easily accumulate in nails, hairs, skin and bones. Subjection to chronic exposure, arsenic via food stuffs and through air leads to thickening of stratum corneum (the outermost layer of the epidermis), hyper coloration of skin, heart diseases, disruption of nervous system, fragile lose nails with Mee's lines and various types of renal and hepatic diseases. (Vahter and Envall, 1983; Hindmarsh and Mc Curdy, 1986; Lu, 1990; Hall, 2002).

Severe arsenic toxicity results in vomiting, mouth and throat desiccation, muscle stiffness, stomach pain, feet and hand shivering and nervous dizziness. Incurable collapse may occur due to kidney disruption and if death does not occur within 24 h than non- recoverable organ disfunctioning might happen. Death may ultimately occur in next 24 h due to liver and kidney failure or due to heart stroke (Reichert and Gruber, 1979; Gorby, 1994).

### 1.9. Arsenic toxicity

Arsenic toxicity and availability in living tissues is dependent on the chemical form, valence state (Cullen and Reimer, 1989). Other factors like its physical state, particle size, absorption and excretion, composition of chemical compounds and the overall health condition of patient, also determine the net toxic effect induced by arsenic exposure (Hindmarsh and McCurdy, 1986).

The most common mechanism through which an element can induce toxicity is via inactivating the enzymatic pathways thus affecting the cellular metabolism (Dhar et al., 1997). The pentavalent arsenic is not reactive for active sites of enzymes (Johnstone, 1963). It is first reduced into trivalent arsenic within the body before it is able to exert deleterious effects (Ginsburg and Lotspeich, 1963; Pauwels et al., 1965). It is postulated that, in cells, arsenic detoxification is mediated through binding of sulfhydryl groups to trivalent arsenic (Aposhian, 1989). Enzymes having hydroxyl and sulfhydryl groups

are specifically vulnerable to trivalent arsenic as it has the tendency to interfere with bonding of these groups. Such enzymes include those that catalyse reactions of citric acid cycle. The mechanism of inhibition involves pyruvate dehydrogenase inactivation by forming complexes with trivalent arsenic. This process inhibits the ATP synthesis. The enzyme catalyzed systems are comprised of several enzymes and additional cofactors. Continued enzymatic activity is halted via trivalent arsenic which substitutes the hydrogen atoms of thiol group and makes dihydrolipoylarsenite chelate complex thus preventing oxidation of dihyrolipoyl group. Once this step is blocked, pyruvate concentration is increased in blood with decreased ATP synthesis and ultimately cellular damage (Wolochow et al., 1949; Belton et al., 1985). Affinity for sulfur is the reason that arsenic readily concentrates within keratinized tissues like nails and hair. It is proposed to form stable bonds with dithiol found in hair keratin (Peters, 1949).

Interestingly, selenium (Se) exhibits antagonistic effects against arsenic within the body thus counteracting each others' toxicity. Unluckily, arsenic is able to interfere with beneficial role of selenium in human metabolism (Kenyon et al., 1997). It is involved in inhibiting the selenium activity through decreasing the glutathione peroxidase activity which is dependent on selenium (Mandal and Suzuki, 2002).

Methylated and di-methylated trivalent derivatives of arsenic are known to exert greater toxic effects at cellular and genetic level and block the enzymatic activities as compared to their pentavalent counterparts in the form of arsenic. This leads to the conclusion that methylation of arsenic results in increased toxicity rather than its detoxification (Thomas et al., 2001). Arsenite acts as the inhibitor of several biochemical pathways via binding the sulfhydryl groups of different proteins whereas, arsenate is found to disturb the phosphorylation reactions. Arsenite in its trivalent form is more active as compared to pentavalent arsenate which indicates its ability to significantly amplify the genes in mammalian cells. However, the effects of both arsenite and arsenate becomes similar due to the reduction of arsenate into arsenite in the blood (Mure et al., 2003).

Arsenic may prove lethal if ingested in large concentrations (70-180 mg). The symptoms of arsenic toxicity include sudden illness, pyrexia, anorexia, liver enlargement, melanosis and arrhythmias leading to cardiac failure. In addition, pathological effects are noted on several physiological functions including neuronal activity, respiration, gastrointenstinal and cardiovascular events etc. Initial symptoms of acute ingestion are apparent as irritated, vesicular and damaged mucous membranes. Pathological effects on nervous system include desensitization in peripheral nerves

preceded by generous exposure to arsenic. This is shown by axonal degeneration which can be reversed in many cases. Other signs appearing a few days after exposure include leukemia, anemia and granulocytopenia. Both chronic and acute toxicity of arsenic is found to exert similar hematological effects including disturbed heme generation and an increase in porphyrin levels in urine samples. Neurotoxicity affecting both central and peripheral nerves has been reported in cases of chronic exposure. The symptoms include general weakness characterized by malfunctioned senses and muscle tenderness. Peripheral nerves are progressively affected which is observed as axonal demyelination. These effects are dose dependent such as, a single high dose can elicit motor dysfunction and sensory loss within duration of 10 days. In contrast, chronic exposure at work is known to exert harmful effects over the years gradually which can be related to dose concentrations (Murphy et al., 1981). Exposure for longer periods can lead to liver damage which is initially manifested as jaundice and ultimately leads to cirrhosis. At cellular level, parenchymal cells of liver are affected which causes increased secretion of liver enzymes into circulation. Moreover, mitochondrial ultrastructure is also altered with granulation and loss of glycogen within cells (Goyer and Clarkson, 1996).

It has also been observed that arsenic exposure leads to tumor formation in several organs including the liver, lungs, kidney, skin and bladder (Waalkes et al., 2004). Nevertheless, arsenic compounds are used for their medicinal properties against acute promyeloticleukaemia (Bode and Dong, 2002; Puccetti and Ruthardt, 2004).

### 1.9.1. Arsenic induced free radicals

Several free radical species are produced from arsenic metabolism which include superoxides, nitric oxide, peroxy radicals and di-methyl arsenic peroxyl radicals (Rin et al., 1995). Despite free radical synthesis mechanism is not clearly known but initial findings suggest that arsenic derivatives mediate their synthesis (Yamanaka et al., 2001). The resulting state of oxidative stress is thought to be the primary cause of several diseases including cancer. These free radicals include not only reactive oxygen but also nitrogen species which deteriorate the lipid, protein and DNA structure in arsenic treated cells. Several experimental observations support the arsenic induced cellular toxicity (Shi et al., 2004). Neurotoxic effects of arsenic are also found to be induced by synthesis of hydroxyl radicals (Garcia-Chavez et al., 2003).

Furthermore, synthesis of free radicals including hydrogen peroxide and superoxide have been detected using techniques like Electron Paramagnetic Resonance (EPR),

EPR spectroscopy and Spin trapping in vascular endothelial cells (Barchowsky et al., 1999) and human keratinocyte cell line (Huang et al, 2002). Previous studies have confirmed that arsenic promotes free radical production during metabolic process (Shi et al., 2004). *In vitro* exposure of U937 cells to arsenite from 1–10 μM concentrations resulted in the synthesis of superoxide free radicals (Iwama et al., 2001). Likewise, similar results were obtained for hybrid cells of humans and hamsters and as well as vascular human smooth muscle cells at arsenic doses 50 μM (Kessel et al., 2002), and 16 μM (Lynn et al., 2000) respectively.

It was found that arsenite promotes the synthesis of hydrogen peroxide which induces apoptosis (Wang et al., 1996; Ma et al., 1998). Arsenic oxide promotes the synthesis of mitochondrial hydrogen peroxide resulting in synthesis of hydroxyl ions which ultimately cause oxidative stress damaging the cellular proteins. Considering the cellular toxicity induced by $NaAsO_2$ (Wang et al., 1996), it is hypothesized that hydrogen peroxide produced from arsenite reaction along with Fenton reaction leading to hydroxyl ion synthesis, promotes the apoptotic process cells via activation of several protein kinases. These findings indicate that arsenite is more potent in inducing cellular toxicity through indirectly deactivating specific enzymes via protein oxidation rather than binding directly to thiol groups of proteins.

Additionally, hydrogen peroxide is known to be synthesized by conversion of trivalent arsenic to pentavalent form *in vivo*.

$H_3AsO_3 + O_2 + H_2O = H_3AsO_4 + H_2O_2$ (– 40.82kcal/mol) (Aposhian et al., 2003).

Both ROS, and reactive nitrogen species are generated following arsenic exposure (Shi et al., 2004). Reactive nitrogen oxide is known to mediate regulatory role in important functions like vasodilation, nerve impulse conduction and immune response. Its synthesis is catalyzed by nitric oxide synthase enzyme. However, some contradictory result has reported nitric oxide production after arsenic exposure. Both the time and concentration dependent elevation of nitrite concentrations have been observed in media of CHO-K1 cells treated with arsenite 20 μM dose (Lynn et al., 1998). Increased nitrite levels are indicators of higher nitric oxide levels in cells which may mediate the genotoxic effects of arsenite (Liu and Jan, 2000). Comparative analyses revealed that arsenite and its methyl derivatives induces DNA damage in human cell cultures and isolated DNA fragment. They observed the DNA strand breaks only in isolated PM2 DNA preceded by treatment with di-methylarsinous. Likewise, treatment of HeLaS3

cells with low doses of 10 nM of arsenite for short duration (0.5-3 h) leads to increased frequency of Fp-g sensitive sites. These findings indicate that even at very low concentrations, both arsenite and its methylated forms are able to induce oxidative stress leading to DNA damage in human cells lines. This also suggests that arsenic is methylated in vivo thus contributing to exert genotoxic and carcinogenic effects. Conversion into trivalent form increases the toxicity of arsenic rather than its detoxification such as observed for arsenate. In conclusion, change of oxidation state from pentavalent to trivalent is more dangerous as compared to its methylation (Schwerdtle et al., 2003).

DNA damage caused by free radicals can be determined through 8-Hydroxyl-2-deoxyguanosine (8-OH-dG) which acts as a good biomarker. Both animal models and human cell cultures treated with arsenic demonstrate the higher levels of 8-OHdG (Schwerdtle et al., 2003). A study revealed higher frequency of 8-OHdG in skin neoplasms caused due to arsenic exposure as compared to Bowen's disease which does not involve arsenic (Matsui et al., 1999). Likewise, 8-OH-dG synthesis in rat liver followed by administration of DMA was significantly increased (Wanibuchi et al., 1996). Similar increase was observed in rats exposed to DMA for 3-9 h (Wei et al., 2002). Antioxidant enzymes are known to decrease the arsenite induced DNA damage. Murine keratinocyte line JB6 showed dose dependent DNA strand breaks after arsenic exposure. In view of these outcomes, it can be deduced that higher frequency of DNA adducts and protein-DNA cross linkage induced by arsenic toxicity are the primary mode of appearance of strand breaks (Bode and Dong, 2002). Antioxidant enzymes are known to mitigate this genotoxic effect of arsenic which highlights dominant role of ROS in inducing strand breaks (Nesnow et al., 2002). DNA strand breaks triggers cell death by activation of poly (ADP-ribose) polymerase (PARP). In addition, these strand breaks can also induce chromosomal rearrangements (Valko et al., 2005).

### 1.9.2. Effect on membrane proteins and lipids

It has been observed in previous investigations that arsenite promotes the lipid peroxidation. Recently, an epidemiological study in China has reported the different levels of lipid peroxidation in response to high and low arsenic exposure via drinking water (Pi et al., 2002). Presence of lower levels of non-protein sulfhydryl levels in blood samples of groups exposed to high concentrations of arsenic as compared to the group exposed to low arsenic concentrations. This indicates arsenic induced oxidative stress

in populations exposed via drinking water led to increased lipid peroxidation. Moreover, Zhang et al. (2000) analysed the comparative effects of different forms of arsenic on lipids and proteins of erythrocyte membrane. They found that arsenites get attached to inner side the plasma membranes while arsenates bind from the outside. This binding results in fluctuations in lipid fluidity of membranes leaving the outer membrane surfaces with negative charge density.

### 1.9.3. Therapeutic effect of arsenic in cancer treatment

Despite carcinogenic properties of arsenic medicinal use of arsenic against promyeloticleukemia (APL) has also been reported. Antilukemic properties of arsenic oxide have been observed in leukemia cell lines with genetically altered mitochondrial DNA (Pelicano et al., 2003). It was found that arsenic trioxide blocks the mitochondrial respiration and promotes synthesis of free radicals thus enhancing the superoxide activity against leukemia cells isolated and cultured *in vitro* This mode of action could increase the potential of arsenic oxide to be used to enhance the efficacy of anticancer drugs through inducing free radical synthesis (Pelicano et al., 2003).

### 1.9.4. Hematological effects

Both acute and chronic exposures to arsenic are known to exert toxic effects on hematopoietic system. These include various forms of anemia and leucopenia which are caused by acute, intermediate, and chronic arsenic poisoning through ingestion (Glazener et al., 1968; Armstrong et al., 1984; Franzblau and Lilis, 1989). These effects are mediated through induction of hemolysis and cyto-toxicity in blood cells which leads to reduced level of erythropoiesis (Lerman et al., 1980). It has been found that arsenates are 1000 times more toxic for human erythrocytes as compared to arsenites. Hemolysis is induced by depleted stores of glutathione within cells which promotes sulfhydryl group oxidation from $Fe^{2+}$ to $Fe^{3+}$ in experimental rats and mice. Moreover, oxygen uptake by cells is significantly reduced by binding of hemoglobin with arsenic (Saha et al., 1999).

### 1.9.5. Renal effects

Kidneys have been found to accumulate the inorganic forms of arsenic upon continuous exposures. These are involved in excretion of arsenic and also its conversion into pentavalent form (Schoolmeester and White, 1980; Armstrong et al., 1984). Compared to other organs, kidneys are considered to have less sensitivity for arsenic. In general, capillaries, tubules and glomeruli are affected the most, this leads to proteinuria,

hematuria, dehydration, shock, oliguria and renal failure (Giberson et al., 1976), cortical necrosis (Gerhardt et al., 1978), and cancer (Hopenhayn-Rich et al., 1998).

### 1.9.6. Gastrointestinal effects

Inorganic arsenic compounds are effectively absorbed through the gastrointestinal tract due to its water solubility. Toxicity becomes apparent in the form of several symptoms if heavy doses are ingested. These include heartburn, nausea, dry-mouth, diarrhea and abdominal discomfort followed by sub-acute exposure, whereas, exposure to low levels of arsenic remains asymptomatic or shows mild irritation and discomfort of lower and upper abdomen (Nriagu, 1994). However, weight loss, malabsorption of food and anorexia may appear with time (Goebel et al., 1990).

### 1.9.7. Respiratory effects

Respiratory problems in humans exposed to inorganic forms of arsenic through natural and occupational means include laryngitis, rhinitis, perforation and congestion in nasal septum, wheezing and shortness of breath etc (Gerhardsson et al., 1988; Nriagu, 1994; Milton et al., 2001).

### 1.9.8. Hepatic effects

Liver becomes concentrated with arsenic when exposed for longer periods such as for months or years. The pathological changes induced followed by chronic exposure include liver cirrhosis, hypertension in portal vein, fat degradation and primary hepaticneoplasia (Carter, 1998).

### 1.9.9. Dermal effects

Various skin conditions arise followed by chronic exposure to arsenic either through inhalation or ingestion. These include spotted melanomas, keratosis, lecuomelanosis etc. These problems have been well documented through previous investigations on populations exposed to arsenic through drinking water (Mazumder et al., 1999; Milton and Rahman, 1999; Tondel et al., 1999; Ahsan et al., 2000; Paul et al., 2000; Smith et al., 2000; Mazumder, 2001; Saha and Chakraborti, 2001; Smedley and Kinniburgh, 2001). However, no such results have been reported after consumption of arsenic doses up to 0.01 mg/kg/day for longer duration (Southwick et al., 1982; Valentine et al., 1985). Body areas where skin is already darker become hyper-pigmented (Shannon and Strayer, 1989). Initial symptoms include actinic keratosis, hyperkeratosis of palms and soles, episodic pruritic urticaria (Cannon, 1936), cellular carcinomas of certain types which can be distinguished through histological examinations from tumors induced via non-arsenic means (Nriagu, 1994).

### 1.9.10. Neurological effects

Previous studies frequently reported arsenic induced neuronal damage. Encephalopathy along with fatigue, confusion, headaches, seizures, hallucinations and even coma were reported following an acute exposure to arsenic (Grantham and Jones, 1977). Whereas, intermediate and chronic exposures (0.05– 0.5 mg/kg/day) of arsenic leads to damage to peripheral nerves initiated with sensory loss in hands and feet which later develops into pins and needle sensation (Wagner et al., 1979) and dropping of ankle and wrist (Chhuttani et al., 1967), asymmetric bilateral phrenic nerve (Bansal et al., 1991), muscle weakness, loss of reflexes, and peripheral neuropathy (including sensory and motor neurons) (Morton and Caron, 1989; Basu, 1996).

### 1.9.11. Arsenic induced developmental toxicity

Different epidemiological research showed that there is a statistically significant relationship between pre-birth arsenic toxicity and harmful infant outcomes, like newborns deaths, birth abnormalities, and low infant body weight (Ahmad et al., 2001; Milton et al., 2005; Rahman et al., 2007). Arsenic have the potential to transfer to fetus through placenta. Experimental studies in animals have demonstrated that the pre-birth exposure to arsenic results in arsenic storage in fetuses even in the brain of developing fetus (Rodriguez et al., 1999; Miyazaki et al., 2005). Monomethyl and dimethyl arsenicals residues have been seen in fetuses (Hall et al., 2007). The arsenite and arsenate are the inorganic arsenic compounds and are highly toxic as compared to organic arsenites to the developing embryos/fetuses. Injection of methylated arsenic and inorganic arsenic in mice/ rats around the lethal doses resulted in developmental toxicity in the infants. However, in mice and rats repeated and single arsenic exposure does not result in gross abnormality (Stump et al. 1999; Holson et al., 2000a). Continuous maternal oral exposure of dimethylarsinic acid increases the absorption rate and reduced fetal weight. However, no net malformation was documented in rats but resulted in increase of cleft palate in mice (Rogers et al., 1981). If the pregnant women intakes higher arsenic concentrations by drinking water it will result into pregnancy problems, like premature births and miscarriages (Chakraborti et al., 2003), while low concentration of arsenic shows harmful effects on uterus and placental growth which severely effects newborn birth weights (Hopenhayn et al., 2003; Rahman et al., 2008).

Earlier reports have indicated fetal abnormality in initial gestation period of rats and mice after an intraperitoneal (i.p.) or intravenous (i.v.) injections of arsenic (DeSesso et al., 1998; Hood, 1998; Stump et al., 1999; Holson et al., 2000b; DeSesso, 2001). However, no developmental toxicity results after inorganic arsenic or arsine exposure through external respiration (Morrissey et al., 1990). In short, the above studies indicate that the developmental toxicity take place only after inducing maternal arsenic toxicity. The rat RBCs have high binding potential for arsenic therefore they can hold large amount of arsenic in their bodies (Lu et al., 2004).

Fetus abnormalities were the major outcomes when pregnant mice and rats were given i.v. and i.p. injections of inorganic arsenites at early stages of pregnancy (Stump et al., 1999; DeSesso, 2001). If the mother acquired the inorganic arsenic through external respiration and through oral consumption then it resulted in adverse effects on fetus growth and behavior except of fetus abnormalities (Holson et al., 1999; Stump et al., 1999; DeSesso, 2001; Chattopadhyay et al., 2002). The circulation levels of arsenic in the mother blood depend upon the rate of absorption. In case of oral toxicity, the major source of arsenic accumulation in the blood are the intestines. Arsenic is then ultimately moved towards liver where it is metabolized first before entering the uterus (Stump et al., 1999; DeSesso, 2001). It was observed in the mouse that after the inorganic arsenic entry into the liver the maternal liver attempted to eliminate it through first phase of defence (Hood et al., 1987). While the i.p. injection provides the direct entry of arsenic into the blood vessels. It was also observed that a portion of arsenic can get access towards the blood vessels of abdominal cavity after passing through the first phase of its metabolism in the liver (Stump et al., 1999; DeSesso, 2001).

About 90% of arsenic compound, dimethylarsinic acid was detected in the blood plasma of mothers and their newborns. Similar form of 60 to 70% arsenic was detected in the urine of most of the population. While the urine of pregnant ladies accumulates about 90% of arsenic compound in the form of dimethylarsinic acid which indicates that the arsenic methylation is higher in pregnancy period and it is the one of the prominent forms of arsenic having quick access to fetus (Concha et al., 1998b).

### 1.9.12. Arsenic and male reproductive toxicity

Arsenic is responsible for gonads dysfunctioning due to reduction of testosterone synthesis, gonadal cell death and necrosis (Davila-Esqueda et al., 2012; Shen et al., 2013). Antigonadal mechanism of arsenic indicates that can as an inhibitory agent on testes or alter the normal function of the hypothalamic-pituitary axis which causes alteration in the plasma concentration of LH and FSH. Impairment of Leydig cells are the ultimate consequences of reduced levels of LH and testosterone production. The normal testosterone level is an essential requirement for normal spermatogenesis (Jana et al., 2006). The additional studies suggested that the cause for decreased serum testosterone, FSH, and LH are the elevated level of corticosterone in serum which may result in the reduction of serum testosterone and gonadotropins concentrations (Vreeburg et al., 1988; Hardy et al., 2005). This has been also studied in male rats treated with arsenic (Biswas et al., 1995).

The sufficient level of testosterone is necessary for healthy spermatogenesis, sperm maturity, normal sperm shape and other important morphological stages in the seminiferous tubules (Sharpe et al., 1988; Sharpe et al., 1992; Anahara et al., 2006). The basic function of 3β-HSD is to change the pregnenolone to progesterone and the major function of 17β-HSD is to change the androstenedione into testosterone. The cholesterol present in the membranes of the growing cells of seminiferous tubes of testes affects the gamete's fertility. In testes, cholesterol is a basic requirement for testosterone synthesis. Several studies have suggested that the reduced plasma testosterone level after arsenic treatment is due to the reduced enzymatic conversion by the reduction of 3-beta hydroxy steroid dehydrogenase (3β-HSD) and 17-beta hydroxy steroid dehydrogenase (17β-HSD) instead of deficiency of synthetic precursor of cholesterol (Kabbaj et al., 2003). In a similar way, another study reported that $NaAsO_2$ toxicity results in a reduction of plasma testosterone concentration in mice. It was shown that the reduction of serum testosterone following arsenite toxicity is due to reduced conversion of 17β-HSD, irrespective of the deficiency of synthetic progenitor, the cholesterol (Morakinyo et al., 2010).

It was also observed that along with spermatogenesis, the cholesterol metabolism and testosterone producing assembly in mice were also altered due to arsenic treatment. It was observed that the exposure of arsenic trioxide in male mice for at least 30 days

resulted in a marked increase in cholesterol concentrations and decreased the expression of 3- and 17β-HSD, the major enzymes involved in steroidogenesis (Chinoy et al., 2004).

Arsenic abruptly affects the mitochondrial membrane function and then alters the function of mitochondrial enzymes. This enzymatic change is responsible for the alteration of mitochondrial structure and also disturbs the integrity of mitochondria of sperm (De Vizcaya-Ruiz et al., 2009). Seminal fluid quality assessment parameter are very important indices of male reproductive system. These include sperm membrane intactness, intactness of sperm DNA, sperm count, sperm contingency and sperm movement (Morakinyo et al., 2010).

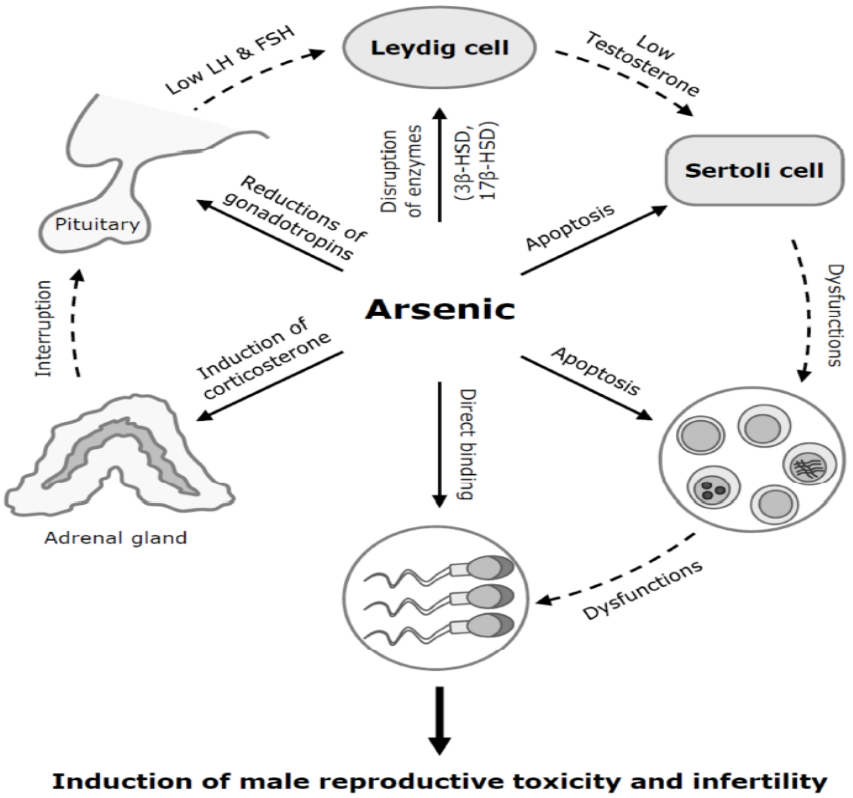


Fig 1.3. Multiple targets sites of arsenic in males and the mechanism of its reproductive toxicity (Kim and Kim, 2015).

Exposure to arsenic results in infertility and also alters the sperms qualitative properties (Nie et al., 2006). Numerous animal researches suggested that arsenic intoxication can cause spermatotoxicity and reduction of testicular steroidogenesis and spermatogenesis (Pant et al., 2001; Sarkar et al., 2003; Pant et al., 2004; Waalkes et al., 2004b). It also changes the functions of enzymes involved in the spermatogenesis (Pant et al., 2001; Chinoy et al., 2004; Pant et al., 2004).

Sperm movement is essential to achieving the ultimate goal of egg fertilization (Yousef et al., 2003). Two major factors used for the identification of male fertility and infertility conditions are the sperm structure and sperm sensitivity (Guzick et al., 2001). Sperm movement is also showed to decrease after $NaAsO_2$ toxicity (Momeni and Eskandari, 2012). The binding of arsenic with thiol proteins is one reason behind reduced sperm movement (Uckun et al., 2002). The thiol protein is known to be crucial for the sperm movement (Pant et al., 2004). The decreased sperm motility can also occur as a result of direct binding of arsenic to sperm. Sperm nuclear chromatin and flagellum both are rich in thiol-rich protamines and these thiol rich regions are the preferred binding regions for arsenic (Uckun and Liu, 2001). Another possible reason behind the reduction in the sperm movement is the oxidative degeneration of lipids in the sperm cell membrane (Das et al., 2009a). The stress of male reproductive toxicity totally depending upon the route of arsenic exposure (Holson et al., 2000b). The major drawback observed of arsenic toxicity in rats was that it altered the sperm structure (Sarkar et al., 2003; Jana et al., 2006). ROS generation due to arsenic stress is also responsible for the alteration of sperm structure and sperm morphology (Kumar et al., 2002).

A reduced sperm count has been reported observed in the mouse (Im Chang et al., 2007). It was shown that when arsenic was given to male mouse during transplacental and lactational stages, the sperm membrane integrity was lost (Reddy et al., 2011). Arsenic trioxide exposure to male rabbit buck resulted in damage of the sperm membrane (Seadawy et al., 2014).

Many several studies approved the noticeable arsenic deposit in male accessory sex organs and testes (Danielsson et al., 1984; Pant et al., 2001). The inorganic retaining potential in rats was 20 times greater as compared to similar exposure to mice (Vahter 1983). It was also observed that the arsenic toxicity also involves in weight loss of testes

and accessory sex organs (Sarkar et al., 2003). If arsenic is administered to male mice or rats through drinking water it has been demonstrated that it causes reproductive toxicity (Pant et al., 2001; Chinoy et al., 2004; Pant et al., 2004). Similar toxicity was caused by arsenic if it was given through i.p route (Sarkar et al., 2003). It was also observed that the arsenite results in the reduction of gonadotrophin and testosterone (Sarkar et al., 2003; Chinoy et al., 2004). All above studies clearly demonstrated effect of arsenic on brain, germ cells, and pituitary (Sarkar et al., 2003).

Arsenite treatments in mouse alters cholesterol metabolism, spermatogenesis, and testicular testosterone concentrations (Chinoy et al., 2004). Similarly, male mice exposed to oral doses of arsenic trioxide at concentration of 0.5 mg/kg induces significant increase in cholesterol levels along with reduction in testicular proteins. The structure of the testes was also damaged, testicular function of testicular enzymes, and serum testosterone concentrations were reduced (Kabbaj et al., 2003).

It was observed that the male mice who acquired arsenic in drinking water exhibited reproductive toxicity (Pant et al. 2001). Adverse effects of arsenite on male reproductive system after prolong exposure through drinking water was observed by Pant et al. (2004). When rats, and mice were exposed to sodium arsenite 533.90 μmol/L through drinking water for 35 days then the process of spermatogenesis, concentration of testosterone and gonadotropins were altered in the blood plasma of exposed rats (Sarkar et al. 2003). When wistar rats were given different concentrations of sodium arsenite for 26 days through i.p injections, higher doses caused prominent weight reduction in the testes and accessory sex organs along with reduction in the epididymal sperm count. There also occurred a prominent reduction in the concentration of FSH, testosterone and LH in blood plasma. Reduction of sperm count was due to the fact that the spermatogenesis stages are much susceptible to testosterone concentration. Higher doses of arsenic also induced substantial degeneration of sperms at stage VII. It appears therefore that arsenic toxicity is responsible for the reduction of blood levels of LH and FSH which may cause the alterations in the testosterone synthesis and reduced testosterone concentration is responsible for sperm degradation. Besides these, arsenite acts on the pituitary gland and brain to alter the FSH and LH levels and affects the process of sperm formation. It was also observed that the arsenic treated rats have high corticosterone level in the serum, and it may be a possible reason for reduced serum

concentrations of testosterone, FSH, and LH (Vreeburg et al., 1988; Biswas et al., 1995; Hardy et al., 2005).

Swiss albino mice were exposed $NaAsO_2$ (53.39 μmol/L) through drinking water for one year. A significant reduction occurred in the testicular weight along with no significant decrease in the weight of accessory sex organs and epididymis. Overall, sperm motility and sperm count were reduced, and the amorphous sperm count was high. Besides all these alterations the function of testicular enzymes also changed. For example, enzymes involved in the post-meiotic spermatogenic cells exhibited alterations. The functions of acid phosphatase and sorbitol dehydrogenase were reduced. It has been suggested that the reduced function of 17β-HSD might have been decreased level of plasma gonadotrophins which was also observed in rats exposed to $NaAsO_2$ via i.p injection (Sarkar et al., 2003).

Sub-chronic exposure of male mice to $NaAsO_2$ through drinking water resulted in a reduction of testicular weight and epididymal sperm counts (Im-Chang et al., 2007). In the same way, arsenic sub-chronic exposure in rats induced reduction in testicular, and accessory sex organs weight, epididymal sperm count was decreased along with the degeneration of germ cells in spermatogenic cycle stage VII (Jana et al., 2006). In many studies, the dose dependent reduction in seminiferous tubular diameter was also seen. Besides this, the reduction was observed in different gametogenic cell populations, for example pachytene spermatocyte, resting spermatocyte and mature sperm (Sanghamitra et al., 2008). Arsenic toxicity in male mice and rat is also related to reduction in plasma testosterone concentration in gonadotropin levels (Sarkar et al., 2003; Chinoy et al., 2004; Pant et al., 2004).

When $NaAsO_2$ (4, 5, or 6 mg/kg) was administered to male rat through i.p injection for 6 days caused a significant decrease in the sperm count, testicular, and accessory sex organ weights. The level of plasma testosterone LH, and FSH concentrations were also reduced in arsenic treated rats. This proved that along with sex organs arsenic also effects the pituitary gland, brain and germ cells (Jana et al., 2006).

*In vitro* reports demonstrated that major outcome of arsenic exposure is apoptosis in the somatic sertoli cells and testicular germ cells (Celino et al., 2009; Kim et al., 2011). Studies on Japanese eel showed that low dose of arsenic (0.1~1 μM) hinders the steroidogenesis and spermatogenesis, whereas high dose (100 μM) of arsenic results in

germ cell apoptosis induced by oxidative stress (Celino et al., 2009). Mouse TM4 sertoli cells exposed to 10 µM concentration of arsenic trioxide systematically results in ROS-related apoptotic cell death and cytotoxicity (Kim et al., 2011).

To date, the information on *in vitro* reproductive effects of $NaAsO_2$ are missing on both prepubertal and adult male mice. Therefore, this study was designed to assess rapid and short-term effects induced by $NaAsO_2$ on the reproductive organs of male mice. Similarly, the postnatal development and function of mice were studied after sub-chronic and chronic oral exposure of $NaAsO_2$.

## 1.10. Aim

The aim of the present study was to investigate the reproductive toxicity following exposure to $NaAsO_2$ under *in vitro* and *in vivo* conditions in prepubertal (including infantile) and adult male mice.

## 1.11. Objectives

- Analyses of *in vitro* toxic effects and alterations induced by $NaAsO_2$ in prepubertal (infantile) and adult male mouse testis and comparing the toxicity in immature vs. adult testis cultures.
- Analyses and comparison of *in vivo* sub-chronic and chronic exposure of $NaAsO_2$ in male mice from PND 25.

# CHAPTER # 2

# *IN VITRO* PREPUPERTAL (INFANTILE) MOUSE TESTICULAR CULTURES

## Abstract

Arsenic exposure has greatly increased over the past few years, particularly contamination of drinking water with arsenic has caused numerous problems. In the present study, the direct effects of $NaAsO_2$ on prepubertal mouse testis using an *in vitro* testicular organ culture system were investigated. Three experiments were conducted using PND 5 Balb-c mice testes in an *in vitro* culture system, each experiment was repeated six times. Prepared sodium arsenite solution (Merck, Germany) was used to prepare the final concentrations through serial dilutions in the medium. Approximately 1 $mm^3$ PND 5 testicular pieces were placed on nucleopore membranes, floating on 1ml of α-MEM and 10% KSR. The culture plates were incubated at 34°C in 5% $CO_2$ incubator. Cultures were left untreated for 24 h (Day1). Media were changed after 24 h to provide fresh nutrients to the growing culture. First experiment comprised of 1-day (24 h) exposure of 0.01, 0.05, 0.1, 0.5, and 1 µM doses of $NaAsO_2$. Whereas in the second experiment and third experiment $NaAsO_2$ doses were administered for 6 days in the prepubertal (infantile) testicular cultures. In second experiment 0.1, 0.5, and 1 µM doses of $NaAsO_2$ were used while in the third experiment, the cultures were exposed to 10, 50, and 100 µM $NaAsO_2$ doses. All cultures were fixed on day 7 and proceeded for hematoxylin and eosin (H&E) staining and double immunohistochemistry for cleaved caspase-3 (CC3) and Mouse Vasa homologue (MVH) for determining apoptosis and germ cells respectively. Results showed that *in vitro* exposure of $NaAsO_2$ for 1 and 6 days in experiment 1 and 2 did not cause any change in the testicular morphology, germ cells density, or cleaved caspase3 expression. However, exposure of PND 5 testis to high concentrations of $NaAsO_2$ in third experiment (10, 50, and 100 µM) induced drastic changes. Germ cells number was reduced in a dose dependent manner, while CC3 expression increased hundred-fold upon exposure to 50 µM $NaAsO_2$ exposure. It is concluded that $NaAsO_2$ treatment at higher concentrations induces a dose-dependent loss of germ cell and apoptosis induction in prepubertal (infantile) mouse testis.

## 2.1. Introduction

Arsenic is a natural metalloid ubiquitously present in soil, water and food (Huang et al., 2002; Duker et al., 2005). Toxicity of arsenic is defined by its binding state. Organic arsenic compounds are less toxic than inorganic arsenic compounds. Usually, natural water reservoirs contain trivalent ($As^{3+}$) and pentavalent arsenic forms ($As^{5+}$) (Feng et al., 2001). Out of these, trivalent arsenic is more toxic (Styblo et al., 2000; Vega et al., 2001).

In various developing countries arsenic poisoning has become severe and escalating problem. Contamination of drinking waters with arsenic occurs from natural environmental sources seeping into aquifers, mining and other industrial activities (Sulanjari et al., 2015). Over the past few decades, arsenic exposure through drinking contaminated water has vastly increased in Bangladesh, India, China, and Cambodia (Smith et al., 2003; Xie et al., 2011). Another latest report has shown that the risk of arsenic exposure has become worse in Pakistan, exposing nearly 50 million people to elevated concentrations of arsenic through groundwater used for drinking purpose (Podgorski et al., 2017).

Exposure of males to inorganic arsenic has been shown to produce reproductive dysfunction in both humans and animals. Inorganic arsenic alters spermatogenesis, interrupts steroidogenesis and decreases blood gonadotropins and testosterone concentrations (Kim and Kim, 2015) causing reproductive dysfunctions (Zubair et al., 2017). Most studies investigating the reproductive toxicity of arsenic focused upon its *in vivo* effects on adult mouse reproductive system (Reddy et al., 2011; Li et al., 2015). Some *in vitro* studies have assessed the effects of arsenic on embryonic development and reproduction. For instance, TM4 Sertoli cells have shown to undergo apoptosis upon arsenic trioxide exposure (Kim et al., 2011). Another *in vitro* study exhibited toxic effects of $NaAsO_2$ on mouse embryonal development (Muller et al., 1986). Chaineau et al. (1990) found that $NaAsO_2$ induced more embryotoxic effects *in vitro* as compared to sodium arsenate.

At present, there are no available studies that have examined the effects and targets of arsenic on prepubertal testes. Prepubertal testicular development is a highly sensitive stage and toxicants such as arsenic may likely act differently on young gonads as compared to adult testes. It is therefore essential to understand arsenic toxicity in

premature testes in terms of its effect on testes. In this study, the effects of $NaAsO_2$ exposure on immature PND 5 mouse testes using an *in vitro* culture system.

## 2.2. Materials and Methods

### 2.2.1. Animals

This part of the research work was carried out at The University of Edinburgh, Scotland U.K. It was approved by the Local Ethical Review Committee of the University of Edinburgh and conducted according to the UK Home Office regulations. Balb-c male mice were kept at 14 h:10 h light and dark photoperiod. They were provided food and water ad libitum. Mice were dissected on PND 5. The day of the pup delivery was considered as post-natal day 0 (PND 0).

### 2.2.2. Tissue culture

Testicular tissues were cultured as described by Lopes et al. (2016). In each culture run, at least two PND 5 mice were dissected and testes were placed into dissection medium Leibovitz L-15 (Invitrogen, UK) containing 3 mg $ml^{-1}$ bovine serum albumin (BSA; Sigma-Aldrich Ltd, UK) at 37°C and further sliced into approximate pieces of 0.5 $mm^3$. Each piece was moved to Whatman nucleopore membrane placed in a 24 well plate (Greiner Bio-one, UK). Each well contained 1ml α-MEM medium (Invitrogen, UK) additionally supplemented 10% Knockout Serum Replacement (Invitrogen, UK). The culture plates were placed in a humidified incubator at 34°C and 5% $CO_2$. Cultures were kept untreated for first 24 h (Day1).

On day 2 of culture, the testicular tissues were treated with different concentrations of sodium arsenite solution (Merck, Germany). Sodium arsenite solution was dissolved into culture medium and final concentrations were prepared through serial dilutions. Media were changed after 24 h to provide fresh nutrients to the growing culture. An *in vitro* study reported by Muller et al. (1986) on mouse embryos was used as a reference for the selection of doses.

Cultures were fixed on day 7. For determination of cell proliferation, 15 µg $ml^{-1}$ Bromodeoxyuridine (BrdU, Sigma Aldrich, UK) were added to culture media three hours before fixing. Cultures were then fixed for 60 min in 10% neutral buffered formalin (Sigma Aldrich, UK) for Immunohistochemistry (IHC), and in Bouin's fluid

for histological examination. Tissues were placed in 3% agar (Sigma Aldrich Ltd, UK) and embedded in paraffin wax.

### 2.2.3. Tissue Histology

Prepubertal (infantile) testicular tissues cultures were prepared for histological analysis. Following steps were performed

#### 2.2.3.1 Fixation

Bouin's solution (Sigma Aldrich, UK) was used for fixing cultures for 60 min. Dehydrated in ascending grades of ethanol (70%, 90%, 95% and 100%) by keeping them for 60 min in each grade at room temperature. Tissues were finally placed in absolute ethanol for 120 min, cleared for 60 min in Xylene I, then 120 min each in Xylene II and III. Paraffin wax (pre melted to 60°C) infiltration was carried out three times for 60 min each and finally embedded in fresh molten paraffin wax.

Tissues were embedded to make blocks, which were trimmed and thin sections (5 µm) were cut on a microtome (Leica RM2255, Germany). Tissues sections were taken on clean glass slides and kept in an incubator at 37°C.

#### 2.2.3.2. Staining

For Hematoxylin and Eosin staining (H&E), sections were dewaxed in xylene I and II for 20 and 5 min respectively. These were then hydrated in decreasing alcohol grades (100%, 90%, and 70% alcohol) for 1 min dipped in lithium carbonate (in 70% alcohol) for 1 min, washed for 5 min in running tap water, and stained with Hematoxylin for 3 min. After washing in running water and the sections were kept in acidified alcohol for 1 min, washing was done again for 5 min and dipped for 3 min in Scott's tap water substitute (STWS). Washing was again done in water for 3 min, the slides were dipped in 1% Eosin for 2 min and then dipped in running water. Slides were kept in Potassium Alum for 2 min and again washed for 5 min in running water. These slides were then dehydrated in increasing grades of alcohol (70%, 90% 95%, and absolute alcohol) for 1 min each, cleared in xylene, and finally mounted in DPX and cover slipped.

#### 2.2.3.3. Photomicrography of H & E slides

Sections were photographed using a light microscope, (Leica Microsystems Ltd, UK) observed at 20x, and 40x magnification. Sections were examined for

histomorphometric analysis, the seminiferous tubular diameter, area of seminiferous tubules, lumen area were examined using the Image J software (image4j-0.7.jar, USA).

### 2.2.4. Immunohistochemistry

For immunohistochemistry, tissues were fixed in 10% neutral buffered formalin (Sigma Aldrich, Co. UK) for 60 min. After setting the tissues in 3% agar, these were processed for embedding as described above. Tissue sections (5 µm thick) were cut on a microtome and taken onto charged polysine slides (Thermofisher, UK). Slides were left overnight in an incubator. Double immunohistochemistry was carried out to study:

(a) Apoptosis by using cleaved caspase-3 (CC3) marker and,
(b) all germ cell population in the testicular fragment by using MVH markers (Table: 1 and 2).

#### 2.2.4.1. Double immunohistochemistry

For performing double immunohistochemistry, small tissue sections were encircle using a diamond pen. After dewaxing in Xylene-I and II for 10 min each, slides were dipped in decreasing alcohol grades (100%, 95%, 90%, and 70%) for 1 min in each. Slides were rehydrated in phosphate-buffered saline (PBS) (Fisher Scientific UK Ltd, UK). After rehydration, 0.01 M of citrate buffer, pH 6.0 (Sigma Aldrich Ltd, UK) was used to equilibrate slides for 5-10 min in and then microwaved for 4×5 min on 'high' setting (double distilled $H_2O$ 50 ml was added between each run). The slides were allowed to cool down for at least 20 min and then equilibrated in PBS with 0.1% Triton X (PBSTx). Tissue sections were isolated using a liquid blocker PAP pen (Abcam, UK). In a moist atmosphere, blocking solution (5% BSA, 20% normal goat serum, in PBST) was applied to the sections and kept at room temperature for 1 h. Slides were then quickly rinsed in PBS and primary antibodies (i) Cleaved Caspase 3 (CC3 Cell Signaling Technology, USA) and (ii) MVH/VASA (DDX4, Abcam, UK), diluted in 5% BSA and 10% Goat serum were applied and overnight incubated in dark at 4°C. On the next day, slides were rinsed 2 × 5 min in PBST and secondary antibodies (i) Polyclonal goat anti-rabbit Ab biotinylated (DakoCytomation, Denmark) and (ii) Alexa Flour Goat anti-mouse-568 (Invitrogen, UK) were applied onto the sections taking care to protect from light as much possible. After 1 hour, slides were washed 2 × 5 min in PBST. After washing, Alexa flour streptavidin (Life technologies, USA) was applied on the slides for 30 min at room temperature. After this, the slides were again washed

2 × 5 min in PBST. After washing, the slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, UK) at 1:10,000 dilution for 2 min. The slides were rinsed in PBS for 5 min and mounted with Vectashield hard-set mounting medium (Vector Laboratories, USA).

Table 1: Primary antibodies used for double immunofluorescence

| **Primary antibody** | **Catalogue** | **Species** | **Dilution** |
|---|---|---|---|
| Cleaved Caspase3 (CC3) | Cell Signaling Technology, USA. ASP 175 | Rabbit | 1:500 |
| MVH/ VASA(DDX4) | Abcam, UK Ab27591 | Mouse | 1:100 |

Table 2: Secondary antibodies used for Double immunofluorescence

| Secondary antibody | Catalogue | Dilution | Visualization |
|---|---|---|---|
| Polyclonal goat anti-rabbit Ab biotinylated | Dako Cytomation, E0432 | 1:200 | Streptavidin Alexa Fluor 488nm conjugate (Life technologies, USA, S11223) |
| Alexa Fluor Goat anti-mouse- 568 | Invitrogen, UK-A11004 | 1:200 | N/A |

### 2.2.4.2. Imaging and analysis

Images were captured using DFC369FX camera on a Leica DM5500B microscope (Leica, Germany). Image J software (image4j-0.7.jar, USA) was used for image analysis by using blind to treatment assessment. For CC3, fluorophore area evaluated was measured as a percentage of section or tubule, as appropriate. Germ cells $MVH^+$ cells were analyzed by manual counting.

### 2.2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism (5.01, CA USA). Statistically significant difference between control group and arsenic treated groups was determined by one-way ANOVA followed by Dunnett's post-hoc test. For analyses including only 2 groups, paired sample t-test was performed. Data are expressed as mean ± S.E, $p < 0.05$ was considered statistically significant difference.

### 2.3. Experiment-I

In this first experiment, testicular cultures were prepared according to the method explained in the methodology section above. On culture day 2, testicular fragments were exposed to 0.01, 0.05, 0.1, 0.5, and 1 µM $NaAsO_2$ concentrations for 1 day. After 24 h of arsenic exposure, the nucleopore membranes carrying the testicular fragments were shifted to a new plate containing drug free media. Media were changed after every 24 h in order to provide fresh nutrients to the growing culture. Cultures were fixed on day 7 and proceeded for histological and immunohistochemistry analysis. This experiment was replicated six times.

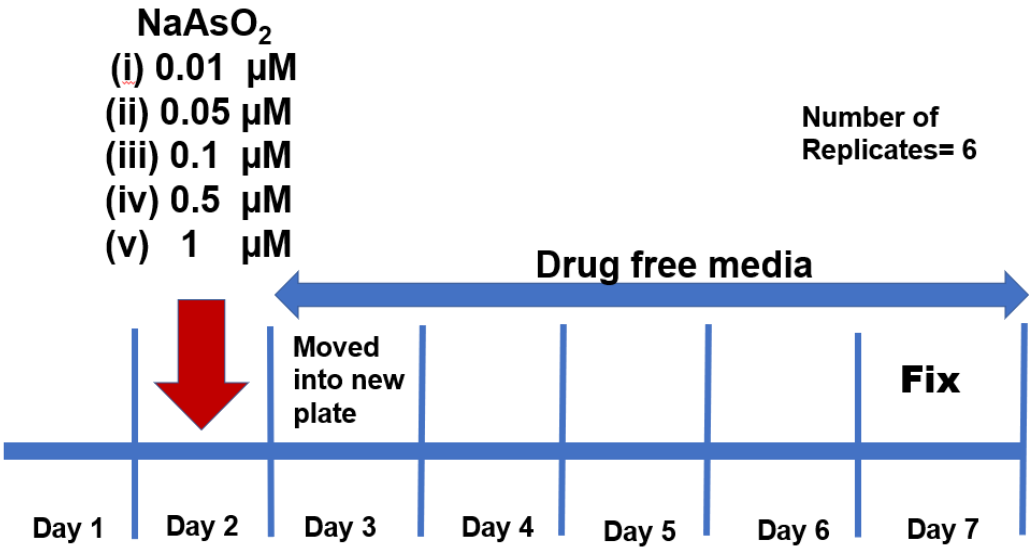


Fig 2.1. Schematic diagram of *in vitro* (infantile) mouse testis cultures with 1-day (24 h) $NaAsO_2$ exposure in a 7 days culture (blue arrow shows: drug free media, red arrow shows $NaAsO_2$ exposure).

### 2.3.1 Results

#### 2.3.1.1. Morphological analysis

Testicular fragments exposed to different concentrations (0.01, 0.05, 0.1, 0.5, 1 µM) of $NaAsO_2$ for 1day (24 h) showed normal testicular morphology with numerous germ cells present in the seminiferous tubules. None of the tested concentration of $NaAsO_2$ caused any damage to testes after 24 h exposure. In every treated testicular fragment, normally growing and dividing germ cells was observed (Fig. 2.2). Mean tubular diameter of seminiferous tubules was found non-significant (P = 0.6997) among all groups (Fig 2.3). Similarly, no change was noticed in the interstitial space of the tissues when compared to the control group (P = 0.88) (Fig. 2.4).

#### 2.3.1.2. Germ cells density

Germ cells in the prepubertal (infantile) testicular culture are represented by red color in Fig. 2.5 In comparison to the control tissue, the germ cell density per tubule and section area of treated tissues remained unaffected in experiment 1 (P-value 0.91 and 0.99 respectively) (Fig. 2.6 and 2.7). No statistical difference was detected in germ cells density after 24 h exposure of $NaAsO_2$ to an *in vitro* prepubertal testicular culture.

#### 2.3.1.3. Analysis of apoptosis

In experiment 1, the CC3 expression was not noticeable in the treated tissues and no difference could be determined when compared with the control tissues (Fig. 2.5). Image analysis revealed that 0.06, 0.053, 0.057, 0.066, and 0.061% of the seminiferous tubule area was covered by CC3 in the treated tissues with $NaAsO_2$ at 0.01, 0.05, 0.1, 0.5, and 1 µM concentrations respectively (Fig. 2.8). Similarly, the percentage section area covered by CC3 was 0.04 % in 0.01 µM treatment, 0.03% in 0.05 µM, 0.04% in 0.1, 0.5, and 1 µM $NaAsO_2$ doses (Fig. 2.9). P value of percentage CC3 expression per tubule area was 0.87, whereas the CC3 expression per section area was 0.95.

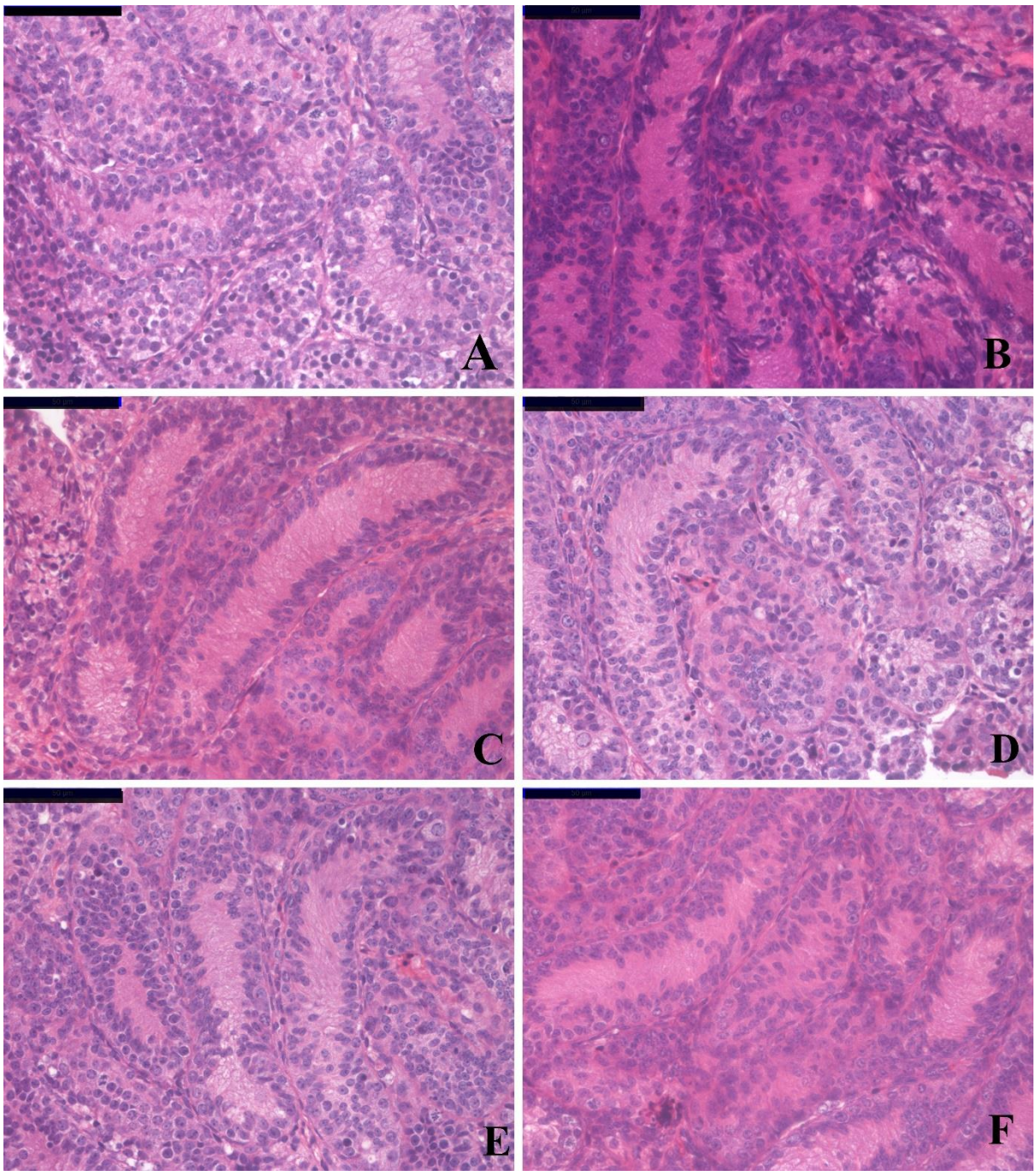


Fig. 2.2 H & E stained cultured (infantile) mouse testis fragments showing the effects of 1-day (24 h) exposure to different concentrations of $NaAsO_2$ on tissue morphology. (A) control tissue (B) 0.01 µM $NaAsO_2$ (C) 0.05 µM $NaAsO_2$ (D) 0.1 µM $NaAsO_2$ (E) 0.5 µM $NaAsO_2$ (F) 1 µM $NaAsO_2$. Scale bar = 50 µm.

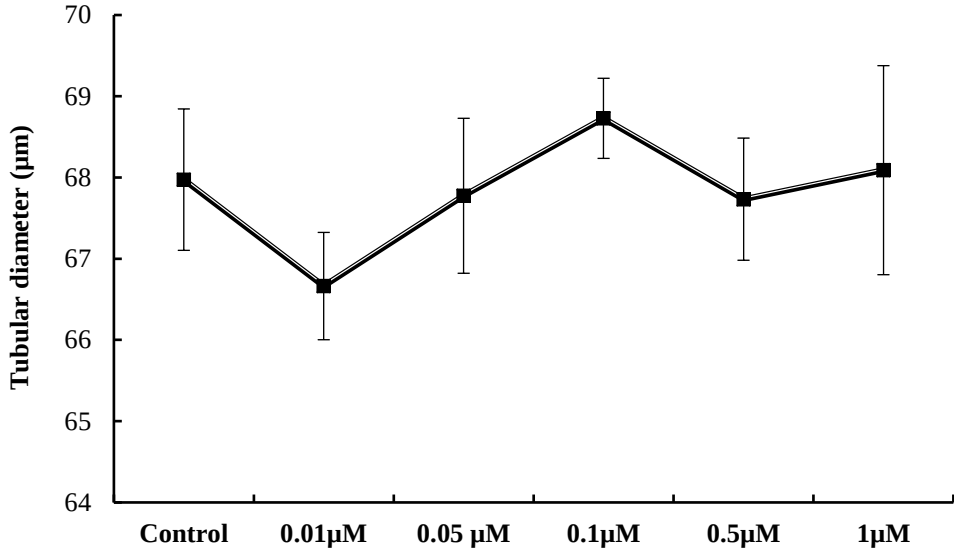


Fig. 2.3 Mean tubular diameter (µm) of seminiferous tubules in the prepubertal (infantile) mouse testicular culture tissues after 24 h exposure to different concentrations of $NaAsO_2$.

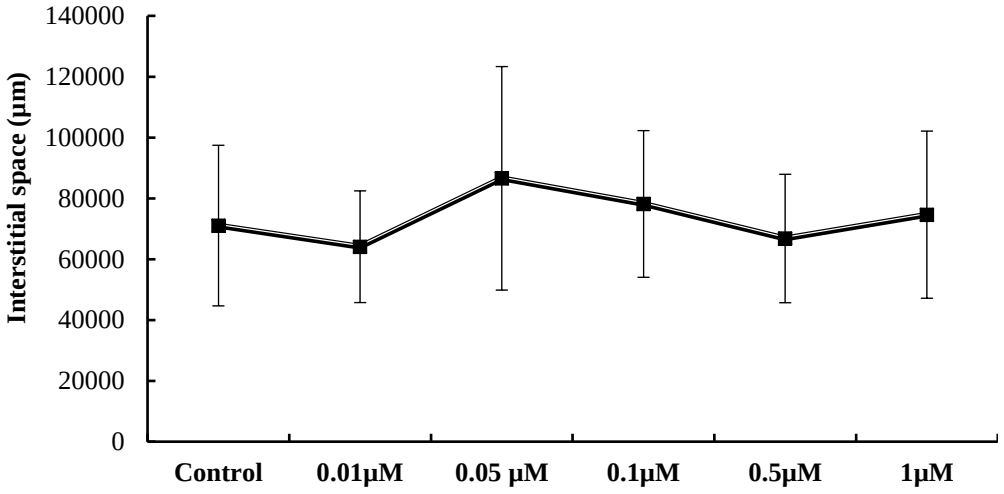


Fig. 2.4 Mean interstitial space (µm) of prepubertal (infantile) mouse testicular culture tissues after 24 h exposure of different concentrations of $NaAsO_2$.

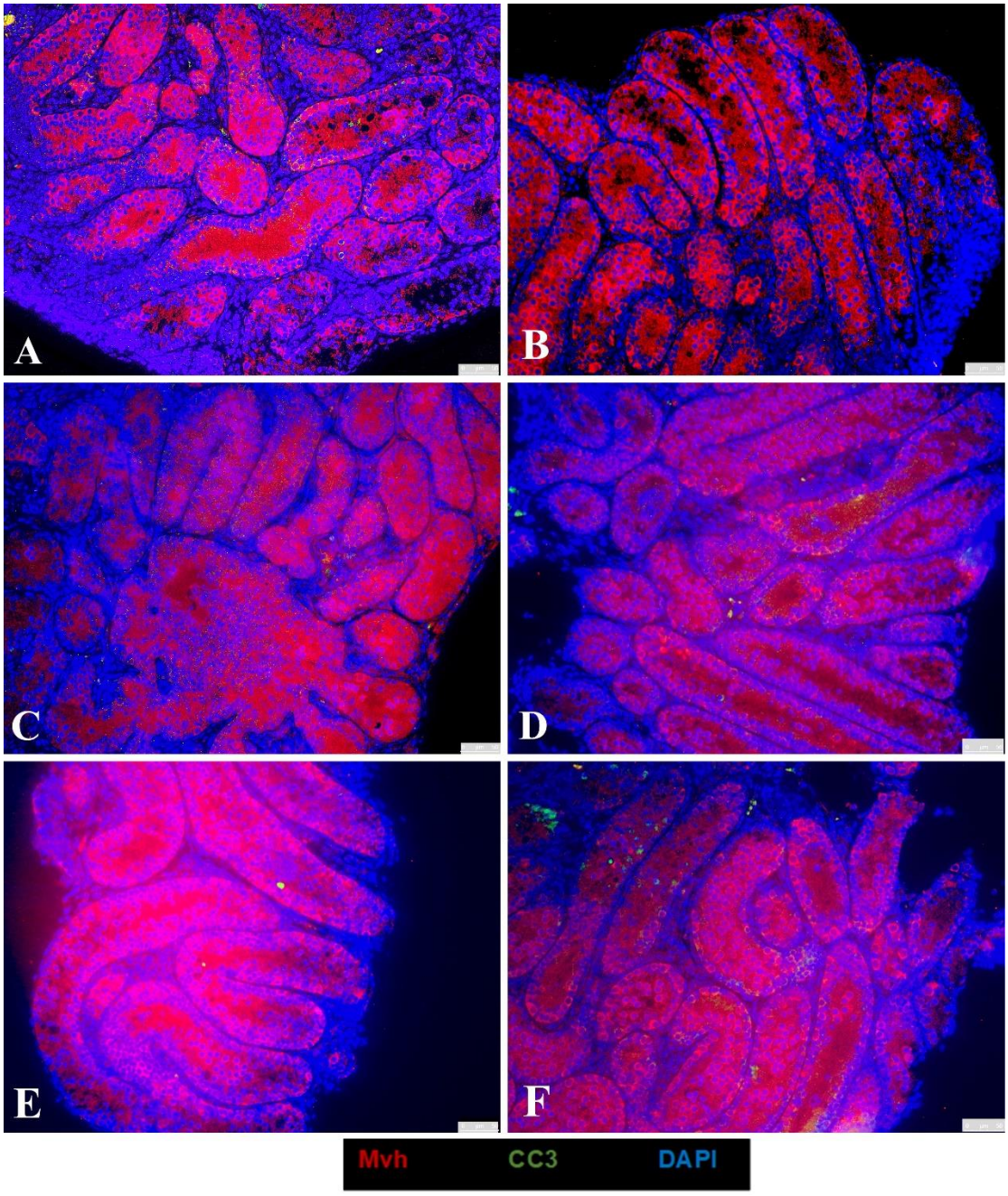


Fig. 2.5 *In vitro* exposure of prepubertal (infantile) mouse testis to different concentrations of $NaAsO_2$ for 1 day (24 h). Photomicrographs show immunohistochemical localization of MVH (red) and CC3 (green), counterstained with DAPI (blue) (A) Control (B) 0.01 µM $NaAsO_2$ (C) 0.05 µM $NaAsO_2$ (D) 0.1 µM $NaAsO_2$ (E) 0.5 µM $NaAsO_2$ (F) 1 µM $NaAsO_2$. Scale bar = 50 µm.

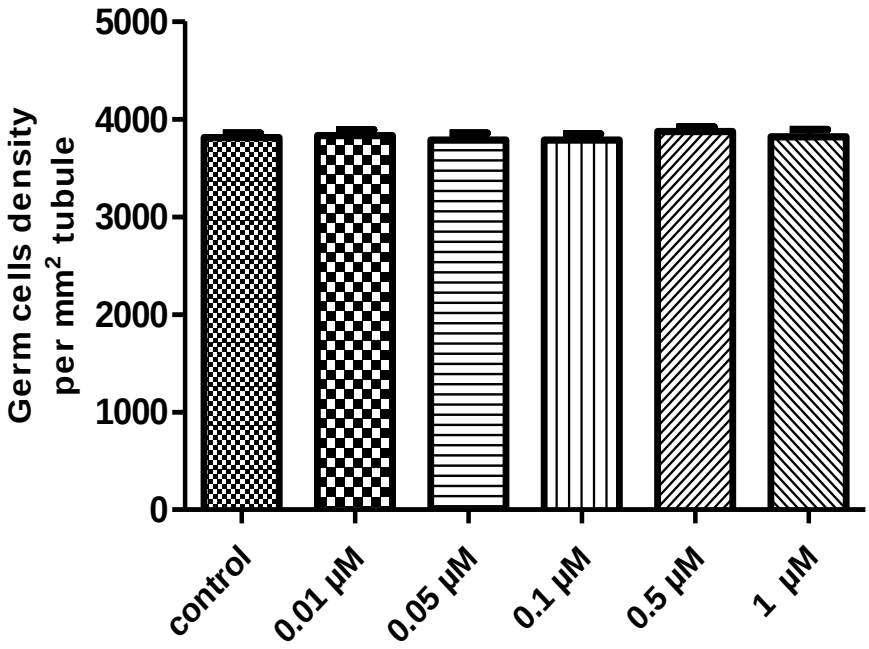


Fig. 2.6 Germ cells density per tubule area of seminiferous tubules after 24 h exposure of different concentrations of $NaAsO_2$ in prepubertal (infantile) mouse testes cultures.

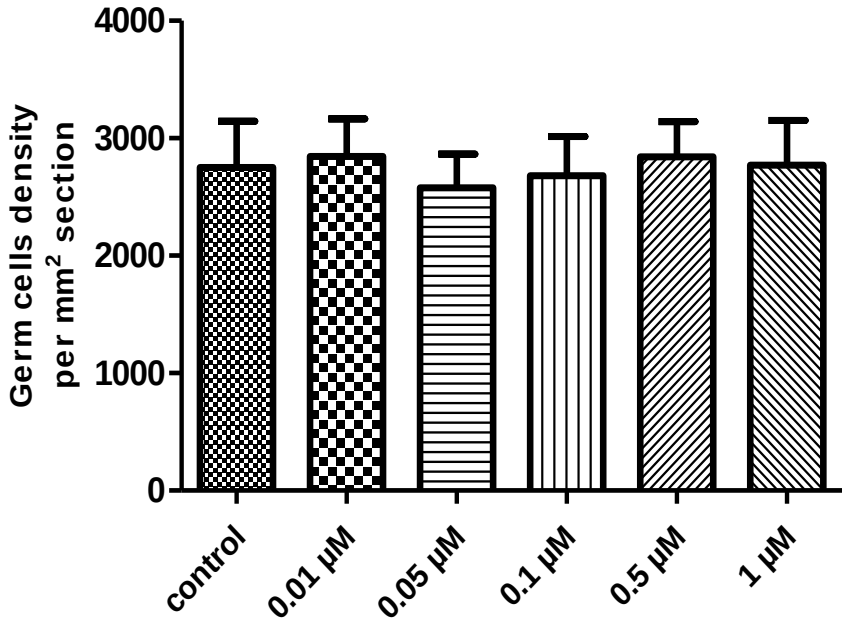


Fig. 2.7 Germ cells density per section area of testicular fragments after 24 h exposure of different concentrations of $NaAsO_2$ in prepubertal (infantile) mouse testes cultures.

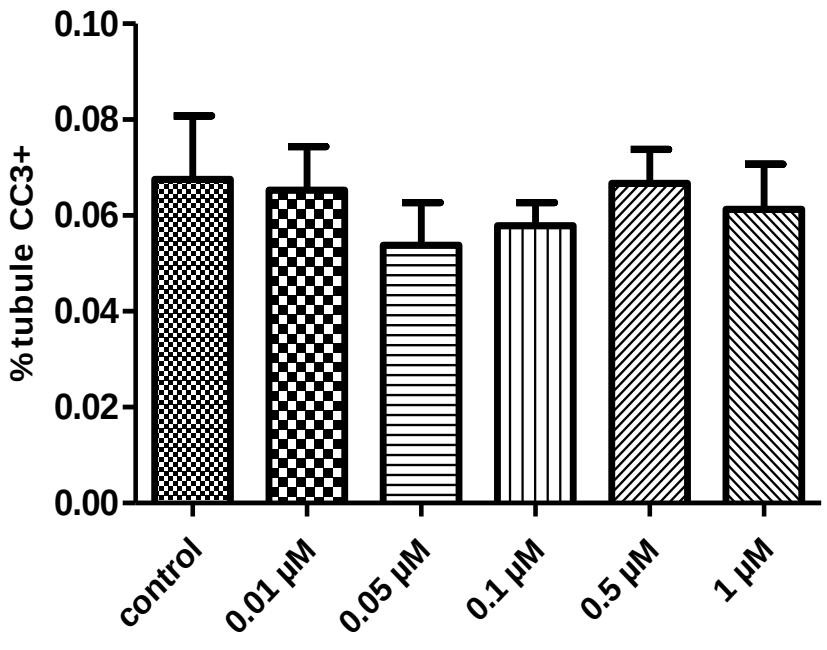


Fig. 2.8 Percentage CC3 expression per tubule area of seminiferous tubules after 24 h exposure of different concentrations of $NaAsO_2$ in prepubertal (infantile) mouse testes cultures.

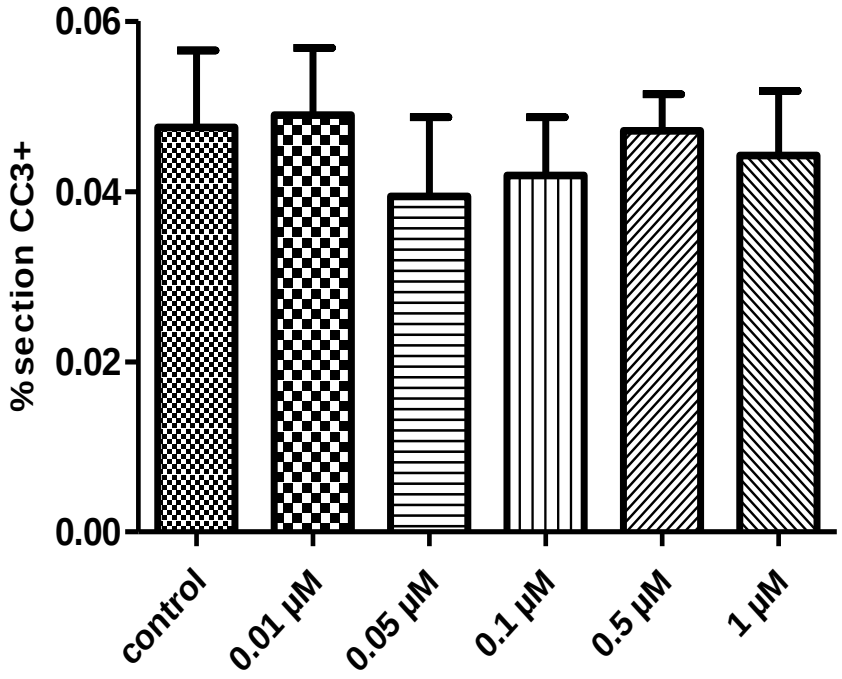


Fig. 2.9 Percentage CC3 expression per section area of testicular fragments after 24 h exposure of different concentrations of $NaAsO_2$ in prepubertal (infantile) mouse testes cultures.

## 2.4. Experiment- II

In the light of the results of experiments conducted above, another experiment was designed to observe the effects of previously used highest three doses of $NaAsO_2$ (i.e. 0.1, 0.5, and 1 µM). Testicular fragments were exposed to $NaAsO_2$ for 6 days in order to observe the effects caused on the prepubertal (infantile) testicular growth and compare it with the short term (1day) exposure. Cultures were set up and left in toxicant-free media for 24 h. On day 2, testicular fragments were exposed to different concentrations of $NaAsO_2$ (i) 0.1 µM, (ii) 0.5 µM, and (iii) 1 µM. Media were changed after every 24 h in order to provide fresh nutrients and $NaAsO_2$ to the testicular tissues. Cultures were fixed on day 7. Experiments were repeated six times. Histological and immunohistochemistry parameters were analyzed for its comparison with 1-day arsenic exposure effects.

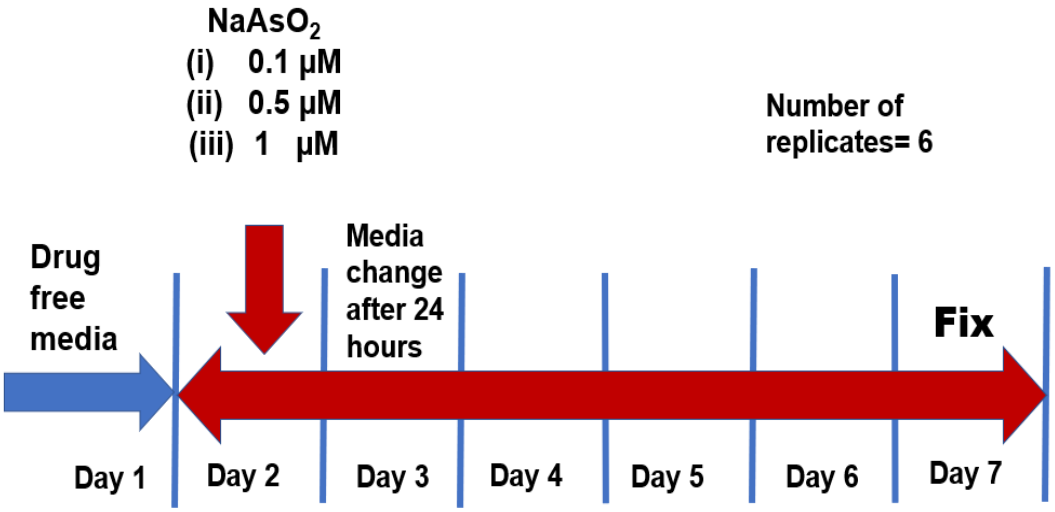


Fig. 2.10 Schematic diagram of *in vitro* (infantile) mouse testis cultures with 6 days $NaAsO_2$ exposure in a 7 days culture (blue arrow shows: drug free media, red arrow shows $NaAsO_2$ exposure).

## 2.4.1. Results

### 2.4.1.1. Morphological analysis

The morphology of control testicular fragments was well preserved at the end of seven days culture period. Seminiferous tubules structure and integrity were kept, with many germ cells lined along the basement membrane (Fig. 2.11). Exposure of prepubertal (infantile) testis culture to different $NaAsO_2$ concentrations for 6 days in this experiment did not cause any morphological changes in seminiferous tubules when compared to the control group. Seminiferous tubules maintained a physiological shape with spermatogonial germ cells abundantly present in tissues treated with arsenic doses (i.e. 0.1, 0.5 and 1 µM $NaAsO_2$) for six days. Seminiferous tubules diameter of the treated testicular fragments did not appear any different from the control group ($P = 0.59$) (Fig. 2.12). The interstitial space of the treated tissues was similar to the control tissue ($P = 0.79$) (Fig. 2.13).

### 2.4.1.2. Germ cell density

Several germ cells were also observed in the treated tissues (Fig. 2.14). No statistical difference between the germ cells density in the control and the treated groups was evident. Germ cells density per tubule area of the seminiferous tubules ($P = 0.73$; Fig. 2.15), and germ cells density per section area of the testicular fragments showed non-significant difference as compared to the control cultures ($P = 0.97$; Fig. 2.16).

### 2.4.1.3. Analysis of apoptosis

In this second experimental design with six days exposure to 0.1, 0.5 and 1 µM $NaAsO_2$ concentrations, the expression of CC3 was extremely low and similar to control tissue (Fig. 2.14). Image analysis revealed that 0.02 % of seminiferous tubule area in 0.1 µM dose and 0.03 % in 0.5 and 1 µM concentrations of $NaAsO_2$ was covered by CC3 ($P = 0.99$, Fig. 2.17). Similarly, analysis of percentage CC3 expression per section area of the tissues revealed that 0.02% of the testicular section expressed CC3 expression in all three $NaAsO_2$ doses ($P = 0.99$, Fig. 2.18).

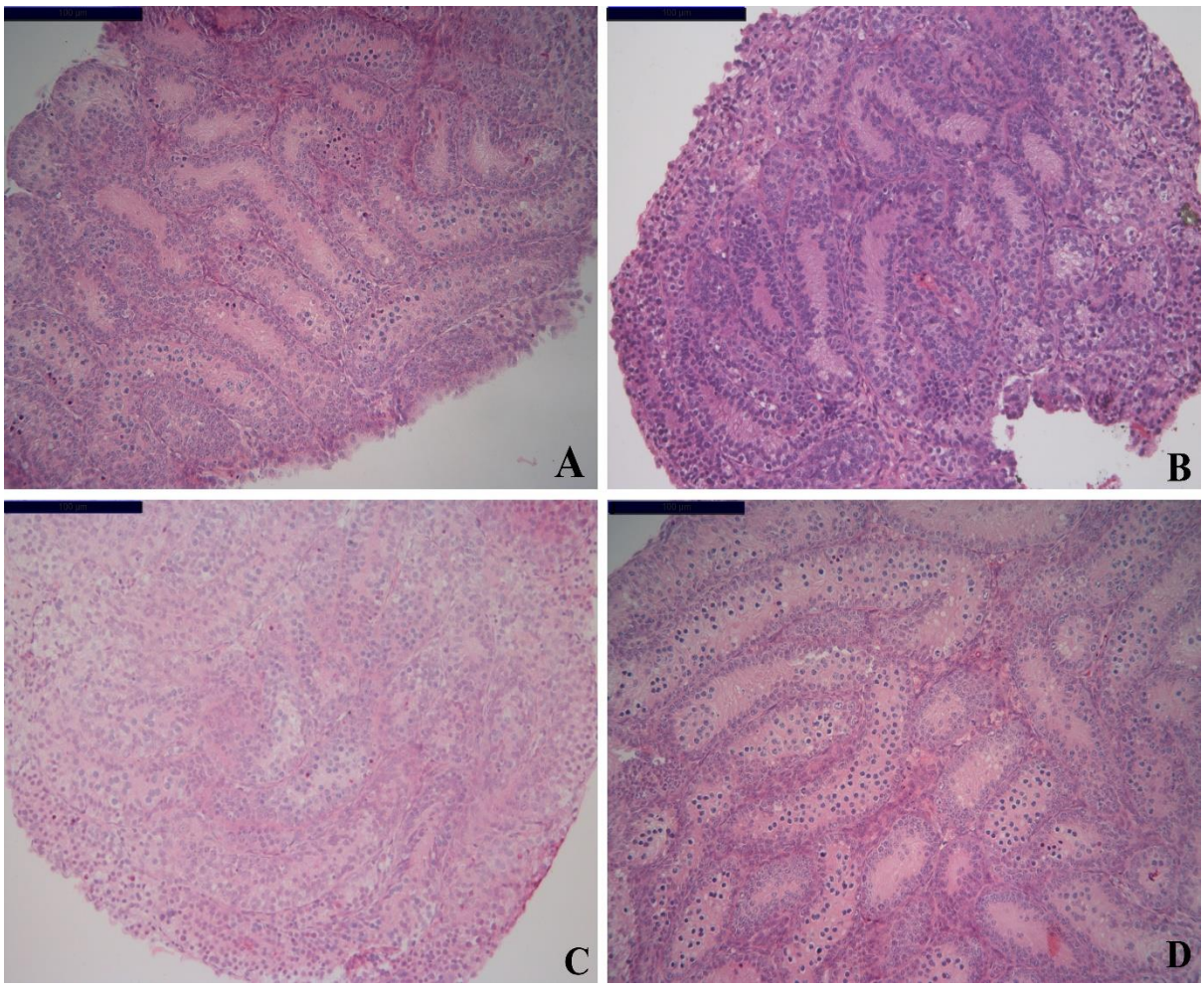


Fig. 2.11 H & E stained cultured (infantile) mouse testis fragments showing the effects of 6 days exposure of different concentrations of $NaAsO_2$ on tissue morphology. (A) control tissue (B) 0.1 µM $NaAsO_2$ (C) 0.5 µM $NaAsO_2$ (D) 1 µM $NaAsO_2$. Scale bar =100 µm.

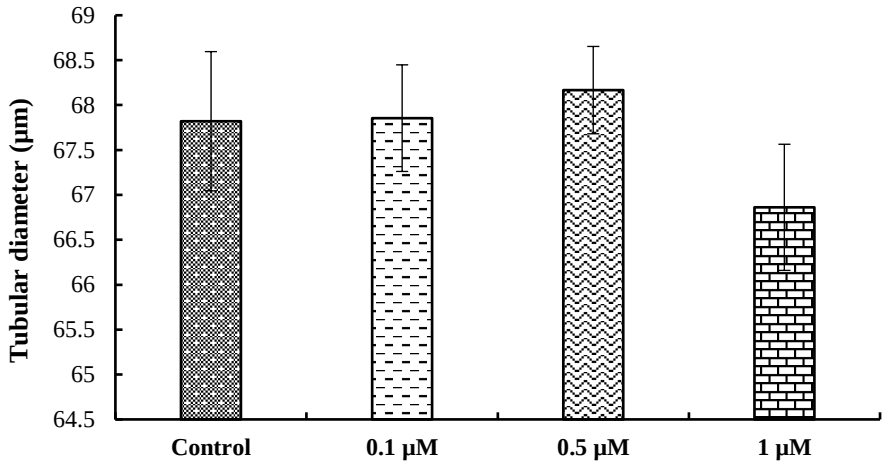


Fig. 2.12 Mean tubular diameter (µm) of seminiferous tubules after 6 days $NaAsO_2$ exposure in prepubertal (infantile) mouse testes cultures

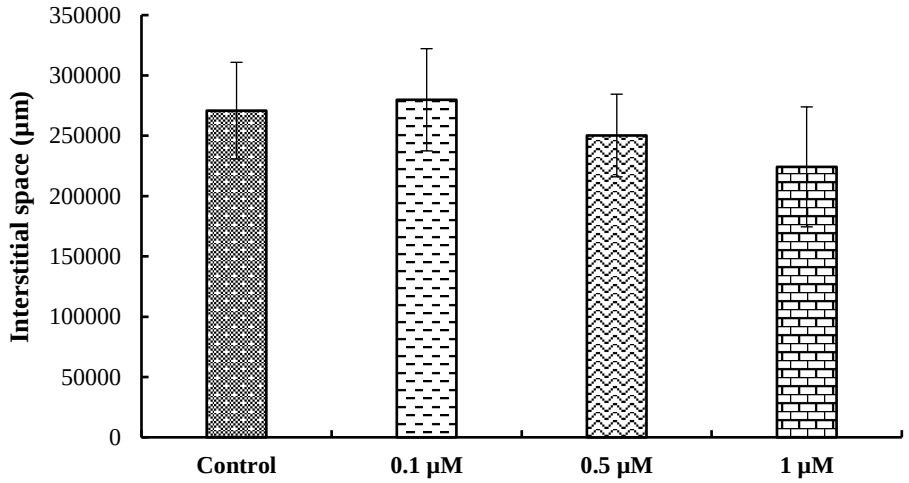


Fig. 2.13 Mean interstitial space (µm) of prepubertal (infantile) mouse testicular culture tissues after 6 days exposure of different concentrations of $NaAsO_2$.

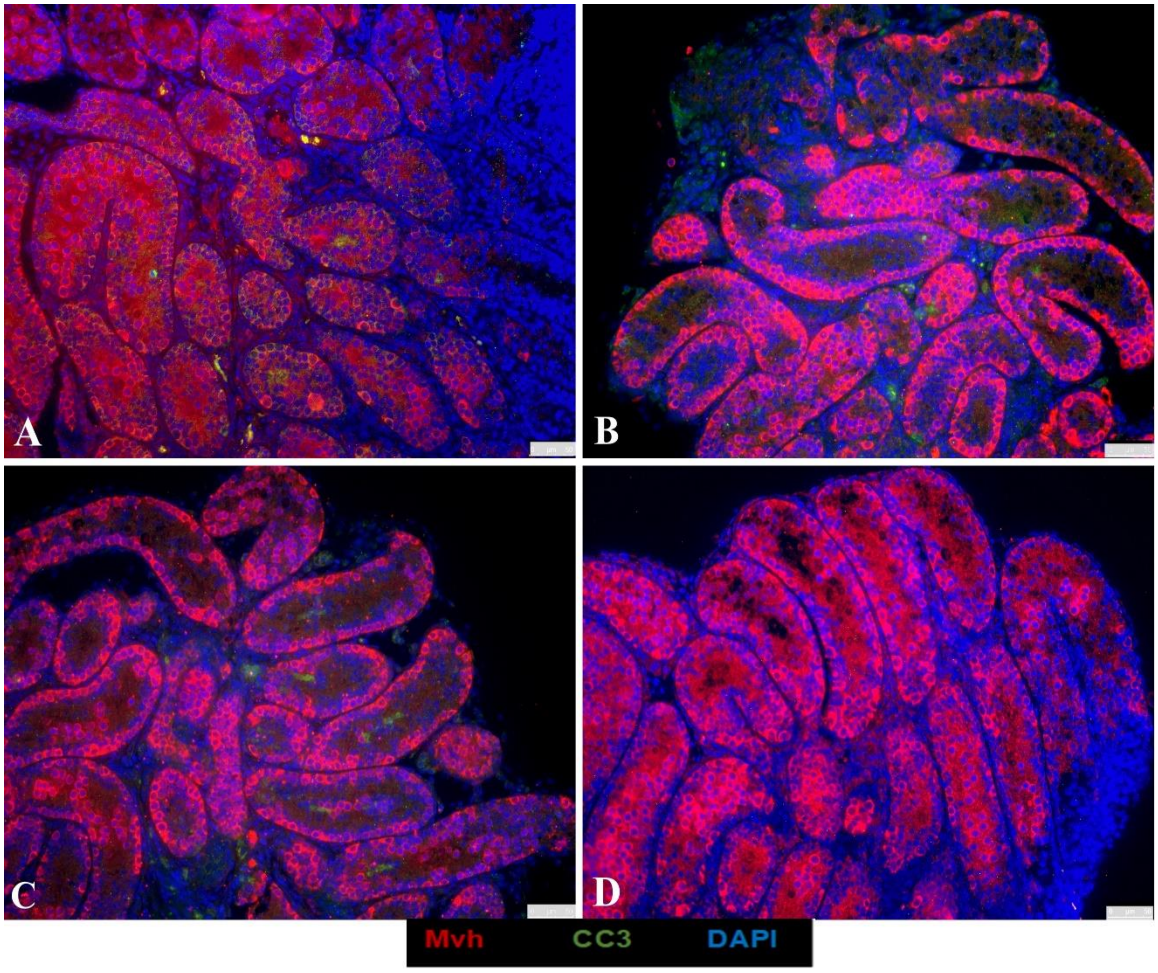


Fig. 2.14 Exposure of prepubertal (infantile) mouse testis to different $NaAsO_2$ concentrations for 6 days. Photomicrographs showing immunohistochemical localization of MVH (red) and CC3 (green), counterstained with DAPI (blue) (A) Control (B) 0.1 µM $NaAsO_2$ (C) 0.5 µM $NaAsO_2$ (D) 1 µM $NaAsO_2$. Scale bar =50 µm.

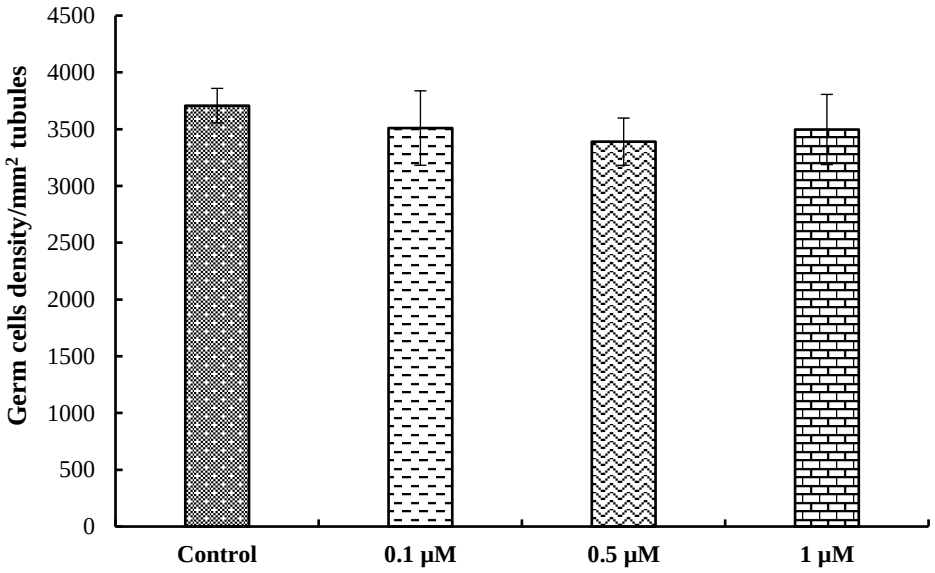


Fig. 2.15 Germ cells density per tubule area of seminiferous tubules in the prepubertal (infantile) mouse testicular culture after 6 days $NaAsO_2$ exposure.

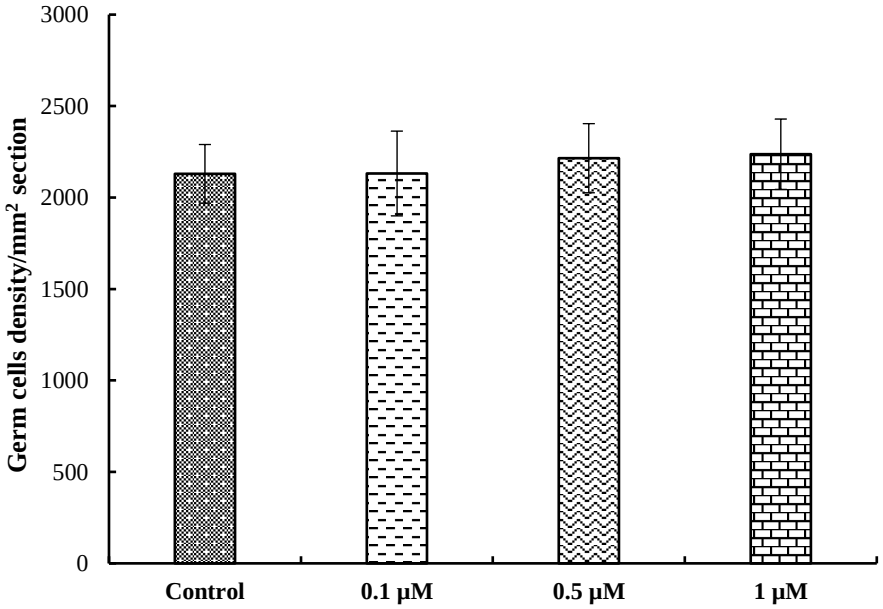


Fig. 2.16 Germ cells density per section area of prepubertal (infantile) mouse testicular fragments after 6 days $NaAsO_2$ exposure.

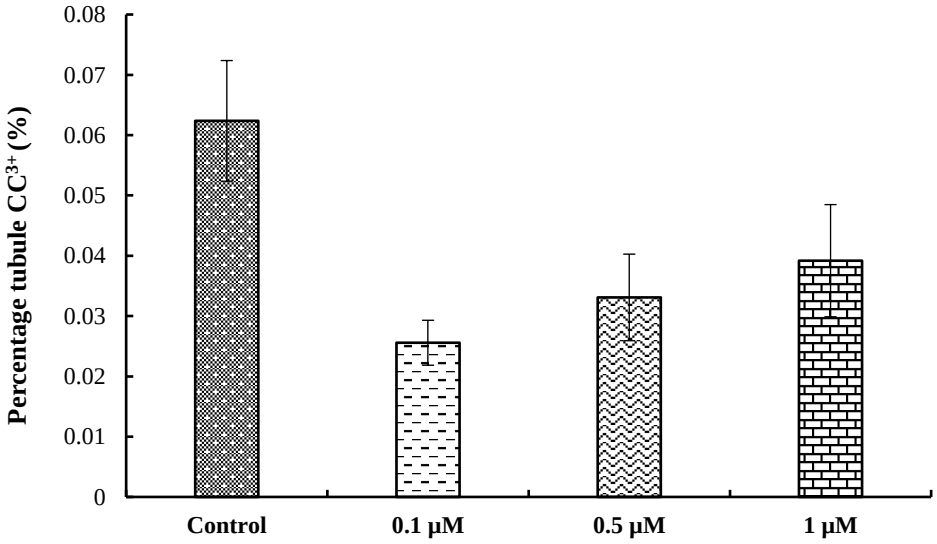


Fig. 2.17 Percentage CC3 expression per tubule area of seminiferous tubules after 6 days *in vitro* exposure of different concentrations of $NaAsO_2$ in prepubertal (infantile) mouse testes cultures.

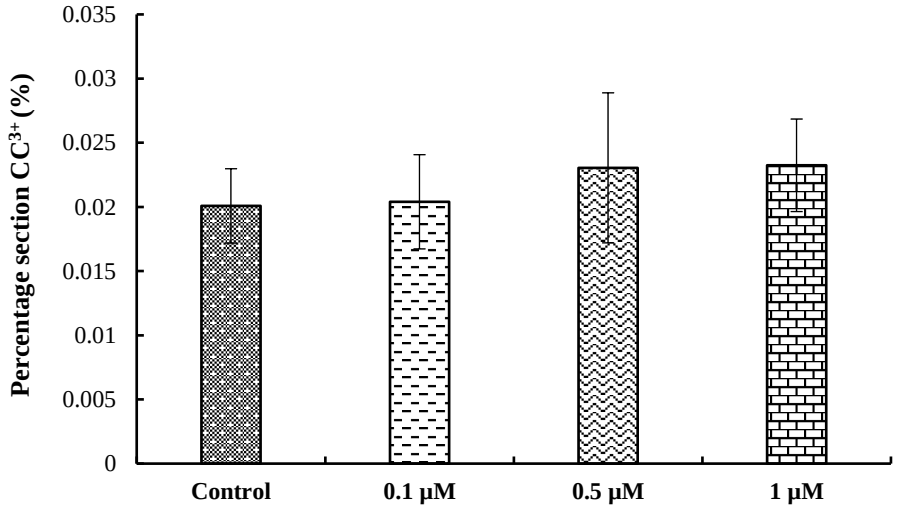


Fig. 2.18 Percentage CC3 expression per section area of prepubertal (infantile) mouse testicular fragments after 6 days *in vitro* exposure of different concentrations of $NaAsO_2$.

## 2.5. Experiment-III

Keeping in view the results of the above two designed experiments, a third experiment was designed to find out the effects of even higher doses (10 µM, 50 µM, and 100 µM) of $NaAsO_2$ on the prepubertal (infantile) mouse testis. Cultures were set up for 7 days with six days exposure to high doses of $NaAsO_2$. Testicular fragments were exposed on day 2 to high concentrations of $NaAsO_2$ for 6 days and media were changed after 24 h. Prepubertal (infantile) testis cultures were fixed on day 7. Experiments were repeated six times.

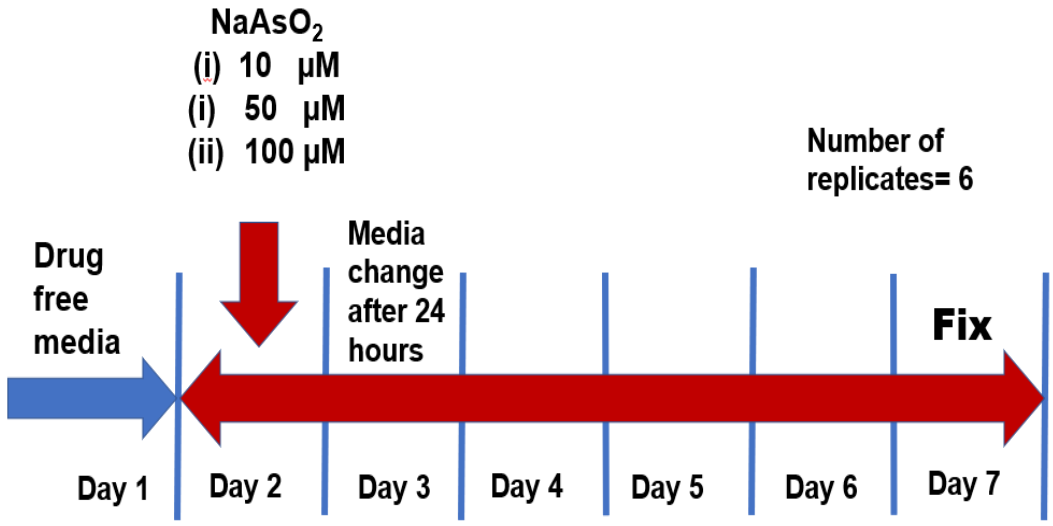


Fig. 2.19 Schematic diagram of *in vitro* (infantile) mouse testis cultures with 6 days exposure of high concentrations of $NaAsO_2$ in a 7 days culture (blue arrow shows: drug free media, red arrow shows $NaAsO_2$ exposure).

## 2.6. Results

### 2.6.1. Morphological analysis

High concentrations of $NaAsO_2$ caused damage to the prepubertal (infantile) testicular fragments. Histology showed reduced seminiferous tubular diameter after 10 µM $NaAsO_2$ exposure for 6 days, while higher concentrations of $NaAsO_2$ (50 and 100 µM) severely disrupted the normal testicular morphology. Seminiferous tubules were irregularly outlined with diffuse apoptotic/necrotic cells as evidenced by the dense dark staining of the chromatin and more intense pink/red staining of the cytoplasm (Fig. 2.20).

As the seminiferous tubules were destroyed upon exposure to higher $NaAsO_2$ doses (50 and 100 µM). The seminiferous tubules diameter could be determined for 10 µM concentration only and was compare with the control. Independent samples t-test presented highly significant difference between the two groups ($P < 0.001$; Fig. 2.21). However, a non-significant difference ($P = 0.44$) was noticed in the interstitial space of the control with the 10 µM $NaAsO_2$ treated prepubertal testicular tissues (Fig. 2.22).

### 2.6.2. Germ cells density

Upon exposure to high concentrations of $NaAsO_2$ for 6 days caused decrease in the germ cells density (Fig. 2.23). The germ cells and seminiferous tubules were distorted with 50 and 100 µM arsenic treatments. As the seminiferous tubules were destroyed after 50 and 100 µM $NaAsO_2$ exposure, it was not possible to determine germ cell density per unit tubule area. Germ cell density was therefore assessed relative to the whole testis section for these doses. Compared to control group germ cells density per tubule area was significantly reduced in 10 µM treated tissues ($P < 0.01$, Fig. 2.24). On the other hand, the germ cells density per testis section was approximately 2-fold decreased after 10 µM arsenic treatment while after 50 and 100 µM exposure, a 3-fold decrease was noticed ($P < 0.001$, Fig.2.25).

### 2.6.3. Analysis of apoptosis

Compared to the control tissue, a greater CC3 expression with a hundred-fold increase was observed at 50 µM $NaAsO_2$ dose (Fig. 2.23). Results showed that 1.9 % of seminiferous tubule area was covered by CC3 in 10 µM treatment group ($P < 0.001$). Whereas, 3.1 %, 10.5 %, and 2.9 % of the section area in 10, 50, and 100 µM treatment

groups respectively was covered by CC3, as compared to 0.1 % CC3 expression in the control tissue ($P < 0.001$). Peak CC3 expression, with a hundred-fold increase, was observed after 50 µM $NaAsO_2$ exposure (Fig. 2.27).

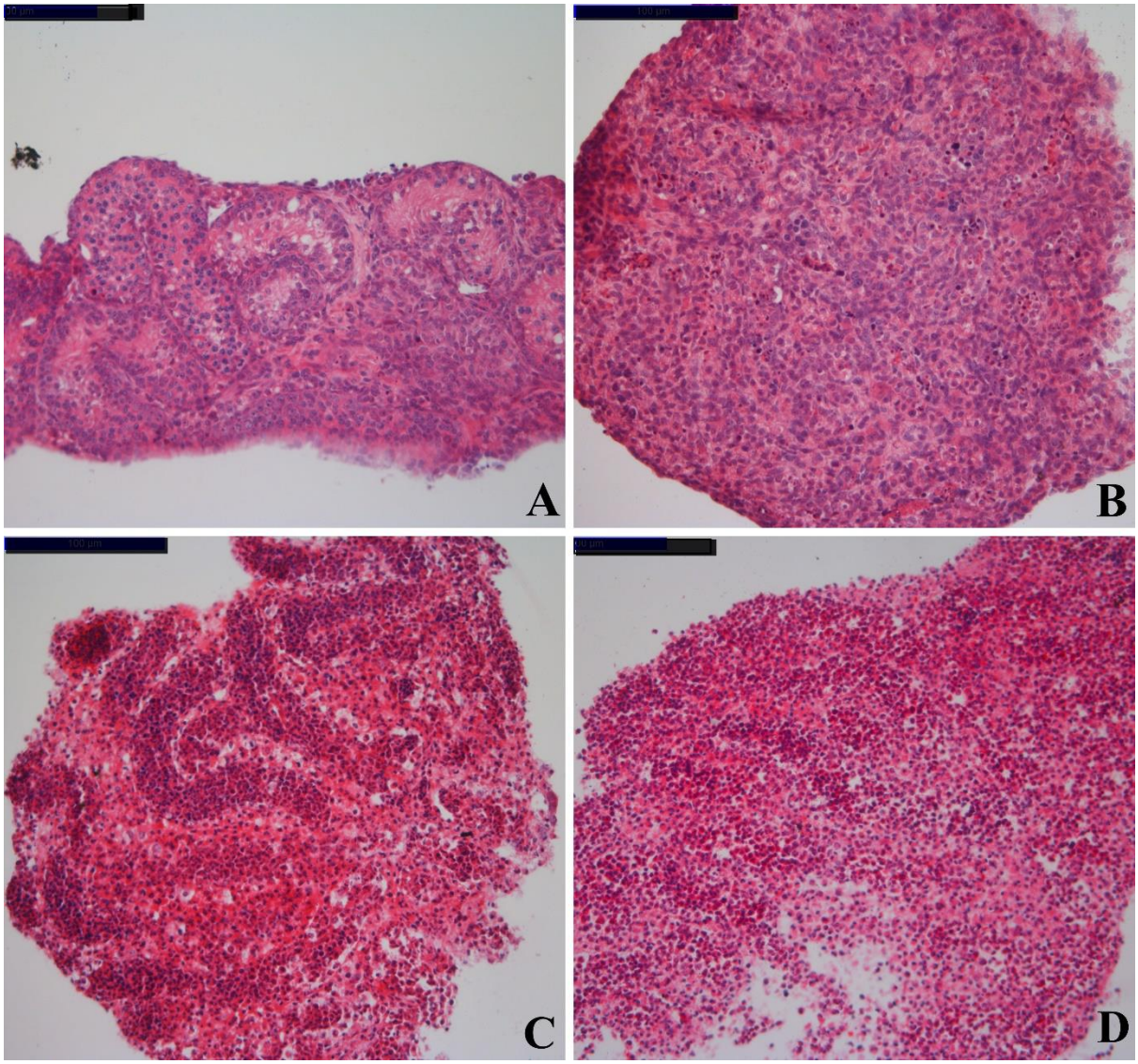


Fig. 2.20 H & E stained cultured prepubertal (infantile) mouse testis fragments showing the effects of 6 days exposure of high concentrations of $NaAsO_2$ on tissue morphology (A) control tissue, (B) 10 µM $NaAsO_2$ (C) 50 µM $NaAsO_2$ (D) 100 µM $NaAsO_2$. Scale bar = 100 µm.

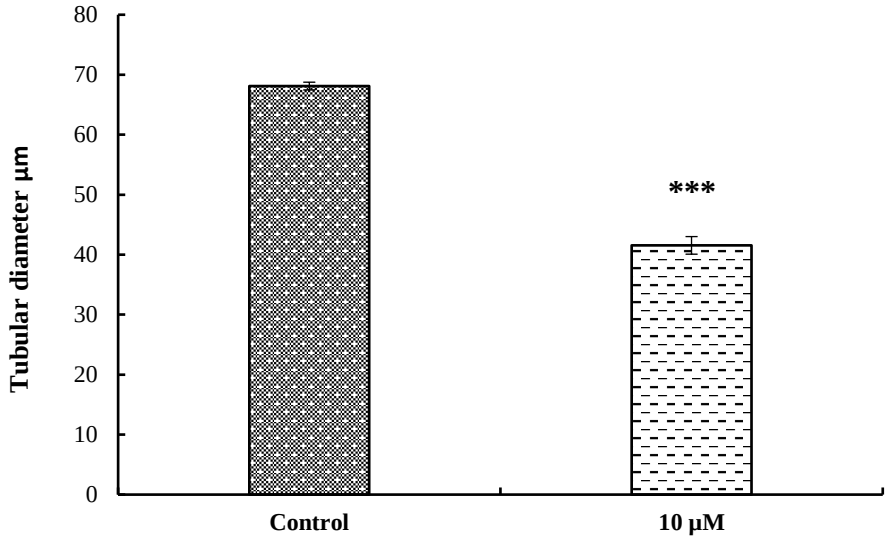


Fig. 2.21 Mean tubular diameter (µm) of seminiferous tubules after 6 days exposure to high $NaAsO_2$ concentration in a 7 days prepubertal (infantile) mouse testicular cultures.

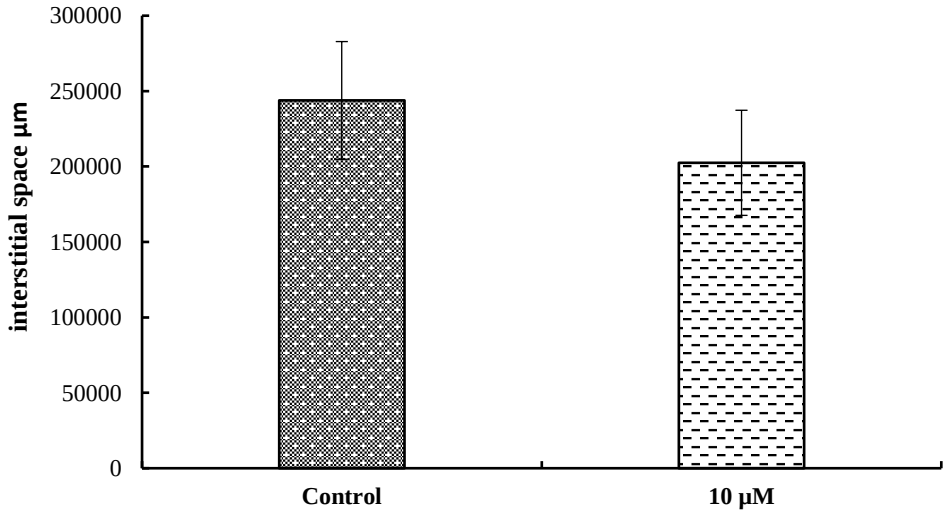


Fig. 2.22 Mean interstitial space (µm) of prepubertal (infantile) mouse testicular culture tissues after 6 days exposure of high concentrations of $NaAsO_2$.

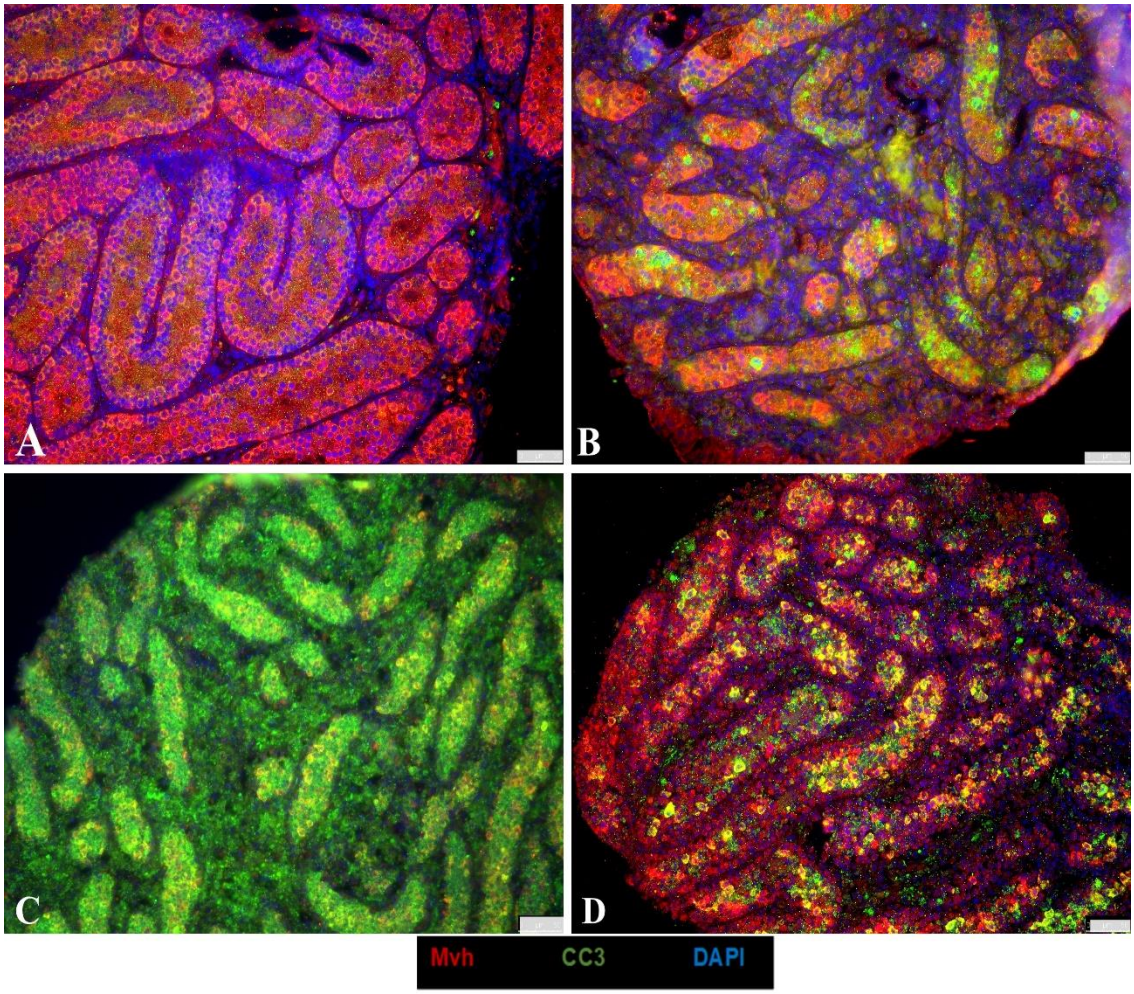


Fig. 2.23 Exposure of prepubertal (infantile) mouse testis to high $NaAsO_2$ concentrations for 6 days. Photomicrographs show immunohistochemical localization of Mvh (red) and CC3 (green), counterstained with DAPI (blue) (A) Control (B) 10 µM $NaAsO_2$ (C) 50 µM $NaAsO_2$ (D) 100 µM $NaAsO_2$. Scale bar = 50 µm.

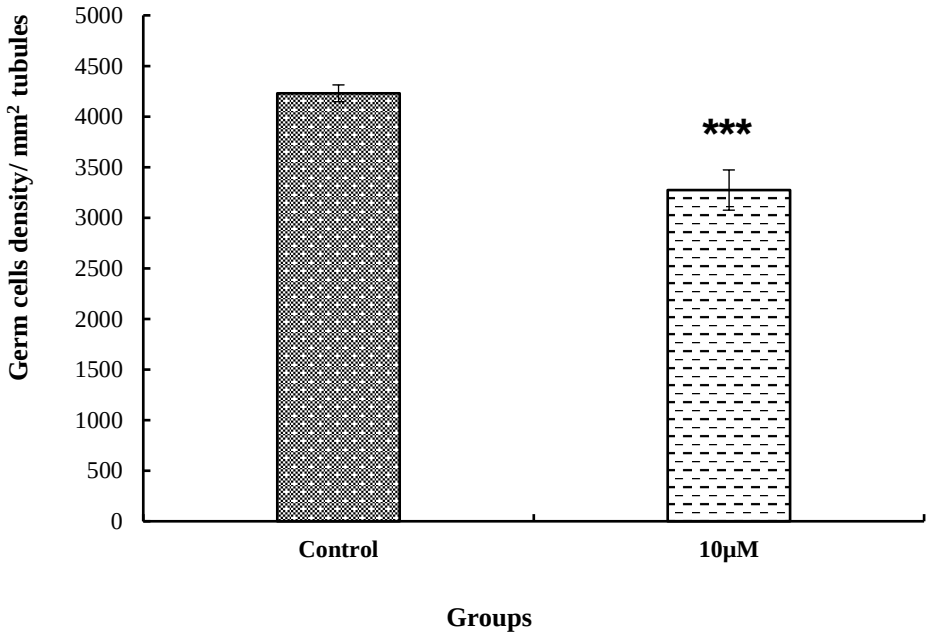


Figure: 2.24 Germ cells density per tubule area of seminiferous tubules after 6 days exposure of prepubertal (infantile) mouse testicular cultures to high concentrations of $NaAsO_2$. *** shows $P < 0.001$.

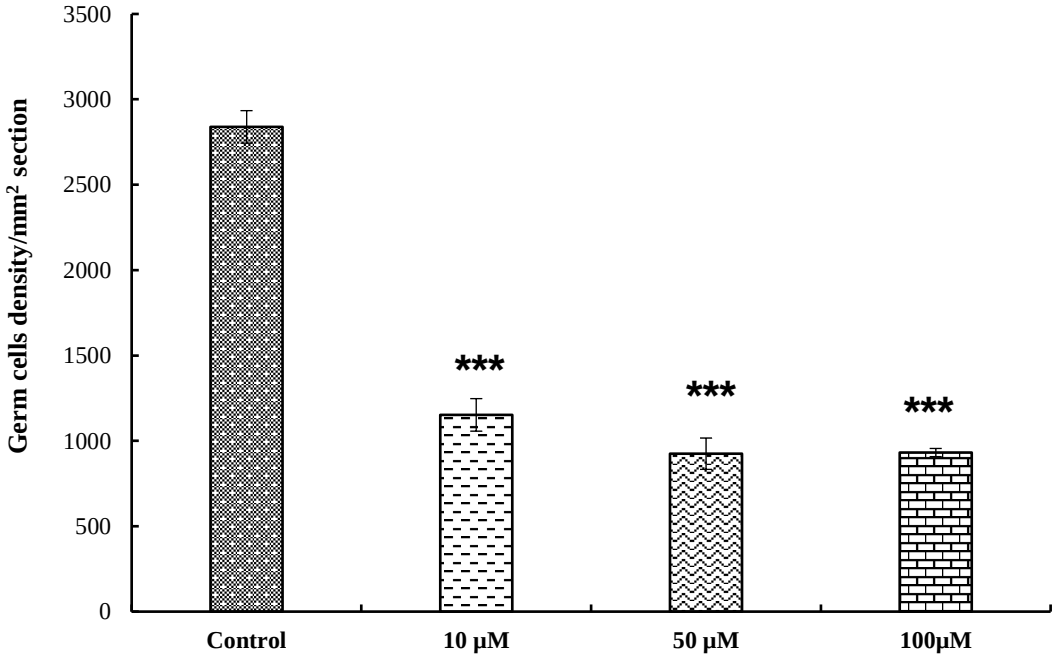


Fig. 2.25 Germ cells density per section area of testicular section after 6 days *in vitro* exposure of prepubertal (infantile) mouse testes cultures to high concentrations of $NaAsO_2$. *** shows $P < 0.001$.

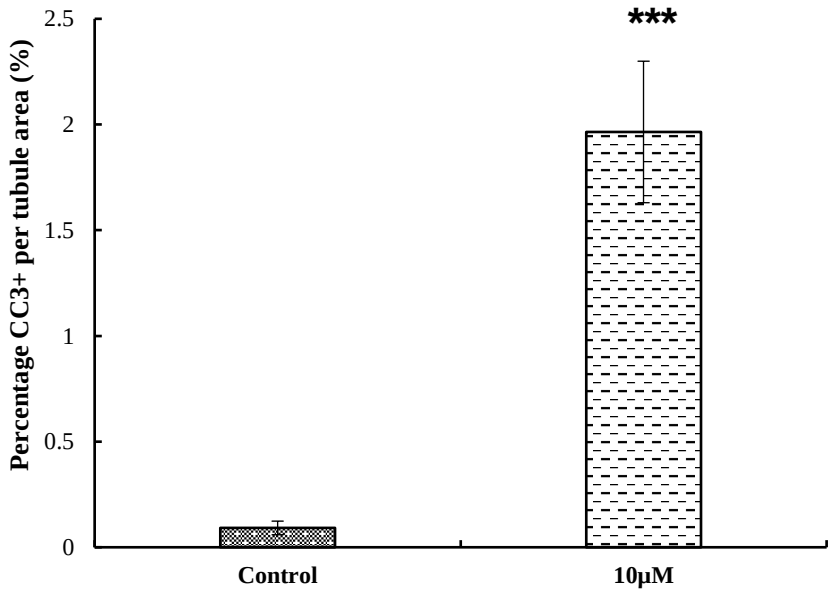


Figure: 2.26 Percentage CC3 expression per tubule area of seminiferous tubule after 6 days *in vitro* exposure of prepubertal (infantile) mouse testes cultures to high concentrations of $NaAsO_2$. *** shows $P < 0.001$.

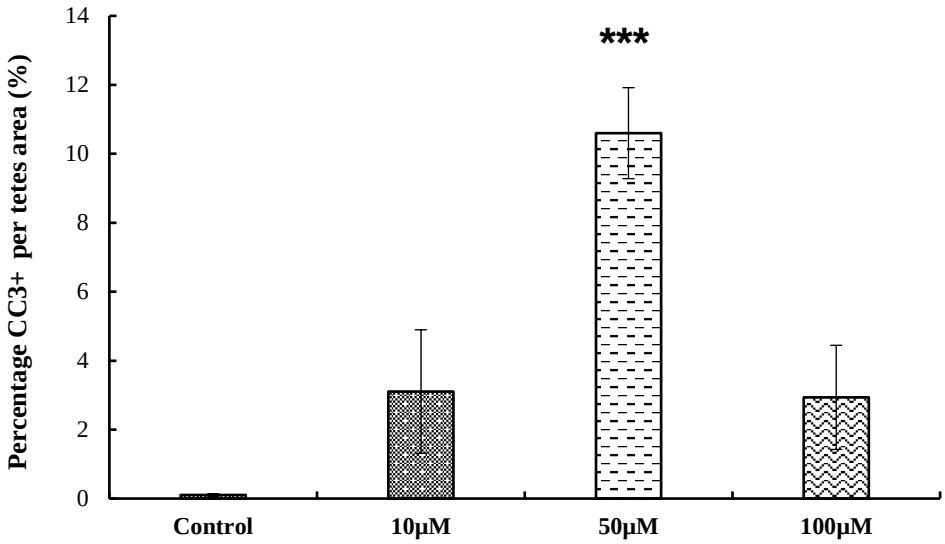


Fig. 2.27 Percentage CC3 expression per section area of testicular tubules after 6 days *in vitro* exposure of prepubertal (infantile) mouse testes cultures to high concentrations of $NaAsO_2$. *** shows $P < 0.001$.

## 2.7. Discussion

The chemical element namely arsenic found naturally in the earth's crust is a well-known carcinogen. Chronic or acute exposure to arsenic can contribute to a spectrum of diseases via consumption of food, water and occupational resources as well. Effects of arsenic on male reproduction has been extensively studied over the past few years. It has been reported that arsenic inhibits steroidogenesis, causes spermatotoxicity, and adversely impact weight of the rat reproductive organs (Sarkar et all., 2003; Pant et al., 2004). Similarly, *in vivo* arsenic exposure induces biochemical and morphological changes in adult rat testis (de Araujo Ramos et al., 2017). Despite several studies that focused attention on the effects of arsenic on adult testis, little information is available as regards the gonadotoxicity of $NaAsO_2$ on the developing testis of immature prepubertal males. The present work used an *in vitro* culture system of prepubertal (infantile) mouse testes as a model to highlight arsenic effects that may occur to human males, especially children and young boys. This system is capable of supporting normal testicular development and has already been used by The University of Edinburgh's Spears research group (Smart et al., 2018) as well as others (Nakamura et al., 2019) for toxicological studies on the testicular tissue.

Different sets of experiments were designed to assess the effects of $NaAsO_2$ concentrations on the testicular fragments, starting from exposure of a range of low concentrations (0.01, 0.05, 0.1, 0.5, and 1 µM) for 1 day. The lowest dose tested, i.e. 0.01 µM, in this experiment is equal to 1.29 ppb whereas 0.05 and 0.1 µM corresponds to 6.49 and 12.9 ppb respectively. These doses were chosen as they are under the WHO declared safe limits i.e. 10 ppb and upper save limit declared as 50 ppb. This experiment was used initially as a baseline to assess the toxicity caused by arsenic in prepubertal (infantile) mouse testes. It was expected that testicular cultures will be highly sensitive to arsenic exposure and that even a short-term exposure (24 h or 1-day) will cause a damage to the growing testis. However, concentrations up to 1 µM (equals to 129 ppb) did not cause any change to the testicular morphology, apoptosis induction or germ cells density of seminiferous tubules, after 24 h $NaAsO_2$ exposure. All treated tissues were healthy and plenty of dividing germ cells were observed.

In the second experiment, time of arsenic exposure was raised to 6 days to assess the toxicity of 0.1, 0.5, and 1 µM $NaAsO_2$ concentrations on mouse testicular tissues. Histology and immunohistochemistry results showed that the prepubertal (infantile)

testis were unaffected even after 1 µM *in vitro* arsenic exposure for 6 days. In contrast, in the third experiment higher arsenic concentrations (10, 50, or 100 µM) caused significant changes in the testis culture, with a clear dose response pattern. $NaAsO_2$ at 10 µM concentration (equals to 1299 ppb) induced moderate damage despite leaving the seminiferous tubule area reasonably well distinguishable, while both 50 and 100 µM $NaAsO_2$ treatments caused distortion of seminiferous tubules structure. In all three high arsenic doses, the germ cell population was severely impaired. The extensive disorganization along with the loss of germ cells, shape, as well as shrinkage of the seminiferous tubule were observed upon high $NaAsO_2$ exposure confirmed similar observations as previously reported in the adult testis after *in vivo* arsenic exposure (Fouad et al., 2011; Ferreira et al., 2012; Momeni et al., 2012; Baltaci et al., 2016; Uygur et al., 2016).

Furthermore, increased apoptotic cells were found within the seminiferous tubules at 10 µM $NaAsO_2$. Apoptosis was also evident in the overall testicular section at 50 µM $NaAsO_2$. Paradoxically, the increased expression of CC3 at 100 µM of $NaAsO_2$ did not reach statistical significance. One possible explanation could be that at the top dose i.e. 100 µM concentration of $NaAsO_2$, the apoptotic cells in the prepubertal (infantile) testis culture died shortly after arsenic administration and the signals had disappeared relatively rapidly, long before the six days culture end point. It has been previously shown that apoptosis can begin and quickly complete within 2-3 h (Elmore, 2007). Similarly, in another *in vitro* study on Japanese eel the testis showed germ cell apoptosis after 100 µM disodium arsenate exposure (Celino et al., 2009).

WHO has declared that arsenic concentrations of less than 10 ppb are safe for drinking purposes (Brown and Ross, 2002). Several studies on rodents have revealed arsenic accumulation in testis and other accessory sex organs (Pant et al., 2001; Dua et al., 2015; Prathima et al., 2018). Arsenic intoxication can occur through drinking contaminated water. Arsenic concentrations in the drinking water of the developing countries like Pakistan are above 50 ppb (Sanjrani et al., 2017). Chronic exposure to high arsenic concentrations through drinking water and its persistent nature after its accumulation in the reproductive organs can badly affect the developing testis of both human and animals. Apoptosis is a self-destruction process which can occur either or by both the intrinsic and extrinsic pathways (Dua et al., 2015). ROS produced as a result in the mitochondria are considered as the biochemical mediators of apoptosis (Uygur

et al., 2016). Arsenic is known to cause oxidative stress by escalating ROS production and reducing antioxidant defense systems (Yamanaka et al., 1991). Previous literature indicates that increased testicular oxidative stress can cause protein and DNA damage after *in vivo* arsenic exposure (Uygur et al., 2016; Fouad et al., 2011; Reddy et al., 2011; Das et al., 2009a; Manna et al., 2008). Therefore, in the present study it is supposed that *in vitro* exposure of the prepubertal (infantile) testis to high arsenic concentrations might have induced ROS generation and oxidative stress, which resulted in cleaved caspase-3 mediated apoptosis.

Earlier, it was reported that *in vitro* application of 100 µM arsenic concentration immediately killed mouse embryos, whereas 1 µM dose entirely inhibited blastocyst formation (Müller et al., 1986). However, comparing the effects of arsenic concentrations in the present study on prepubertal (infantile) mouse testis culture, the mouse testicular fragments were unaffected at 1 µM arsenic dose after both 1day (24 h) and 6 days exposure, while the 10 µM treatment for 6 days started inducing changes in the infantile mouse testis.

## 2.8. Conclusion

In conclusion, using a short-term *in vitro* culture system of mouse prepubertal (infantile) testis, the present study showed that exposure to high levels of arsenic for six days caused a dose dependent reduction of spermatogonial germ cells and a marked increase in testicular cell apoptosis.

# CHAPTER # 3

# *IN VITRO* ADULT MOUSE TESTICULAR CULTURES

## Abstract

Arsenic poisoning is well-known for its innumerable toxic and carcinogenic effects. *In vivo* data on reproductive toxicity are available but *in vitro* data are scant. Presently, the *in vitro* toxic effects of $NaAsO_2$ on adult mice testes and epididymal tissues using the organ cultures and sperm incubation were investigated. Testicular and epididymal fragments were incubated at 37°C and 33°C respectively with 1, 10, 50, and 100 µM concentrations of $NaAsO_2$. Cultures were allowed to incubate for 2 h and 24 h. Levels of oxidative stress markers, the ROS and TBARS, antioxidant enzymes, testosterone concentrations, and the extent of sperm DNA damage were estimated. Sperms were incubated with Human tubal fluid at 37°C in a 5% $CO_2$ incubator, and sperm motility, viability, and morphology were assessed after 2 h. Results were analyzed statistically at $P < 0.05$. Results demonstrated both time and dose-dependent alterations, whereby, following 24 h incubation with $NaAsO_2$, substantial increases were noticeable in ROS and TBARS levels, and sperm DNA damage ($P < 0.001$), while decreases ($P < 0.001$) occurred in catalase, peroxidase, superoxide dismutase, and reduced glutathione levels at 10, 50 and 100 µM concentrations. Incubations for 2 h revealed similar but relatively low toxic effects. Testosterone concentrations decreased significantly only after 24 h of incubation with 50 (1.95 vs 2.93 ng/g; $P < 0.01$) and 100 µM (1.32 vs 2.93 ng/g; $P < 0.001$) $NaAsO_2$ concentrations. Sperm motility, and viability was substantially reduced with increasing arsenic concentrations. Similarly, sperm morphology was affected by higher arsenic doses (50 and 100 µM). The study concludes that exposure of testicular and epididymal tissue fragments, as well as sperms to arsenic under *in vitro* conditions induces rapid and immediate metabolic and genotoxic damage at higher concentrations.

## 3.1. Introduction

Arsenic is a poisonous heavy metal that is ubiquitously found in natural environments (Vahidnia et al., 2007). In nature, organic and inorganic forms of this metalloid occur in various oxidation states like, -3, 0, +3, and +5 (Bissen and Frimmel, 2003). Of variable oxidation states, trivalent arsenic is more toxic than the pentavalent form (Hughes et al., 2011). Exposure of life forms to arsenic occurs mainly through the air, food, drinking water, and industrial sources (Feng et al., 2001). WHO has declared that < 10 µg/l arsenic concentrations in the drinking water are safe, while amounts nearing or > 50 µg/l are considered maximum contamination level (Brown and Ross, 2002). Over the past few years, arsenic concentrations have increased to alarmingly high levels due to the rampant release of industrial waste into natural soils, open fields, and freshwater bodies. Drawing of groundwater for drinking and irrigation purposes leads to further exposure of living organisms (including humans) to arsenic concentrations above permissible limits (Kumar et al., 2016).

Contamination of drinking water with arsenic has generated serious health-related problems in human beings across the world. The issue is more serious in developing Asian countries. For instance, in Pakistan arsenic levels in the drinking water have risen to > 200 µg/l or ppb in the most populous areas of Sindh and Punjab provinces, whereas in most other areas of the country levels have been found > 50 µg/l (Sanjrani et al., 2017). As the groundwater, especially in the developing countries, contains significantly high levels of arsenic, therefore, it seems at present, impossible to avoid exposure to arsenic. Human mortalities have been reported in Bangladesh after chronic arsenic exposure by drinking contaminated water (Argos et al., 2010). It has been demonstrated that arsenic induces toxicity through the generation of ROS and reactive nitrogen species (RNS). Increased ROS generation causes cellular and tissue necrosis by causing oxidative damage to lipids, DNA, and proteins (Kitchin and Conolly, 2009). Arsenic can also induce alterations in DNA repair mechanisms (Duker et al., 2005), and the antioxidant defense system of the animals (Shi et al., 2004). Arsenic toxicity is related to different kinds of cancers, cardiovascular diseases, dermal lesions, organ damage, anemia, diabetes, and neurological disorders (Izah and Srivastav, 2015).

Reproductive toxicity following exposure to arsenic has also been documented (Kim and Kim, 2015). It inhibits testicular steroidogenesis, reduces accessory sex organs and testicular weight, and causes spermatotoxicity. Moreover, this heavy metal accumulates

in the epididymis and testicular tissues, which indicates its persistent nature (Pant et al., 2004). Since arsenic is an endocrine disruptor and reduces testosterone, LH, and FSH, concentrations, arsenic poisoning might cause human infertility (Sengupta et al., 2013; Zubair et al., 2014).

Previously reported studies have focused on assessing the *in vivo* reproductive toxicity of arsenic. However, no data are available on the *in vitro* toxic effects of $NaAsO_2$ on male mouse reproductive organs. The present *in vitro* study was conducted on adult mouse testicular and epididymal organ cultures, and sperm *in vitro* incubation to assess the extent of biochemical and genotoxic damage caused by $NaAsO_2$.

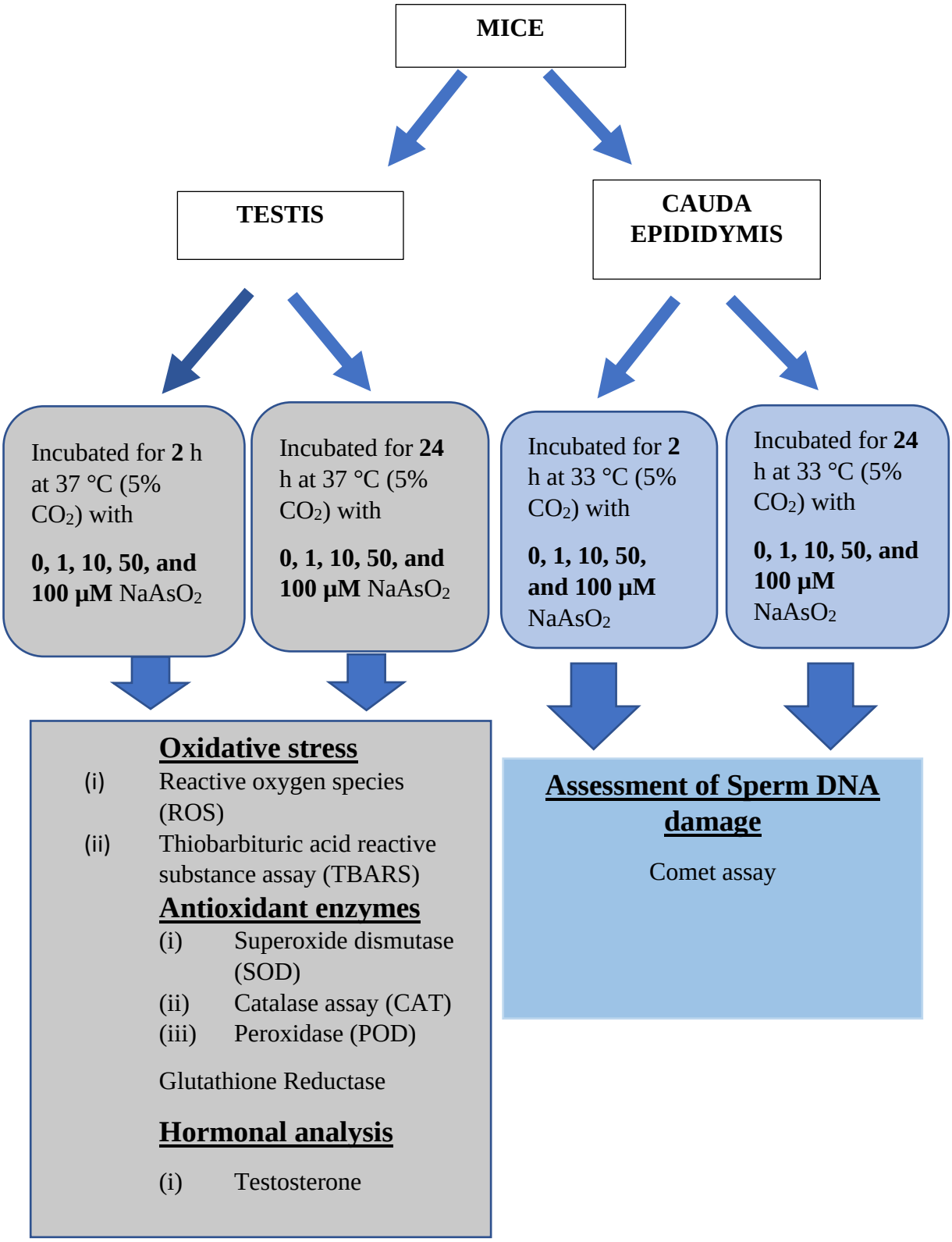


Fig. 3.1 Schematic representation of *in vitro* $NaAsO_2$ exposure to adult mouse testis and epididymis organ cultures.

## 3.2. Materials and Methods

### 3.2.1. Animals and maintenance

Adult male Swiss albino mice were obtained from the National Institute of Health (NIH), Islamabad and kept in the Animal Facility of Department of Zoology, Quaid-i-Azam university, Islamabad. Temperature was maintained at 22-24 °C with 16:8 h light and dark photoperiod. Animals were provided standard rodent feed and drinking water *ad libitum*. The present study design and animal handling was approved by the Bioethical Committee of The Faculty of Biological Sciences.

### 3.2.2. Chemicals

A stock solution of sodium arsenite-$NaAsO_2$ (Sigma-Aldrich, UK) was prepared in distilled water, and doses were freshly prepared through serial dilutions with media.

### 3.2.3. Experimental design

*In vitro* experiments were performed to assess the effects of $NaAsO_2$ on oxidative stress, antioxidant enzymes parameters and production of testosterone using the organ culture. Six adult swiss albino mice were used for obtaining testes and epididymes. Each testis was cut in 4 pieces (6 × 2 testes × 4 pieces= 48 pieces). $NaAsO_2$ doses were selected according to an earlier study on mice embryos (Muller et al., 1986). Different concentration of $NaAsO_2$ (0, 1, 10, 50, and 100 µM) were tested *in vitro* on adult mouse testes for short duration i.e. 2 and 24 h (Fig. 3.1). Therefore, the number of testis explants per data-point was 4-5 (48 pieces / 10 doses of two incubation periods).

### 3.2.4. Testis preparation for incubation

*In vitro* testicular culture was performed following the procedures of Moundipa et al. (2006) and Freyberger et al. (2010) with some modifications. Mice were decapitated and testes were taken out. These were washed with physiological saline and decapsulated. Testes were cut into four nearly equal sized pieces and placed randomly in culture tubes. $NaAsO_2$ doses (0, 1, 10, 50, and 100 µM) were prepared through serial dilutions in Dulbecco's modified eagle's medium/Ham F12 (DMEM/ Ham F12 mixture medium 1:1 ratio, Caisson Labs, CA, USA). Two (ml) of media were added to each labelled culture tube and 50 IU/ml Penicillin and 50 µg/ml streptomycin were

supplemented. Organ cultures were incubated at 37 °C with 5% $CO_2$ in air (Sanyo $CO_2$ incubator MCO175, Japan).

Following 2 and 24 h of incubation period, tissues were taken out of media and washed with physiological saline and homogenized in 1ml of PBS and centrifuged for 30 min at 30,000 rpm for supernatant separation. Supernatant were stored at -80 °C and used for antioxidant assays and hormone concentrations.

### 3.2.5. Epididymal fragment incubation

For culturing of sperms *in vitro,* epididymes were immediately separated out after dissection and washed with chilled physiological saline. Cauda epididymes were crushed in 1 ml PBS having pH =7.4 (150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris base and 10% glycerol). Supernatant was removed after centrifugation (30,000 rpm for 10 min at 4 °C). Sperm pellets were incubated at 33 °C in a 5% $CO_2$ humidified incubator with 2 ml of Ham's F12 medium (Sigma-Aldrich, Germany), while Bovine serum albumin (BSA, Sigma-Aldrich, Germany) was added as a supplement. After the desired incubation period (2 and 24 h), supernatants were centrifuged at 1000 rpm for 15 min and sperm pallets were removed and diluted with 1ml of PBS for comet assay.

### 3.2.6. Biochemical analysis

Testicular supernatants were processed for the determination ROS and lipid peroxidation levels, antioxidant enzymes, the superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and non-enzymatic enzyme, reduced glutathione (GSH).

#### (i) Reactive oxygen species (ROS)

ROS production in the testis homogenate was determined following the protocol of Hayashi et al. (2007). One mg of N.N-Diethyl para phenylene diamine sulfate (DEPPD) was dissolved in 10 ml of distilled water for reagent 1 preparation. $FeSO_4$ stock solution contained 50 mg ferrous sulfate dissolved in 10 ml sodium acetate buffer having pH 4.8. Fifty (µl) $FeSO_4$ stock solution was mixed with 100 ml of sodium acetate buffer for making reagent 2. Both reagents were mixed in a 1:25 ratio and placed in darkness 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 Ultraviolet-Visible Spectrophotometer (Agilent 8453, USA) at 505 nm. For each sample, three readings were taken at an interval of 15 sec intervals.

**(ii) Thiobarbituric acid reactive substance assay (TBARS)**

Malondialdehyde in the homogenate was determined following Iqbal et al. (1996) through its reaction with thiobarbituric acid (TBA). Oxidative stress produced due to lipid peroxidation was determined indirectly by this method. Reaction mixture was made in a test tube by adding 0.1 ml of ascorbic acid (1.5 mM), 0.1 ml of 50 mM Tris -HCL, 0.1 ml of $FeSO_4$ (1mM), 0.6 ml of distilled water, and 0.1ml of testis homogenate. After vigorous mixing, it was incubated for 15 min at 37 °C. After adding 1 ml of thiobarbituric acid and tricholoroacetic acid (0.375% and 10% respectively), this mixture was mixed and boiled on a water bath for 15 min at 100 °C and centrifuged at 2500 rpm for 10 min. Supernatant's optical density was calculated on a spectrophotometer at 535 nm.

**(iii) Measurement of Reduced glutathione (GSH)**

Jollow et al. (1974) method was followed for the measurement of reduced glutathione. 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.

**(iv) Superoxide dismutase (SOD)**

It was determined according to Kakkar et al. (1984) method. Reaction mixture was made by adding 1.5 ml of L-Mehionine, 0.7531 of Triton X-100, and 1ml of nitroblue tetrazolium (NBT). By adding 50 mM PBS (pH 7.8), the final volume was brought to 30 ml. One milliliter of above prepared mixture was separated into test tubes and 20 µl of sample was added into each. Sample tubes were illuminated with fluorescent lamp for 7 min and the contents were then incubated at 37 °C for 5 min. For reaction initiation, riboflavin (10 µl) was added and the contents were again incubated at 40 °C for 8 min. Three readings were noted during one minute at 560 nm on a spectrophotometer.

**(v) Catalase (CAT)**

CAT activity in the samples was assessed following Maehly and Chance (1955) method with some alterations. Mixture was prepared by adding 1.99 ml of potassium phosphate buffer (pH 7), 1000 µl $H_2O_2$ (5.9 mM), and 100 µl testis homogenate. Three readings

were noted after 1 min interval at 240 nm on a spectrophotometer. 1 unit CAT activity was 0.01 absorbance change unit/min.

**(v) Peroxidase (POD)**

Activity of POD in the testis homogenate were determined according to Maehly and Chance, (1955) method. Mixture was made by 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. Optical density was noted after 1 min at 470 nm. One unit of POD activity was reflected as 0.01absorbance change as unit/min.

### 3.2.7. Hormonal analysis

#### 3.2.7.1. Testosterone- method, principle, and procedure

Testosterone concentration in the testis homogenate was determined quantitatively by using EIA kits, following the manufacturer's instructions (Calbiotech, CA USA). Determination of testosterone is based on competitive binding of sample testosterone and testosterone conjugated enzyme (Horseradish peroxidase). Concentration of sample testosterone is inversely proportional to the measured color intensity of the reaction mixture. For the measurement of testosterone concentration in the testicular tissue homogenate, 50 µL of kits control, standards, and testicular homogenate were inserted into the streptavidin coated wells. In each well, 100 µL of Testosterone-enzyme conjugate reagent and 50 µL of biotin reagent were added. After gentle mixing for 30 sec, the ELISA plate was incubated for 1 h at room temperature. Incubation period is vital for the competitive binding of labelled testosterone and sample with antibodies coated in the well. After incubation wells were washed thrice with washing buffer for the removal of any unbound enzyme conjugate. Following this, TMB substrate reagent (100 µL) was added in the wells and incubation was done for 30 min. In each well stop solution (50 µL) was added and mixed for 20 sec. ELISA plate reader (Via Medical Pro reader-96, Germany) was used to measure the reaction mixture absorbance at 450 nm. Testosterone in the sample was calculated as ng/ml and expressed as ng/ml of tissue.

### 3.2.8. Sperm DNA damage assessment through comet assay or single cell gel electrophoresis (SCGE)

Comet assay was carried out on mice epididymes (incubated for 2 and 24 h) as described by Hughes et al. (1998) protocol. Slides were first coated with 1% normal melting agarose (NMA), cover slipped and allowed to solidify at 4 °C for at least 20 min. After gently removing the cover slips, 85 µl of low melting agarose (including sperm suspension-20 µl + 1% Low melting agarose-65 µl) was spread over the first layer. After solidification, cell lysis was done by dipping the slides for 2 h in freshly prepared chilled lysis buffer. Electrophoresis was performed by dipping slides uniformly in neutral buffer in electrophoresis tank. Electrophoresis was done at 24 V for 30 min. Slides were dipped 2-3 times in the neutralizing buffer followed by staining with 80 µl ethidium bromide. Slides were observed under a fluorescent microscope (Nikon AFX-1 Optiphot, Japan) at 40X magnification. For each sample fifty to hundred cells were observed. Following sperm DNA comet parameters were determined: Percentage Tail DNA, and Tail length (µm).

### 3.2.9. Statistical analysis

GraphPad Prism (5.01, CA USA) software was used for statistical analysis. For the comparison of experimental groups with the control, one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test was applied on the data. Level of significance was considered as $P < 0.05$. Data are expressed as mean ± S.E. DNA damage in sperms was evaluated by using CASP-Lab image analysis software (CASP Lab version 1.2.3.beta2, Poland).

## 3.3. Results

### 3.3.1. ROS and antioxidant enzymes

A substantial increase was observed in ROS ($P < 0.01$; Fig. 3.2) and lipid peroxidation (TBARS) ($P < 0.001$; Fig. 3.3) after 2 h of mice testis organ cultures. Whereas, CAT and SOD enzymes showed significant decrease ($P < 0.05$; Figs. 3.4 and 3.5 respectively) in 50 µM and 100 µM treatment groups when compared to the control group. POD concentration decreased significantly after 2 h *in vitro* treatment with 100 µM $NaAsO_2$ (Fig. 3.4). In contrast, a non-significant decrease was observed in GSH in testicular culture homogenate (Fig. 3.6). The effect of $NaAsO_2$ exposure on cellular antioxidant enzymes was assessed *in vitro* in mice testis cultures (N = 4-5) and are represented in Table 3.1.

In contrast after 24 h of culturing the testicular fragments with 10, 50, and 100 µM $NaAsO_2$, a highly significant increase occurred in ROS (Fig. 3.7) and TBARS (Fig. 3.8), but a highly significant decrease was noticeable in CAT, and SOD and GSH (Figs. 3.9; 3.10; 3.11 respectively). Considerable decrease in POD was also detected at 50 and 100 µM $NaAsO_2$ concentrations ($P < 0.01$; Fig. 3.9) (Table 3.2).

### 3.3.2. Testosterone concentration

A non-significant decrease occurred in testosterone concentration after 2 h of incubation of testicular cultures with different concentrations of $NaAsO_2$ (Fig. 3.12). In contrast, significant decrease was noticeable after 24 h of incubation with 50 µM ($1.95 \pm 0.22$ vs $2.93 \pm 0.14$ ng/g; $P < 0.01$) and 100 µM ($1.32 \pm 0.05$ vs $2.93 \pm 0.14$ ng/g; $P < 0.001$) $NaAsO_2$ doses as compared to control samples (Fig. 3.13).

### 3.3.3. Sperm DNA damage

DNA damage in sperm was assessed by comet assay. Its basic principle includes the migration of damaged DNA fragments during electrophoresis. Comet assay demonstrated significant sperm DNA damage after 2 h of incubation of epididymal sperms with 50 and 100 µM $NaAsO_2$ concentrations (Figs. 3.14; 3.15; 3.16). On the other, after 24 h of incubation, a highly significant increase was observed in Tail length at 10 ($P < 0.01$), 50 and 100 µM ($P < 0.001$) $NaAsO_2$ concentrations (Figs. 3.17; 3.18; 3.19). However, the cultures exposed to 1 µM arsenic doses remained indifferent from controls after both 2 and 24 h incubations (Table: 3.3).

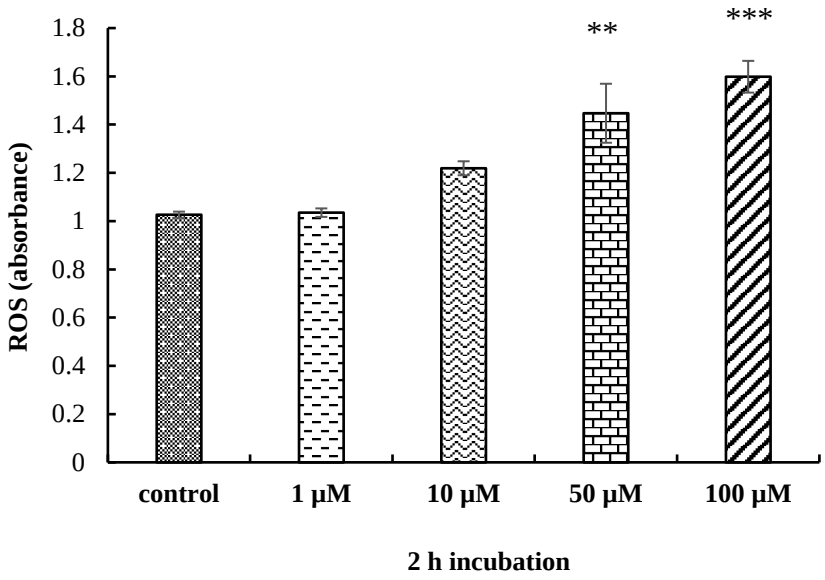


Fig. 3.2 Concentrations of ROS (absorbance) in mouse testicular homogenate after 2 h incubation with different concentrations of $NaAsO_2$. ** and *** indicates $P < 0.01$ and $P < 0.001$ respectively.

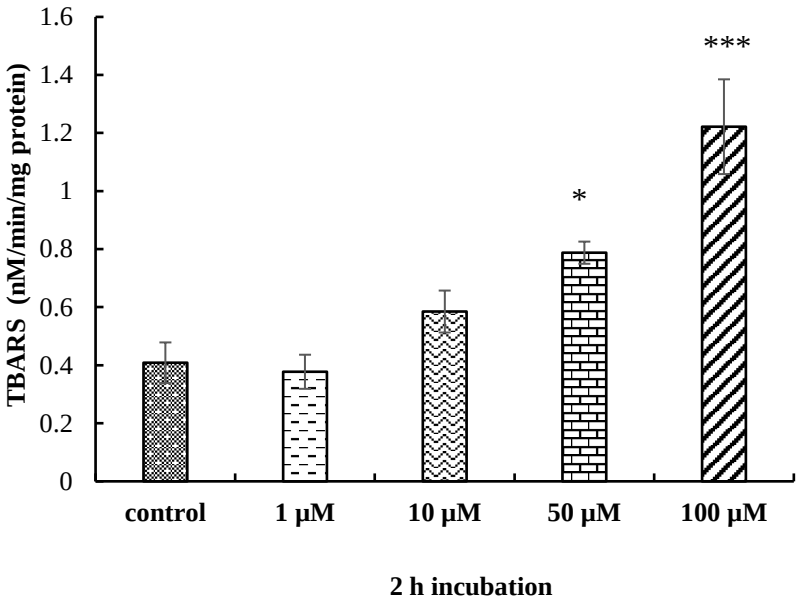


Fig. 3.3 Concentrations of TBARS (nM/min/mg protein) in mouse testicular homogenate after 2 h incubation with different concentrations of $NaAsO_2$. * and *** shows $P < 0.05$ and $P < 0.001$ respectively.

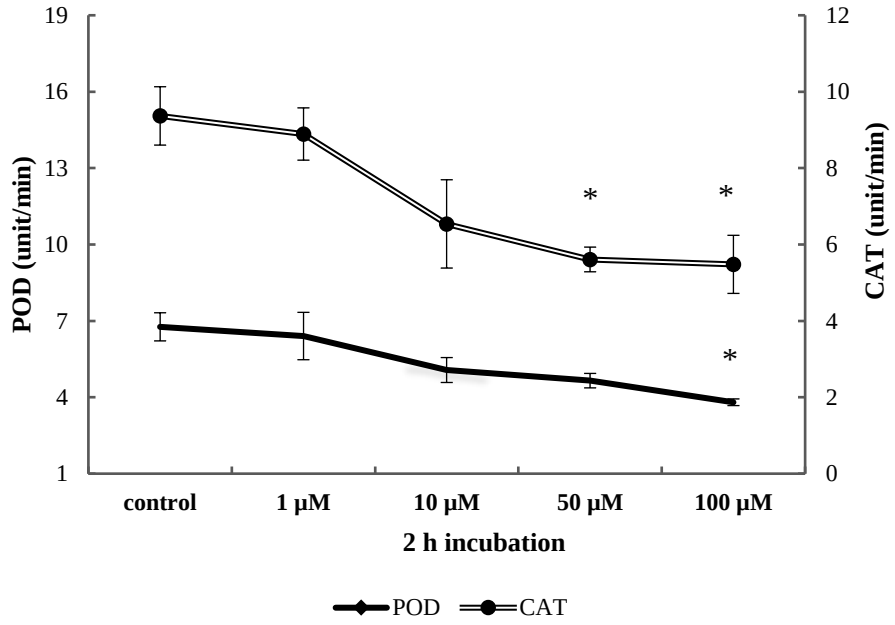


Fig. 3.4 Concentrations of POD and CAT (unit/min) in mouse testicular homogenate after 2 h incubation with different concentrations of $NaAsO_2$. * shows $P < 0.05$.

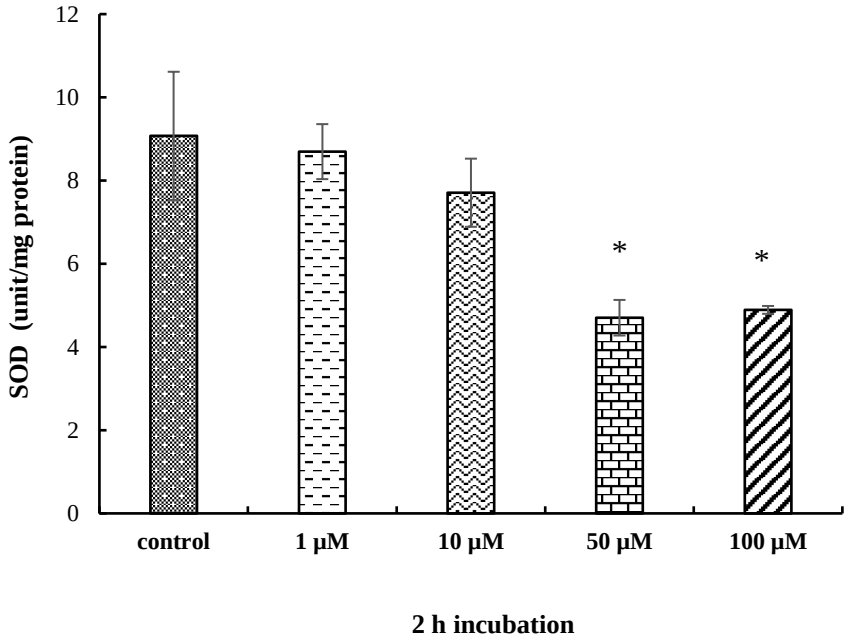


Fig. 3.5 Concentrations of SOD (unit / mg protein) in mouse testicular homogenate after 2 h incubation with different concentrations of $NaAsO_2$. * shows $P < 0.05$.

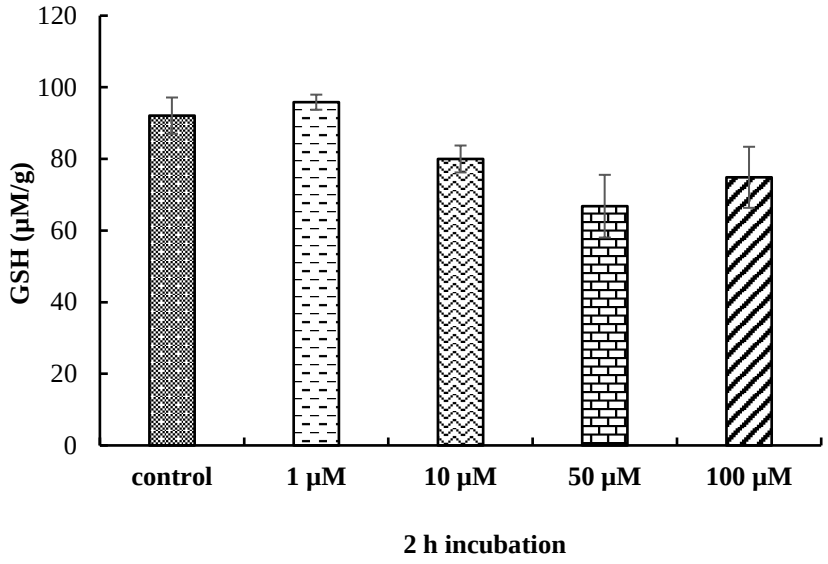


Fig. 3.6 Concentrations of GSH (µM/g) in mouse testicular homogenate after 2 h incubation with different concentrations of $NaAsO_2$.

Table 3.1. Effect on biochemical parameters after exposure of mouse testicular fragments *in vitro* to $NaAsO_2$ concentrations for 2 h

| Parameters | Treatments | | | | |
|---|---|---|---|---|---|
| | Control | 1 µM | 10 µM | 50 µM | 100 µM |
| ROS (abs) | 1.02± 0.01 | 1.03± 0.01 | 1.21± 0.02 | 1.44± 0.12** | 1.59± 0.06*** |
| TBARS (nM/min/mg protein) | 0.40± 0.06 | 0.37± 0.05 | 0.58± 0.07 | 0.78± 0.03* | 1.22± 0.16*** |
| CAT (unit/min) | 9.36± 0.76 | 8.89± 0.68 | 6.53± 1.15 | 5.60± 0.32* | 5.47± 0.76* |
| SOD (unit/mg) | 9.07± 1.54 | 8.69± 0.66 | 7.70± 0.81 | 4.70± 0.42* | 4.89± 0.09* |
| POD (unit/min) | 6.76± 0.55 | 6.40± 0.92 | 5.06± 0.48 | 4.65± 0.28 | 3.80± 0.13* |
| GSH (µM/g) | 9.07± 1.54 | 8.69± 0.66 | 7.70± 0.81 | 4.70± 0.42 | 4.89± 0.09 |

Values are expressed as mean ± S.E. *, **, *** indicate significant difference at probability $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA following Dunnet's multiple comparison test).

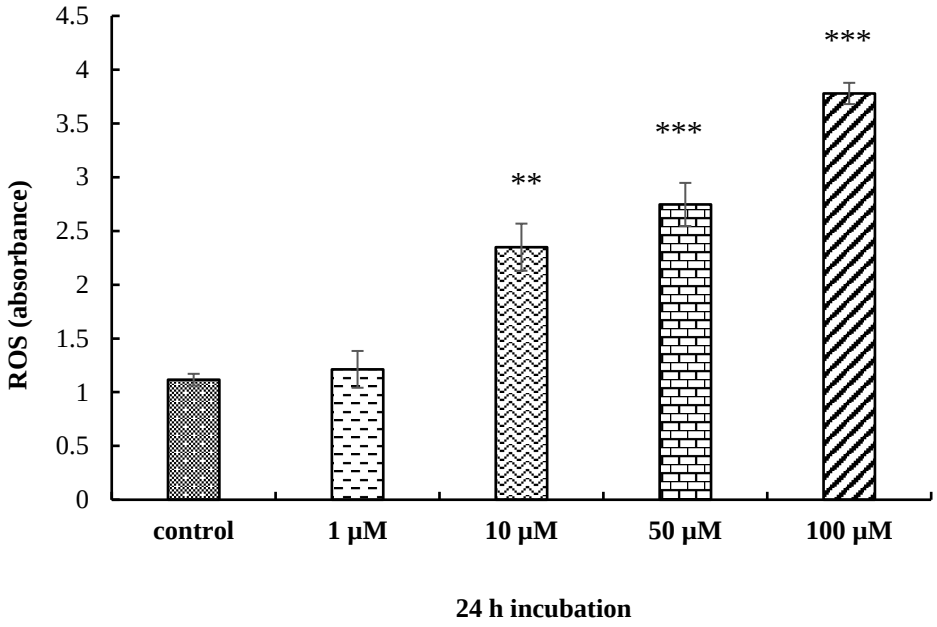


Fig. 3.7 Concentrations of ROS (absorbance) in testicular homogenate after 24 h incubation with different concentrations of $NaAsO_2$. ** shows $P < 0.01$ and *** represents $P < 0.001$.

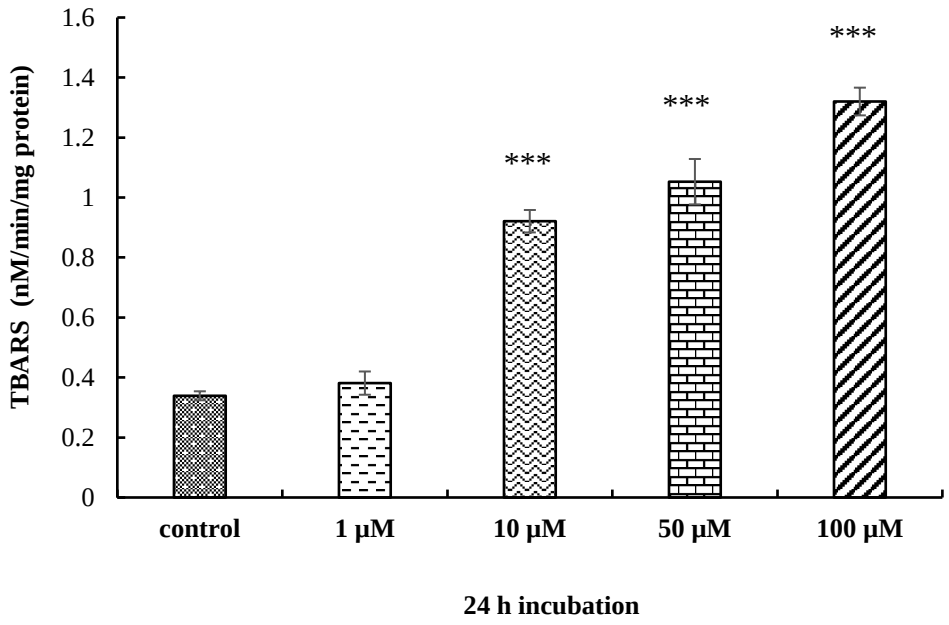


Fig. 3.8 Concentrations of TBARS (nM/min/mg protein) in mouse testicular homogenate after 24 h incubation with different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

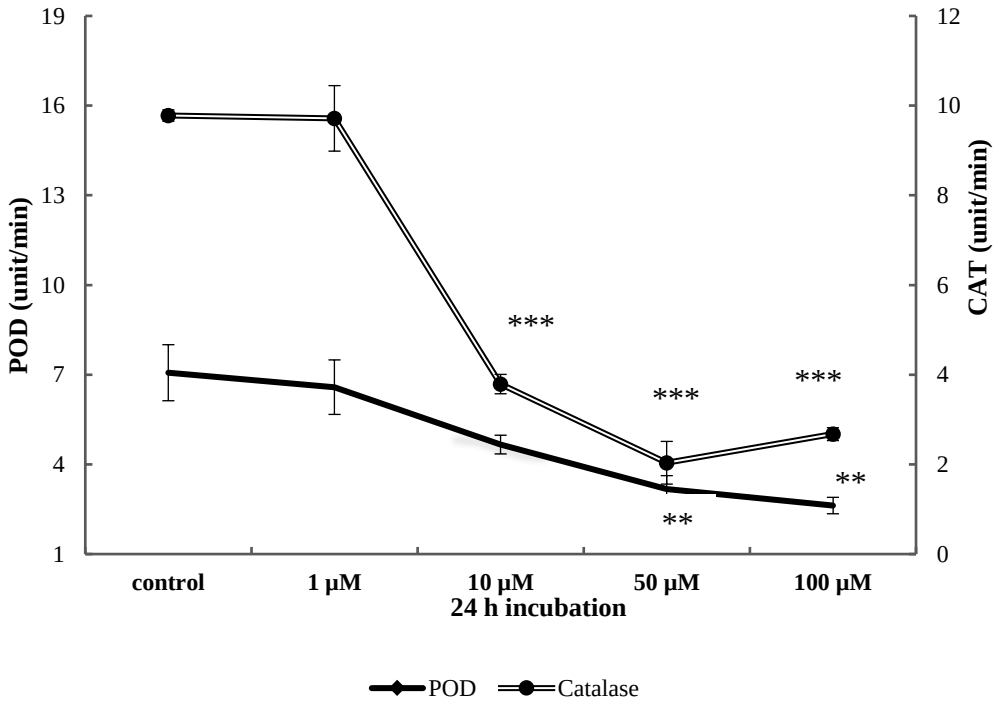


Fig. 3.9 Concentrations of POD and CAT (unit/min) in mouse testicular homogenate after 24 h incubation with different concentrations of $NaAsO_2$. ** shows $P < 0.01$ and *** represents $P < 0.001$.

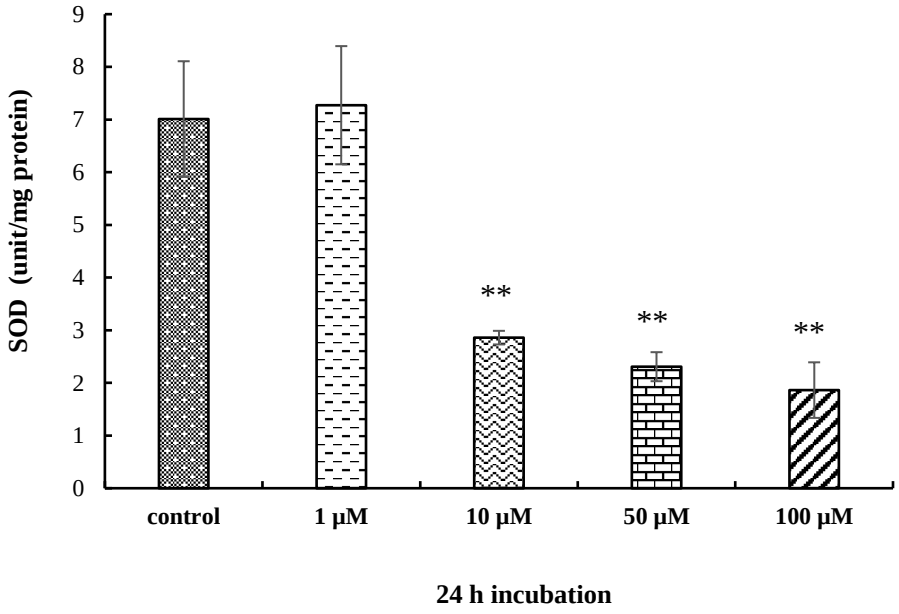


Fig. 3.10 Concentrations of SOD (unit/ mg protein) in mouse testicular homogenate after 24 h incubation with different concentrations of $NaAsO_2$. ** shows $P < 0.01$.

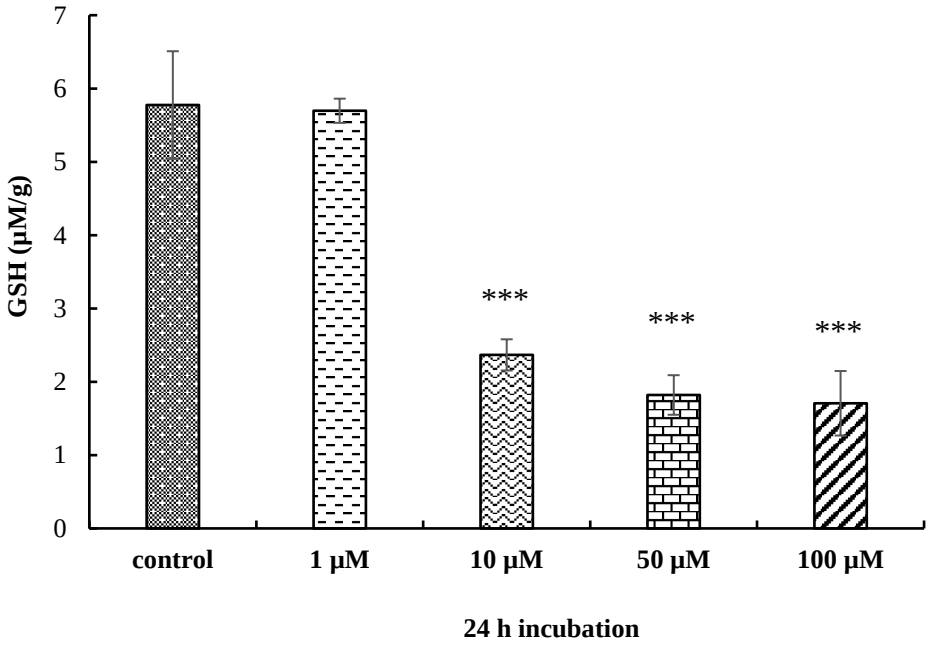


Fig. 3.11 Concentrations of GSH (µM/g) in mouse testicular homogenate after 24 h incubation with different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

Table 3.2. Effect on biochemical parameters after exposure of mouse testicular fragments *in vitro* to $NaAsO_2$ concentrations for 24 h

| Parameters | Treatments | | | | |
|---|---|---|---|---|---|
| | Control | 1 µM | 10 µM | 50 µM | 100 µM |
| ROS (abs) | 1.11± 0.05 | 1.21± 0.17 | 2.34± 0.21** | 2.74± 0.20*** | 3.77± 0.09*** |
| TBARS (nM/min/mg protein) | 0.33± 0.01 | 0.38± 0.03 | 0.92± 0.03*** | 1.05± 0.03*** | 1.32± 0.04*** |
| CAT (unit/min) | 9.77± 0.12 | 9.71± 0.73 | 3.79± 0.21*** | 2.03± 0.47*** | 2.67± 0.14*** |
| SOD (unit/mg) | 7.00 ± 1.09 | 7.27± 1.12 | 2.85± 0.12** | 2.30± 0.27** | 1.86± 0.52** |
| POD (unit/min) | 7.06± 0.93 | 6.58± 0.91 | 4.66± 0.31 | 3.17± 0.44** | 2.62± 0.27** |
| GSH (µM/g) | 5.77± 0.73 | 5.69± 0.16 | 2.36± 0.21*** | 1.82± 0.27*** | 1.70± 0.44*** |

Values are expressed as mean ± SEM.

*, **, *** indicates significant difference at probability $P < 0.05$, $P < 0.01$ and $P< 0.001$ compared to control (ANOVA following Dunnet's multiple comparison test).

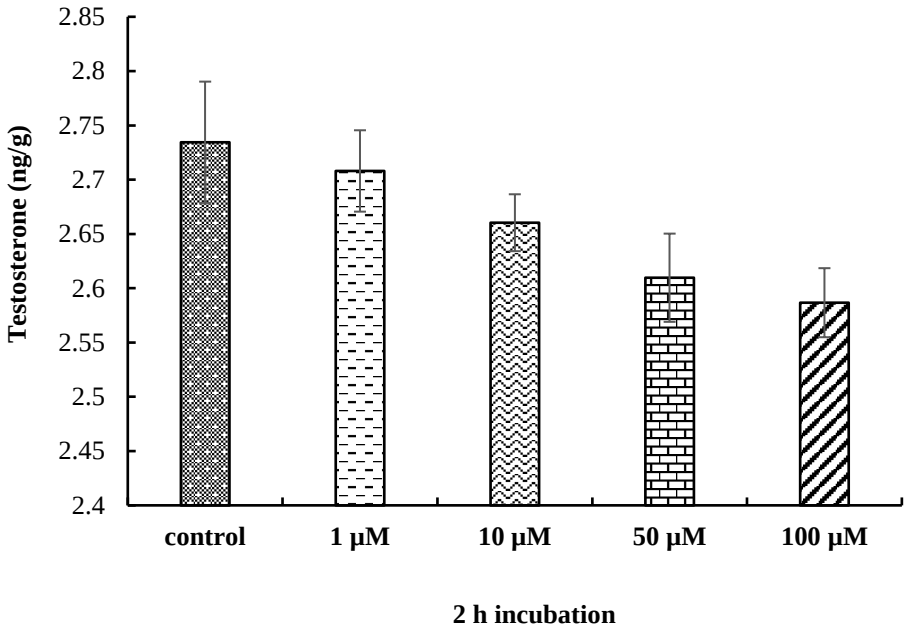


Fig. 3.12 Testosterone concentration (ng/g) in mouse testicular homogenates following 2 h incubation with $NaAsO_2$.

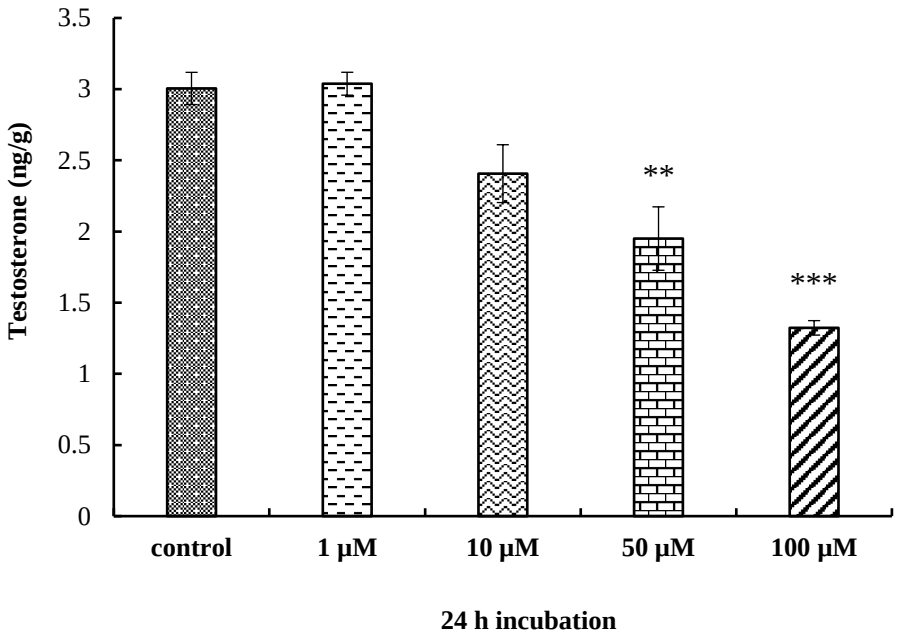


Fig. 3.13 Testosterone concentration (ng/g) in mouse testicular homogenates following 24 h incubation with $NaAsO_2$. ** and *** shows $P < 0.01$ and $P < 0.001$ respectively.

Table 3.3 *In vitro* effect of $NaAsO_2$ on mouse epididymal sperms DNA damage

| Treatment | 2 h incubation | | 24 h incubation | |
|---|---|---|---|---|
| | % Tail DNA | Tail length(µm) | % Tail DNA | Tail length(µm) |
| Control | 6.36±0.72 | 3.0±0.05 | 4.69±2.06 | 3.0±0.11 |
| 1µM | 6.27±0.55 | 3.33±0.33 | 10.68±2.67 | 4.33±1.33 |
| 10µM | 7.62±1.06 | 3.66±0.33 | 18.75±1.57* | 13±2.51** |
| 50µM | 13.22±0.96* | 6.33±0.33* | 39.4±4.69*** | 18.3±2.72*** |
| 100µM | 14.92±0.86* | 8.66±0.33* | 55.2±2.00*** | 24.3±0.88*** |

Values are expressed as mean ± SEM.

*, **, *** indicates significant difference at probability $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

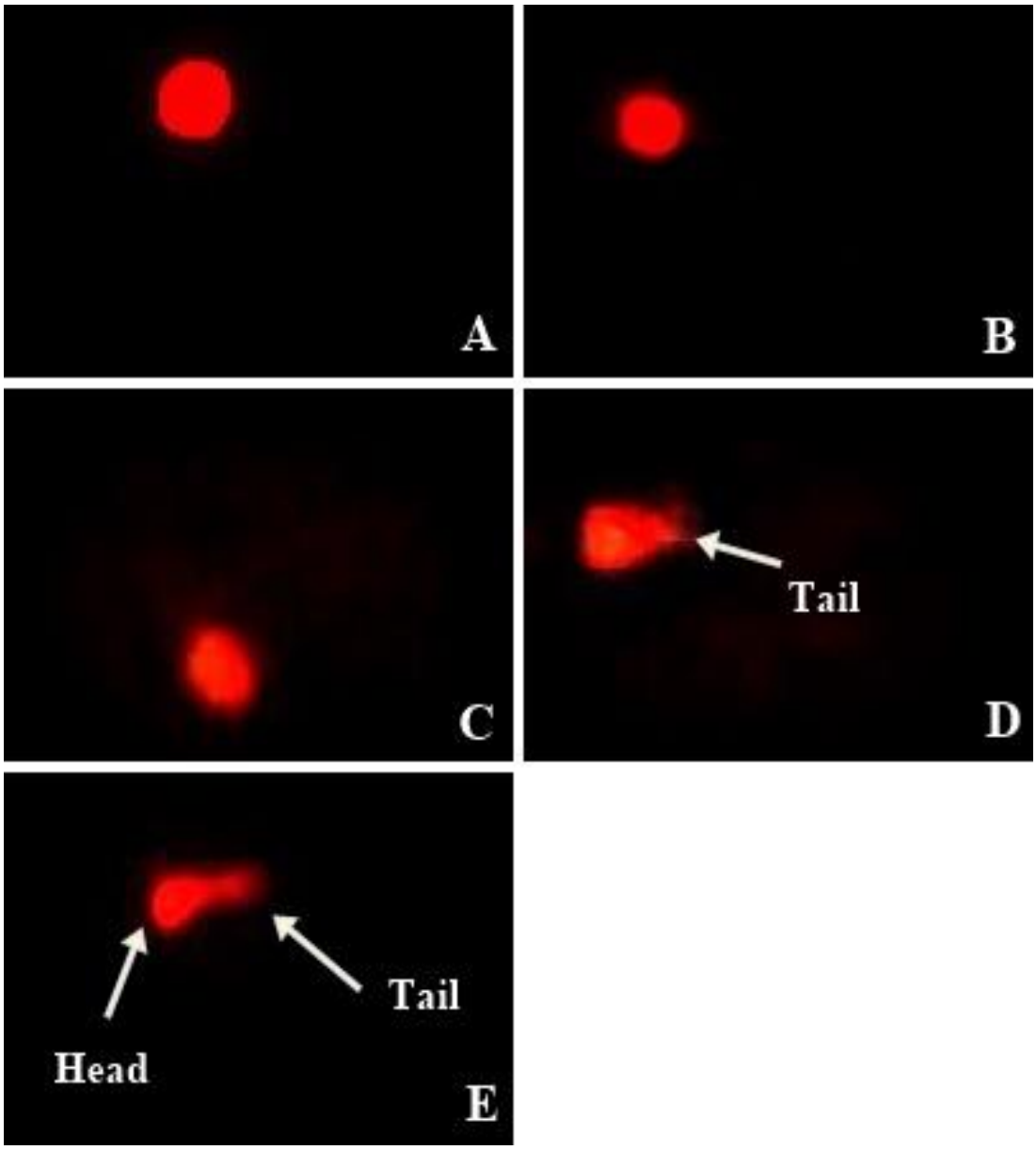


Fig. 3.14 Photomicrographs of Comet assay showing sperm DNA damage after 2 h incubation of mouse epididymes with different $NaAsO_2$ concentrations (N= 4-5) (A) Control with intact DNA (B) 1 µM $NaAsO_2$ and (C) 10 µM $NaAsO_2$ showing no effect on sperm DNA (D) 50 µM $NaAsO_2$ and (E) 100 µM $NaAsO_2$ both depicting sperm DNA damage with short tail (40x).

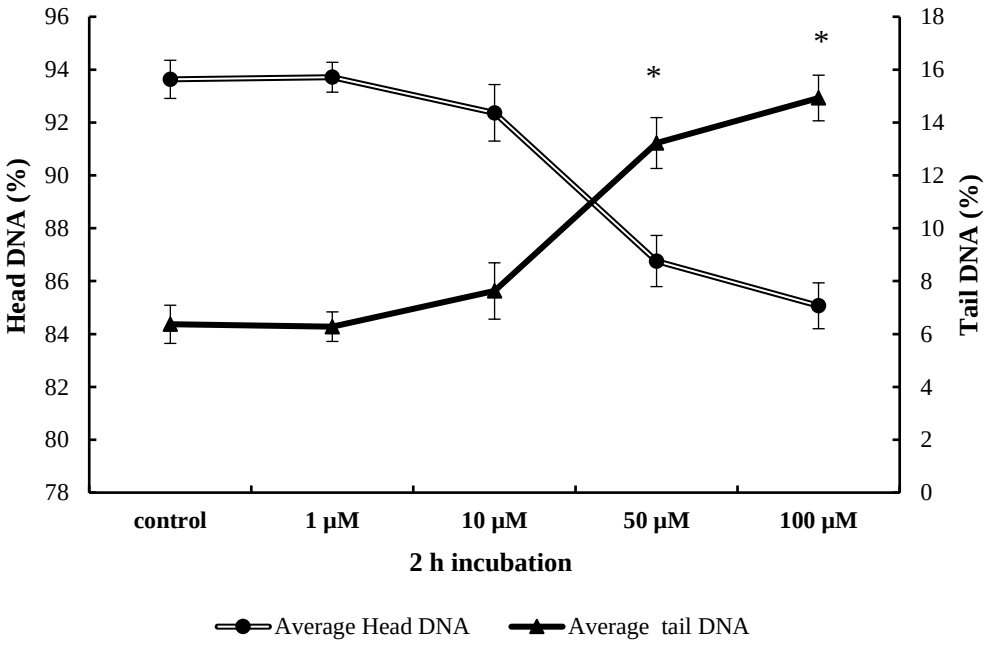


Fig. 3.15 Percentage head and tail DNA of sperms after 2 h incubation with different concentrations of $NaAsO_2$. * shows $P < 0.05$.

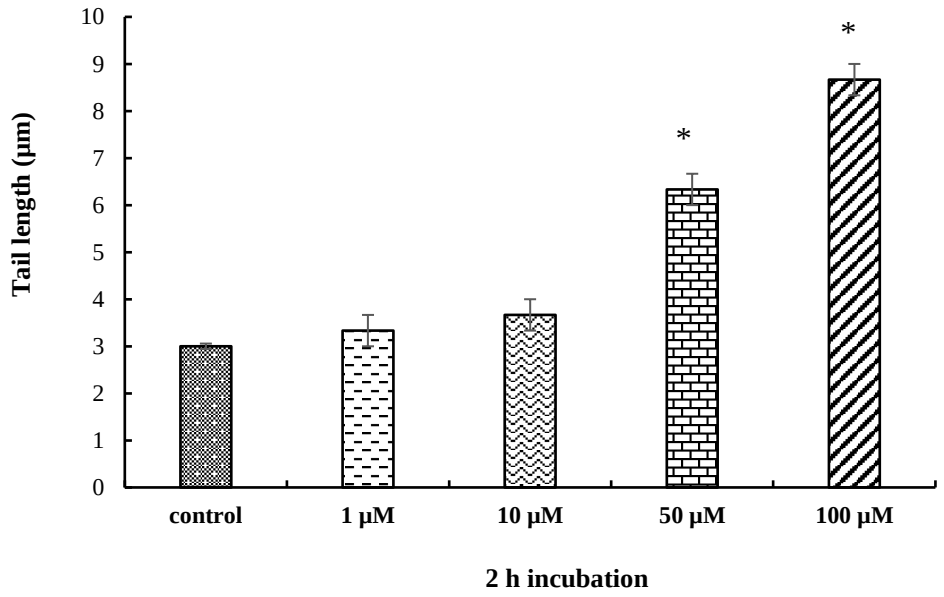


Fig. 3.16 Comet tail length (µm) of sperm comets after 2 h incubation with different concentrations of $NaAsO_2$. * shows $P < 0.05$.

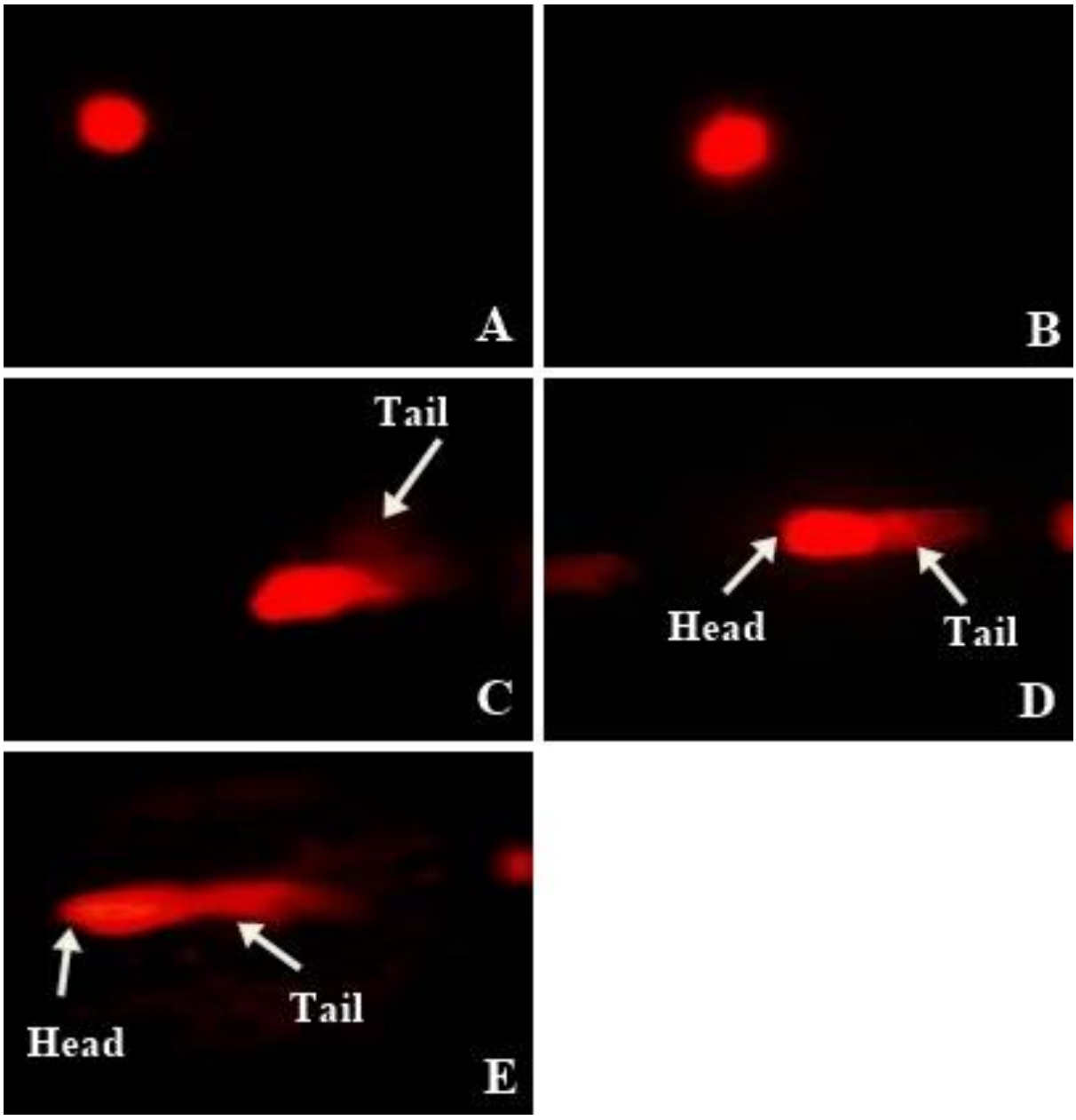


Fig. 3.17 Photomicrographs of Comet assay showing sperm DNA damage after 24 h incubation of mouse epididymes with different $NaAsO_2$ Concentrations (N=4-5) (A) Control with intact DNA (B) 1 µM $NaAsO_2$ with no effect on sperm DNA (C) 10 µM $NaAsO_2$ represents sperm DNA damage with short tail (D) 50 µM $NaAsO_2$ and (E) 100 µM $NaAsO_2$ both showing increased sperm DNA damage with comparatively long tails (40x).

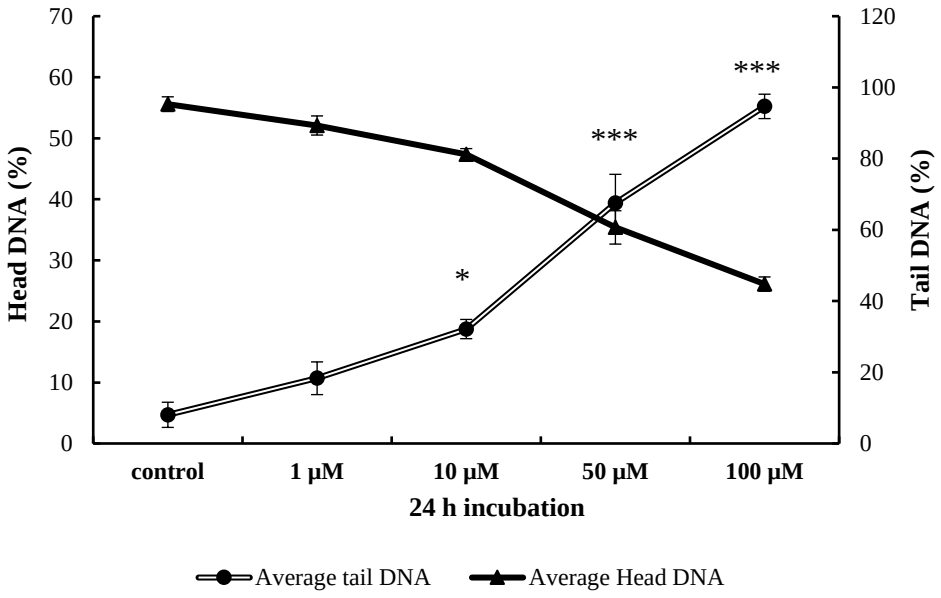


Fig. 3.18 Percentage head and tail DNA of sperms after 24 h incubation with different concentrations of $NaAsO_2$. * represents $P < 0.05$ and *** shows $P < 0.001$.

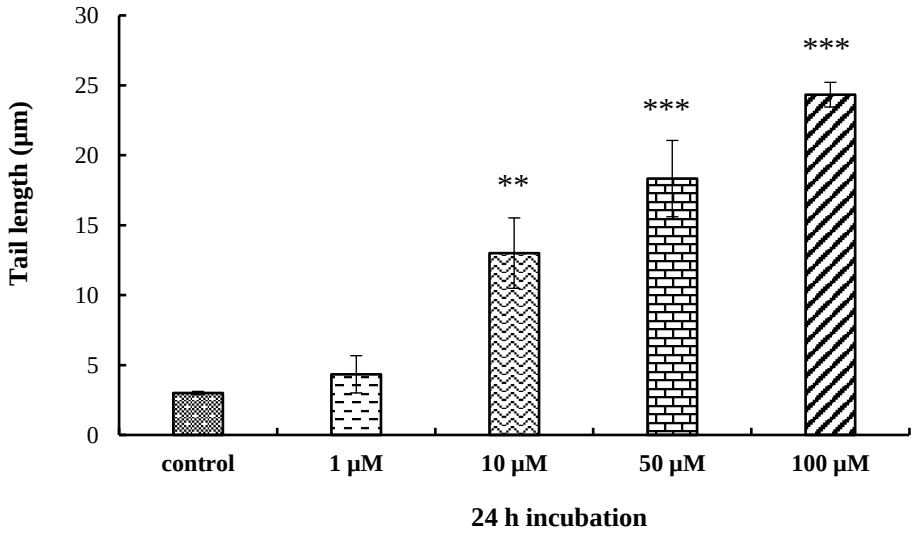


Fig. 3.19 Comet tail length (µm) of sperm comets after 24 h incubation with different concentrations of $NaAsO_2$. ** shows $P < 0.01$ and *** shows $P < 0.001$.

### 3.4. Effect of $NaAsO_2$ on Sperms (Part-II)

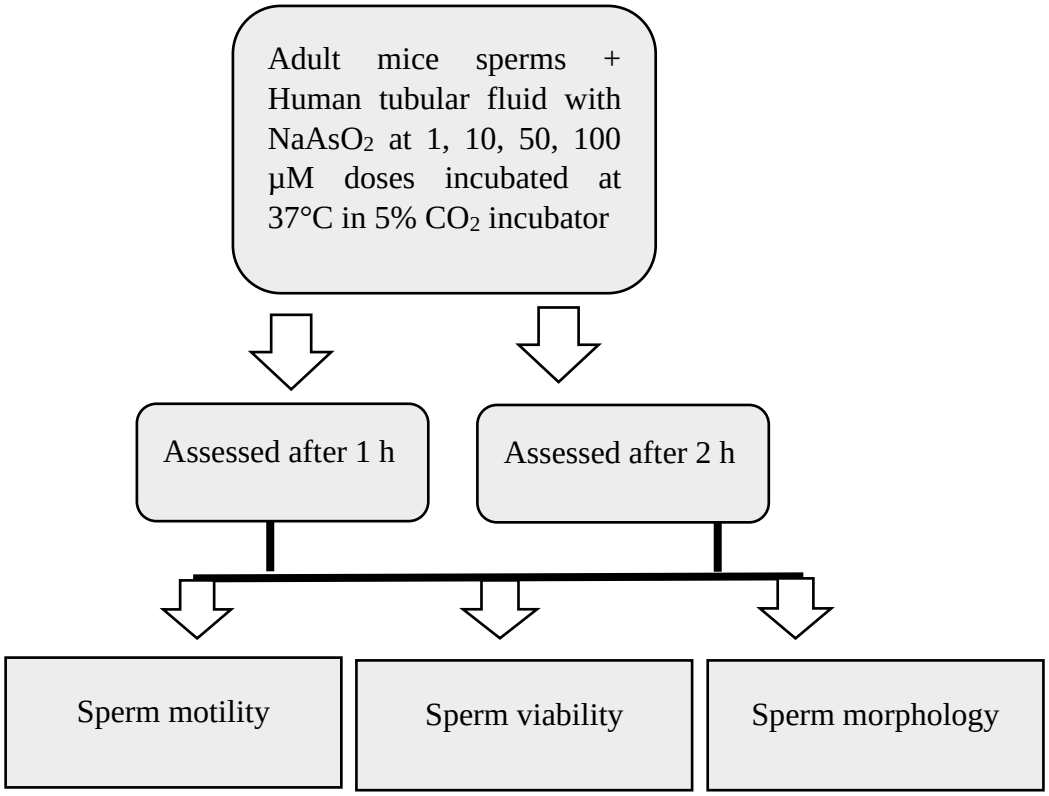


Fig. 3.20 Schematic diagram representing the experimental design of *in vitro* exposure of mouse sperms to different concentrations of $NaAsO_2$.

### 3.4.1. Materials and Methods

Six adult swiss albino mice were taken from the Animal House facility of the Zoology department of Quaid-i-Azam University, Islamabad. Animals were killed by cervical dislocation and cauda epididymis from mice were taken out and placed in Lebovitz-15 medium supplemented with 3 mg/ml BSA. In a culture plate 1ml of EmbryoMax Human Tubal Fluid (HTF) (Sigma-Aldrich, UK) was added and covered with Dow corning 200/50cS fluid (BDH, USA). Cauda epididymis was cut, and sperms were released into the HTF medium and placed inside 5% $CO_2$ incubator at 37 °C for 10 min. Ten µL of sperm suspension was mixed with 30 µL of HTF media containing different concentrations of $NaAsO_2$ (1, 10, 50, 100 µM), (Fig. 3.20). Culture plates were placed in incubator for 2 h. Sperm motility, viability, and dead sperms were counted after 1 h interval using the hemocytometer. Sperm suspension was filled in the hemocytometer counting chamber and analyzed microscopically. Sperm morphology was observed after incubation. Percentage of dead sperms, motility, and viability was also manually calculated. Percentage was calculated according to Seed et al. (1996) by following formula:

$$\frac{No.of\ motile\ or\ stationery\ sperms}{Total\ sperm\ number} \times 100$$

### 3.4.2. Results

#### 3.4.2.1. Percentage sperm motility

Sperm motility was significantly reduced after 1 h incubation of sperms with 50 and 100 µM $NaAsO_2$ concentrations ($P < 0.001$; Fig. 3.21). Following 2 h incubation, a highly significant decrease in the sperm motility was noticed following $NaAsO_2$ exposure. The effects of $NaAsO_2$ aggravated at 50 and 100 µM. In addition, significant decline in sperm motility was also evident after 10 µM treatment of sperms for 2 h ($P < 0.001$; Fig. 3.22).

#### 3.4.2.2. Percentage of dead sperms

The percentage of dead sperms increased almost three-fold after 1 h incubation with 100 µM dose of $NaAsO_2$ ($P < 0.001$; Fig. 3.23). However, after 2 h incubation, a remarkable increase occurred in dead sperm number with approximately 4-fold increase in 50 µM and 5-fold increase in 100 µM $NaAsO_2$ treatments ($P < 0.001$; Fig. 3.24).

#### 3.4.2.3. Sperm morphology

Fig. 3.25 represents the sperm morphology after 2 h incubation with different concentrations of $NaAsO_2$. Sperms incubated with HTF medium alone served as control and showed no changes in the sperm morphology (Fig. 3.25A). Exposure to 1 µM $NaAsO_2$ concentrations did not produce any alteration in the sperm morphology (Fig. 3.25B) However, dramatic changes in the sperm morphology was noticed following 2 h incubation with high concentrations of $NaAsO_2$ (10 µM) which caused curvature abnormalities in the sperms (Fig. 3.25C). Similarly, even greater severe effects were observed in 50 and 100 µM treatments. Sperms became pinhead and few were tailless after exposure to 50 µM $NaAsO_2$ (Fig. 3.25D), while after 100 µM $NaAsO_2$ exposure majority of sperms became tailless (Fig. 3.25E).

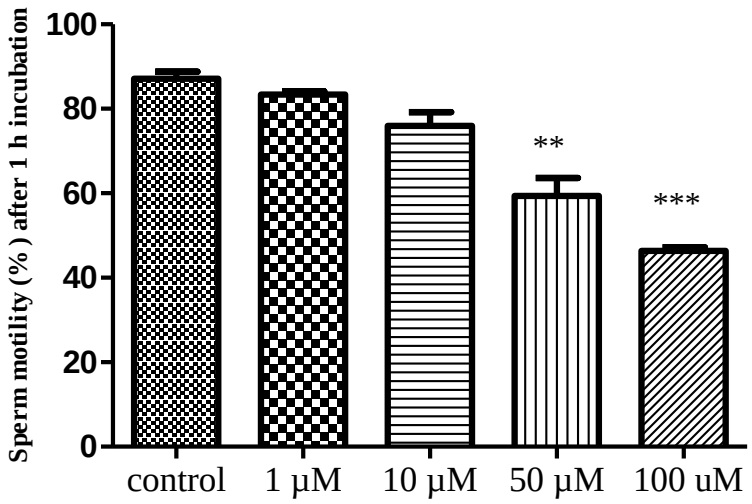


Fig. 3.21 Sperm motility (%) after *in vitro* incubation of sperms with different concentrations of $NaAsO_2$ for 1 h. ** shows $P < 0.01$, and ** shows $P < 0.001$.

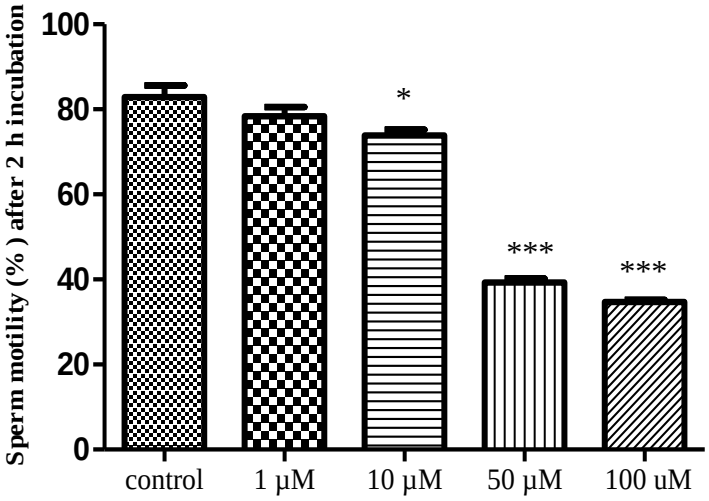


Fig. 3.22 Sperm motility (%) after *in vitro* incubation of sperms with different concentrations of $NaAsO_2$ for 2 h. * shows $P < 0.05$ and ** shows $P < 0.001$.

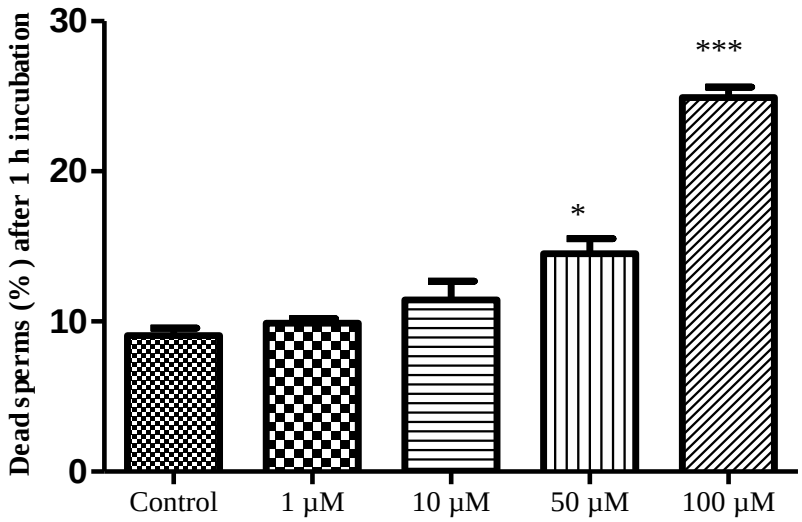


Fig. 3.23 Dead sperms (%) after *in vitro* incubation of sperms with different concentrations of $NaAsO_2$ for 1 h. * shows $P < 0.05$, and *** shows $P < 0.001$.

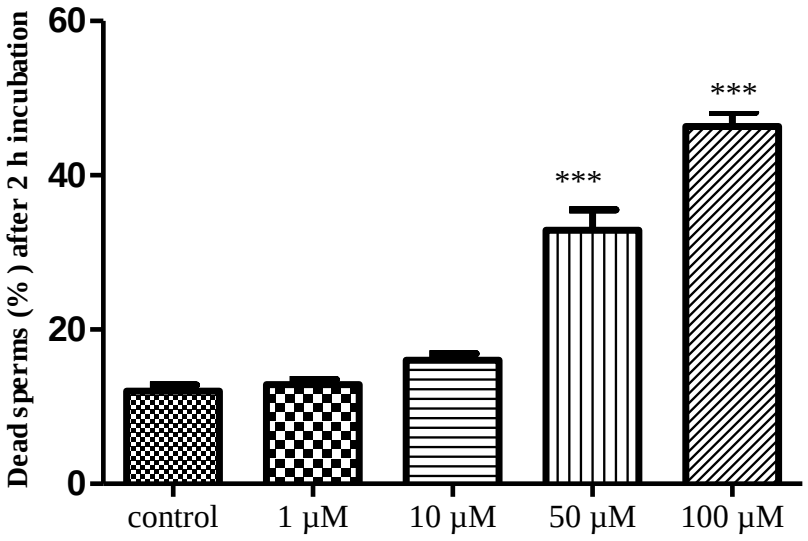


Fig. 3.24 Dead sperms (%) after *in vitro* incubation of sperms with different concentrations of $NaAsO_2$ for 1 h. *** shows $P < 0.001$.

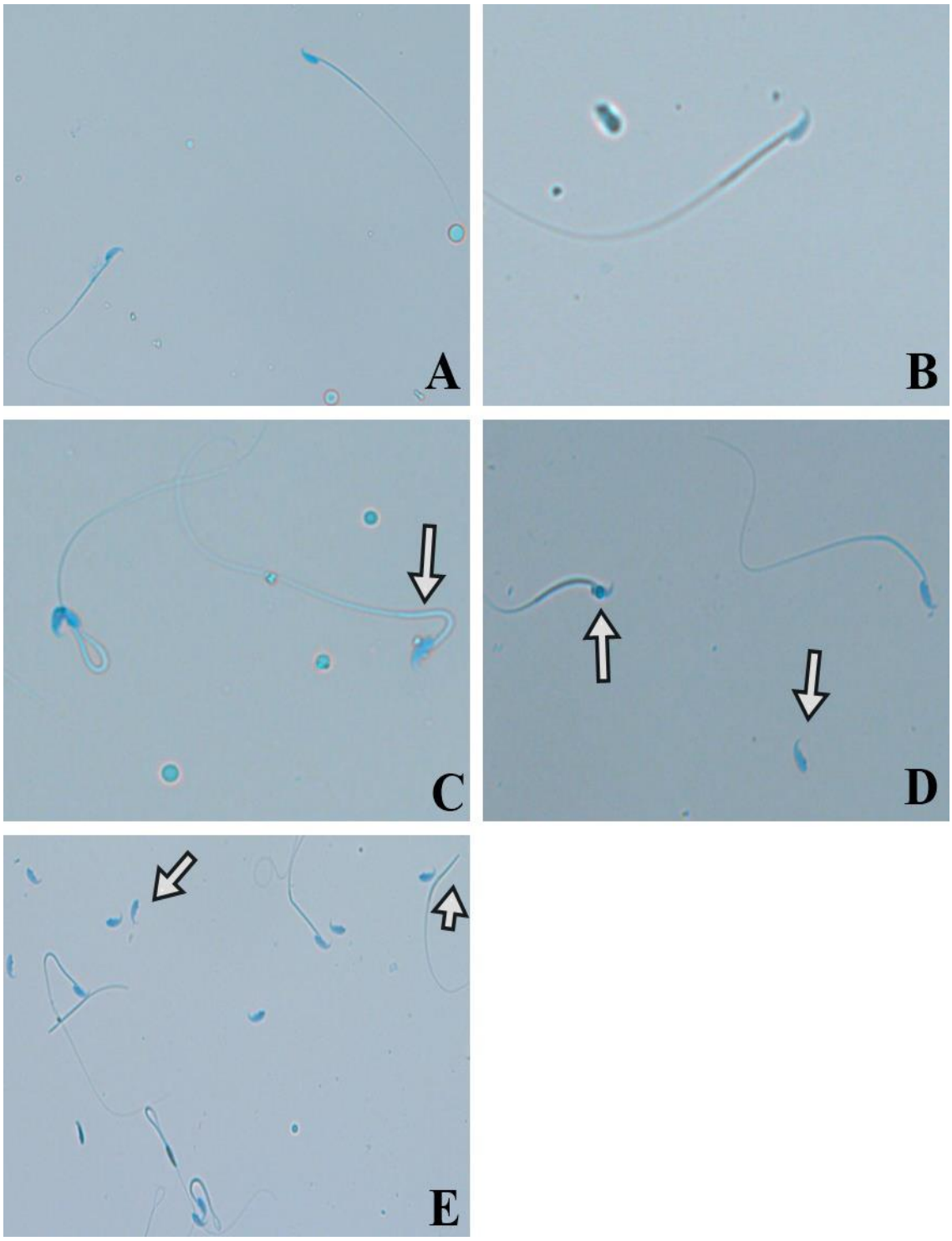


Fig. 3.25 Morphological evaluation of mouse epididymal sperms after 2 h incubation with A: Control, B: 1 µM, C: 10 µM, D: 50 µM, E: 100 µM of $NaAsO_2$ concentrations. Magnification 20x.

## 3.5. Discussion

Contamination of food and drinking water with arsenic, and as well as exposure through occupational resources increases the risk of arsenic poisoning (Jain and Ali, 2000). *In vivo* reproductive toxicity of arsenic in male mice is known (Kim and Kim, 2015) but the *in vitro* effects of $NaAsO_2$ on adult mice testis have not been stated so far. The current study was intended to investigate immediate stress following exposure to $NaAsO_2$. Testicular and epididymal cultures were set up for 2 h and 24 h and incubated with different concentrations of $NaAsO_2$. As the results of chapter 2 have indicated that *in vitro* exposure of high doses (i.e. 50 and 100 µM) for 6 days drastically compromised seminiferous tubule structure. Therefore, the duration of *in vitro* exposure of $NaAsO_2$ was kept short in these experiments to evaluate the early changes induced by arsenic in the adult mice testicular and epididymal tissues.

The study demonstrated *in vitro* biochemical, hormonal, and genotoxic effects upon exposure to arsenic. $NaAsO_2$ induced oxidative stress and genotoxic damage in cultured tissues within 2 h at 50 and 100 µM doses. This effect aggravated by 24 h at 50 and 100 µM doses, and this time it was also evident at 10 µM dose. However, testosterone concentration showed reductions only at 24 h of incubation.

To the best of the author's knowledge, the present *in vitro* study is the first that addressed metabolic and genotoxic stress induced by arsenic in the mice reproductive organs. However, *in vitro* studies conducted on rabbits (Castellini et al., 2009), and Japanese eel (Celino et al., 2009) have also demonstrated the toxic effects of arsenic on sperm characteristics and steroidogenesis respectively. Several *in vivo* studies have however reported reproductive toxicity in male mice upon exposure to arsenic. For instance, sodium arsenite induces oxidative stress and adversely affects sperm functions (Monika et al., 2013), reduces steroidogenesis (Chang et al., 2010), and generates spermatotoxic effects (Pant et al., 2004).

It is very well established that an imbalance between ROS levels and antioxidant enzymes indicates oxidative stress. Usually, CAT, SOD, and POD are considered to be the first line of defense in the antioxidant system (Deyashi and Chakraborty, 2016). Many *in vivo* studies have also reported oxidative stress induced by arsenic (Bashandy et al., 2016; Souza et al., 2016). Increased ROS production causes irreversible oxidation of biomolecules, especially the lipid peroxidation of cell membranes, damage proteins and DNA (Radak et al., 1999). By products of lipid peroxidation are known as TBARS,

which are also considered as oxidative stress index. The antioxidant enzymes act as the self-defense mechanism of the cell and are activated in response to increased ROS production (Shi et al., 2004). Cells become more sensitive to oxidative stress if levels of antioxidant enzymes fall substantially and fail to detoxify ROS production within the cell (Kaul and Forman, 2000). In this aspect, the current study appears similar to the above-mentioned studies, since present testicular cultures also demonstrated significant rise in ROS and TBARS levels and decreases in all three key antioxidant enzymes, CAT, SOD, POD.

Compared to the highly complex *in vivo* environment, the *in vitro* environment is rather simplistic. It can therefore be stated with confidence that no ingredients in the culture medium could induce such alterations, except $NaAsO_2$. This is supported by the control cultures which demonstrated normal levels of ROS and TBARS generated during metabolic activities (Andreyev et al., 2015).

A significant reduction in testosterone concentration that occurred only after 24 h of incubation with $NaAsO_2$ is not unusual. The reason could be increased levels of ROS and TBARS, and a concomitant decrease in the levels of antioxidant enzymes, indicating damage to cellular metabolism. It has been shown previously that testosterone production in the Leydig cells is susceptible to oxidative stress (Aitken and Roman, 2008). Similar to our results, past *in vivo* studies have also reported reduction in testosterone concentrations after arsenic exposure (Jahan et al., 2015; de Araujo Ramos et al., 2017).

The reason why testosterone concentration remained unaffected after 2 h of exposure could be due to a shorter duration of exposure. Since at 24 h, $NaAsO_2$ had enough time to inflict damage to cells and ultimately the normal metabolic activities. Thus, exposure of testicular organ cultures to $NaAsO_2$ concentrations for only 2 h to assess hormonal alterations due to arsenic was perhaps a relatively short time. It is worth mentioning that several past studies have also assessed the *in vitro* reproductive toxicities within 2 h (Ullah et al., 2016; Rehman et al., 2019). In another study, mice neuroblastoma cell cultures were exposed to $NaAsO_2$ for 24 h (Repetto et al., 1994).

Comet assay on epididymal sperm cultures demonstrated substantial increase in comet tail DNA and tail length. This effect is most likely to have been generated by increased ROS that ultimately led to DNA damage. In this study, comet assay parameters like increased tail length and % tail DNA demonstrated DNA damage in sperm after *in vitro*

incubation of sperms with $NaAsO_2$. Similarly, some past reports have also described the genotoxic effects of arsenic (Patlolla et al., 2012; Jahan et al., 2016).

It is also identified that arsenic binds to sulfhydryl groups of proteins residing within sperms (Wang et al., 2007). In addition, sperm nuclear DNA and elasticity of the sperm plasma membrane is adversely affected by the oxidative stress (Farombi et al., 2012). Raised oxidative stress is therefore believed as one of the reasons underlying sperm DNA damage (Kumar et al., 2002). In corroboration with the earlier studies, the current findings also demonstrated that the DNA damage in sperms intensified in a time and dose dependent manner. The results of comet assay revealed that 2 h incubation with higher doses (50 and 100 µM) of $NaAsO_2$ led to significant sperm DNA damage. However, after 24 h incubation even the low dose of 10 µM was capable of inducing sperm DNA damage. Moreover, presently $NaAsO_2$ was used, which is a trivalent state of inorganic arsenic and is highly toxic (Pires Das Neves et al., 2004). Thus, it further justifies that the genotoxic stress observed presently was most likely caused by increased ROS and TBARS production after $NaAsO_2$ exposure.

Many *in vivo* studies have reported reproductive toxicity of arsenic in male mice. Presently no data have reported the in vitro effects of $NaAsO_2$ on adult mice testis. The present study is first time reporting on the biochemical, hormonal and genotoxic effects of different concentrations of $NaAsO_2$ in mice testicular and epididymal culture.

Imbalance between ROS and antioxidant enzymes results in oxidative stress. Usually CAT, SOD, and POD are the first defense boundary in the antioxidant system (Deyashi and Chakraborty, 2016). After 2 h incubation, higher concentrations of $NaAsO_2$ (50 and 100 µM) induced oxidative stress by significant increase in ROS and TBARS, while decreasing the antioxidant enzymes levels in the testis. In contrast, after 24 h incubation highly significant decrease occurred after 10 µM $NaAsO_2$ exposure. Arsenic exposure increased oxidative stress by ROS production, leading towards impaired antioxidant enzymes defence system (Kavitha and Rao, 2008).

Two hours incubation did not affect the testosterone concentrations. However, after 24 h culture incubation, a highly significant reduction was seen in 50 and 100 µM treatments ($P < 0.01$ and $P < 0.001$). Steroidogenesis in the Leydig cells is susceptible to oxidative stress (Aitken and Roman, 2008). *In vivo* arsenic exposure is reported to cause reduction in intratesticular testosterone concentrations (Jana et al., 2006; Jahan et al., 2015).

DNA damage of the epididymal sperms caused by arsenic treatments was analyzed by comet assay. Sperm DNA damage increased in both dose and time dependent pattern. Results of the comet assay showed that after 2 h incubation at higher doses (50 and 100 µM) causes sperm DNA damage, whereas after 24 h even the low dose of 10 µM was capable of inducing sperm DNA damage. Sperm DNA damage is thought to be induced by increasing oxidative stress (Kumar et al., 2002). *In vivo* arsenic treatment is also reported to escalate DNA strand breaks (Balakumar et al., 2010).

Male infertility and fertility states are identification by two major factor such as sperm structure and sperm sensitivity (Guzick et al., 2001). The sperm movement is very much important in achieving its ultimate goal of egg fertilization (Yousef et al., 2003). $NaAsO_2$ toxicity results in the reduction of sperm movement (Momeni and Eskandari, 2012). Guvvala et al. (2015) stated dose dependent decrease in mice sperms kinetics after *in vivo* exposure to $NaAsO_2$ for 40 days and increasing concentration of arsenic caused decline in spermatozoa count. In the present study, application of $NaAsO_2$ *in vitro* to mouse epididymal sperms induced dose dependent sperm motility reduction, whereas sperm death and morphological abnormalities were increased after 2 h incubation. Thiol and sulfhydryl groups in mammalian sperms maintain its stability and motility (Pant et al., 2004). The basic reason behind the reduction of sperm movement in the current study can be the binding property of the arsenic with the thiol proteins or sulfhydryl groups (Uckun, et al., 2002). Normally, the thiol proteins are very considered crucial for the sperm movement (Pant et al., 2004; Wang et al., 2007). Another possible reason behind the reduction of sperm movement is due to the oxidative degeneration of lipids in the cell membranes of the sperm (Das et al., 2009a,b). The sperm morphological abnormalities observed in the present *in vitro* study are curvature abnormalities, pinhead sperms, and tailless sperms after 10, 50, and 100 µM $NaAsO_2$ exposure respectively. Present observations appear parallel to an earlier study by Momeni (2012) which demonstrated reduction in sperm viability and motility along with morphological abnormalities of sperms, like banana head, pin head and bent neck, after 8 weeks *in vivo* exposure of $NaAsO_2$ in rats.

### 3.6. Conclusion

In conclusion, the outcomes of this *in vitro* study indicate that higher concentrations of $NaAsO_2$ (50 and 100 µM) have immediate toxic effects on mice reproductive system while moderate dose (10 µM) requires greater exposure time for causing oxidative stress and alterations in the mice reproductive system. Exposure to 1 µM $NaAsO_2$ dose

caused no changes in the testicular tissues and epididymal sperms. The toxic effects are exhibited in time and dose dependent manner. However, further *in vitro* studies with increased incubation time duration are required to understand detailed mechanism, biochemical and physiological effects of $NaAsO_2$ on male reproductive system.

# CHAPTER # 4

# *IN VIVO* SUB-CHRONIC AND CHRONIC EXPERIMENTATION

## Abstract

At present times exposure of human populations and other organisms to arsenic has considerably increased due to diverse human anthropogenic activities. Various forms of arsenic are reported to induce reproductive toxicity in both humans and animals. Numerous researches have been conducted to evaluate the developmental and reproductive toxicity of arsenic in rodents after in utero exposure. Little information is however available on the reproductive organ development and toxicity induced upon oral exposure of $NaAsO_2$ in prepubertal mice from PND day 25. The present experiments were aimed to evaluate the reproductive and endocrine effects of $NaAsO_2$ following chronic and sub-chronic exposure. Prepubertal male mice were treated with $NaAsO_2$ (0.01, 5, and 10 mg/L) in drinking water from PND 25 to PND 53 in sub-chronic experiment while in the chronic experiment same doses of $NaAsO_2$ were administered orally from PND 25 to PND 114. Histological, hormonal, sperm parameters, oxidative stress, antioxidant enzymes, and DNA damage were assessed. Sub-chronic arsenic exposure at high doses (5 and 10 mg/L) produced significant decrease in the antioxidant enzymes (CAT, SOD, POD), sperm and hormonal parameters (Follicle-stimulating hormone, luteinizing hormone, Testosterone), whereas substantial increases were noted in ROS and TBARS. After sub-chronic exposure of $NaAsO_2$, the testicular morphological changes as well as sperm DNA damage was only evident at the highest dose (10 mg/L). In contrast, chronic exposure of $NaAsO_2$ induced severe harmful effects on mice reproductive system even at the lowest treated dose (0.01 mg/L). Chronic exposure of $NaAsO_2$ in mice induced testicular oxidative stress, reduction in hormonal and sperm parameters, along with substantial changes in mice testis, and body weight, and morphological changes in testis and epididymis. In chronic exposure experiments the sperm DNA damage was noticed at 5 and 10 mg/L $NaAsO_2$ concentration. In conclusion, higher doses of arsenic initiated adverse effects on mice reproductive system after both sub-chronic and chronic exposure. On the other hand, low dose of $NaAsO_2$ (0.01 mg/L) induced significant changes in mice reproductive system only after its chronic exposure.

## 4.1. Introduction

Arsenic, which is ubiquitously present in the atmosphere, is considered as the major toxic metal element (Fu et., 2014). Apart from exposure to arsenic through natural resources, many anthropogenic sources like fungicides, herbicides, rodenticides, and industrial waste have also increased the chances of its occurrence in the environment (Nickson et al., 1998). It is also identified as a widely present environmental waste having potential carcinogenic, mutagenic, and teratogenic properties (Chiou et al., 2008). At the present, the major concern is severe contamination of the natural water reservoirs with arsenic in West Bengal, China, India, Bangladesh, Pakistan, Afghanistan, Mongolia, Myanmar, Cambodia, DPR Korea, and Nepal (Mukherjee et al., 2006).

Chronic arsenic exposure leads to numerous health problems in humans. For instance, hyperkeratosis, peripheral vascular disease, melanosis, gangrene, and lung diseases have been associated with arsenic exposure (Das et al., 2004). Arsenic can potentially induce male reproductive toxicity (Chang et al., 2010; Das et al., 2009ab). Arsenic reproductive toxicity in males depends upon the route of arsenic exposure (Holson et al., 2000ab) Its exposure decreases testes and accessory sex organ weight, and testosterone concentrations eventually causing spermatotoxicity in animals (Sarkar et al., 2003; Waalkes et al., 2003; Pant et al., 2004). A few studies have suggested that arsenic induced necrosis, apoptosis and diminished testosterone production and can cause gonadal dysfunction (Davila-Esqueda et al., 2012; Khan et al., 2013; Shen et al., 2013).

Arsenic exposure induces morphological abnormalities in the male reproductive system by increasing the production of ROS (Kumar et al., 2002). Developmental toxicity caused by arsenic includes, retardation of fetal growth, abnormalities, and mortality (Tabacova et al., 1996; Golub et al., 1998). Numerous studies have reported *in vivo* toxic outcomes of arsenic on adult male rat reproductive system (Mukherjee and Mukhopadhyay, 2009; Morakinyo et al., 2010; Sudha, 2012; Khan et al., 2013; Jalaludeen et al., 2014).

Pre and postnatal developmental time periods are considered to be critical as regards exposure to a toxicant and can cause severe adverse health effects in the later life (Barker, 2007). A number of adverse effects has been reported in mouse after prenatal

exposure to high arsenic concentrations (Waalkes et al., 2003; He et al., 2007; Srivastava et al., 2007; Hill et al., 2008). Similarly, low dose of arsenic (50 ppb) in mice induced learning impairments (Martinez-Finley et al., 2009) and liver dysfunctions (Lantz et al., 2009). Adverse outcomes of low doses of arsenic on pre-and post-natal development of mouse have also been reported by Kozul-Horvath et al. (2012). Prepubertal arsenic exposure is reported to generate delay in sexual maturation in female rats (Reilly et al., 2014).

Postnatal time-period is considered extremely perilous for the development of reproductive system, and it is suspected that any toxicant exposure during this phase can cause drastic changes in the reproductive organ morphology and function. The present experiments were designed to evaluate the male mouse reproductive system development after postnatal exposure to different concentrations of $NaAsO_2$. Effects on testicular and epididymal morphology, hormones, biochemical analysis, and sperm DNA damage were evaluated after sub-chronic and chronic exposure of $NaAsO_2$ from PND 25.

## 4.2. Materials and Methods

### 4.2.1. Animals and maintenance

Eighty Swiss albino male mice (age 21-25 days old) were obtained from the NIH Islamabad and maintained in the Animal House Facility of Quaid-i-Azam University, Islamabad. Before start of experiment, mice were maintained in small rodent cages under standard laboratory conditions. Ambient room temperature (22-24°C) and photoperiod (14 h light: 10 h dark). Mice were fed standard rodent feed and water *ad libitum.* Animal handling and the experimental design was approved by the Bioethical committee of the Faculty of Biological sciences, Quaid-i-Azam University, Islamabad.

### 4.2.2. Experimental design

The present experiment was designed to assess the *in vivo* effects of $NaAsO_2$ on the prepubertal development and physiology of male mice reproductive system. Mice were divided into four groups, each group had ten mice. $NaAsO_2$ was administered orally at the concentrations of 0.01, 5, and 10 mg/L for 28 days (PND 25 to PND 53) and 90 days (PND 25 to PND 114). Animals were weighed and dissected by decapitation on PND 54 and PND 115 (Fig. 4.1).

Blood was collected and centrifuged at 3000 rpm for 10 min. Plasma was stored at -20 °C for hormonal analysis. Mice reproductive organs were weighed and collected for histology analysis.

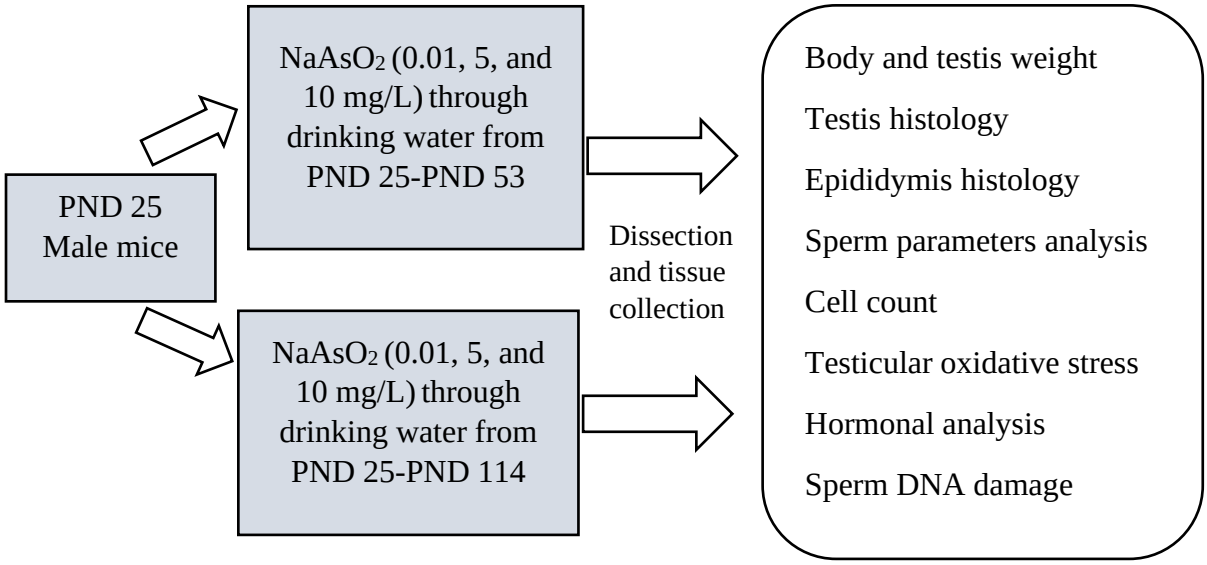


Fig. 4.1 Experimental design for sub-chronic and chronic *in vivo* exposure to $NaAsO_2$ in male mouse from PND 25.

### 4.2.3. Tissue processing for histological and biochemical analysis

After dissection, one testis and epididymis of each mice were washed with saline and stored for biochemical analysis. Other testis and epididymis of each animal was also washed with saline and fixed for histology in 10% formalin.

### 4.2.4. Sperm collection

Cauda epididymis was sliced into pieces in 5 ml HTF medium (Merck, UK) and sperms were allowed to swim out of epididymis. These were left in an incubator at 37°C and 5% $CO_2$ for 30 min.

### 4.2.5. Epididymal sperm count

For sperm counting, 10 µl of sperm mixture was mixed with 190 µl of distilled water to prepare 1:20 dilution. Ten µl of the above prepared dilution was taken and placed in

a Neubauer's chamber and sperms were counted under a light microscope at 40x magnification (Zobeiri et al., 2012).

### 4.2.6. Evaluation of sperm motility

For evaluation of sperm motility, a preheated slide was taken and 10 µl of sperm suspension was placed and covered with a cover slip. Motility of the epididymal sperms was evaluated at 200x magnification under a photomicroscope (Nikon, Japan).

### 4.2.7. Evaluation of sperm viability

Sperm viability was assessed following the method of Wyrobek et al. (1983). Twenty µl of the sperm suspension was combined with the same volume of Eosin-nigrosin stain (0.05%). Following incubation for 2 min, 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 in color. Percentage of sperm viability was counted after observing 200 sperms in each sample.

### 4.2.8. Antioxidant enzymes

Testes were homogenized in 1 ml PBS and centrifuged at 3000 rpm for 15 min and supernatant was collected and stored for determination of hormonal analysis, antioxidant enzymes and lipid peroxidation. Oxidative stress parameters (ROS and TBARS) and antioxidant enzymes (CAT, SOD, POD, and GSH), were determined following the standard protocols with slight modifications (methods described in detail in Chapter 3).

### 4.2.9. Hormonal analysis

#### 4.2.9.1. Testosterone

Intra-testicular and plasma testosterone concentrations were measured as described in Chapter 3.

#### 4.2.9.2. Luteinizing hormone (LH)

Quantitative measurement of plasma LH was done following the manufacturer's provided protocol using a commercial kit (Reddot Biotech Inc. USA).

#### 4.2.9.2.1 Principle and procedure

Enzyme linked immune sorbent assay used for determination of the plasma LH is principally based on competitive binding of sample's plasma LH and biotin labelled LH, with coated LH antibody. LH determination in the samples were done according to protocol provided in the kit. Fifty µl of standards, blank, and samples were added into the wells coated with LH antibody. Immediately after this, in each 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. Later, washing was done with 350 µl of washing solution for three times and inverted on an absorbing paper. Following drying, 100 µl of reagent B was put into each well and incubated for 60 min. After 1 h wells were washed 5 times with the washing solution. Following this, 90 µl of Horseradish Peroxidase (HRP) was added into each well and incubated for 15-25 min at 37°C. HRP gave blue color to the wells. There is an inverse relationship between the developed color intensity and LH concentration in the sample. Fifty µl of stop solution was then added into each well and mixed thoroughly, it turned blue color into yellow. Absorbance was taken at 450 nm on an ELISA plate reader. Lowest LH detection limit of the kit was 0.11 ng/ml.

### 4.2.9.3 Follicle stimulating hormone (FSH)

FSH was quantitatively measured using a commercial kit and following the manufacturer's protocol (Mybiosource, USA).

#### 4.2.9.3.1 Principle and procedure

It detects FSH by using double antibody sandwich method. Principally, it can simultaneously locate the coated antibody and detection of the antibody. In each well, hundred µl of standards and samples were added, after sealing it was incubated for 90 min at 37°C. Subsequently, washing was done twice and 100 µl of biotinylated FSH antibody was added and incubated for one hour at 37°C. Washing of the plate was done thrice. With the exception of blank wells, 100 µl of enzyme conjugate was added and incubated for 30 min. After 30 min, 5 times washing was performed. After washing, 100 µl of color reagent was added and incubated at 37°C in dark. After appearance of darker color, 100 µl of Color reagent C was added to stop the reaction mixture. The contents were mixed thoroughly, and absorbance at 450 nm was taken within 10 min.

### 4.2.10. Tissue histology

Testis and epididymis histology were performed as described in Chapter 2.

#### 4.2.10.1 Microscopy and image analysis

Prepared H & E slides of testis and epididymis were observed using a photomicroscope (Leica, Germany). Pictures were captured at 20x and 40x using digital camera (Canon, Japan) attached to the microscope. Image analysis was done using Image J software.

Area was measured in $\mu m^2$ for seminiferous tubules, interstitial space, and epididymal tubules. The percentage area was calculated by using the following formula:

$$\%A(st)= \frac{A\,(st)\times 100}{T}$$

Here, 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 there mean per tubule seminiferous were calculated.

### 4.2.11. Statistical analysis

Data values are shown as mean ± S.E. For comparing the control group with different treated groups, one-way ANOVA following Dunnet's post-hoc test was applied on the data using the GraphPad Prism (5.01, CA USA). P-value was < 0.05.

## 4.3. Results

### 4.3.1. Sub-chronic exposure

#### 4.3.1.1. Body and testis weight

After *in vivo* sub-chronic exposure to arsenic mice testis (Fig. 4.2) and body weight (Fig. 4.3) were determined on postnatal day 54. No statistical difference was found between the control and treated group.

#### 4.3.1.2. Sperm parameters

Sperm count, motility, and viability were reduced ($P < 0.001$) in 5 and 10 mg/L treated groups when compared to the control group. Low $NaAsO_2$ treatment (0.01 mg/L) did not induce any change. Fig. 4.4 represents sperm motility and viability, whereas Fig. 4.5 shows sperm count after sub-chronic exposure of $NaAsO_2$.

#### 4.3.1.3. Testicular biochemical parameters

Sub-chronic oral arsenic exposure to mice from PND 25-53 caused significant changes in the biochemical parameters (Table 4.1). ROS increased significantly after arsenic exposure ($P < 0.001$; Fig. 4.6), while lipid peroxidation was also significantly induced ($P < 0.001$; Fig. 4.7) in the higher treatment groups (5 and 10 mg/L).

Significant reduction was observed in the antioxidant enzymes levels. CAT, POD ($P < 0.001$) (Fig. 4.8), and SOD (Fig. 4.9). Similar trend was noticed in the reduced glutathione (GSH) concentrations (Fig. 4.10). $P < 0.001$ was observed after 5 and 10 mg/L $NaAsO_2$ treatments, while 0.01 mg/L treated group remained unaffected when compared with the control group.

#### 4.3.1.4. Effect of *in vivo* sub-chronic $NaAsO_2$ administration on hormonal concentrations of mice

Hormone concentrations are presented in Table 4.2. Fig. 4.11 and 4.12 show intratesticular and plasma testosterone concentrations respectively. Substantial reduction ($P < 0.001$) occurred in the intratesticular testosterone and plasma testosterone levels after 5 and 10 mg/L $NaAsO_2$ treatments, while 0.01 mg/L treatment group showed no statistical difference, when compared to the control.

Plasma FSH and LH concentrations were significantly reduced ($P < 0.001$) upon 5 and 10 mg/L $NaAsO_2$ treatment. Low dose $NaAsO_2$ treatment group (i.e. 0.01 mg/L) did not cause any change in plasma LH and FSH concentrations (Fig. 4.13).

#### 4.3.1.5. Morphological changes induced in mice testis and epididymis after sub-chronic $NaAsO_2$ exposure

Fig. 4.14 represents the testicular morphology after *in vivo* sub-chronic $NaAsO_2$ exposure. In control group, seminiferous tubules were compactly arranged, the tubular lumen were filled with sperms and tubules had thickened epithelium. In 0.01 mg/L $NaAsO_2$ treatment group, no morphological changes were observed, and the seminiferous tubule were similar in appearance to the control group. In contrast, 5 and 10 mg/L treatment groups showed thin epithelium, and loss of spermatocyte population. The seminiferous tubule of both treatment groups also showed reduced sperm number in the luminal space.

The cauda epididymis has no morphological alterations in treated and control group. However, the number of sperms in the lumen was decreased in 5 and 10 mg/L treatment groups (Fig. 4.15).

#### 4.3.1.6. Morphometry of mice testis and epididymis

Table 4.3 represents the morphometrical analysis of the mice testis after in vivo sub-chronic exposure to $NaAsO_2$. Area of seminiferous tubules and cauda epididymis exhibited no statistically significant difference between treated and control group. Diameter of seminiferous remained unaffected after $NaAsO_2$ treatment. Epithelial height was significantly reduced in 5 and 10 mg/L treated groups ($P < 0.001$). Lumen diameter of the seminiferous tubules showed significant increase only after 10 mg/L treatment ($P < 0.05$). Fig. 4.16 shows the number of spermatocytes and spermatids in the testis after *in vivo* sub-chronic exposure of different concentrations of $NaAsO_2$. Number of spermatocytes reduced significantly in mice testis after 10 mg/L $NaAsO_2$ treatment ($P < 0.01$). Similarly, spermatid numbers were decreased substantially ($P < 0.001$) in the testis after highest dose treatment (10 mg/L). In contrast, non-significant changes were observed in the mice testicular seminiferous tubule and interstitial space (%) (Fig. 4.17).

Morphometry of cauda epididymis revealed no statistical difference in the lumen and tubular diameter (Fig. 4.18), and epithelial height (Fig. 4.19) of the treated group when compared with the control.

### 4.3.1.7. Sperm DNA damage

Postnatal *in vivo* sub-chronic $NaAsO_2$ exposure in mice caused sperm DNA damage at high dose (10 mg/L) (Fig. 4.20). Fig. 4.21 shows percentage head and tail DNA, whereas tail length is presented in Fig. 4.22. Tail DNA, and Tail length were significantly increased ($P < 0.05$) after 10 mg/L $NaAsO_2$ treatment as compared to the control (Table 4.4).

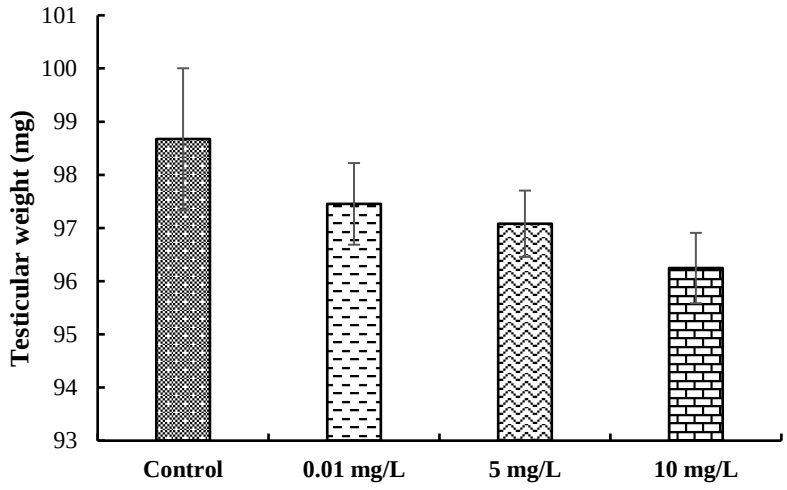


Fig. 4.2 Mouse testis weight (mg) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$.

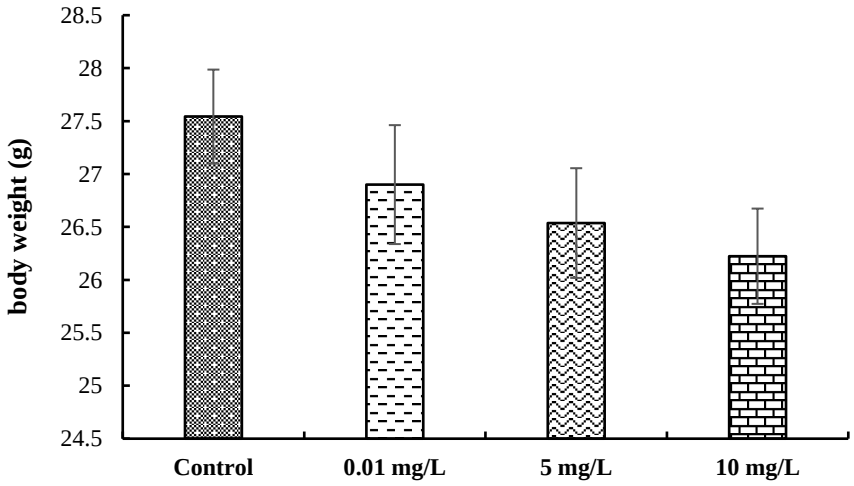


Fig. 4.3 Mouse body weight (g) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$.

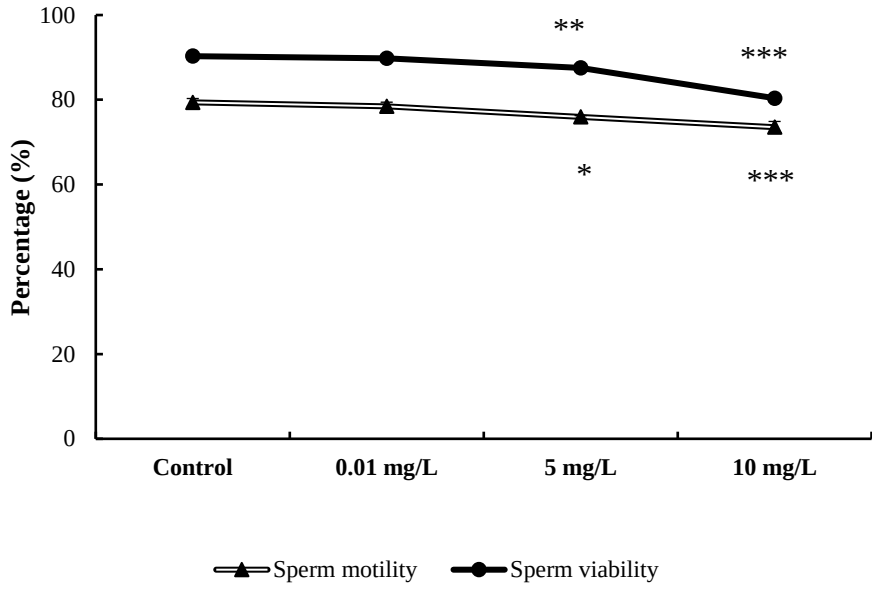


Fig. 4.4 Sperm motility and viability (%) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. *, **, and *** shows $P < 0.05$, 0.01, and 0.001 respectively.

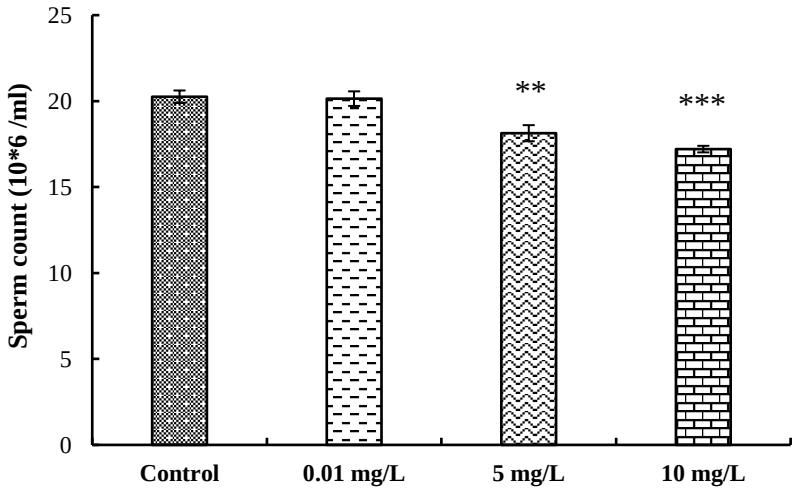


Fig. 4.5 Sperm count ($10^{\times}6$/ml) in mouse after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows $P < 0.01$, and 0.001 respectively.

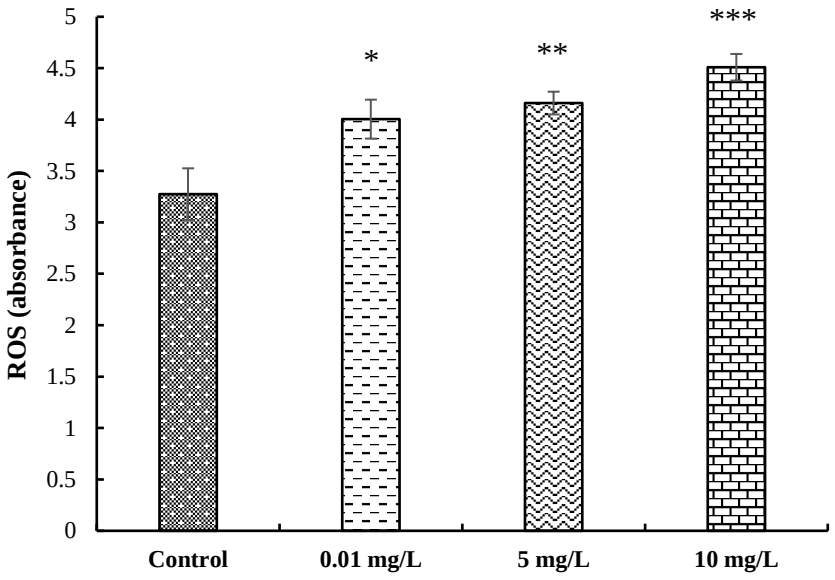


Fig. 4.6 ROS (absorbance) levels in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. *, **, and *** shows $P < 0.05$, 0.01, and 0.001 respectively.

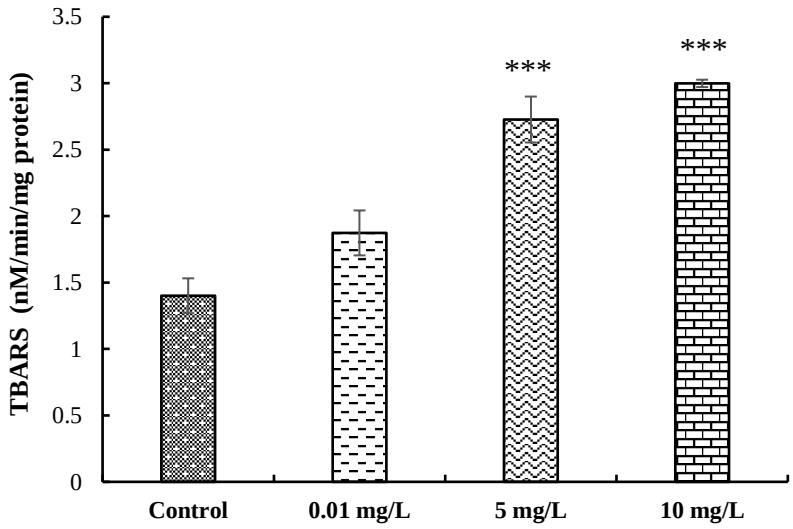


Fig. 4.7 TBARS (nM/min/mg protein) levels in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

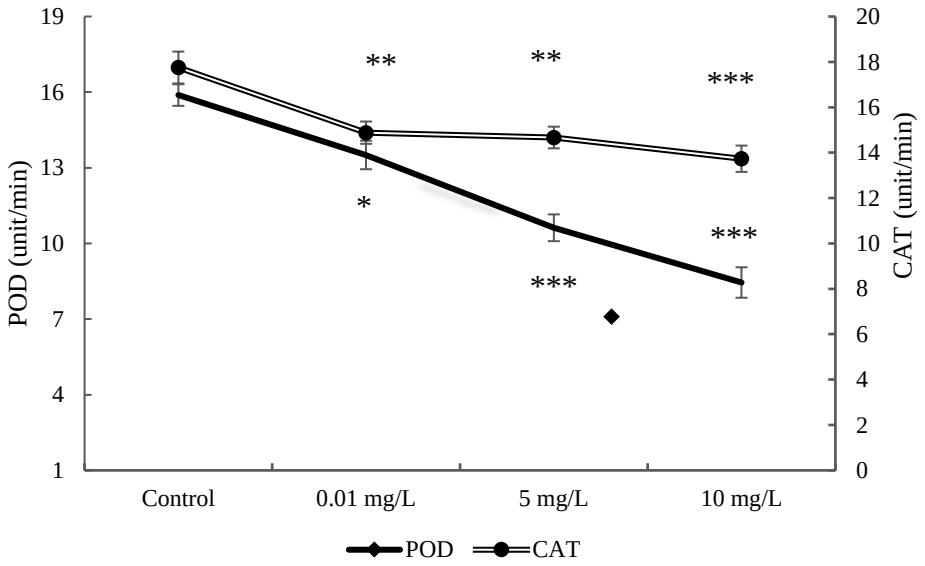


Fig. 4.8 Concentrations of CAT and POD (unit/min) in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. *, **, and *** shows $P < 0.05$, 0.01, and 0.001 respectively.

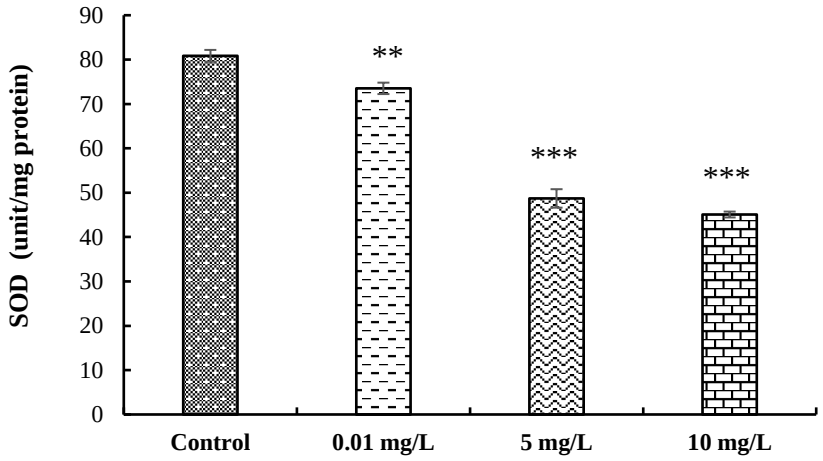


Fig. 4.9 Concentrations of SOD (unit/mg protein) in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows $P < 0.01$, and 0.001 respectively.

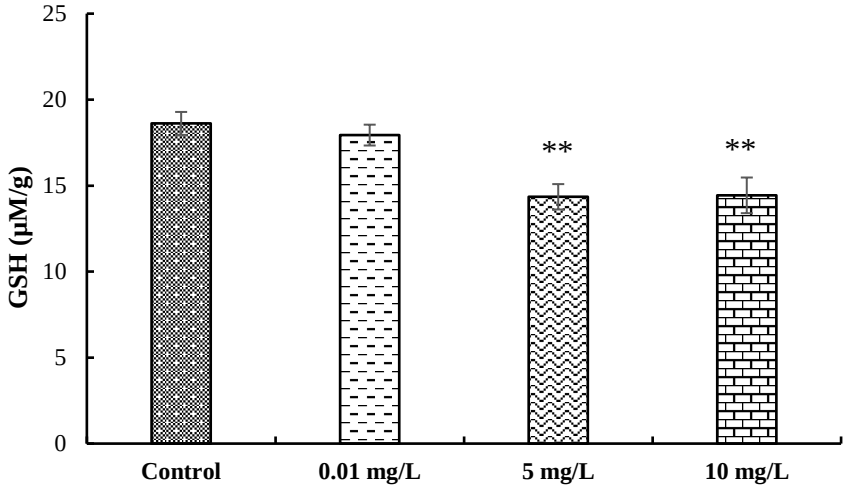


Fig. 4.10 Concentrations of GSH (µM/g) in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. ** shows $P < 0.01$.

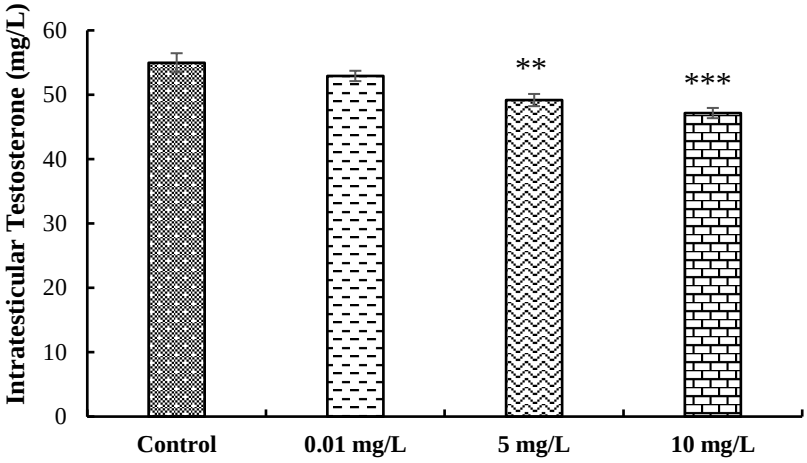


Fig. 4.11 Intratesticular testosterone concentration (mg/L) in mouse testicular homogenate after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows $P < 0.01$ and 0.001 respectively.

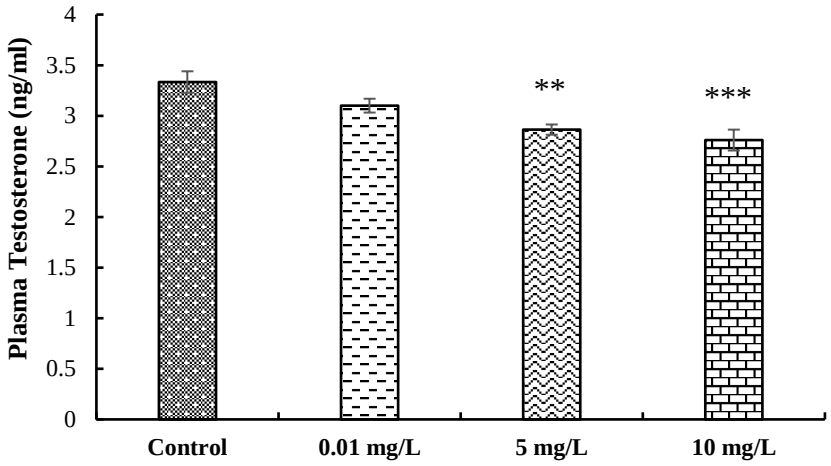


Fig. 4.12 Plasma testosterone concentrations (ng/ml) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows P < 0.01 and 0.001 respectively.

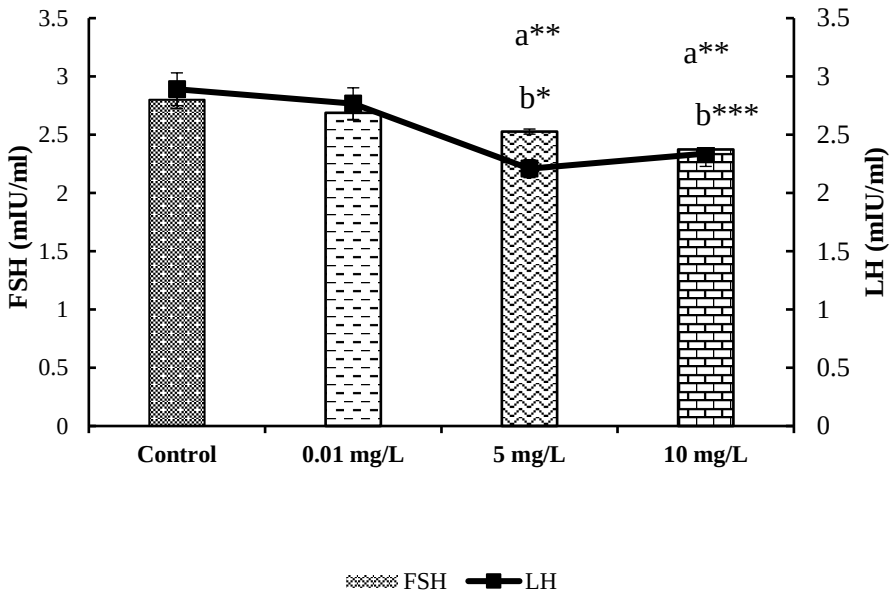


Fig. 4.13 Plasma FSH and LH concentrations (mIU/ml) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. a** shows $P < 0.01$ of LH concentrations, while b* and b*** shows $P < 0.05$ and 0.001 of FSH concentrations.

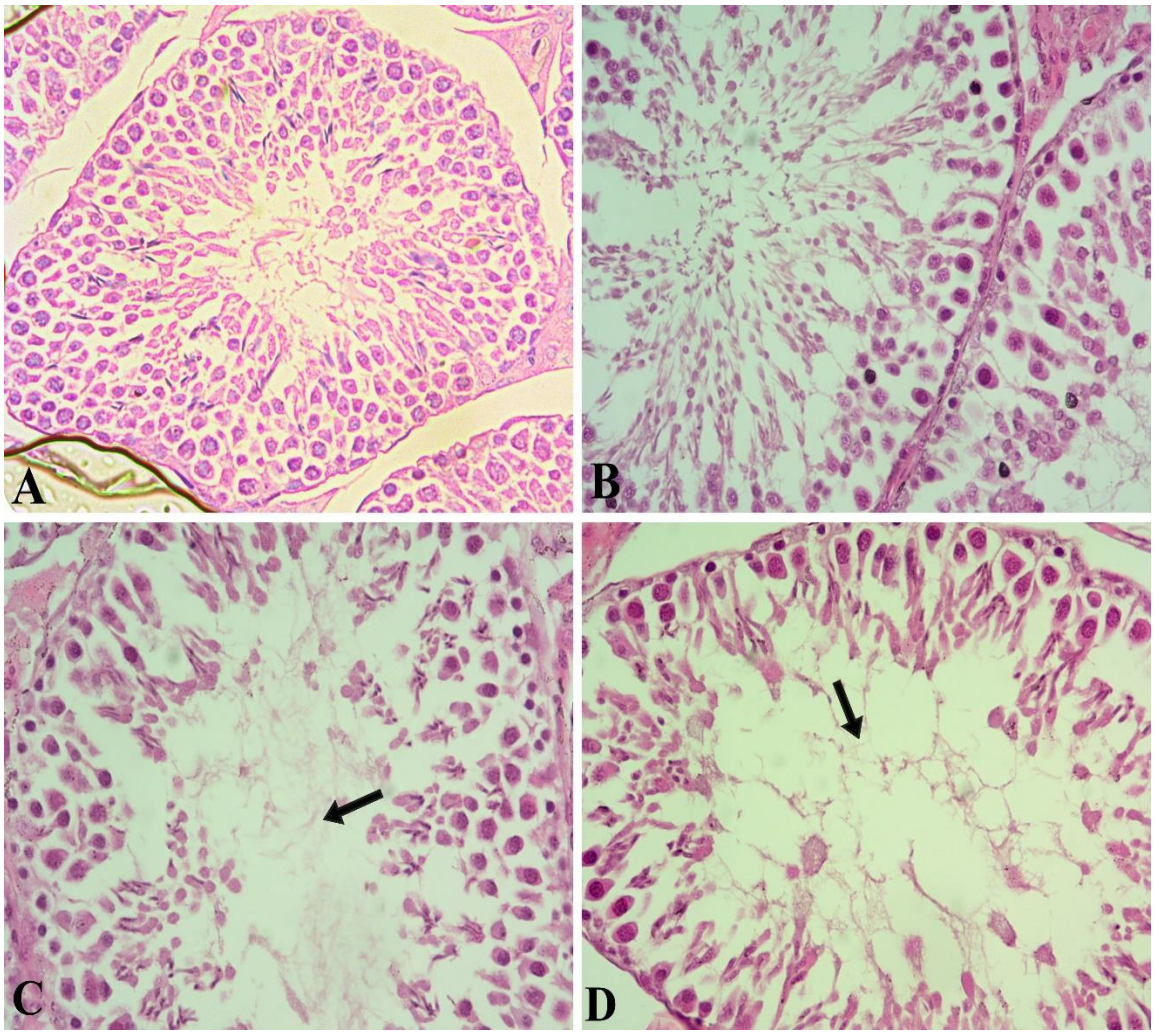


Fig. 4.14 Photomicrograph of mouse testicular tissue after *in vivo* sub-chronic $NaAsO_2$ exposure (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Hematoxylin and eosin stained (40x).

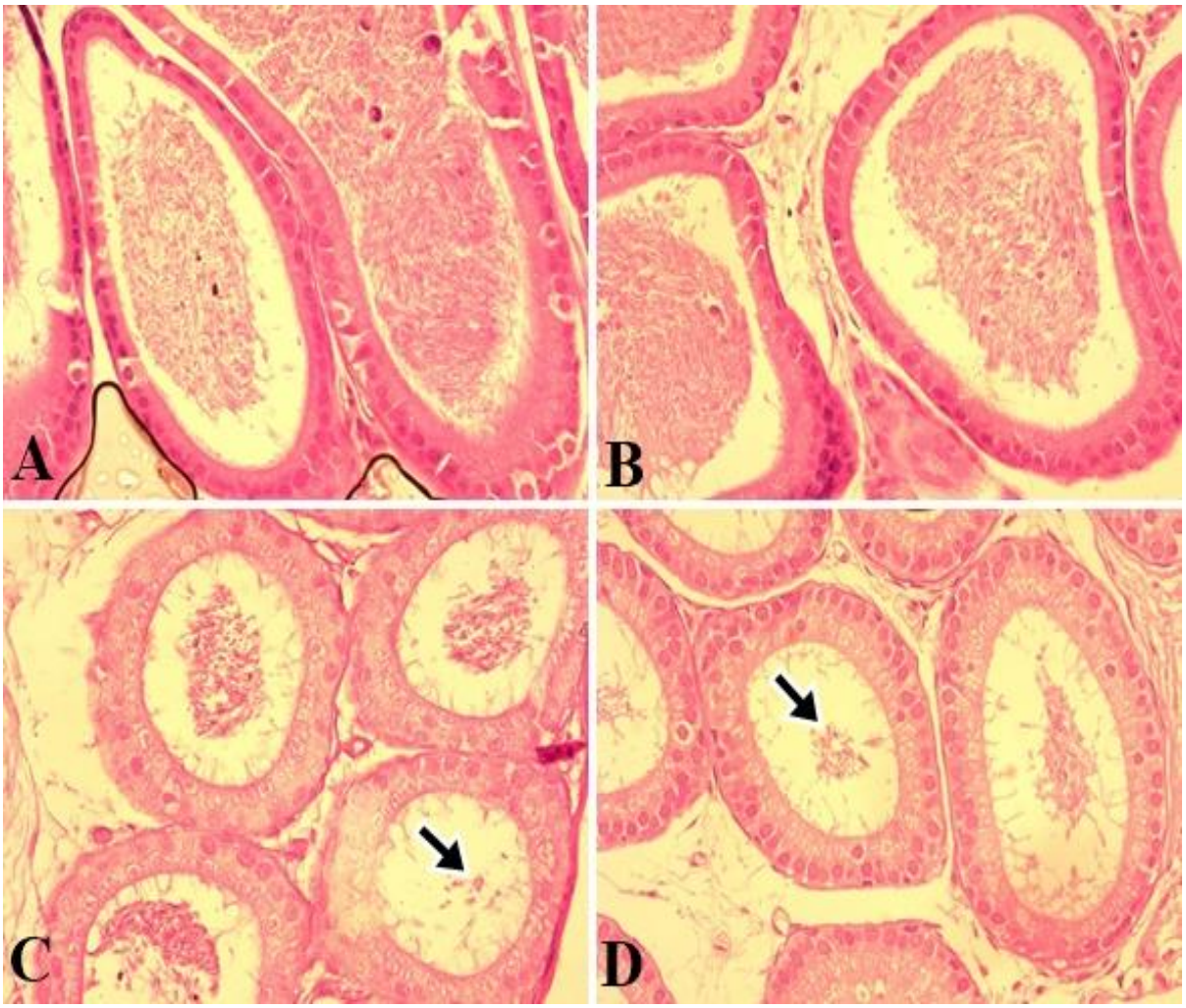


Fig.4.15 Photomicrograph of mouse epididymal tissue after *in vivo* sub-chronic $NaAsO_2$ exposure (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Hematoxylin and eosin stained (40x).

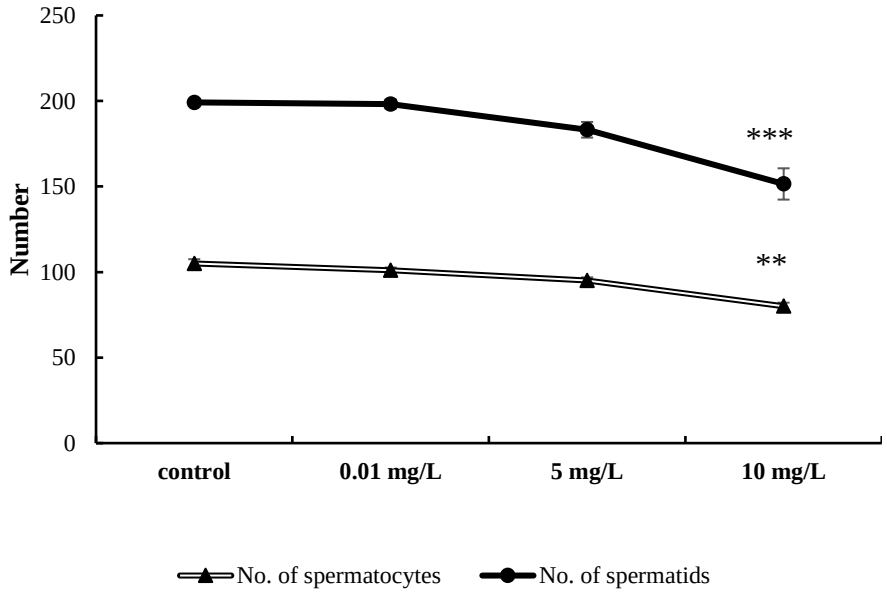


Fig. 4.16 Number of spermatocytes and spermatids in the mouse testis after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. ** and *** shows $P < 0.01$ and 0.001 respectively.

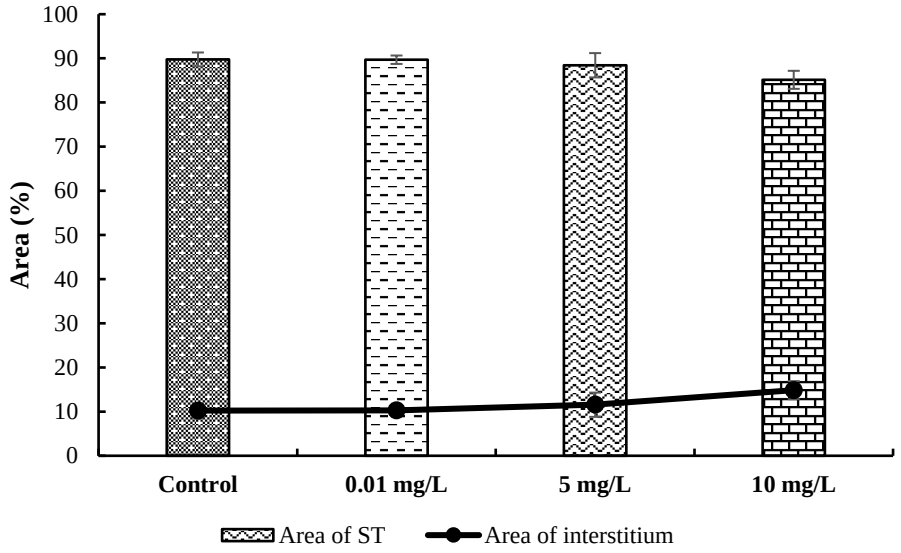


Fig. 4.17 Area of mouse testis seminiferous tubules and interstitial space (%) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$.

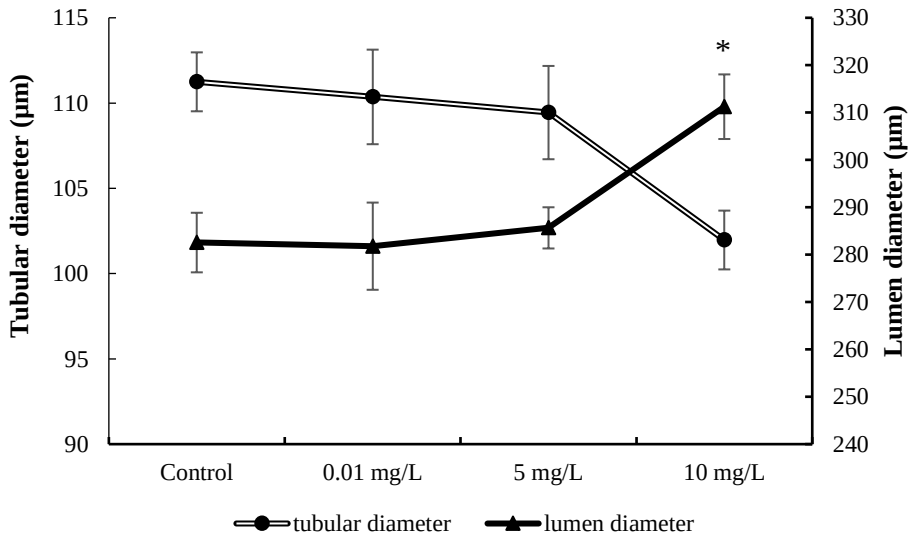


Fig. 4.18 Tubular and lumen diameter of mouse epididymis (µm) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$.

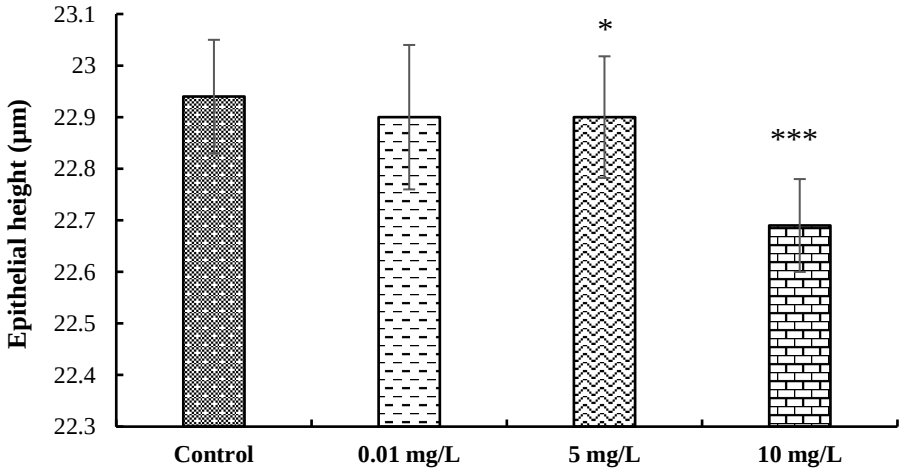


Fig. 4.19 Epithelial height of mouse epididymis (µm) after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$.

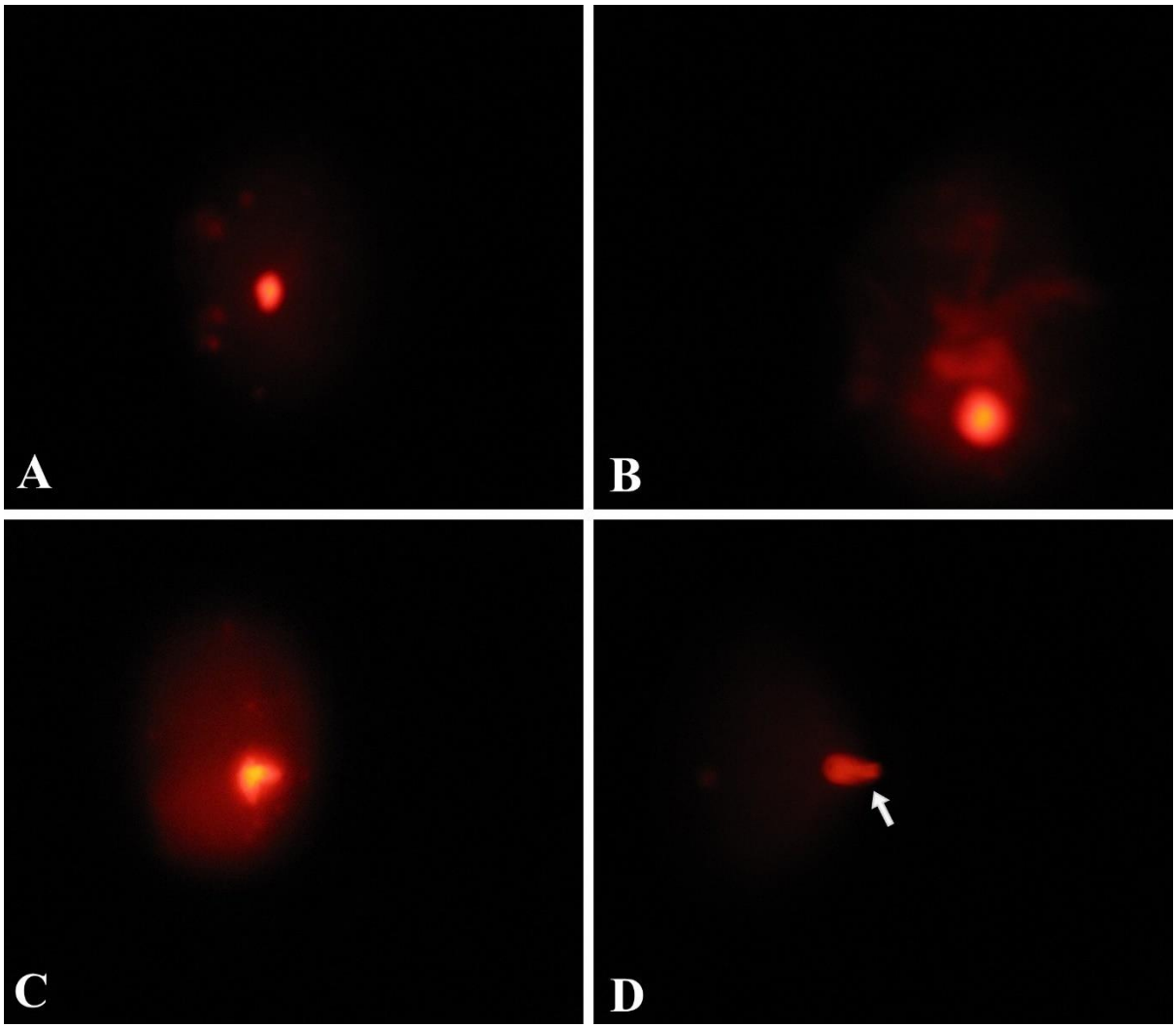


Fig. 4.20 Total DNA damage in the sperm tissue *in vivo* sub-chronic $NaAsO_2$ exposure (A) Control (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L. Arrow shows increased tail length after 10 mg/L $NaAsO_2$ treatment. 40x.

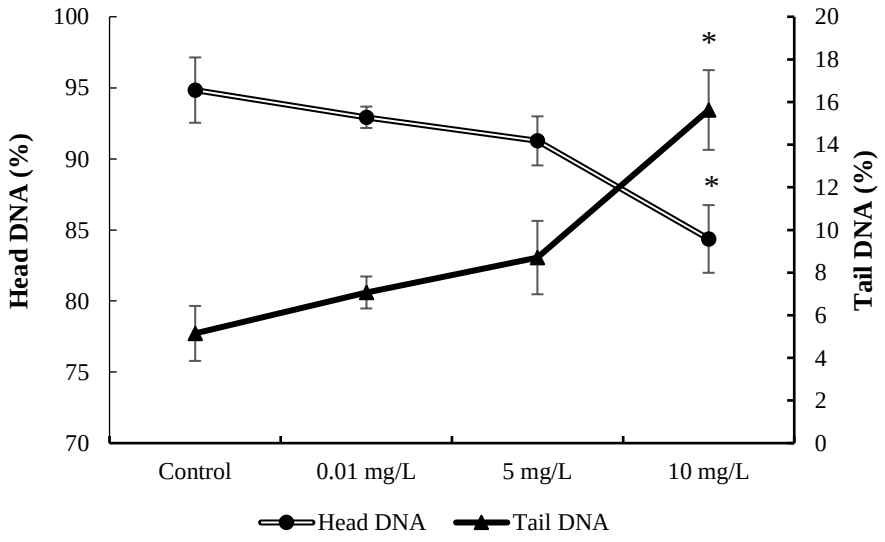


Fig. 4.21 Sperm head and tail DNA (%) determined through comet assay after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. * shows $P < 0.05$.

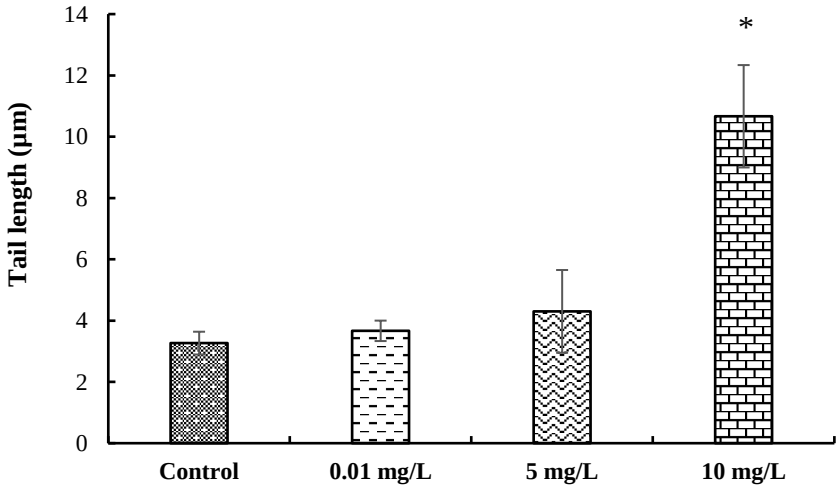


Fig. 4.22 Sperm comet tail length (µm) determined through comet assay after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. * shows $P < 0.05$.

Table: 4.1 Oxidative stress parameters in mouse testicular homogenate after *in vivo* sub-chronic $NaAsO_2$ exposure.

| | **Parameters** | | | | | |
|---|---|---|---|---|---|---|
| **Groups** | **ROS (Absorbance)** | **TBARS (nM/min/mg protein)** | **CAT (unit/min)** | **SOD (unit/mg protein)** | **POD (unit/min)** | **GSH (µM/g)** |
| **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** | 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 mg/L** | 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 mg/L** | 4.5±0.12*** | 2.99±0.02*** | 13.7±0.57*** | 45.0±0.66*** | 8.4±0.60*** | 14.4±1.03** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA following Dunnet's multiple comparison test).

Table: 4.2 Hormonal concentrations in mouse after *in vivo* sub-chronic $NaAsO_2$ exposure

| **Treatments** | **Plasma testosterone (ng/ml)** | **Intra-testicular testosterone (ng/g)** | **LH (mIU/mL)** | **FSH (mIU/mL)** |
|---|---|---|---|---|
| **Control** | 3.33±0.10 | 54.96±1.48 | 2.89±0.14 | 2.80±0.07 |
| **0.01 mg/L** | 3.10±0.06 | 52.92±0.80 | 2.76±0.13 | 2.68±0.06 |
| **5 mg/L** | 2.86±0.05** | 49.17±0.94** | 2.20±0.07** | 2.52±0.02* |
| **10 mg/L** | 2.76±0.10*** | 47.15±0.79*** | 2.33±0.11** | 2.37±0.07*** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

Table: 4.3 Morphometry of mouse testis after *in vivo* sub-chronic $NaAsO_2$ exposure

| | Parameters | | | | |
|---|---|---|---|---|---|
| **Groups** | **Area of seminiferous tubules (%)** | **Area of interstitium (%)** | **Seminiferous tubule diameter (µm)** | **Epithelial height** | **Lumen diameter (µm)** |
| **Control** | 89.76±1.56 | 10.23±1.56 | 201.78±1.85 | 56.91±0.92 | 31.57±0.76 |
| **0.01mg/L** | 89.69±0.95 | 10.30±0.95 | 199.45±0.95 | 56.57±0.82 | 31.38±0.83 |
| **5 mg/L** | 88.44±2.75 | 11.55±2.75 | 197.78±0.65 | 53.62±0.57* | 33.23±0.67 |
| **10mg/L** | 85.14±2.04 | 14.85±2.04 | 197.87±0.86 | 51.83±0.80*** | 34.64±0.80* |

Values are expressed as mean ± S.E.

*, *** indicate significant difference at $P < 0.05$, and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

Table: 4.4 Sperm DNA damage after *in vivo* sub-chronic $NaAsO_2$ exposure.

| | **Parameters** | | |
|---|---|---|---|
| **Groups** | **% Tail DNA** | **Tail length (µm)** | **% Head DNA** |
| **Control** | 5.14 ± 2.29 | 3.26 ± 0.37 | 94.84 ± 2.29 |
| **0.01 mg/L** | 7.06 ± 0.74 | 3.66 ± 0.33 | 92.92 ± 0.75 |
| **5 mg/L** | 8.70 ± 1.72 | 4.30 ± 1.35 | 91.2 ± 1.72 |
| **10 mg/L** | 15.6 ± 2.37* | 10.6 ± 1.67* | 84.36 ± 2.38* |

Values are expressed as mean ± S.E.

*, *** indicate significant difference at $P < 0.05$, and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

### 4.3.2. After chronic $NaAsO_2$ exposure

#### 4.3.2.1. Body and testis weight

After chronic $NaAsO_2$ exposure of mice from PND 25 to PND 114, significant reductions ($P < 0.001$) were observed in testis and body weights among the treated groups (Fig. 4.23 and 4.24 respectively).

#### 4.3.2.2. Sperm parameters

Fig. 4.25 shows the sperm motility and viability after *in vivo* chronic treatment of $NaAsO_2$. Sperm motility was substantially decreased in all treatment groups ($P < 0.001$), whereas sperm viability was significantly reduced ($P < 0.001$) in the 5 and 10 mg/L treatment groups as compared to the control group. When compared to the control group, significant decline ($P < 0.001$) was observed in sperm count in all three treatment groups (Fig. 4.26).

#### 4.3.2.3. Testicular biochemical parameters

Table 4.5 represents levels of oxidative stress parameters in the testicular homogenates after *in vivo* chronic exposure to $NaAsO_2$. ROS and TBARS increased significantly ($P < 0.001$) in all dose groups of $NaAsO_2$ (Fig. 4.27 and 4.28 respectively). However, antioxidant enzymes CAT, SOD, and POD were significantly reduced ($P < 0.001$) in all treatment groups. Similarly, substantial decreases were noticed in GSH ($P < 0.001$). Fig. 4.29 shows the concentrations of CAT and POD, Fig. 4.30 represents SOD concentrations, and GSH levels in the testis homogenate after chronic exposure of $NaAsO_2$ is shown in Fig. 4.31.

#### 4.3.2.4. Hormonal parameters

Hormonal concentrations are presented in Table 4.6. When compared to the control group, the intra-testicular testosterone levels were found significantly decreased (Fig. 4.32; $P < 0.001$) in all treatment groups. The plasma testosterone concentrations were substantially reduced after 5 and 10 mg/L treatments, while comparatively less reduction was observed after 0.01 mg/L chronic $NaAsO_2$ treatment (Fig. 4.33; $P < 0.001$). Similar reductions ($P < 0.001$) were also observed in plasma LH and FSH concentrations after chronic exposure of $NaAsO_2$ (Fig. 4.34).

### 4.3.2.5. Morphological changes in mice testis and epididymis

Fig. 4.35 shows testicular morphology after *in vivo* chronic exposure of $NaAsO_2$. Histology of control testis shows sperm filled lumen, and compactly arranged cells in the seminiferous tubules. Conversely, in 0.01 mg/L treatment group the luminal space was increased, and the number of sperms were reduced. Similarly, excessive degeneration of germ cells and reduced sperm numbers were observed in a dose-dependent manner in 5 and 10 mg/L chronic $NaAsO_2$ treatments. On the other hand, figure 4.36 illustrates the morphological changes and dose dependent reduction in sperm number in the mice epididymis after chronic exposure of $NaAsO_2$.

### 4.3.2.6. Morphometry of mice testis and epididymis

Morphometrical analysis of mice testis showed significant changes following chronic exposure to $NaAsO_2$ (Table 4.7). In comparison to the control group, spermatocytes and spermatid numbers were significantly decreased ($P < 0.001$) in all treatment groups in a dose dependent manner (Fig. 4.37). On the other hand, the percentage area of interstitium was increased ($P < 0.05$), while percentage seminiferous tubule diameter was significantly decreased ($P < 0.001$) in highest treated groups (5 and 10 mg/L), as compared to the control (Fig. 4.38). Epithelial height and seminiferous tubule diameter were substantially decreased in the all treatment groups ($P < 0.001$; Fig. 4.39 and 4.40 respectively). The lumen diameter of seminiferous tubules showed significant increase ($P < 0.001$; Fig. 4.39), in comparison to the control group.

A significant decrease ($P < 0.001$) in epididymal epithelial height was noticed. Tubular diameter of cauda epididymis was significantly decreased ($P < 0.001$) only in 5 and 10 mg/L $NaAsO_2$ treatment groups in comparison to the control group. In contrast to this, luminal diameter of cauda epididymis increased substantially ($P < 0.001$) in all treatments (Fig. 4.41).

### 4.3.2.7. Sperm DNA damage

Fig. 4.42 represents sperm DNA damage after chronic *in vivo* $NaAsO_2$ exposure. Table 4.8 shows that the percentage tail DNA and tail length were significantly increased ($P < 0.01$) in 5 and 10 mg/L treated groups (Fig. 4.43 and 4.44 respectively).

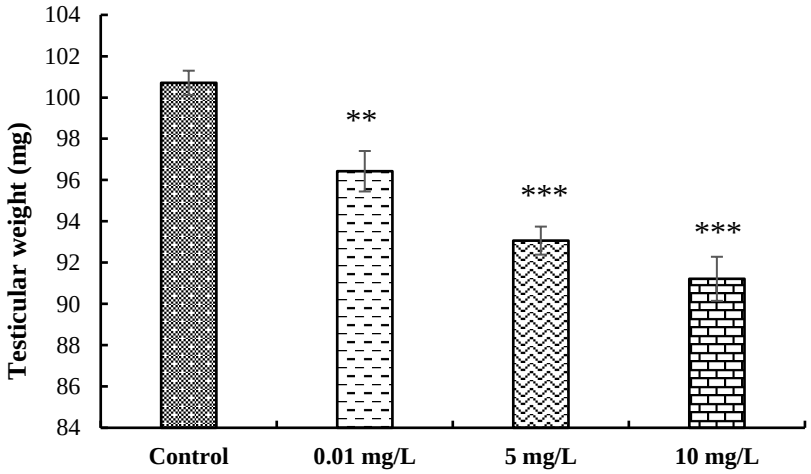


Fig. 4.23 Mouse testis weight (mg) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows P < 0.01 and 0.001 respectively.

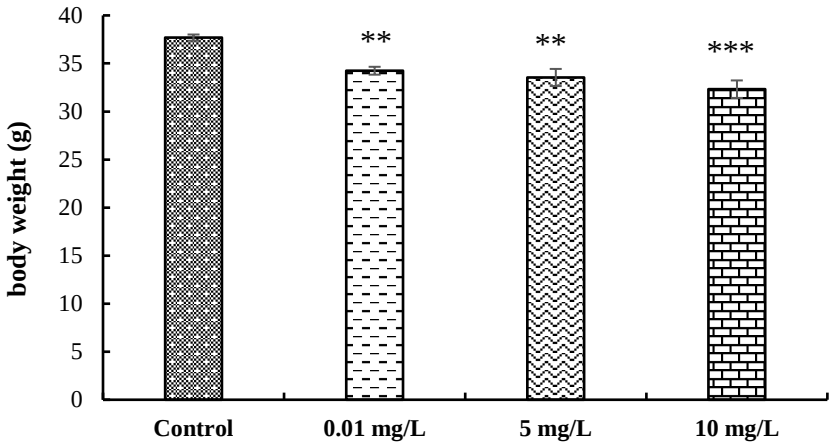


Fig. 4.24 Mouse body weight (g) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows P < 0.01 and 0.001 respectively.

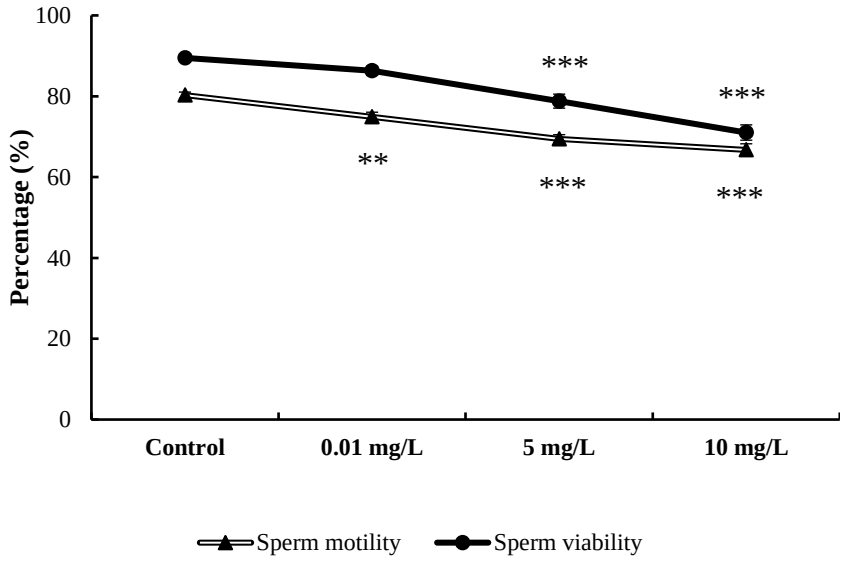


Fig. 4.25 Sperm motility and viability (%) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows P < 0.01, and 0.001 respectively.

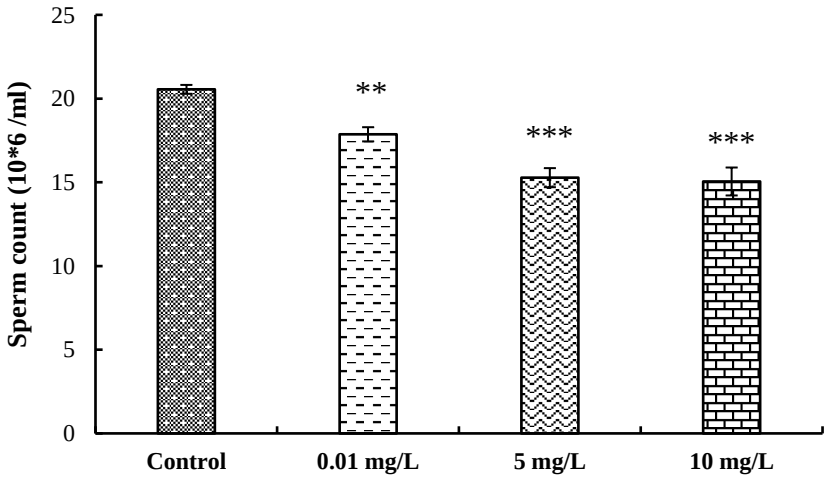


Fig. 4.26 Sperm count ($10^{\times}6$/ml) in mouse after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. **, and *** represents $P < 0.01$, and 0.001 respectively.

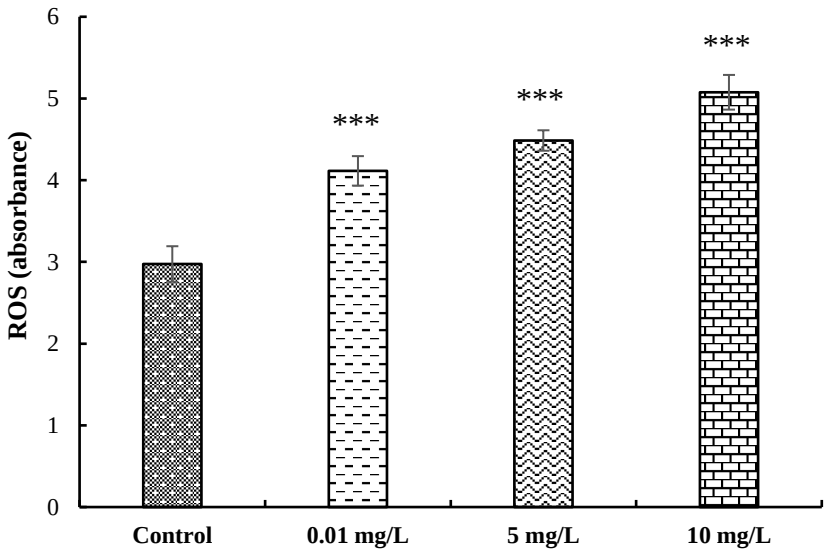


Fig. 4.27 ROS (absorbance) levels in testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

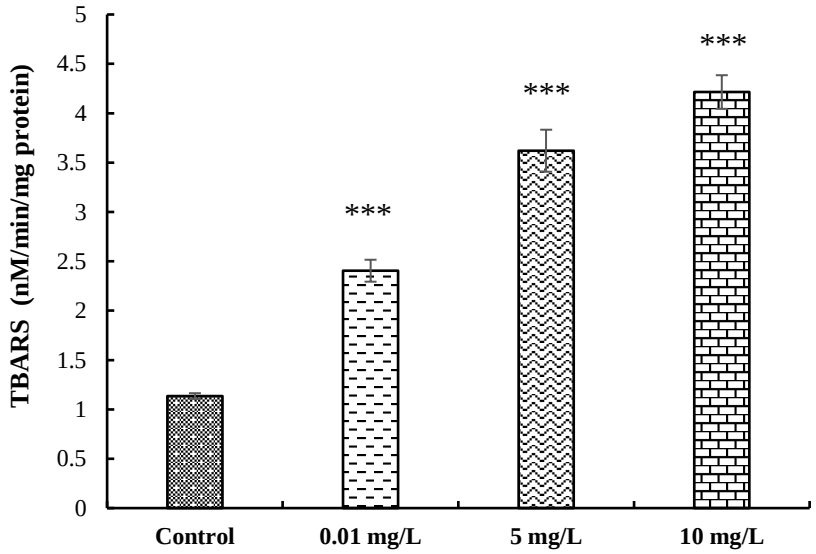


Fig. 4.28 TBARS (nM/min/mg protein) levels in mouse testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

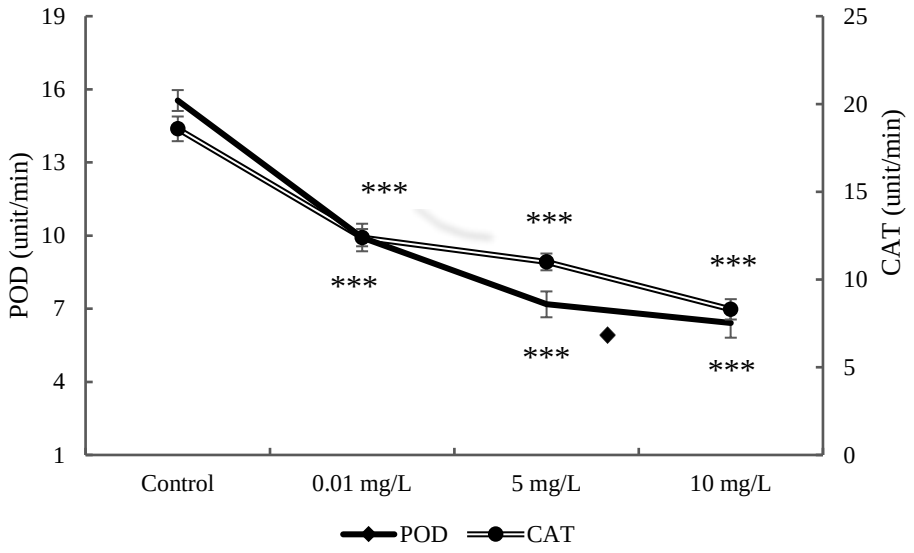


Fig. 4.29 Concentrations of CAT and POD (unit/min) in mouse testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

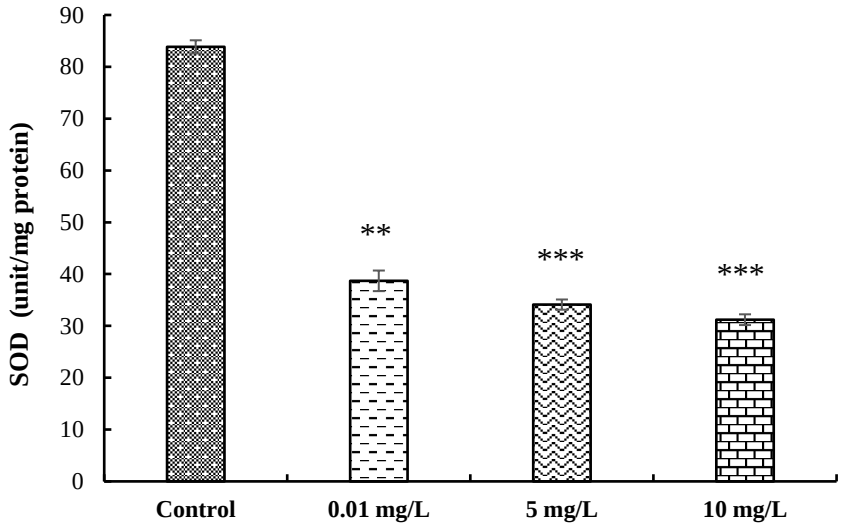


Fig. 4.30 Concentrations of SOD (unit/mg protein) in mouse testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. **, and *** shows $P < 0.01$, and 0.001 respectively.

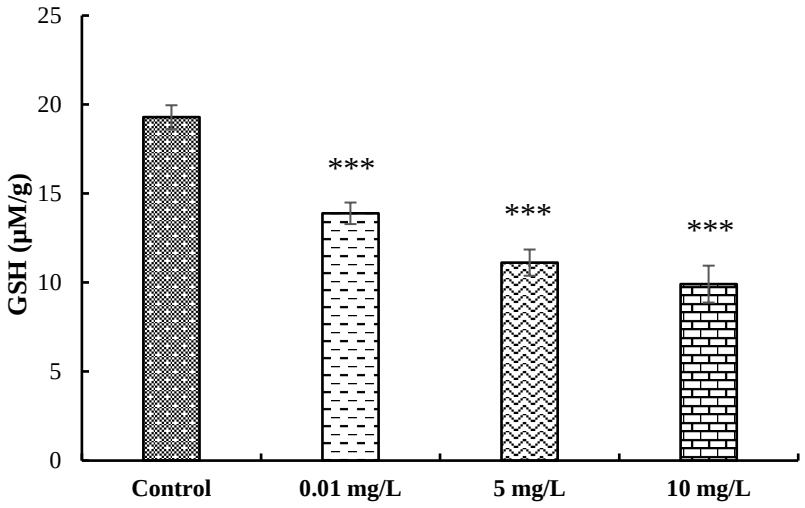


Fig. 4.31 Concentrations of GSH (µM/g) in mouse testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

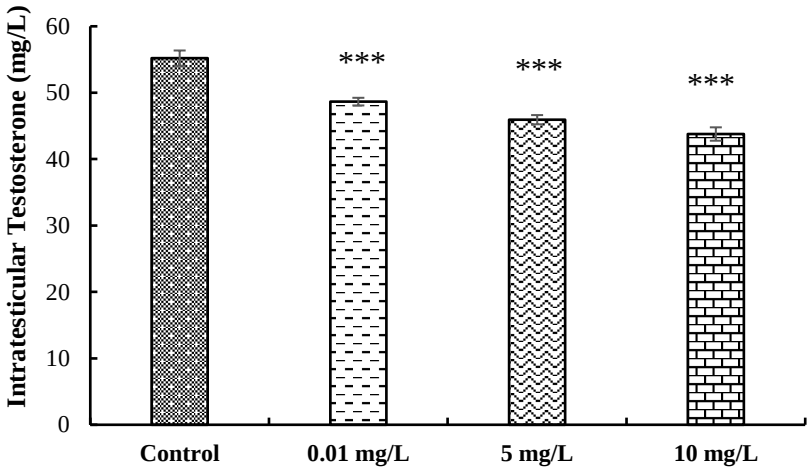


Fig. 4.32 Intratesticular testosterone concentration (mg/L) in mouse testicular homogenate after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

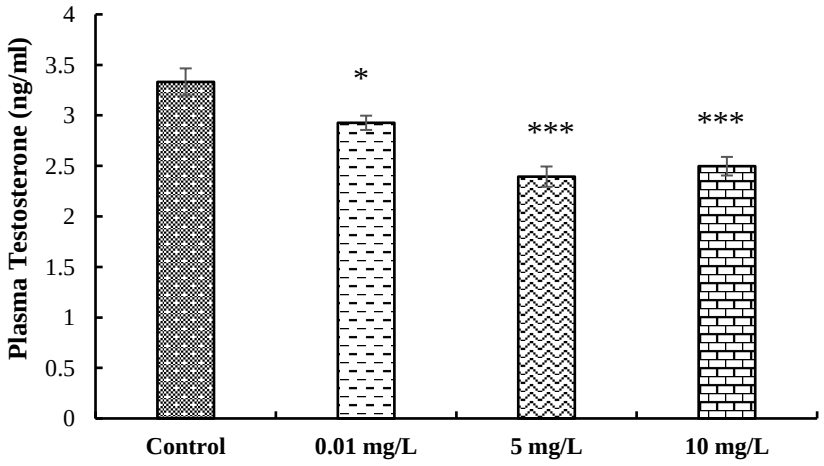


Fig. 4.33 Plasma testosterone concentrations (ng/ml) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *, and *** shows $P < 0.05$ and 0.001 respectively.

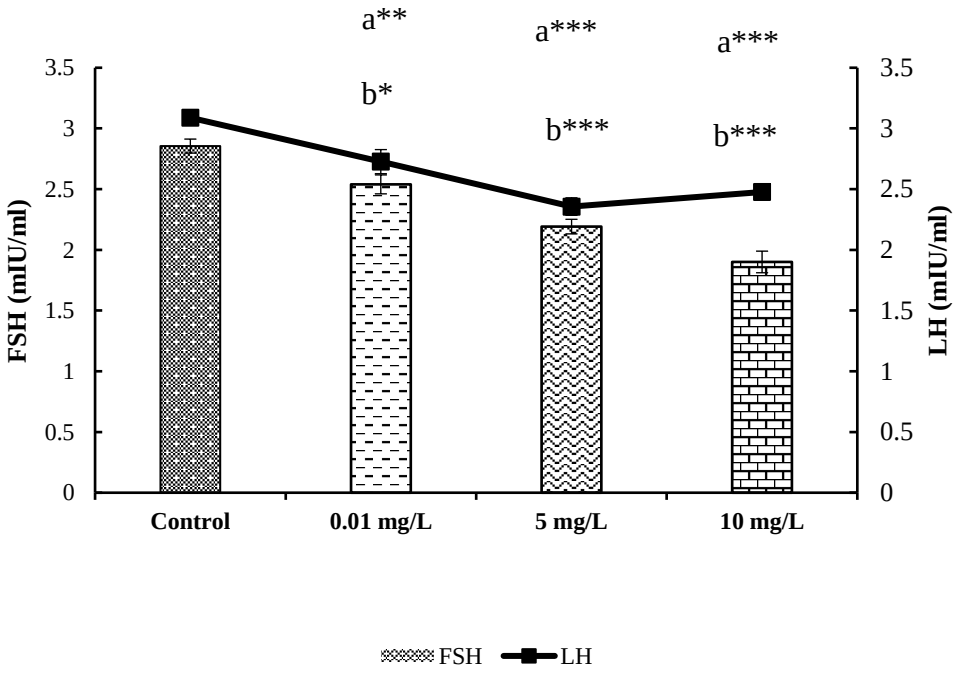


Fig. 4.34 Plasma FSH and LH concentrations (mIU/ml) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. a** and a*** shows LH concentrations at P < 0.01, and 0.001 respectively, while b* and b*** shows FSH concentrations at P < 0.05 and 0.001 respectively.

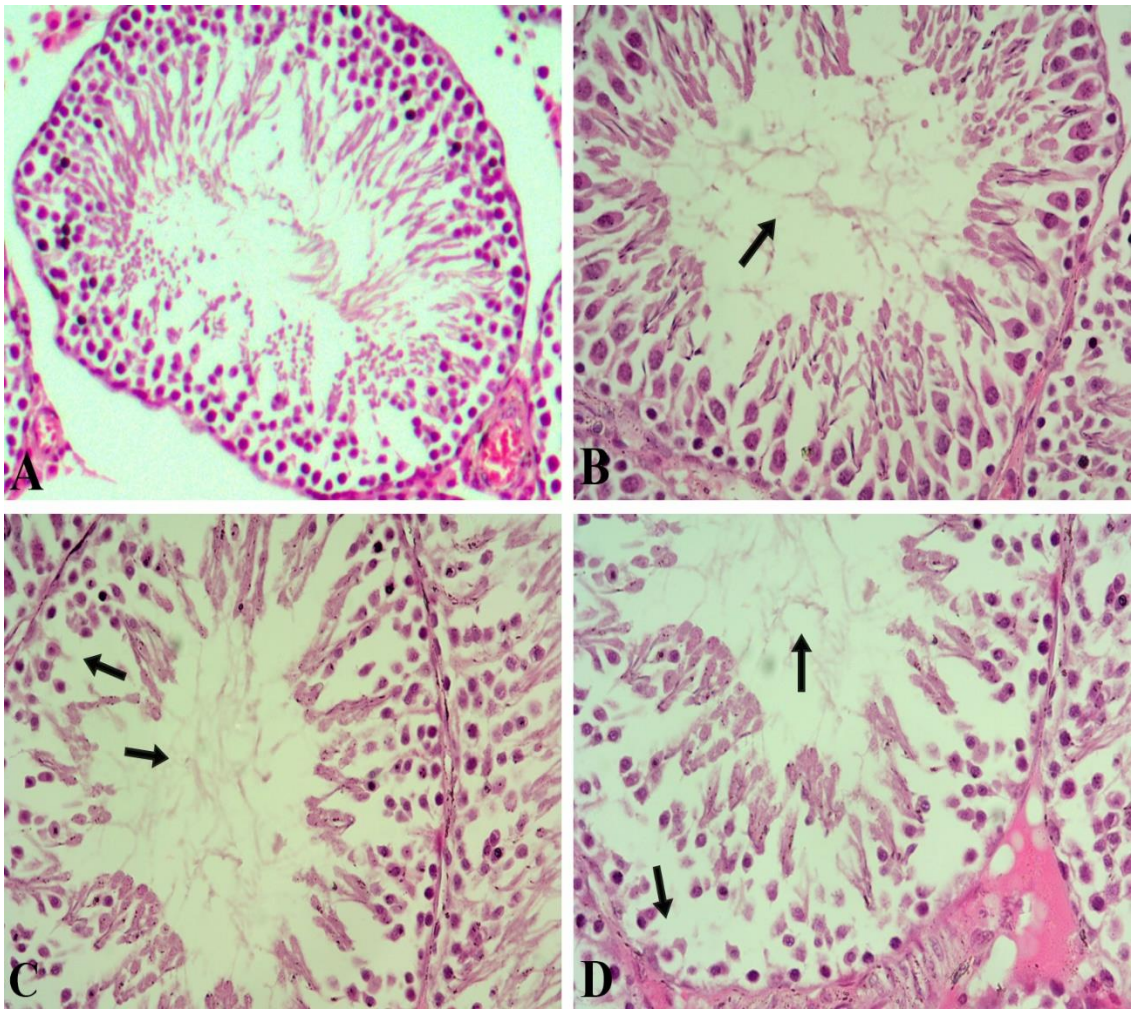


Fig. 4.35 Photomicrograph of mouse testicular tissue after *in vivo* chronic $NaAsO_2$ exposure (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Hematoxylin and eosin stained (40x).

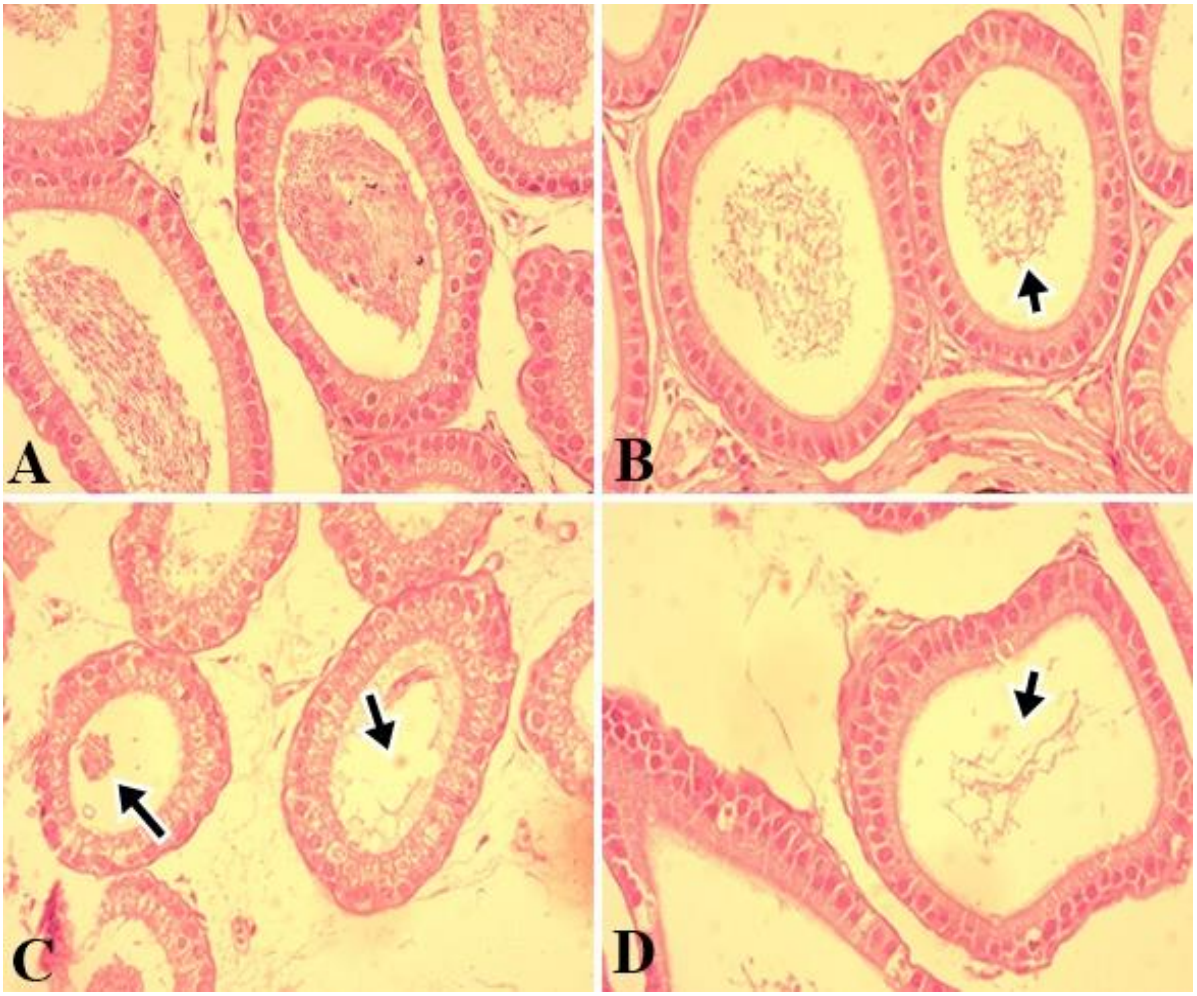


Fig. 4.36 Photomicrograph of mouse epididymal tissue after *in vivo* chronic $NaAsO_2$ exposure (A) control tissue (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L $NaAsO_2$. Hematoxylin and eosin stained (40x).

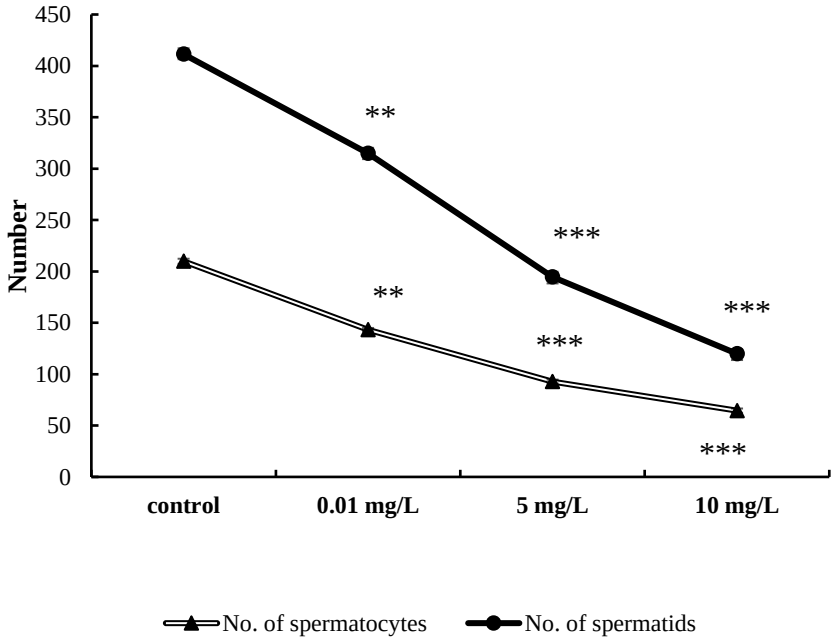


Fig. 4.37 Number of spermatocytes and spermatids in the mouse testis after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. ** and *** shows $P < 0.01$ and 0.001 respectively.

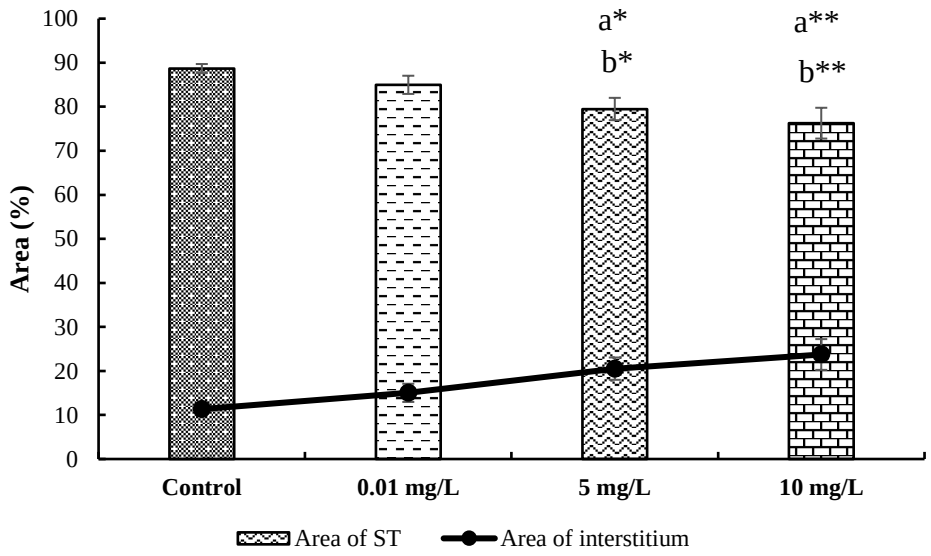


Fig. 4.38 Area of mouse testis seminiferous tubules and interstitial space (%) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. a*, and a** represents % ST area at $P < 0.05$ and 0.01 respectively. Whereas, b*, and b** shows % interstitium area at $P < 0.05$ and 0.01 respectively.

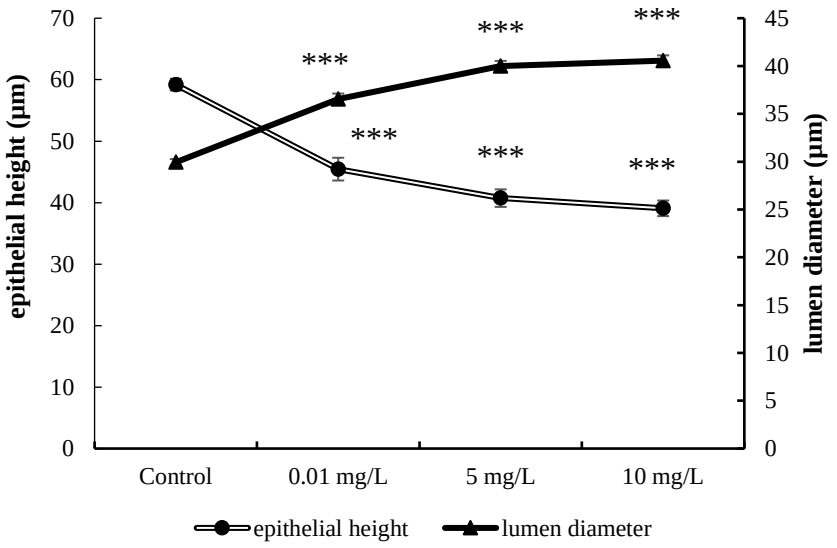


Fig. 4.39 Mean epithelial height and lumen diameter (µm) of mouse testis after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

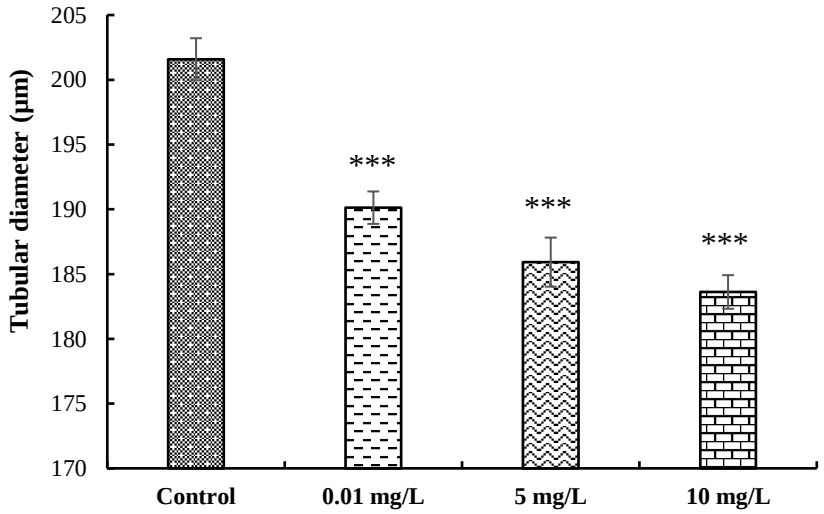


Fig. 4.40 Mean seminiferous tubule diameter (µm) of mouse testis after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows P < 0.001.

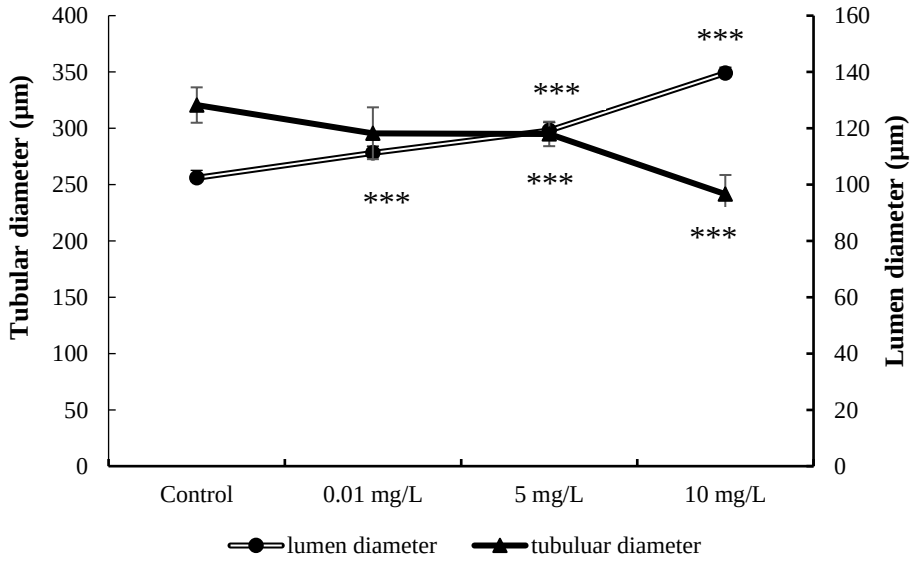


Fig. 4.41 Tubular and lumen diameter of mouse epididymis (µm) after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. *** shows $P < 0.001$.

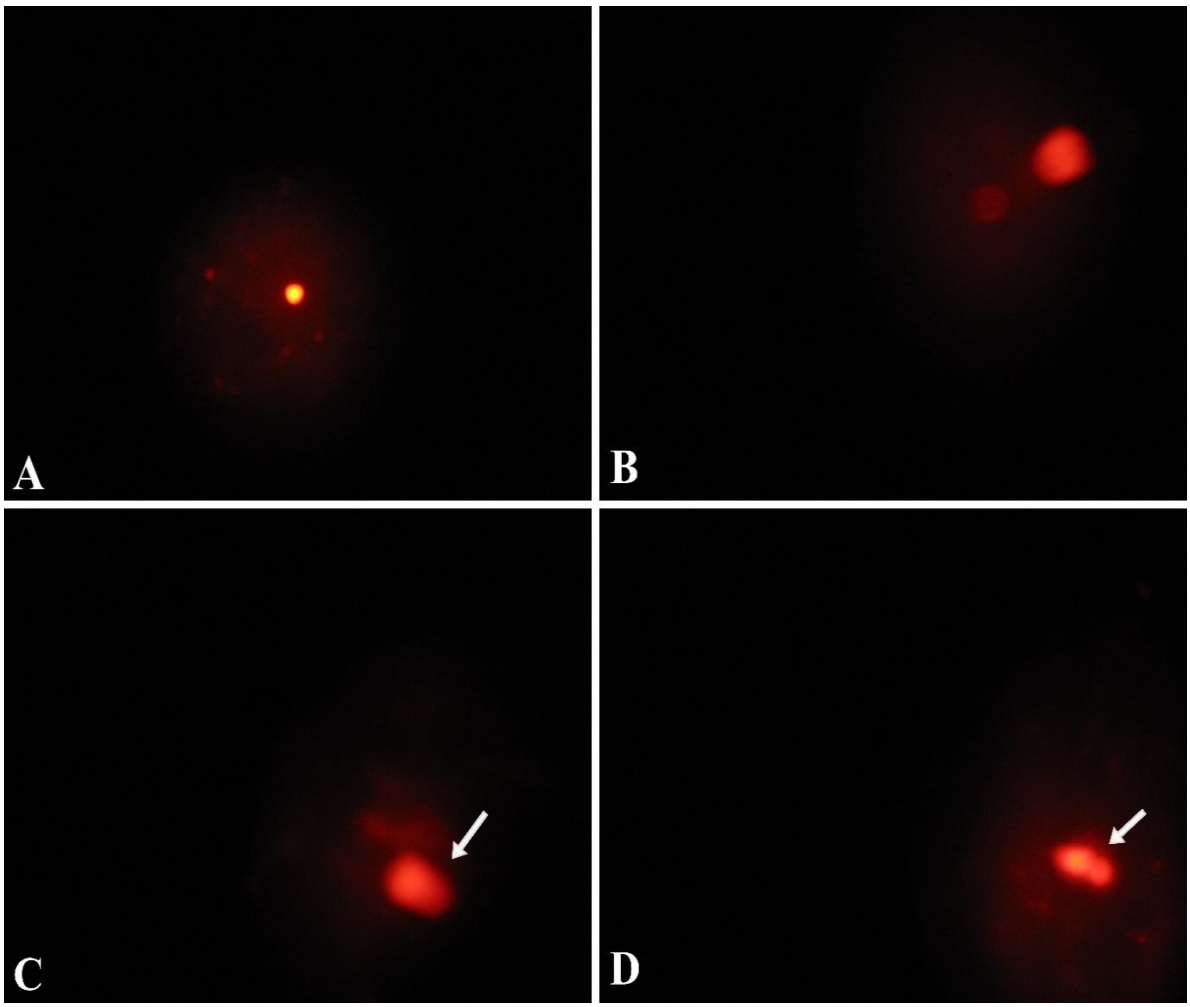


Fig. 4.42 Total DNA damage in the sperm tissue *in vivo* chronic $NaAsO_2$ exposure (A) Control (B) 0.01 mg/L (C) 5 mg/L (D) 10 mg/L. Arrow shows increased tail length after 5 and 10 mg/L $NaAsO_2$ treatments (40x).

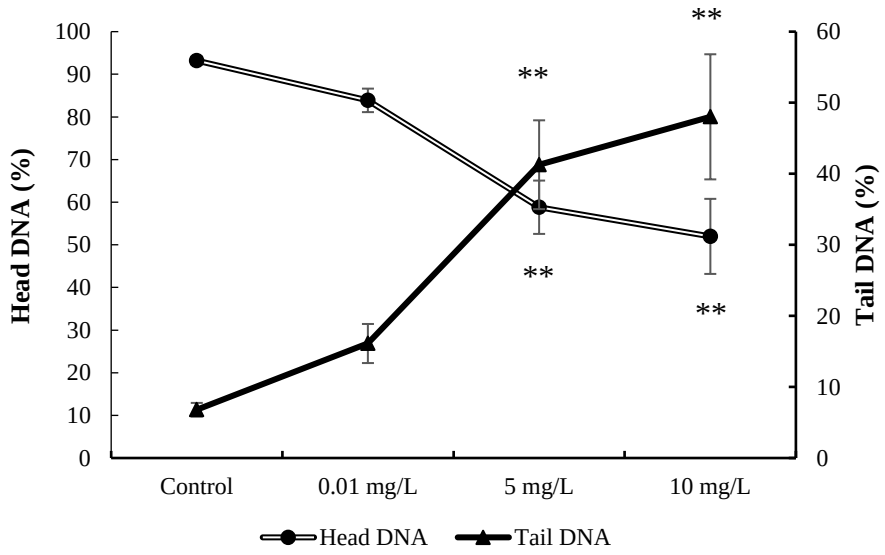


Fig. 4.43 Sperm head and tail DNA (%) determined through comet assay after *in vivo* chronic exposure to different concentrations of $NaAsO_2$. ** shows $P < 0.01$.

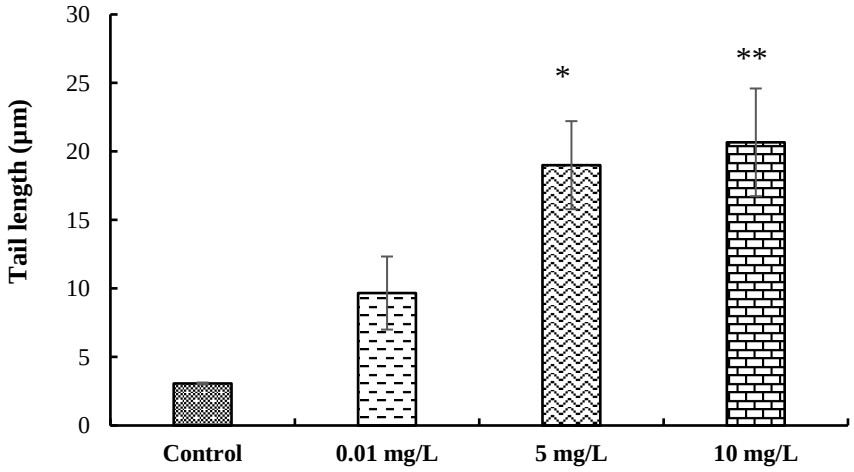


Fig. 4.44 Sperm comet tail length (µm) determined through comet assay after *in vivo* sub-chronic exposure to different concentrations of $NaAsO_2$. *, ** shows P < 0.05 and 0.01

Table: 4.5 Oxidative stress parameters in mouse testicular homogenate after *in vivo* chronic $NaAsO_2$ exposure.

| | Parameters | | | | | |
|---|---|---|---|---|---|---|
| **Groups** | **ROS (absorbance)** | **TBARS (nM/min/mg protein)** | **CAT (unit/min)** | **SOD (unit/mg protein)** | **POD (unit/min)** | **GSH (µM/g)** |
| **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** | 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 mg/L** | 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 mg/L** | 5.07±0.21*** | 4.21±0.17*** | 8.2±0.55*** | 31.2±1.0*** | 6.4±0.5*** | 9.91±0.6** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA following Dunnet's multiple comparison test).

Table: 4.6 Hormone concentrations in mouse after *in vivo* chronic $NaAsO_2$ exposure

| **Treatments** | **Plasma testosterone (ng/ml)** | **Intra-testicular testosterone (ng/g)** | **LH (mIU/mL)** | **FSH (mIU/mL)** |
|---|---|---|---|---|
| **Control** | 3.33±0.13 | 55.2±1.15 | 3.08±0.03 | 2.85±0.05 |
| **0.01 mg/L** | 2.92±0.07* | 48.6±0.57*** | 2.72±0.09** | 2.53±0.07* |
| **5 mg/L** | 2.39±0.10*** | 45.93±0.69*** | 2.35±0.07*** | 2.19±0.06*** |
| **10 mg/L** | 2.49±0.09*** | 43.77±1.00*** | 2.47±0.02*** | 1.90±0.08*** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

Table: 4.7 Morphometry of mouse testis after *in vivo* chronic $NaAsO_2$ exposure

| | **Parameters** | | | | |
|---|---|---|---|---|---|
| **Groups** | **Area of seminiferous tubules (%)** | **Area of interstitium (%)** | **Seminiferous tubule diameter (µm)** | **Epithelial height (µm)** | **Lumen diameter (µm)** |
| **Control** | 88.66±1.03 | 11.33±1.03 | 201.6±1.62 | 59.19±0.96 | 29.93±0.32 |
| **0.01mg/L** | 84.97±2.06 | 15.02±2.06 | 190.1±1.25*** | 45.45±1.84*** | 36.55±0.56*** |
| **5 mg/L** | 79.48±2.53* | 20.51±2.53* | 185.9±1.89*** | 40.73±1.42*** | 40.00±0.52*** |
| **10mg/L** | 76.26±3.49** | 23.7±3.49** | 183.6±1.29*** | 39.08±1.26*** | 40.56±0.54*** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

Table: 4.8 Sperm DNA damage after *in vivo* chronic $NaAsO_2$ exposure

| | **Parameters** | | |
|---|---|---|---|
| **Groups** | **% Tail DNA** | **Tail length (µm)** | **% Head DNA** |
| **Control** | 6.79 ± 0.95 | 3.06 ± 0.06 | 93.1 ± 0.96 |
| **0.01 mg/L** | 16.10 ± 2.75 | 9.66 ± 2.66 | 83.8 ± 2.75 |
| **5 mg/L** | 41.27 ± 6.24** | 19.0 ± 3.21* | 58.8 ± 6.25** |
| **10 mg/L** | 48.01 ± 8.8** | 20.6 ± 3.93** | 51.98 ± 8.8** |

Values are expressed as mean ± S.E.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control (ANOVA followed by Dunnet's multiple comparison test).

## 4.4. Discussion

In the present study, doses were selected within a range in which the lowest dose, i.e. 0.01 mg/ L is equal to 10 ppb, which is WHO declared safest limit of arsenic in drinking water. However, the highest dose used in this experiment (i.e. 10 mg/L) is equal to 10000 ppb. After sub-chronic exposure of $NaAsO_2$ from PND 25-53, no significant difference was detected in the testis and body weight of mice. However, after chronic exposure from PND 25-114, significant reduction in body and testis weight was noticed. Similarly, a previous study is seen in agreement with the current findings. The study shows that chronic arsenic exposure caused substantial decline in the body weight of the young rats (Rodriguez et al., 2002). In contrast, several studies demonstrated no effects of arsenic exposure in adult animals (Sarkar et al., 2003; Jana et al., 2006; Chiou et al., 2008). Dose dependent decline in rat testis weight was observed to by Sarkar et al. (2003). Considerable reduction in rat testis and volume following $NaAsO_2$ have also been demonstrated in previous studies (Soleimani-Mehranjani and Hemadi, 2007; Dhar et al., 2010).

On the other hand, despite the direct toxic effects of arsenic it is also possible that these changes in testicular markers and body/testes weight might be produced and aggravated because of indirect toxic effects of $NaAsO_2$. Chronic exposure of arsenic induces heart diseases, disruption of the nervous system, and several renal and hepatic diseases. Similarly, it can also cause weakness, tiredness, inflammation of colon and stomach linings (Hall, 2002). Weight loss, malabsorption of food and anorexia is also reported to appear with time after arsenic exposure (Goebel et al., 1990). Therefore, it is presumed that these indirect systemic and resulting metabolic factors could also have played contributing role in the current *in vivo* findings.

Sperm parameters for example, sperm motility, viability and count were reduced after sub-chronic and chronic $NaAsO_2$ treatment in male mice. During sub-chronic exposure, low concentration of $NaAsO_2$ (0.01 mg/L) did not induce any alterations in the sperm parameters, whereas after chronic exposure, significant toxic effects of arsenic on sperm parameters were observed by all treatment groups, exhibiting that the toxicity of arsenic depends on the dose and time of exposure. Correspondingly, in agreement with the present results, postnatal arsenic exposure in rats were shown to cause significant reduction in the sperm number (Kaushal et al., 2012). Similar results have been demonstrated in mice, where arsenic exposure induced changes in sperm morphology

and number (Pant et al., 2004). Declined numbers of sperms after exposure of animals to toxicant might have been due to germ cell loss in the seminiferous tubules (Kaushal et al., 2012). This decrease in sperm number is also related to its reduced production in the seminiferous tubules, and any blockage or phagocytosis occurring in the ducts (Goyal et al., 2001).

$NaAsO_2$ treatment in both sub-chronic and chronic experiments induced severe morphological changes in mouse epididymis and testis. In general, arsenic induced shrinking of seminiferous tubule diameter, decrease in epithelial height, number of germ cells and mature sperms in the testis. Lumen space was increased considerably with respect to the arsenic dose. Similarly, a substantial reduction in the number of sperms stored in the epididymal tubules was also evident after both experiments. Low dose arsenic treatment (0.01 mg/L) did not induce morphological changes in the testis and epididymis after sub-chronic exposure. However, chronic treatment of this dose initiated many harmful effects on the reproductive organ's histology. Numerous studies have also stated parallel results in rats (Sarkar et al., 2003; Momeni et al., 2012; Fouad et al., 2014) and male goats (Wares et al., 2015) after arsenic treatment. Similarly, germ cells diminution and reduction in seminiferous tubule diameter have also been demonstrated (Ahmad et al., 2008; Mukherjee and Mukhopadhyay, 2009; Sharma and Kumar, 2011).

Increased ROS and TBARS with an accompanying reduction in antioxidant enzymes were observed after both treatments, sub-chronic and chronic. ROS formation by arsenic damage proteins, lipids, and interstitial tissues (Chang et al., 2010). It can upsurge collagen synthesis and in response the testis interstitial tissue becomes fibroid (Yamanaka et al., 2000). Glutathione plays crucial role in the mammalian antioxidant defence system. Marked increase in lipid peroxidation, ROS generation, and GSH depletion in rat brain, liver, and RBCs after 12 weeks arsenic exposure has also been reported (Flora, 1999).

After sub-chronic arsenic exposure, testosterone (intratesticular and plasma), LH, and FSH concentrations were found significantly reduced in the highest treatment groups (5 and 10 mg/L). However, after chronic exposure of $NaAsO_2$ from PND 25-114, low dose 0.01 mg/L also caused considerable decrease in the hormonal profile. Similar reductions in the plasma testosterone and LH in rats after arsenic exposure has also

been reported (Sarkar et al., 2003; Jana et al., 2006). Significant reduction in plasma testosterone levels were noticed after 1month exposure to 20 and 40 mg/L $NaAsO_2$ in adult mouse, however the LH concentrations remained unaffected (Chang et al., 2010).

Testosterone is crucial for sustaining the normal functioning of the seminiferous tubules and consequently, any reduction in this steroid may cause disintegration of the germinal epithelium (Kim et al., 2001). 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 (Chowdhury, 1979; Sarkar et al., 2003; Kaushal et al., 2012).

DNA damage in rats has been strongly linked to the free radicals generated after arsenic exposure (Kadirvel et al., 2007). Similarly, DNA damage in human cells via ROS has also been demonstrated (Li, et al., 2001). Arsenic induces genotoxicity in different animals (Piao et al., 2005; Kadirvel et al., 2007; Mishra and Flora, 2008) and cell types (Mouron et al., 2001; Guillamet et al., 2004; Ding et al., 2005; Partridge et al., 2007). In the present study, exposure of postnatal mouse to $NaAsO_2$ induced DNA damage. After sub-chronic exposure, only 10 mg/L treatment was able to cause substantial damage to the DNA, whereas after chronic exposure even 5 mg/L concentration of $NaAsO_2$ also induced DNA breaks.

### 4.5. Conclusion

In conclusion, the present study indicates that high doses of $NaAsO_2$ induce toxic effects in mouse from postnatal day 25 after both sub-chronic and chronic exposure. Arsenic intake is capable of inducing considerable morphological, biochemical, hormonal, and DNA disruptions during the postnatal reproductive development. Even the low dose of 0.01 mg/L (10 PPB) of arsenic induced considerable changes in the mouse reproductive system after chronic exposure.

# CHAPTER # 5

## *GENERAL DISCUSSION*

# General Discussion

Arsenic is the twentieth copious natural metalloid present in the earth layers. Its exposure can occur through natural and anthropogenic sources (Mandal and Suzuki, 2002). Chemical state of arsenic determines its availability along with its toxicological and physiological effects (Cullen and Reimer, 1989). Inorganic arsenic compounds are more toxic as compared to organic ones (Smedley and Kinniburgh, 2001; Maeda,1994). The inorganic arsenic is the major contaminant in drinking water and much greater adverse effects as compared to arsenic contaminants in foods such as grains and vegetables (Akter et al., 2005).

According to the WHO, arsenic concentrations less than 10 ppb have been declared safe for drinking purposes (Brown and Ross, 2002). However, the concentrations of arsenic in the drinking water in some areas of developing countries like Pakistan, Bangladesh, India (Bihar) are above this limit (Brammer and Ravenscroft 2008; Sanjrani et al., 2017). For instance, in Bangladesh arsenic limit in drinking water is set at 50 ppb. However, in a survey 25% of wells providing domestic water exceeded such level (Ravenscroft et al., 2005). The analysis of water from irrigation shallow tube wells in Bangladesh has revealed that the majority produced water above 100 ppb arsenic with several going above 200 ppb arsenic (Islam et al., 2005). Top arsenic concentration of 1891ppb have been found in Bihar-India (Ghosh et al., 2007), with the highest in Nepal of 2629 ppb arsenic (Shresta et al., 2003).

Numerous past studies have reported significant arsenic accumulation in the testes, epididymis, prostate glands and seminal vesicle (Danielsson et al., 1984; Pant et al., 2001). Free radical generation by arsenic exposure causes cellular damage (Barchowsky, 1996; Schinella et al., 1996). Oxidative stress induced by arsenic trioxide exposure in human hepatocellular carcinoma cell caused apoptosis and DNA damage (Alarifi et al., 2013). In the same way, *in vitro* exposure to arsenic in testicular germ cells and Sertoli cell is known to induce apoptosis (Celino et al., 2009; Kim et al., 2011). Six days *in vitro* fish testicular culture caused germ cell death after 100 µM arsenic (Celino et al., 2009). Mouse Sertoli cells exposed to 10 µM arsenic trioxide proficiently increased ROS mediated apoptosis or cell death (Kim et al., 2011). Arsenic toxicity is

assumed to cause spermatogenic dysfunction by directly damaging the testicular cells (Kim and Kim, 2015).

Arsenic is the 12$^{th}$ abundant element found in the human body (Woolson, 1975). In the past it has been extensively used in medicine, industry, livestock, metallurgy, and agriculture (Nriagu and Azcue, 1994). The abundance of arsenic on land is up to 3 mg/kg. Its exposure can occur either or both through natural and synthetic sources. Oral, dermal, and respiratory are its three routes of exposure for both humans and animals (Mandal and Suzuki, 2002). The physiological and toxic effects of arsenic, as well as its biological accessibility are determined by its chemical state (Cullen and Reimer, 1989). $NaAsO_2$ is considered more toxic and biologically active than sodium arsenate (Mandal and Suzuki, 2002).

In the recent years, arsenic contamination through drinking water has immensely enlarged in the sub-continent, especially in Pakistan. The most populated provinces of Pakistan (Punjab and Sindh) are contaminated with 50 ug/L arsenic in the drinking water (Sanjrani et al., 2017). Some major cities of these provinces have arsenic exposure up to 200 ug/L (Toor and Tahir, 2009). Adult male reproductive toxicity of arsenic has been widely reported in the past, however little information is present as regards the *in vitro* gonadotoxicity of $NaAsO_2$ in prepubertal (infantile) and adult mouse.

Due to declined antioxidant capability and excessive levels of polyunsaturated fatty acids, germ cells become highly prone to damage caused by oxidative stress (Vernet et al., 2004; Nishigori et al., 2004). Oxidative stress is proposed to be a chief mechanism of arsenic toxicity (Mishra and Flora, 2008). During apoptosis, genes direct the cells to actively die in an organized manner. This process can be initiated either impulsively or may be due to some external agent (Das et al., 2009). Various cells, including those involved in spermatogenesis, have been linked to apoptosis induced by ROS and oxidative stress (Kasahara et al., 2002). Oxidative stress is sensed by the mitochondria of the cell, and as they get damaged. They release pro-apoptotic factors and cytochrome C in the cytosol. After apoptosome formation the caspase cascade is activated (Gao et al., 2003). Bcl-2 controls the mitochondrial membrane permeability. Anti-apoptotic proteins counter the release of cytochrome C into cytosol. Whereas, the pro-apoptotic

proteins for example Bad, Bik, and Bid promote the cytochrome C movement (Budihardjo et al., 1999; Hengartner, 2000).

Arsenic has been reported to stimulate the Bad expression while decreasing the Bcl-2 function. This leads towards activation of Caspase-3. Cleaved caspase-3 is a product that is formed during the activation of caspase-3, and it can be used to assess its activation (Das et al., 2009).

In the current study, prepubertal (infantile) mouse testis were exposed to different concentrations of $NaAsO_2$ in *in vitro* cultures. Three different sets of experiments were conducted. First experiment comprised of 24 h exposure of arsenic doses up to 1 µM. The lowest tested dose (0.01 µM) in this experiment of chapter 2 corresponds to 1.29 ppb, whereas 1 µM equals to 129 ppb which is above the WHO declared safest limit of arsenic in drinking water i.e. 50 ppb. However, in this experiment, all treated groups showed normal testicular morphology with plenty of growing germ cells. The exposure duration of these doses was increased to six days in the second experiment. Histology and immunohistochemistry showed that the prepubertal (infantile) mouse testes were unaffected even after 1 µM *in vitro* arsenic exposure for 6 days. Doses administered for a short period of six days under culture conditions did not affect the testicular development. It is however possible that chronic exposure of these low dose may be damaging to the prepubertal (infantile) testis, for which further longer-term studies are necessary. Furthermore, it is not possible to exclude that other physiological processes which could have been compromised and might influence fertility in the adulthood. In the third experiment, *in vitro* 6 days exposure to high arsenic concentrations (10, 50, and 100 µM) caused significant changes in the infantile testes. It was found that the lowest concentration of 10 µM equals to 1299 ppb arsenic was highly detrimental for the prepubertal (infantile) testes, as indicated by increased cell death and reduction in spermatogonial germ cell population. Such dose is below the top concentrations of arsenic found in irrigation shallow tube wells, which are also used as source of drinking water for local populations in countryside areas of developing countries.

However, at 50 and 100 µM concentrations, seminiferous tubules were completely distorted along with reduction in germ cells number and increased expression of CC3. Apoptosis signals were more excessive at 50 µM $NaAsO_2$ concentration. Earlier, it has been reported that *in vitro* 100 µM arsenic concentration immediately killed mouse

embryos, whereas 1 µM dose entirely inhibited blastocyst formation (Muller et al., 1986). Another study demonstrated apoptosis of germ cells in an *in vitro* testicular culture of Japanese eel (Celino et al., 2009).

Increased ROS production causes irreversible oxidation of the biomolecules especially the lipid peroxidation of cell membranes, damaging DNA and proteins (Radak et al., 1999). By products of lipid peroxidation are known as TBARS, it is also considered as the oxidative stress index (Kaul and Forman, 2000). In the testis, antioxidant system (including, CAT, SOD, POD) is present that counteracts the toxic effects of ROS (Vernet et al., 2004). During oxygen metabolism hydrogen peroxide, hydroxyl radical, and superoxide anion are formed as intermediates (Flora et al., 2007). In general, SOD acts first for detoxifying the oxyradicals detrimental effects by superoxide radicals dismutation to hydrogen peroxide. Further CAT and GSH acts on hydrogen peroxide for its degradation (Imlay and Linn, 1988; Halliwell, 1996; Zhou et al., 2005; Pineda et al., 2013). GSH is a non-antioxidant enzyme, which via chelation reduces the toxicity of arsenic (Huang et al., 1995). It acts as a cofactor for biomethylation of arsenic. Arsenic intermediates bond with GSH, directly or indirectly by glutathione-S transferase to produce oxidized glutathione (Zakharyan et al., 1996). Arsenic produces oxidative stress by elevating ROS production and reducing antioxidant enzymes levels (Flora et al., 2007; Kavitha and Rao, 2008). This decrease also leads towards declined non-enzymatic antioxidant enzymes such as GSH. The imbalance between ROS and antioxidant enzymes generates oxidative injury of the cells (Flora et al., 2007).

Adult male mouse testis and epididymis were incubated with $NaAsO_2$ for 2 and 24 hours. After 2 h incubation, higher concentrations of $NaAsO_2$ (50 and 100 µM) induces oxidative stress by significant increase in ROS and TBARS, while decreasing antioxidant enzymes in the testicular tissue. In contrast, after 24 h incubation highly significant decrease started occurring after 10 µM $NaAsO_2$ exposure. Analogous results of in vitro reports have corroborated elevated ROS and lipid peroxidation, along with GSH diminution (Han et al., 2008; Kim et al., 2011). Similarly, increased ROS and TBARS accompanying reduction in antioxidant enzymes were observed after *in vivo* sub-chronic and chronic arsenic exposure. Numerous studies have reported *in vivo* oxidative stress in testes after arsenic exposure (Jana et al., 2006; Das et al., 2009; Biswas et al., 2010; Uygur et al., 2016; Jahan et al., 2015; Guvvala et al., 2016; Souza

et al., 2016). The decreased concentrations of antioxidant enzymes depict the malfunction of the defensive mechanism against arsenic exposure.

In rodents, oxidative stress induced by arsenic in testes causes morphological changes such as, decrease in tubular diameter, epithelial height, increase in lumen diameter, and spermatogenic disintegration (Jana et al., 2006; Sanghamitra et al., 2008; Uygur et al., 2016; Jahan et al., 2015; Guvvala et al., 2016; Souza et al., 2016). Similar results were observed in sub-chronic and chronic *in vivo* experiments, where rigorous detrimental changes in testes morphology were observed with increasing dose and time duration.

Testosterone concentration is crucial for sustaining the normal functioning of the seminiferous tubules and consequently, any reduction in it might cause disintegration of the germinal epithelium (Kim et al., 2001). Testosterone induces germ cells connection to the seminiferous tubules. Declined testosterone concentration in the testis stimulates germ cell disengagement in the seminiferous tubules and induction of apoptosis (Blanco-Rodriguez and Martinez-Garcia, 1997). Testosterone concentrations decline by either through direct inhibition of the steroidogenic enzymes or by decreased sensitivity to LH (Ali et al., 2013). Arsenic has been indicated to reduce steroidogenic enzymes (Sarkar et al., 1991; Ali et al., 2013). Plasma LH and FSH are regulators of these enzymes, and their activity can be reduced by diminished LH and FSH levels (Odell et al., 1963). Furthermore, Leydig cells are susceptible to oxidative stress and thus it can reduce steroidogenesis (Aitken and Roman, 2008). The current *in vitro* and *in vivo* studies are in accordance with these facts as testosterone concentration in adult mouse testis was substantially reduced after 24 h incubation with 50 and 100 µM $NaAsO_2$ doses. Similarly, postnatal *in vivo* results exhibited significant reduction in testosterone concentration. The decrease in testosterone concentration is assumed to be oxidative stress induced changes in steroidogenesis. Chronic exposure to arsenic is proposed to induce toxic effects by free radical generation (Ito et al., 1998; Flora, 1999; Flora et al., 2005).

The sperm movement is very much important in achieving its ultimate goal of egg fertilization (Yousef et al., 2003). The $NaAsO_2$ toxicity result in the reduction of sperm movement (Momeni and Eskandari, 2012). The basic reason behind the reduction of sperm movement is the binding property of the arsenic with the thiol proteins in sperm nuclear chromatin and flagellum (Uckun, et al., 2002; Guvvala et al., 2016). Thiol

proteins are very crucial for the sperm movement (Pant et al., 2004). The other possible reason behind reduction of sperm movement is due to the oxidative degeneration of lipids in the cell membranes of the sperm (Das et al., 2009). Sperm structure and sperm sensitivity are the two major factors used for the identification of male fertility and infertility conditions (Guzick et al., 2001). The male reproductive toxicity stress totally depends upon the route of acquiring the arsenic stress (Holson et al., 2000ab).

Arsenic effects the sperm motility by directly binding to the thiol or sulfhydryl groups or indirectly by averting the enzymes necessary for sperm motility (Uckun et al., 2002; Pant et al., 2004). Oxidative stress induced hydrogen peroxide is postulated to reduce sperm motility by crossing the sperm membrane and disturbing the sperm enzymes (Makker et al., 2009). Sperm motility is also sustained by the antioxidant enzymes (Hughes et al., 1998; Mata-Campuzano et al., 2012). Various studies have reported reduced sperm motility after arsenic exposure (Pant et al., 2001; Das et al., 2009; Mukherjee and Mukhopadhyay, 2009; Reddy et al., 2011; Momeni and Eskandari, 2012; Ince et al., 2016; Guvvala et al., 2016). In the present study, both *in vitro* and *in vivo* experiments revealed that arsenic potentially reduced the sperm motility. With increasing the exposure duration, moderate doses can also considerably affect the sperm motility.

Arsenic effects the sperm morphology by two mechanisms: one through the oxidative stress and secondly by directly binding to the sperm thiol groups (Yamanaka et al., 1990; Aitken et al., 1993). Prolonged arsenic exposure and its binding with the thiol groups can cause sperm tail defects (Pant et al., 2004). Oxidative stress induced by arsenic leads to breaks in the DNA (Momeni and Eskandari, 2012). This damage to the DNA can raise the quantity of head abnormalities in sperms (Guvvala et al., 2016). Similarly, in the current *in vitro* study 2 h incubation of sperms with $NaAsO_2$ induced curvature abnormalities, pinhead and tailless sperms.

Declined sperm number can occur due to reduced production in the seminiferous tubules, retaining of sperms in the tubule epithelium, or duct blockage (Goyal et al., 2001). Loss of germ cells and ineffective discharge of sperms into the lumen can also cause considerable drop in the sperm count (Kaushal et al., 2012). FSH and testosterone sustains normal spermatogenesis in the seminiferous tubules. Arsenic induced decline in FSH and testosterone concentrations can stimulate loss of germinal epithelium, and

spermatogenesis inhibition (Chowdhury, 1979; Sharpe et al., 1988; Sharpe et al., 1992; Sarkar et al., 2003). The current *in vivo* studies are in accordance with these findings, as dose dependent decrease in FSH and testosterone concentrations were accompanied by substantially decreased sperm number. Post-natal chronic exposure even at the lowest dose (0.01 mg/L), which is equal to 10 ppb concentration, induced toxic effects on male mouse reproductive system.

Carcinogenesis is caused by ROS induced DNA damage (Imlay and Linn, 1988). Oxidation of DNA leads to excessive production of 8-hydroxy2-deoxyguanosine, causing DNA mutations (Cheng et al., 1992). Arsenic stimulation of NADH oxidase, generates superoxide ions which are known to induce oxidative damage in the DNA (Lee and Ho, 1995; Barchowsky et al., 1999ab). Arsenic is described to cause DNA damage by generating ROS (Schwerdtle et al., 2003; Momeni and Eskandari, 2012), and by inhibiting enzymes involved in DNA repairing (Yager and Wiencke, 1997; Andrew et al., 2006). DNA is supercoiled and negatively charged, and any damage in it causes breaks in the strand thus during electrophoresis it moves towards anode (Klaude et al., 1996). DNA strand breaks can be directly represented by the range of DNA migration during electrophoresis (McCarthy et al., 1997).

The results of the current study revealed that 1 µM dose of $NaAsO_2$ equals to 129 ppb did not cause any toxic changes in the prepubertal (infantile) *in vitro* testicular cultures, both after 1 day and 6 days exposure of $NaAsO_2$. Similarly, this dose was unable to induce biochemical changes and DNA damage in *in vitro* adult mouse testicular cultures. However, *in vitro* toxic effects both in prepubertal (infantile) and adults were observed at 10 µM dose which is 1299 ppb. Such dose is below the top concentrations of arsenic found in irrigation shallow tube wells, wells that are used as a source of drinking water for local populations in rural areas of developing countries. The highest dose used in *in vivo* experiments i.e. 10 mg/L and the *in vitro* top dose (100 µM) used in chapter 1 and chapter 2 are approximately similar; 10000 ppb and 12990.9 ppb respectively. These doses were highly toxic to prepubertal (infantile) and adult mouse reproductive system. However, it is also possible that some additional physiological processes have been compromised except of those assessed in the current study that might influence fertility in adulthood.

Several studies on rodents have revealed arsenic accumulation in testis and other accessory sex organs (Dua et al., 2015; Prathima et al., 2018). Such persistent nature after accumulation in the reproductive organs can have adverse impact on the developing testis of both human and animals. Present data raised a concerning scenario for preadolescent and adolescent males living in the highly arsenic contaminated areas, as exposure to arsenic may compromise the fertility. In addition, in areas where animal husbandry is an important economic factor, arsenic-induced infertility of farm animals could represent a further financial burden for the economy of farmers in developing countries.

# *GENERAL CONCLUSION*

# General conclusion

In conclusion, the study overall demonstrated that PND-5 prepubertal (infantile) mouse testis cultured *in vitro* undergo germ cell loss and apoptosis induced testicular damage after 6 days exposure to 10 µM $NaAsO_2$ concentration. Similarly, the toxic effects caused by $NaAsO_2$ to adult mouse testes and epididymes were rapid and, both time and dose dependent. Higher doses and longer duration of exposure led to greater toxicity to reproductive tissues and sperms. *In vitro* sperm incubation with arsenic led to decreased sperm viability, motility, and morphological effects. These effects were more pronounced with increasing the time duration.

In context with the *in vitro* studies, both sub-chronic and chronic *in vivo* postnatal arsenic exposure caused disruption of reproductive function in male mouse. Although, more deleterious effects were detected after *in vivo* chronic arsenic exposure. However, low dose of 0.01 mg/L or 10 ppb, which is described safe for drinking by WHO can generate considerable changes after *in vivo* chronic exposure in male mouse reproductive system.

As observed, the lower doses (i.e. upto 1 µM) administered for short period of six days did not affect the testicular development in prepubertal (infantile) mouse. However, these results cannot exclude the possibility that *in vitro* chronic long-term exposure to such concentrations of $NaAsO_2$ may be detrimental to the prepubertal (infantile) testis. Hence, in light of the limitations discussed above further studies with increased incubation time durations are required in order to confirm the alterations in signaling systems, and translational risk of future fertility impairment for young boys. Such investigations should be extended to the fertility of large animals; in areas where animal husbandry is an important economic factor, arsenic induced infertility of farm animals could represent a further financial burden for the unstable economy of farmers in developing countries. Overall, the study uncovers a concerning situation for immature and adult animals as well as human populations living in the severely arsenic contaminated zones.

# *PUBLICATIONS*

1. **Anwar, N. & Qureshi, I. Z.** (2019). *In vitro* application of sodium arsenite to mice testicular and epididymal organ cultures induces oxidative, biochemical, hormonal, and genotoxic stress. *Toxicology and Industrial health*, 35(10): 660-669.
2. **Anwar, N., Qureshi, I. Z., Spears, N. & Lopes, F**. (2020). *In vitro* administration of sodium arsenite in prepubertal testis induces germ cell loss and apoptosis. *Toxicology in Vitro*, 67 (2020): 1-8.

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