REPRODUCTIVE TOXICITY OF SODIUM ARSENITE IN SPRAGUE DAWLEY MALE RAT

ISLAMABAD

BY

OMMIA KALSOOM

FACULTY OF BIOLOGICAL SCIENCES
DEPARTMENT OF ANIMAL SCIENCES

QUAID-I- AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
2009

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE THESIS REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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CERTIFICATE

This thesis submitted by Ms Ommia kalsoom is accepted in its present form by the Department of Animal Sciences as satisfying the thesis requirement for the degree of Doctor of Philosophy in Reproductive Physiology.

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In the name of Allah, the Beneficent, the Merciful.
Praise to be Allah, Lord of the world,

The Beneficent the Merciful:
Owner of the Day of Judgment

Thee (alone) we worship:
Thee (alone) we ask for help

Show us the straight path

The path of those whom thou has favored.
Not (the path) of those who earned
Thine anger,
Nor of those who go astray.

(Al-Fateha)

All the efforts, the entire honor and all the achievements are dedicated to my loving parents
and
grand mothers

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ACKNOWLEDGEMENTS

All the praises to ***"Almighty Allah"***, the most beneficent, Who is the source of all the wisdom and knowledge and bestowed me with potential and ability to complete the present work. Countless salutation upon the Holy Prophet (peace be upon him), source of knowledge and blessings for entire creation, who has guided His ummah to seek knowledge from cradle to grave and enabled me to win honor of life.

The work in this manuscript was accomplished under the sympathetic attitude, expert supervision of ***Dr Samina Jalali***, Professor, Department of animal Sciences, Quaid-i-Azam University, Islamabad. I feel so richly blessed to have her as my supervisor. Her efforts tower the inculation of spirit of hard work and maintenance of professional integrity besides other valuable suggestions will serve as beacon of light through the course of my life.

I would like to record my sincerest thanks and grateful appreciation to ***Dr. Muhammad Shahab***, Chairman, Department of Animal Sciences, Quaid-I-Azam University Islamabad for providing me all the necessary facilities.

I am extremely grateful to ***Dr. S. A. Shami*** for his encouragement, keen interest and expert advice regarding the manuscript. I can never repay him for his timeless efforts and patience with which he taught me.

I am thankful to ***Dr. Sarwat Jahan*** for her kind behavior, encouragement and suggestions during my research work.

I would like to extend my deepest thanks to ***Higher Education Commission*** for providing me Merit Ph.D. scholarship and for all its financial assistance without which this work would not have been possible.

Words are lacking to express thanks to my friends ***Zertashia Akram*** and ***Sajda Batool*** for their endearment, care, support, cooperation and consolatory behavior during the whole time period of this study. Any attribute will be less for them.

Special thanks for wonderful company and cooperation of my little friend ***Nida Zafar***.

I am thankful to my all ***lab fellows*** for helping me in various ways during my research work.

I must acknowledge ***Rana Mohammad Tariq*** bhai for his care during the crucial moments of thesis write up.

I acknowledge a lot of emotional backing, encouragement care and support of nice friends ***Sadia Almas***, ***Tayyeba Firdos***, ***Nosheen Ilyas***, ***Anna Iqbal***, ***Asma Batool***, ***Jasia Bokhari*** and ***Nazeefa Taqveem***. I am thankful to ***Uzma Khan***, ***Wajiha Khan***, ***Dr. Shakra*** and ***Zarghona***. The good time spent with them can never be erased from my memories.

No acknowledgement could ever adequately express my feelings to my affectionate and adorable family without whom I feel myself incomplete. My ***Parents*** deserve special mention for their inseparable support and prayers. From the start till the accomplishment of this manuscript, they have been my source of strength and love. I am honored to have them as my parents. Thanks to them for giving a chance to prove and improve myself through all walks of life. Words fail to express my appreciation to my dearest sisters ***Safia*** and ***Raheela***, my brother-in-law ***Sahibzada Fazal-e-Subhan*** for their care, love long kindness and support during all circumstances in my journey. Bundles of thanks to my brother, ***Dr. Haroon-ur-Raheed***, for being there always to take the loads off my shoulder with all his love, care and support. Special thanks to my Sweet bhabhi ***Dr. Noshaba***.

Many many thanks to my sweet nephews ***Hamza***, ***Fahad***, cute nieces ***Sana*** and ***Mahnoor***, and my friend's sweet kids ***Amina***, ***Abdullah*** and ***Bilal***, their pleasant, innocent and naughty company enables me to forget all my tensions and worries.

I would like to acknowledge my husband ***Ateeque-ur-Rehman***, who has shared my dreams and gave the best suggestions. Thank him for his love and commitment for which I feel extremely fortunate.

In the end I want to present my thanks to all those hands who prayed for my betterment and serenity.

OMMIA KALSOOM

LIST OF ABBREVIATIONS

Ab	Antibody
AR	Androgen receptor
As	arsenic
$As3+/As^{III}$	Trivalent arsenic (arsenite)
$As5+/As^{V}$	Pentavalent arsenic (arsenate)
AS3MT	Arsenic (+3) methyltransferase
ATP	Adenosine triphosphate
AST	Assisted reproductive techniques
ASTDR	Agency for toxic substances and disease registry
BW	Body weight
$CdCl_2$	Cadmium chloride
CHRIS	Chemical hazard response information system
CNS	Central nervous system
DART	Developmental and reproductive toxicant
DES	Diethylstilboestrol
DMA^{III}	Dimethylarsinous acid
DMA^{V}	Dimethylarsinic acid
DMSO	Dimethylsulfonic acid
DNA	Deoxyribo nucleic acid
DOSE	Dictionary of substances and their effects
ds	Double stranded
DSP	Daily sperm production
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbant assay
EPA	Environmental protection agency
ER	Estrogen receptor
FSH	Follicle stimulating hormone
Gao	General accounting office
G.I.tract	Gastrointestinal tract
GR	Glucocorticoid receptor
GSH	Glutathione
GST	Glutatione-S-transferase
GSSG	Glutathione disulfide
HCl	Hydrochloric acid
HNO_3	Nitric acid
HSD	Hydroxy dehydrogenase
HSDB	Hazardous Substances Data Bank
17β-HSD	17-beta hydroxyl dehydrogenase
^{125}I	Radioactive labeled iodine
ICSI	Intracytoplasmic sperm injection
IgG	Immunoglobin G

IgM	Immunoglobin M
In As	Inorganic arsenic
i.p.	Intraperitoneal
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LMP	Low melting point
MMA^{III}	Mono methylarsonous acid
MMA^{V}	Mono methylarsonic acid
mPCEs	Micronucleated polychromatic erythrocyte
MR	Mineralocorticoid
NaOH	Sodium hydroxide
NAS	National academy of sciences
NADP	Nicotine adenine dinucleotide phosphate
NADPH	Nicotine adenine dinucleotide phosphate oxidase
NCA	Neutral comet assay
NO	Nitric oxide
NRC	National research council
OHM/TADS	Oil and hazardous materials/technical assistance data system
PBS	Phosphate buffer saline
PR	Progesterone
QC	Quality control
RBCs	Red blood cells
RIA	Radio immuno assay
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SCGE	Single cell gel electrophoresis
SCSA	Sperm chromatin structure assay
ss	Single stranded
TdTA	Terminal deoxynucleotidyl transferase assay
$TMAO^{V}$	Trimethylarsine oxide
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
TR	Thioredoxin reductase
Trx (SH)2	Thioredodxin disulfide
USA	United states of America
US FDA	Uninted state of America food and drug administration
WHO	World Health Organization

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ABSTRACT

The present study was designed to evaluate the effect of sodium arsenite on reproductive structure and function in growing age (28-56 days) male rats for 28 days duration.
There was no significant difference in body weight of control and sodium arsenite treated rats at the start of experiment. Body weight decreased highly significantly in sodium arsenite treated rats compared to controls. High dose (200ppm) treated group showed highest decrease in body weight compared to controls and other two treatment groups. Testicular weight showed dose dependent significant decrease in treated rats, the highest decrease being observed in 200ppm treatment group compared to controls and other two treatment groups. Testicular length and width showed non-significant decrease in 50ppm and 100ppm groups, but this decrease was highly significant in 200ppm group. Arsenic treatment caused highly significant dose dependent decrease in weight and length of epididymes and vas deferens compared to controls. Morphometric studies showed highly significant dose dependent decrease in testicular seminiferous tubule diameter and its epithelial height, nuclear diameter of Leydig cell, Sertoli cell, and spermatogenic cells compared to controls as well as in between them. The number of Leydig cell, Sertoli cell and spermatogenic cells decreased with the increase in dose of sodium arsenite. Interstitial spaces extended and thickness of testicular capsule decreased in the treated groups. In 100ppm and 200ppm groups X-XIV stages of spermatogenesis showed impaired spermatogenesis. Epididymal (caput, corpus and cauda) tubule diameter and its epithelial height decreased highly significantly in dose dependent manner in treated rats compared to the controls as well as in between them. The intertubular areas enlarged and the height of principal cells and cilia decreased with the increase in dose of sodium arsenite. Disarrangement of epithelial cells was observed in treated groups. Lumen contained cellular debris in 200ppm group. Vas deferens diameter, its muscular thickness and epithelial height decreased highly significantly in dose dependent manner in treated rats compared to controls as well as in between them. Height of principal cells and cilia decreased dose dependently. Disarrangement of epithelial cells and loosening of muscularis was observed in treated groups. Cellular debris was observed in high dose group. Sodium arsenite treatment caused highly significant dose dependent decrease in plasma testosterone concentration compared to controls. High dose group showed significant decrease in mean testosterone concentration compared to 50ppm group. Treated groups showed dose dependently highly significant decrease in plasma FSH concentration compared to controls. High dose treatment group exhibited highly significant decrease in plasma FSH concentration compared to other two groups. Plasma LH concentration decreased significantly in treated rats compared to the control whereas comparison of treated groups showed no significant difference. Sodium arsenite treatment caused highly significant dose dependent decrease in testicular DSP compared to controls. Treatment of 100ppm and 200ppm sodium arsenite caused highly significant decrease in mean DSP compared to low dose treatment. Mean efficiency of DSP decreased non-significantly in low dose treated group and highly significantly in 100ppm and 200ppm treatment groups compared to controls. Mean efficiency of DSP decreased highly significantly in 100ppm and 200ppm groups compared to 50ppm group. Arsenic treatment caused highly significant dose dependent decrease in mean epididymal sperm count compared to controls as well as in between the groups. Mean testicular and blood

arsenic deposition increased significantly in dose dependent manner in treated rats compared to controls as well as in between the treated groups. Dose dependent significant increase was observed in mean epididymal arsenic deposition in the treatment groups compared to controls, the highest deposition was observed in 200ppm group. Mean vas deferens arsenic deposition increased non-significantly in 50ppm treatment group and significantly in 100pm and 200ppm groups compared to controls. High dose treatment caused significant increase in vas deferens arsenic deposition compared to other two doses. Number of epididymal sperm nuclei with intact DNA decreased and with comets increased highly significantly in dose dependent manner in treated groups compared to controls as well as in between them. Mean epididymal sperm comet length, comet height, comet head diameter and comet head DNA percentage increased highly significantly in a dose dependent manner compared to controls and among the treated groups. Mean comet tail length and comet tail DNA percentage increased non-significantly in 50ppm treatment group and highly significantly in other two treatment groups compared to controls. High dose group showed significant increase compared to 50ppm group. Arsenic treatment caused significant difference in mean comet tail DNA percentage in between treatment groups. Arsenic treatment caused non-significant increase in mean comet tail and olive moment in 50ppm treatment group and significant increase in 100ppm and 200ppm groups compared to controls, the highest increase was observed in 200ppm group. It is concluded that arsenic treatment at all dose levels adversely effect the structure and function of testes, epididymes and vas deferens in rats at 56 days age.

INTRODUCTION

INTRODUCTION

Arsenic is a naturally occurring metalloid element, found in soil, air and water (Huang et al., 2004; Duker et al., 2005; ATSDR, 2007). Environmental arsenic exists in both organic and inorganic states. Organic arsenicals are generally considered nontoxic (Gochfield, 1995), whereas inorganic forms are toxic. Inorganic arsenic exists predominantly in trivalent (As^{III}) and pentavalent (As^{V}) forms, where As^{III} is 60 times more toxic than As^{V} (Malachowski, 1990; Cervantes et al., 1994; Smedley et al., 1996; Csanaky et al., 2003; Ratnaike, 2003; Duker et al., 2005). Both trivalent and pentavalent arsenicals are soluble over a wide pH range (Bell, 1998) and are routinely found in surface and ground water (Feng et al., 2001). Under aerobic conditions, pentavalent arsenic is more stable and predominates, whereas trivalent species predominate under anaerobic conditions (Duker et al., 2005).

KING OF POISONS AND POISON OF KINGS:

Arsenic has been called "king of poison" and "poison of kings". One of the first references to arsenic was in 55 AD, when Nero reputedly used arsenic to poison Brittanicus, to secure his Roman throne (Azcue & Nriagu, 1994). Arsenic compounds were considered favorite murder weapon during the Middle Ages, particularly among the ruling classes in Italy because the poisoning symptoms were similar to those of cholera, which was common at that time. Arsenic poisoning has been implicated in the illness and death of a number of historical prominent people including George III, Napoleon Bonaparte, Charles Francis Hall and Clare Boothe Luce (Bentley and Chasteen, 2002).

EXPOSURE:

Exposure of humans to arsenic may occur from a variety of sources which can be medical, occupational, and environmental in origin (Lee and Fraumeni, 1969; Westervelt

et al., 2001; Watanabe et al., 2004). Arsenic ranks 20th in abundance in relation to other elements in the earth's crust and high concentrations are found in granite and in many minerals including copper, lead, zinc, silver and gold (NAS, 1977). Arsenic naturally accumulates as both organic and inorganic forms in soil, surface groundwater (Attrep and Anirudhan, 1977; Lloyd-Smith and Wickens, 2000), and seawater (Penrose et al, 1977). Soil may contain 0.1-40ppm arsenic (WHO, 1981; Yan-Chu, 1994) provided natural and pedogenic processes do not bring about redistribution or disturbance of underlying bed of rocks (Yan-Chu, 1994). The primary source of arsenic in soil is the parent rock (Smedley and Kinniburgh, 2002). Additionally, volcanoes are a major natural source of arsenic released into the environment (Nriagu and Pacyna, 1988; Nriagu, 1989) that can generate high arsenic concentrations in natural waters (Smedley and Kinniburgh, 2002). Arsenic that is naturally present in soil can be mobilized and transported, leading to increased concentrations of arsenic in aquifers that are sources of drinking water (Harvey et al., 2002). For the general population, arsenic in drinking water is the main exposure source (National research council, 2001), and more harmful than arsenic in food, because the bioavailability (actual amount absorbed into bloodstream) of arsenic from drinking water is greater than that from grains or vegetables (Akter et al., 2005). Arsenic concentrations in lakes are often less than in rivers, due to adsorption by iron oxides, although changes in water levels (Nimick et al., 1998; Smedley and Kinniburgh, 2002) and geothermal activity can enhance concentrations in some cases (Aggett and Kriegman, 1988; Duker et al., 2005). Groundwater from alluvial and deltaic watersheds generally has high arsenic concentration due to predominantly reducing conditions (Smedley and Kiniburgh, 2002). Arsenic compounds may enter the plant food chain from agricultural products or from soil irrigated with arsenic contaminated water (Tamaki and Frankenberger, 1992).

SODOIUM ARSENITE:

Origin of substance

Reaction of caustic soda with arsenious	(HSDB, 1995)

Synonyms

Arsenious acid, sodium salt
Sodium arsenic oxide
Sodium meta-arsenite (DOSE, 1994)

Chemical group

A compound of arsenic, a group VA element

Reference number

CAS 7784-46-5 (DOSE, 1994)
RTECS CG 3675000 (HSDB, 1995)
UN 2027 (solid)
1686 (aqueous solution) (DOSE, 1994)
HAZCHEM 2X (DOSE, 1994)

Physicochemical properties

Chemical structure
$NaAsO_2$ (DOSE, 1994)

Molecular weight (DOSE, 1994)
129.91
Physical state at room temperature
Solid (HSDB, 1995)

Color
White or grayish-white (HSDB, 1995)

Odour

None (CHRIS, 1995)

Ph

Forms basic solution in water (OHM/TADS, 1995)

Solubility

Freely soluble in water, soluble in ethanol (DOSE, 1994)

Chemical interactions

Arsine gas is evolved when sodium arsenite reacts with acids and metals. (OHM/TADS, 1995)

Major products of combustion

Arsenic fumes and sodium oxide may be generated during a fire. (OHM/TADS, 1995)

Flammability

Non-flammable (CHRIS, 1995)

Boiling point

Decomposes when heated (CHRIS, 1995)

Density

1.87 at 25 °C (DOSE, 1994)

Reactivity

Reacts with strong oxidizers (HSDB, 1995)

Hygroscopic

Uses

Insecticide
Acaricide
Arsenical soap manufacture (DOSE, 1994)

Current therapeutic uses of arsenic:

Arsenic trioxide (As_2O_3) is widely used to induce remission in patients with acute promyelocytic leukaemia, based on its mechanism as an inducer of apoptosis (Shen et al., 1997; Zhu et al., 2002).

Arsenic continues to be an essential constituent of many traditional medicine products. Some Chinese traditional medications contain realgar (arsenic sulphide) and are available as pills, tablets, and other preparations. They are used for psoriasis, syphilis, asthma, rheumatism, hemorrhoids, cough and pruritus, and are also prescribed as a health tonic, an analgesic, anti-inflammatory agent, and as a treatment for some malignant tumors (Wong et al., 1998; Shen et al., 1999). Arsenic compounds like potassium arsenite, arsenic iodide or arsenic trichloride were used for treatment of various ailments (WHO 1981; Azcue and Nriagu, 1994). Before pre-antibiotic era savarson was the main medicine used against syphilis. Though toxicity of arsenic compounds has resulted in abandoning the use of drugs containing arsenic but still several arsenic based organic compounds or herbal products are in human use as medicine as Tryparsamide, used for the treatment of African sleeping sickness (Carter and Fairlamb, 1993). Similarly, in acute promyelocytic leukemia, retinoic acid is used (Castaigne et al., 1990). In India, herbal medicines containing arsenic are used in some homoeopathic medicines (Kew et al., 1993) and haematological malignancies (Treleaven et al., 1993). In India Ayurvedic system of medicine is also used to control blood counts of persons with hematological malignancies (Ireleaven et al., 1993). In Korea arsenic is prescribed in herbal medicine for hemorrhoids (Mitchell-Heggs et al., 1990).

However rather than an intended ingredient, arsenic is more often a contaminant, sometimes with mercury and lead (Ko, 1999; Ong et al., 1999; Wong et al., 1998). The Department of Health Services of California tested 251 products in retail herbal stores and detected arsenic in 36 products (14%) in concentrations from of 14.5-53ppm (Ko, 1999). A study in Singapore identified 17 patients during a five year period with cutaneous lesions related to chronic arsenic toxicity, and in 14 (82%) patients toxicity was due to arsenic acquired from Chinese proprietary medicines while the other three consumed well water contaminated with arsenic (Wong et al., 1998).

ANTHOPOGENIC INFLUENCE:

Human activities have intensified arsenic accumulation in the environment (Bell, 1998) such as fossil fuel combustion and metal smelting, as well as the semiconductor and glass industries. Arsenic is also used in metallurgy for hardening of alloys of copper and lead, as a dopant in semiconductor production, in manufacturing of wood preservatives, pigments, insecticides, herbicides, rodenticides and fungicides (Hathaway et al., 1991). Men made arsenic compounds are very effective against pests, parasites and weeds, and due to their extensive use in agriculture; they may accumulate in soil, thereby elevation level of arsenic (WHO, 1981; Yan-chu, 1994; Azcue and Nriagu, 1994). Although most arsenic in soil is derived from the parent rock, the intensified use of arsenic compounds in agriculture and forestry practices may lead to extreme soil contamination and subsequent groundwater contaminati on, while the burning of coal and smelting of metals ma y be major sources of airborne arsenic (Bhumbla and Keefer, 1994). Mining activities of various metals may result in high levels of arsenic contamination in soil, surface water, groundwater and vegetation (Amasa, 1975; Smedley et al., 1996; Smedley and Kinniburgh, 2002). Additionally, human modifications to the natural hydrograph, including the construction of dams (Armah et al ., 1998), waste wate r recycling and irrigation practices (Siegel, 2002), enhances arsenic accumulation in soil and in water supplies.

THE ROLE OF MICROBES:

Many microorganisms have adapted to arsenic-rich environments, including soils and waters (Nakahara et al., 1977; de Vicente et al., 1990; Cervantes and Chavez, 1992; Ahmann et al., 1994; Cervantes et al., 1994; Laverman et al., 1995; Saltikov and Olson, 2002) and may be important factors in arsenic biotransformation (Shariatpanahi et al., 1981) and mobilization (Cummings et al., 1999) in the environment. Bacterial resistance to the toxic effects of arsenic may be due to a specific arsenic-resistance operon, *ars* (Carlin et al., 1995; Cai et al., 1998), and may be facilitated by reduction in arsenic uptake and increased phosphate transport (Willsky and Malamy, 1980). The *ars* operon is conserved in Gram-negative bacteria and detoxifies arsenic (Diorio et al., 1995). Bacterial populations have been associated with both oxidation and reduction of arsenic in soils (Macur et al., 2004). Some microbes can reduce the less toxic arsenate to the more toxic arsenite under anaerobic conditions (Andreae, 1978; Nies and Silver, 1995; Rensing et al., 1999) through an energy-generating process (Ilyaletdinov and Abdrashitova, 1981). Additionally, other microbes are able to methylate arsenic compounds (Gadd, 1993) and detoxifies arsenite by converting it to arsenate via an electron transfer chain (Anderson, 1992). Seasonal variations in water and temperature levels can have strong effects on arsenic concentration and speciation in soil and water due to changes in microbial uptake (Andreae 1978 , 1979). During warm, dry periods arsenic compounds are often oxidized (Maest et al., 1992), potentially increasing toxicity (Savage et al., 2000), while during wet periods oxidized arsenic is solubilized and distributed throughout the environment (McLaren and Kim, 1995; Rodriguez et al., 2004).

BIOACCUMULATION AND METABOLISM:

Arsenic accumulates across highly diverse environments within the soil, water and air where it is taken up and processed by microbes, plants and animals. Soluble arsenic taken up by plants rapidly accumulates in the food chain (Green et al., 2001). Freshwater plants and peat moss have been shown to contain considerable amount of arsenic (Reay, 1972; Minkkinen and Yliruokanen, 1978). Wetlands may have elevated concentrations of arsenic, due to the high metal-binding affinity (Beining and Ote, 1996) when compared

with uplands. High arsenic concentrations have been found in the tissues of wild birds (Fairbrother et al., 1994) and in many marine organisms, including algae (Lunde, 1972 , 1973), crustaceans (Edmonds et al., 1977), cetaceans, pinnipeds, sea turtles and sea birds (Kubota et al., 2003). Ecotoxicants released into the environment, including arsenic, often accumulate most rapidly in aquatic habitats where they enter the biota and are subsequently transferred to higher trophic levels eventually to humans. Extremely high levels of arsenic have been observed in many fish taxa (Bosnir et al., 2003; Juresa and Blanusa, 2003) and have been shown to be toxic (Suhendrayatna et al., 2002; Tisler and Zagorc-Koncan, 2002). Some species possess specific arsenic-binding proteins (Oladimeji, 1985) that may increase bioaccumulation. Monitoring arsenic levels and their associated health effects in aquatic organisms, particularly in taxa at high trophic levels such as fish, may provide insight into overall ecosystem health (Zelikoff et al, 2000) as well as into potential impacts on human health (Zelikoff, 1998; Adams and Greeley, 1999).

The adverse effects of arsenic are dependent, in part, on its chemical form and metabolism (Aposhian, 1997; Vahter, 2002; Mandal et al., 2004). Exposure from air and soil is usually minimal in humans. The major sources of exposure for humans are food and water (Bernstam and Nriagu, 2000). Once ingested, arsenic that is not eliminated from the body may accumulate in the muscles, skin, hair and nails (Ishinishi et al., 1986; Kitchin, 2001). Food contains both organic and inorganic arsenic, whereas water primarily contains inorganic forms. Seafood may provide higher concentrations of arsenic when compared with terrestrial food products (Sakurai et al., 2004), presumably due to increased bioaccumulation through generally longer trophic chains. As elemental arsenic is poorly absorbed, it is predominantly eliminated from the body unchanged (Duker et al., 2005). Humans are exposed primarily to trivalent [arsenite, As^{III}] and pentavalent [arsenate, As^{V}] inorganic arsenicals present in the environment, as well as to organic arsenic [e.g., dimethylarsinic acid, DMA^{V}] in some situations (Kenyon and Hughes, 2001; Shen et al., 2003b). Once arsenate enters the gastrointestinal tract or is absorbed, it is rapidly reduced to arsenite. In vitro studies have shown that arsenate reduction can occur non-enzymatically in the presence of thiols (Delnomdedieu et al., 1993; 1994a, b;

1995; scott et al., 1993) in a reducing (low oxygen) environment, but recent in vitro evidence suggests the reduction also occurs enzymatically (Thomas et al., 2001; 2004). A purine nucleocide phosphorylase catalyzes the in-vitro reduction of arsenate (Gregus and Nemeti 2002; Radabaugh et al., 2002) but its in-vivo relevance is not clear (Nemeti et al, 2003; Nemeti and Gregus, 2004). Whereas As^{III} produced by this reduction or from direct ingestion, is methylated primarily to monomethylarsonic acid [MMA^{V}] by an arsenic methltransferase. MMA^{V} is then reduced to monomethylarsonous acid (MMA^{III}), which is then methylated to dimethylarsinic acid (DMA^{V}). MMA and DMA are the predominant metabolites of inorganic arsenic (Vahter 2002), although DMA may be further methylated to trimethylarsine oxide (TMAO) (Hughes 2002; Yoshida et al., 1998). Individuals whose urine contains relatively lower proportions of DMA have been reported to be at increased risk for skin and bladder cancers (Chen et al., 2003a, 2003b; Hsueh et al., 1997; Yu et al., 2000).

Previously, inorganic arsenicals were thought to be more acutely toxic than organic species, as the methylation of inorganic arsenic was proposed to be a detoxification process. However, recent studies indicate that trivalent organic arsenicals [e.g., MMA^{III} and DMA^{III}] that are metabolic products of inorganic arsenic can be more toxic than the parent compound (Ahmed et al., 1999; Lee et al., 1988; Nesnow et al., 2002; Petrick et al., 2001; Styblo et al., 2000; Del Razo et al., 2001) for certain DNA damage endpoints (Ahmad et al., 2002; Mass et al., 2001). Furthermore, DMA can act as a tumor promoter at various sites and as a complete carcinogen for the urinary bladder in rodents (Wanibuchi et al., 2004; Salim et al., 2003; Wei et al., 2002; Hughes, 2001; Kenyon and Hughes, 2001; Yamamoto et al., 1997). MMA produces preneoplastic changes in liver and urinary bladder but does not produce overt neoplasia (Shen et al., 2003a), whereas TMAO can induce hepatocellular adenomas (Shen et al., 2003b). Therefore, it is important to compare and evaluate the toxicity of As^{III}, As^{V}, MMA^{V}, and DMA^{V} under

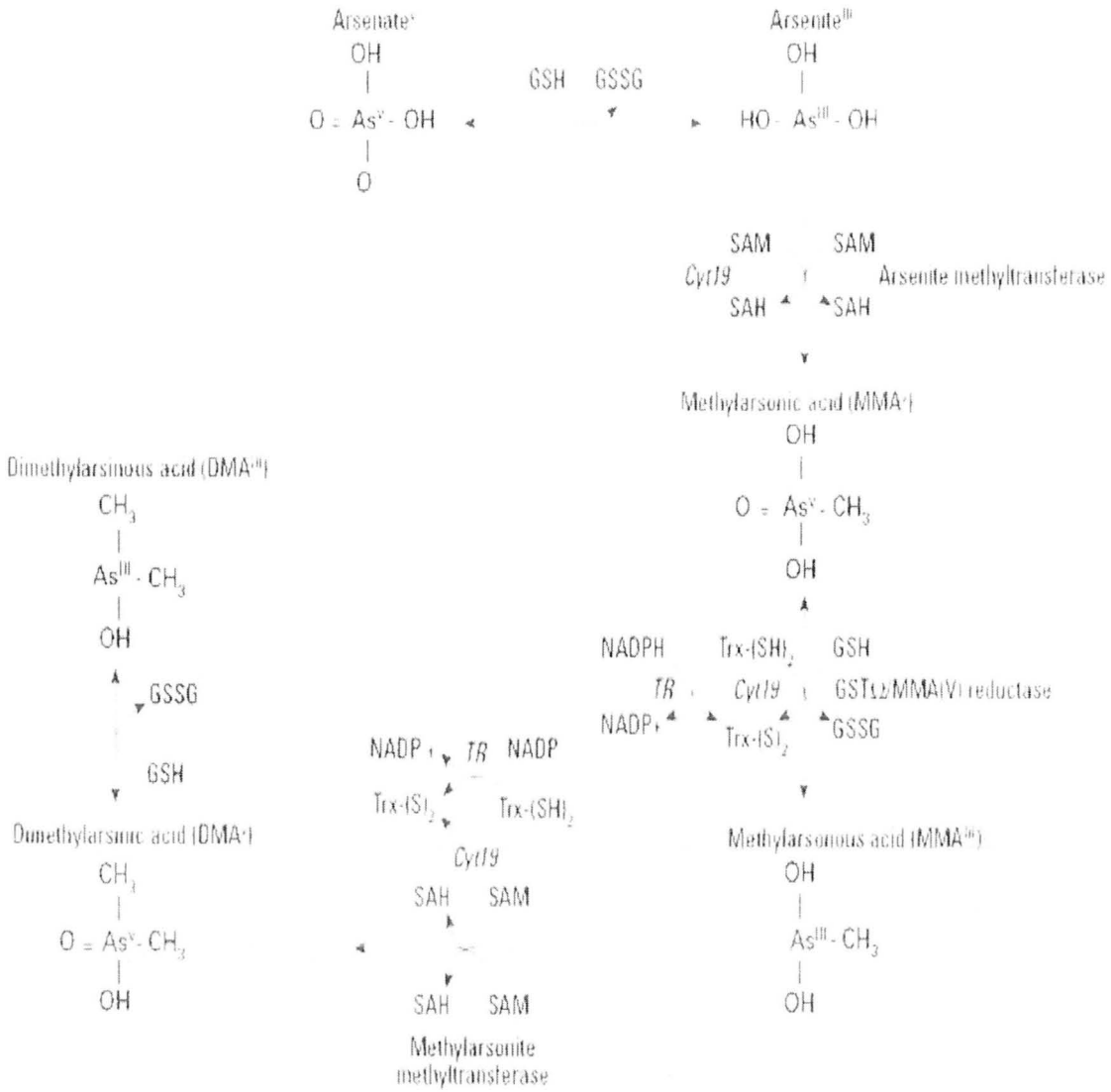


Fig. 1. Arsenic metabolic pathway. Arsenate is reduced to arsenite in a reaction thought to be dependent on GSH or other endogenous reductants. Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-S-transferase;SAH,S-adenosylhomocysteine; SAM, S-adenosylmethionine; TR, thioredoxin reductase. Arsenite then undergoes an oxidative methylation, with SAM as the methyl donor, forming MMA^{V} and SAH. MMA^{V} is reduced to MMA^{III} before a subsequent oxidative methylation step yielding DMAV and SAH. Little is known regarding in vivo reduction of DMA^{V} to DMA^{III}. Enzymes capable of catalyzing the illustrated reactions include Cyt19, arsenite methyltransferase and methylarsonite methyltransferase (two activities of one enzyme, and MMA^{V} reductase (also known as GST-Ω) (Zakharyan et al., 2001).

similar experimental conditions. Some mammals are deficient in arsenite methyltransferases necessary for effective methylation (Aposhian, 1997). These animals include the marmoset and tamarin which are New World monkeys (Vahter and Marafante, 1985; Zakharyan et al., 1996; Aposhian et al., 1997), the chimpanzee, an Old World primate (Vahter et al., 1995), and the guinea pig, a New World rodent (Healy et al., 1997). They may also show different tissue-specific expression (Abernathy et al., 1999).

TOXICOLOGY OF ARSENIC:

Acute and chronic arsenic toxicities have been shown in a variety of organisms, and the data suggest that most inorganic arsenicals are more toxic than organic forms (Abernathy et al., 1999; Duker et al., 2005). Toxic effects of inorganic arsenic include denaturing of cellular enzymes through interaction with sulfhydryl groups (Graeme and Pollack, 1998; Gebel, 2000), causing cellular damage through increased reactive oxygen species (ROS) (Wang et al., 1996; Ahmad et al., 2000), and altering gene regulation (Rossman, 1998; Abernathy et al., 1999). Arsenic is known to inhibit more than 200 enzymes (Abernathy et al., 1999; Styblo et al., 2002) and has been implicated in multisystemic health effects via interference with enzymatic function and transcriptional regulation (NRC, 1995). A variety of inhibitory effects on cellular metabolism have been shown, affecting mitochondrial respiration (Klaassen, 1996; Abernathy et al., 1999) and synthesis of adenosine triphosphate (ATP) (Winship, 1984). Oyeronke et al (2007) observed that sodium arsenite and lead acetate alone and in combination caused a clastogenic effect in bone marrow of rats. It has been demonstrated that sodium arsenite is able to induce apoptosis in both human (Abello et al., 1994) and porcine endothelial cell lines (Wang et al., 1995). Methylated arasenicals containing As^{III} are potent cytotoxins in cultured primary rat hepatocytes (Styblo et al., 1999; 2000). Blood samples obtained from women and children exposed to high levels of arsenic in drinking water show genotoxic effects in peripheral blood lymphocyte, which may be the result of loss of complete chromosomes (Dulout et al., 1996). After metabolic methylation of inorganic arsenic to DMA, DNA damage is induced due to the inhibition of repair polymerization (Zhao et al., 1997; Yamanaka et al., 1997; Hartwig et al., 1997). DMA induces an organ specific lesion and

single strand breaks in DNA of rats, mice and human lung cells in vitro (Kenyon and Hughes, 2001). Other effects of arsenic include activation of the estrogen receptor, inhibition of angiogenesis and tubulin polymerization, induction of heat-shock proteins, and oxidation of glutathione (Bernstam and Nriagu, 2000). Due to its structural similarity to phosphate, arsenate may replace phosphorus in bone (Ellenhorn and Barceloux, 1988).

There is clear evidence that arsenic can disrupt gene expression, particularly through its effects on signal transduction (Abernathy et al., 1999). Arsenic can interact directly with the glucocorticoid receptor (GR), selectively inhibiting GR-mediated transcription (Kaltreider et al., 2001). At low concentrations, arsenic has been shown to affect the DNA-binding capabilities of transcription factors NFB and AP-1, leading to increased gene expression and stimulation of cell proliferation (Chen et al., 2000; Wijeweera et al., 2001). However, at high concentrations, arsenic may lower NFB activation, inhibit cell proliferation and induce apoptosis (Abello et al., 1994; Shumilla et al., 1998; Wei et al., 2005).

It has been suggested that arsenic can disrupt cell division by deranging the spindle apparatus (Abernathy et al., 1999). Arsenic induces large deletion mutations (Hei et al., 1998), chromosome damage and aneuploidy (Abernathy et al., 1999) and, DNA protein cross-linking, and sister chromatid exchange (Huang et al., 2004). It is known to inhibit DNA repair (Lynn et al., 1997; Rossman, 1998; Brochmoller et al., 2000) and even to exacerbate the effects of other mutagenic agents (Abernathy et al., 1999), thereby increasing susceptibility to multiple diseases (Duker et al., 2005).

ARSENIC AND THE IMMUNE RESPONSE:

Chronic exposure to arsenic, in addition to its stimulation of many diseases, may affect lymphocyte, monocyte and macrophage activity in mammals, resulting in immunosuppression (Blakley et al., 1980; Gonsenbatt et al., 1994; Lantz et al., 1994; Yang and Frenkel, 2002; Wu et al., 2003; Duker et al., 2005; Sakurai et al., 2006). Arsenic disrupt glucocorticoid regulation of immune function (Kaltreider et al., 2001) and arsenic-mediated apoptosis may lead to a diminished immune response in mice (Harrison

and McCoy, 2001), rats (Bustamante et al., 1997) and humans (de la Fuente et al., 2002; Gonzales-Rangel et al., 2005). Arsenic has been shown to suppress IgM and IgG antibody-forming cell response in rats and mice following acute oral intratracheal exposure to gallium arsenite (Sikorski et al., 1989; flora et al., 1998). Arsenic exposure in mice reduces macrophage and neutrophil abundance (Patterson et al., 2004), increases susceptibility to infection (Aranyi et al., 1985), and increases mortality due to bacterial infection (Hatch et al., 1985). A field study of the effects of environmental arsenic exposure along a pollution gradient has also been shown to suppress immune function in wood mice (Tersago et al., 2004). Blakley et al., 1980; Savabieasfahani et al., 1998; Nayak et al., 2007 reported that immunosuppressive effects of arsenic were most pronounced at low concentrations of exposure, compared to high concentrations. It has even been reported that in some situations arsenic may enhance certain immune responses (Yoshida et al., 1987).

Reactive oxygen species (ROS) generated during the innate respiratory-burst response destroys invading pathogens; however, they can also cause oxidative stress and tissue damage and affect signal transduction. The introduction of environmental arsenic may lead to the increased production of ROS in laboratory animals (Schinella et al., 1996; Flora 1999; Santra et al., 2000) or in cultured animal and human cells (Lee and Ho, 1995; Barchowsky et al., 1996; 1999a, b; Pineda-Zaveleta et al., 2004) through various pathways. Arsenic activate NADPH oxidase and induces the production of O_2 and H_2O_2 (Chen et al., 1998), thereby increasing the production of the intercellular messenger NO (Gurr et al., 1998), and uncoupling mitochondrial oxidative phosphorylation (Klaassen, 1996). ROS have been implicated in the induction of single-strand (ss) breaks in DNA (Yamanaka et al., 2002) from the lungs of dimethylarsinic acid treated mice and rats (Yamanaka et al., 1989). Low levels of ROS can modulate gene expression and induce apoptosis (Chen et al., 1998), whereas high levels of ROS can result in oxidative damage and cell death (Iwama et al., 2001; Nandi et al., 2005). DNA damage and micronucleus formation caused by ROS may also potentiate cancer by enhancing cell proliferation (Hei et al., 1998).

(Breukink et al., 1980). In acute poisoning the best indicator of recent ingestion (1-2 days) is urinary arsenic concentration.

Chronic poisoning:

Long term arsenic toxicity leads to multisystem disease and the most serious consequence is malignancy. The clinical features of arsenic toxicity vary between individuals, population groups, and geographic areas. The onset is insidious with non-specific symptoms of abdominal pain, diarrhoea, and sore throat.

Skin:

Numerous skin changes occur with long term exposure (Lien et al., 1999). Dermatological changes are a common feature and the initial clinical diagnosis is often based on hyperpigmentation, palmar and solar keratosis. The keratosis may appear as a uniform thickening or as discrete nodules (Guha et al., 1998; Mazumder et al., 1998). It is emphasised that both palmar and solar keratosis are a significant diagnostic criterion. Hyperpigmentation occurs as diffuse dark brown spots, or less discrete diffuse darkening of the skin, or has a characteristic "rain drop" appearance (Smith et al., 2000). Arsenic associated skin cancer, Bowen's disease, is an uncommon manifestation in Asians and may be due to the high skin melanin content and increased exposure to ultraviolet radiation. Arsenic may cause a basal cell carcinoma in a non-melanin pigmented skin (Abernathy et al., 1999).

Large population based studies from West Bengal in India show a relationship between arsenic concentration in tube well water, dose per body weight, and hyperpigmentation and keratosis, and those persons with a poor nutritional status were more susceptible. However the study by Smith et al (2000) reports that arsenic induced skin lesions occur among Atacameno people in northern Chile, despite a good nutritional status.

Gastrointestinal system:

Though diarrhoea is a major and early onset symptom in acute arsenic poisoning, in chronic toxicity diarrhoea occurs in recurrent bouts and may be associated with vomiting. Suspicion of arsenic ingestion should be aroused if other manifestations such as skin changes and a neuropathy are also present (Poklis and Saady, 1990). Mild oesophagitis or colitis with respective upper and lower abdominal discomfort, may be accompanied by anorexia, malabsorption and weight loss, result from chronic low dose arsenic ingestion (Morton and Dunnette., 1994). In 248 patients with evidence of chronic arsenic toxicity from West Bengal, India who consumed arsenic-contaminated drinking water for one to 15 years, hepatomegaly occurred in 76.6%, and of the 69 who were biopsied, 63 (91.3%) showed non-cirrhotic portal fibrosis (Santra et al., 1999). In another study, arsenic was considered the aetiological agent in five of 42 patients with incomplete septal cirrhosis, an inactive form of macronodular cirrhosis, characterised by slender, incomplete septa that demarcate inconspicuous nodules, and an unusually high incidence of variceal bleeding (Nevens et al., 1994).

The incidence of liver injury is, in fact, higher in Guizhou, in southwest China where exposure comes from high-arsenic coal than in areas where arsenic poisoning is caused by contaminated drinking water, such as in Xinjiang and Inner Mongolia (Wang et al., 1993; Wang et al., 1996), or in West Bengal, India (Mazumder et al., 1998). The liver injury is clinically manifested as liver enlargement (hepatomegaly), abdominal pain, loss of appetite, chronic indigestion with portal hypertension, with or without elevations in serum aminotransferases (indicative of hepatocellular death) (Zhou, 1993; Zhang et al., 2000; Zhou et al., 1994; Zhou et al., 1994; Zhou et al., 1998).

Cardiovascular system:

Arsenic exposure via drinking water has been associated with coronary heart diseases, stroke, and peripheral vascular diseases in humans (Wang et al., 2002; Navas Acien et al., 2005; Duker et al., 2005). In individuals exposed to arsenic via artesian in Taiwan

increased mortality from cardiovascular desease has been reported (Chen et al., 1996). In a study in Millard County, USA, based on a matrix for cumulative arsenic exposure, a significant increase in mortality in both males and females from hypertensive heart disease occurred (Lewis et al., 1999). In Bangladesh, Rahman et al in 1999 reported an increased incidence of hypertension in a large study of 1481 subjects exposed to arsenic in well water. Seventy four Taiwanese patients with ischaemic heart disease in "arseniasis-hyperendemic villages" were studied and a link between ischaemic heart disease and long term arsenic exposure was suggested (Hsueh et al., 1998; Tsai et al., 1999).

Arsenic causes direct myocardial injury and cardiomyopathy (Benowitz, 1994). Black foot disease is a unique peripheral vascular disease, causing gangrene of the foot unique to a limited area on the south western coast of Taiwan, due to long term exposure to high arsenic in artesian well water (Tsai et al., 1999).

Neurological system:

The neurological system is the major target for the toxic effects of arsenic both in children and adults (Wasserman et al., 2004). The neurological effects are many and varied. The most frequent finding is a peripheral neuropathy mimicking Guillain-Barré syndrome with similar electromyographic findings (Goddard et al., 1992). The neuropathy is initially sensory with a glove and stocking anaesthesia. Neurotoxicity, manifested as loss of hearing, loss of taste, blurred vision, and tingling and numbness of the limbs frequently occurs (Zhou, 1993; Liu, 1992). Corneal inflammation, tearing eyes, and blurred vision become more frequent and severe as exposure levels to arsenic increase (Zhou, 1993; Zhang et al., 2000; Liu, 1992).

An increased prevalence of cerebrovascular disease, especially cerebral infarction, was observed in a large study of 8102 men and women who experienced long term arsenic exposure from well water (Chiou et al., 1997).

Genitourinary system:

The Millard County study reported an increased mortality fro m nephritis and prostate cancer (Lewis et al., 1999). Guo et al in 1997 analysed cancer registry data (1980 87) of tumours of the bladder and kidney in Taiwan and reported that high arsenic levels in drinking water from wells were associated with transitional cell carcinomas of the bladder, kidney, ureter and all urethral cancers in both males and females, and adenocarcinomas of the bladder in males (Guo et al., 1997). The authors suggest that the carcinogenicity of arsenic may be cell-type specific. In contrast, a study from Finland found an association with bladder cancer risk but not kidney cancer, despite very low arsenic concentrations in the drilled wells (Kurttio et al., 1999).

More data are required to establish a firm causal relationship between arsenic ingestion and adverse outcomes during pregnancy and on neonatal morbidity and mortality. In pregnant Andean women who consumed water with arsenic concentrations of about 200 µg/l, arsenic in cord blood (9 µg/L) was almost as high as in maternal blood (11 µg/L). In the same group placental arsenic was 34 µg/L compared with 7 µg/L in women unexposed to arsenic (Concha et al., 1998).

The results of studies by Concha and colleagues in the Andes in Argentina add another dimension to this problem (Concha et al., 1998). The fetus, infants and children who are breast fed, are exposed to arsenic toxicity from the mother.

Respiratory system:

Inhalation of arsenic in the indoor air can cause respiratory symptoms including persistent cough, chronic bronchitis, reduced residual volume and vital capacity, and X-ray abnormalities (Zhou, 1993; Zhang et al., 2000; Liu, 1992). Studies from West Bengal, India draw attention to both restrictive and obstructive lung disease (Mazumder et al.,

1998). Respiratory disease was more common in patients with the characteristic skin lesions of chronic arsenic toxicity (Mazumder et al., 2000). Similar association between skin manifestations and lung disease was reported in Chilean children (Borgono et al., 1977). The possibility of increased deposition of arsenic in the lung, although the reason is not known, is supported by necropsy studies in a limited number of patients (Saady et al., 1989; Quatrehomme et al., 1992). An increased incidence of bronchitis occurs in a study on patients with black foot disease in Taiwan (Tsai et al., 1999).

Endocrine and haematological systems:

Exposure to high concentrations of arsenic is associated with an increased risk of diabetes mellitus (Tsai et al., 1999; Rahman et al., 1998). Tseng et al., (2000) in a study in Taiwan have found that there is a link between exposure level of arsenic and incidence of diabetes. In a case control study conducted in Northern states of Mexico in which link between arsenic and diabetes 2 was found. It was found that the individuals with intermediate amount of urinary arsenic, had two fold more risk of developing diabetes while those having higher concentration, the risk became three times greater (Gonzales et al., 2007). In chronic arsenic toxicity neutropenia occurs (Poklis and Saady, 1990).

Malignant diseases:

The relationship between arsenic and malignancy is of growing concern as many millions of people are potential victims. long-term exposure to inorganic arsenic is associated with certain forms of cancer of the skin, lung, colon, bladder, liver and breast (Nemery, 1990; Abernathy et al., 1999; Huang et al., 2004; Duker et al., 2005).In Bangladesh and India arsenic is associated with skin, lung, liver, kidney, and bladder cancers (Rahman et al., 2001). There is evidence from other countries that arsenic exposure causes malignancies of lung (Axelson et al., 1978; Hopenhayn et al., 1998), liver (Tsai et al., 1999), kidney (Lewis et al., 1999; Hopenhayn et al., 1998), and bladder (Guo et al., 1997). Data from Taiwan also documents malignancies of the bladder, kidney, skin, lung, nasal cavity, bone, liver, larynx, colon, and stomach as well as lymphoma (Tsai et al., 1999). In

addition, inorganic arsenic (InAs) has long been considered to be a teratogen in multiple mammalian species (Shalat et al., 1996; Wlodarczyk et al., 2001).

It has been suggested that arsenic may act as a carcinogen through DNA hypomethylation and overexpression of protooncogenes (Zhao et al., 1997). Cancer may induce by alteration of DNA-repair mechanisms, thus interfering with cell division, differentiation and tumor suppression (Chen et al., 1996; Goering et al., 1999). Additionally, arsenic may induce certain forms of cancer by enhancing the carcinogenic effects of other substances and by affecting metabolic pathways (Huang et al., 2004).) Structural chromosome aberrations were studied in a group of individuals who consumed arsenic from well water in Finland and the association was stronger in current users than in the 10 subjects who had stopped using the contaminated well water for 2–4 months before sampling (Maki-Paakkanen et al., 1998).

Confirmation of Poisoning:

Measurement of 24-hour urinary excretion of arsenic (micrograms per day) is the most common way to confirm excessive absorption and is the preferred method to follow serial levels and evaluate chronic exposure (Malachowski, 1990; Fesmire et al., 1998).
Methods to determine blood arsenic concentration are available; however blood levels tend to poorly correlate with exposure except in the initial acute phase (Fesmire et al., 1998; Wagner and Weswig 1974). Arsenic excretion above 100 µg/day should be viewed with suspicion and the test should be repeated. Excretions above 200 µg/day reflect a toxic intake, unless seafood was ingested (Fesmire et al., 1998; Wagner and Weswig 1974; Buchet et al., 1994; Braselt and Cravey, 1990). Diets rich in seafood, primarily shellfish in the previous 48 hours, may generate 24-hour urine excretion levels as high as 200 µg/day and sometimes more (Campbell and Alvarez, 1989; Buchet et al., 1994). The majority of marine arsenic that is excreted is in the methylated form (arsenobetaine) and is not considered acutely toxic. However, one study supports that some of the arsenic released from mussels may contain higher amounts of arsenic trioxide than previously thought (Buchet et al., 1994).

Concentrations of arsenic in blood, urine, or other biologic materials can be measured by either wet or dry ashing, followed by colorimetric or atomic absorption spectrometric analysis. The latter method is preferred. Blood concentrations in excess of about 100 µg/L probably indicate excessive intake or occupational exposure; provided that seafood was not ingested before the sample was taken (Campbell and Alvarez, 1989; Fesmire et al., 1998; Wagner and Weswig 1974; Braselt and Cravey, 1990). Blood samples tend to correlate with urine samples during the early stages of acute ingestion (Fesmire et al., 1998), but because arsenic is rapidly cleared from the blood, the 24-hour urine sample remains the preferred method for detection and for ongoing monitoring (Malachowski, 1990; Fesmire et al., 1998; Wagner and Weswig 1974). Hair has been used for evaluation of chronic exposure. Levels between 0.1 and 0.5 mg/kg on a hair sample indicate chronic poisoning while 1.0 to 3.0 mg/kg indicates acute poisoning. Hair samples should be viewed with caution because external environmental contamination such as air pollution may artificially elevate arsenic levels.

DEVOPMENTAL AND REPRODUCTIVE TOXICITY:

Arsenic is a metalloid that is typically grouped with metals in considering its developmental and reproductive toxicity (Ferm, 1972; Domingo, 1994). Arsenic was the highest ranking of 164 developmental and reproductive toxicant (DART) candidates compiled for consideration under Proposition 65 (A prioritization scheme was developed by the California Department of Health Services to select chemicals for consideration by the Proposition 65 Scientific Advisory Panel. Four ascertainment methods were used to identify and construct a master list of 164 candidate agents in 1992 (Donald et al., 1992). This ranking was based on production, usage, and exposure data, inclusion on published lists of reproductive toxicants, and nomination by experts in reproductive toxicity. Arsenic was also ranked first as the agent that "posed the greatest reproductive or developmental hazard to humans" based on responses of a Delphi committee of experts organized by RCHAS to prioritize candidate DARTs. At the federal level, arsenic was 1 of 30 chemicals identified as of greatest concern for regulation as a reproductive toxicant (GAO, 1991).

DEVELOPMENTAL TOXICITY:

Developmental toxicity can have consequences ranging from death, structural abnormality to altered or retarded growth and functional deficiency (US FDA 2000, review). In a brief report of an ecological study, spontaneous abortion and still birth were examined in a Hungarian population (n=25, 648) exposed to elevated Arsenic in drinking water from regional wells (Borzsonyi et al., 1992).

Organic arsenicals are less toxic than inorganic arsenicals. The teratogenic potential of As^{III} is greater than As^{V} (Hunter, 2000; Lammon et al., 2003). There are three case reports of inorganic Arsenic poisoning in pregnant women. In all cases, the mothers survived under clinical case. In one case, the mother ingested Arsenic at 30 weeks gestation and delivered a premature infant 3 days later which died the same day (Lugo et al., 1969). In the second case, 28 weeks pregnant, intrauterine death occurred 4 days after poisoning and toxic levels of Arsenic were found in fetal tissues (Bollinger et al., 1992). In the third, 20 weeks pregnant, chelation therapy was immediately initiated and a healthy infant was delivered at 36 weeks (Daya et al., 1989).

Intraperitoneal injections of sodium arsenate to rats on gestational day 9, increases the incidence of resorption and produces cranial, neural tube, eye, skeletal, renal and gonad defects as well as cleft lip and micrognathia were observed, though dosing on day 8 or 10 was effective(Beaudoin, 1974; Burk and Beaudoin, 1977; Stump1998). In one study pregnant rats receiving intraperitoneal injections of sodium arsenite caused an increase in resorption and malformation in rats. Brain and eye defects were seen after treatment on gestational day 7, 8 or 9 (Umpierre, 1981). Pregnant rats receiving sodium arsenite i.p. on gestational day 9 had fetuses with cranial neural tube and eye defects. Arsenic exposure is also reported to result in structural changes in the thymus of pregnant and newborn mice (Skal'naia et al., 1995), and longterm exposure of arsenic is associated with abortion, low birth weight, and reduced lactation (Donald et al., 1995) as well as with embryonic cells toxicity in vitro (Lee et al., 1985). When pregnant rats were fed with arsenic in drinking water at a dose of 0.03, 0.3 and 3ppm, it was found that at dose of 3ppm there was a 25% neonatal death as compared to 0.3ppm, where rats were able to

complete gestation and parturition on schedule. Other effects included decreased spontaneous behavior of mother and 1 day old pups at both 0.3 and 3ppm. There was also an increase in the generation of ROS, nitric oxide and decreased DNA and protein synthesis (Chattopadhyay et al., 2002).

Embryotoxicity induced by arsenicals is concentration, embryonic stage and time dependent (Tabadova et al., 1996). The mechanism proposed to explain the arsenic teratogenicity include cytotoxicity, inhibition of condrogenic differentiation, mutagenicity, cell cycle arrest, apoptosis and enhanced ROS production (Kanti and Smith, 1997; Hunter, 2000; Liu et al., 2003; Navarro et al., 2004).

Arsenic has an ability to get through the placenta to the developing fetus. When pregnant mice inhaled arsine gas, a higher concentration of arsenic in the fetal brain and liver as compared to maternal liver and placenta (Miyazaki et al., 2005) was observed. In the drinking water of a pregnant rat the presence of inorganic arsenic results in the accumulation of arsenic in fetus including the fetal brain (Rodriguez et al., 2002).

REPRODUCTIVE TOXICITY:

Many heavy metal contaminants of the environment, including arsenic, have the potential to disrupt reproductive physiology and development in widely disparate species from laboratory rodents to humans (Bell and Thomas 1980; Shalat et al., 1996). Occupational, industrial, environmental, therapeutic and dietary exposures to wide range of chemicals and heavy metals have harmful effects on male fertility (Cheek and Mclachlan, 1998; Pant et al., 2003).

In studies of pregnancy outcome at the Ronnskar smelter (Ronnskar smelter in northern Sweden produces a number of metallurgical and chemical products from complex ores with high contents of arsenic), paternal effects on spontaneous abortion were investigated. When both mother and father worked at the smelter, the abortion rate was higher than if only the mother was employed there (19.4 vs 13.5%) (Beckman, 1978). A higher abortion rate was found for parity >2 (n=117) but not for parity 1 or 2 (Nordstram et al., 1979a). Survey reports from the Ukraine, Taiwan, and Bangladesh revealed that the intake of arsenic-contaminated drinking water caused reproductive disturbances in

women (Zadorozhnaja et al., 2000), adverse pregnancy outcomes (Yang et al., 2003), and spontaneous abortion (Ahmad et al., 2001).

The effect of sodium arsenite on male reproductive system is meager. In rodents, high levels of radioactive arsenic have been detected in the epididymis and seminal duct (Danielsson et al., 1984) and caused pathological changes in the structure of testes (Pires et al., 2004). Sodium arsenite itraperitoneally administered to rats caused suppressive influence on spermatogenesis, gonadotropin and testosterone release, induced inhibitory effect on testicular steroidogenic enzymes and showed reduction in the weight of testes and accessory sex organs (Sarkar et al., 1991). Some of the related studies in adult animals are:

- Pant et al (2001) administered male mice (20±2 g) sodium arsenite at a dose of 53.39, 133.47, 266.95 and 533.90 μmol/L in drinking water for 35 days. They observed a decrease in the activity of 17 β HSD along with increase in LDH, γGT at high dose level. Epididymal sperm count, motility and morphological abnormalities in sperm also decreased significantly at high dose. Significant accumulation of arsenic in testes and accessory sex organs was also found at all the doses except the low (53.39 μmol/L) dose.
- In another study Pant et al (2004) studied the effect of chronic (365 days) oral sodium arsenite (53.39 μmol/L) exposure to Swiss male albino mice of 8-10 weeks old and found a decrease in testicular weight, activities of marker testicular enzymes such as sorbitol dehydrogenase, acid phosphatase and 17 β HSD with a significant decrease in sperm count and sperm motility. They also observed a significant accumulation of arsenic in testes, epididymis and accessory sex organs.
- Sarkar et al (2003) investigated the effect of 4, 5 or 6 mg/kg/day sodium arsenite for 26 days in 4 months old male Wistar rats. In the 5 and 6 mg/kg groups, there were significant dose dependent decrease in the accessory sex organ weights, epididymal sperm count and plasma concentrations of LH, FSH and testosterone with massive degeneration of spermatogenic cells.
- Sarkar et al (2008) studied the effect of 30 mg/L and 40 mg/L sodium-meta-arsenite in adult male Swiss albino mice for 30, 45 and 60 days. They observed a

significant reduction in testicular weight, seminiferous tubule diameter, various spermatogenic cell population and Leydig cell atrophy.

However, the actual molecular events resulting in male reproductive toxicity from exposure of inorganic arsenic remain unclear. There are several possible mechanisms for the antigonadal activities of different chemicals. They may exert direct inhibitory action on the testis, they may affect pituitary causing changes in gonadotrophins concentrations and thus spermatogenesis impairment (Jana et al., 2006).

SPERMATOGENESIS:

Testis is representative of the major system at risk when animals are exposed to environmental genotoxic agents which cause DNA damage, due to potential inheritable mutations (Atorino et al., 2001). In the testis, spermatogenesis occurs in three major phases, mitotic proliferation of spermatogonia, reductional meiotic spermatocyte division and haploid spermatid differentiation to form mature spermatozoa (Handel, 1998). Several unique events take place during meiosis that are not found in somatic cells, the two most prominent being chromosome pairing and recombination during prophase I (Hawley, 1988).

Spontaneous germ cell death during spermatogenesis is an important event that takes place mostly at some specific stages of the spermatogenic cycle (Kerr, 1992' Blanco-Rodriguez and Martinez-Garcia, 1996b). The occurrence of increased germ cell apoptosis is under several circumstances, such as hormone deprivation particularly testosterone and gonadotropins (Tapananinen et al, 1993; Henriksen et al, 1995a, 1995b; Sinha-Hikim et al, 1995; Blanco Rodriguez and Martinez-Garcia, 1996a; 1997a), and experimental chryptorchidism (Shikone et al., 1994) have also been reported.

Throughout their development from spermatogonia into fully differentiated spermatozoa, the germ cells are nursed by the enveloping Sertoli cells. From Sertoli cell reconstruction studies, it has been calculated that about 50 germ cells of four different cell types are embedded in the surface of each Sertoli cell (Weber et al., 1983). Sertoli cells are the main target of FSH and testosterone. LH and FSH are required for the initiation and maintenance of spermatogenesis in prepubertal and pubertal rats (Chowdhury, 1979) and

for quantitatively normal spermatogenesis in pubertal rats (Russell et al., 1987). High level of testosterone is required for the maintenance of structural morphology and normal physiology of seminiferous tubule (Sharpe et al., 1988; Sharpe et al., 1992; Blanco-Rodriguez and Martinez-Garcia, 1998; Santen, 1999; Weinbauer et al., 2001; Perez et al., 2006).

Arsenic is a potent endocrine disruptor (Sakurai and Himeno, 2006), altering gene regulation by the closely related receptors of glucocorticoids (GR), mineralocorticoids (MR), progesterone (PR), androgens (AR), and estrogens (ER) at concentration as low as 0.01 μM or 0.7ppb (Davey et al., 2007). Arsenic intoxication is associated with spermatotoxicity (Waalkes et al., 2003; Pant et al., 2004), inhibition of testicular androgenesis and reduction of the weight of the testes and accessory sex organs (Sarkar et al., 2003) in experimental animals. Arsenic causes testicular toxicity by germ cell degeneration and inhibits androgen production in adult male rats. Co-administration of hCG with sodium arsenite was found to partially prevent the deleterious effects of arsenite whereas estradiol was found to enhance the effects of arsenite, so the toxic effects of arsenic might involve pituitary gonadotrophins and that it might act through an estrogenic mode of action (Jana et al., 2006).

DNA DAMAGE:

Paternal exposure to the toxicant may cause changes in sperm quality, there by contributing to infertility and adverse progeny outcomes. Evidence is now accumulating about the importance to embryogenesis of genetically competent spermatozoa during both natural and assisted fertilization (Alexis et al., 2004). Several authors have reported significant correlations between sperm DNA damage and effects on fertilization as well as embryo cleavage and pregnancy rates (Sun et al., 1997; Lopes et al., 1998; Larson et al., 2000; Morris et al., 2002).

The recent increasing popularity of assisted reproduction techniques (ART) including itra-cytoplasmic sperm injection (ICSI) demands sensitive estimation of sperm DNA integrity in order to ensure the genetic health of resultant offspring. Full sperm DNA

integrity usually is defined as the absence of DNA nicks or single strand (ss) breaks, double strand (ds) breaks and chemical modifications of the DNA (Hoeijmakers, 2001).

Four main methods are mostly used to detect the integrity of DNA individual spermatozoa: in situ nick translation, the terminal deoxynucleotidyl transferase assay (TdTA or TUNEL), the sperm chromatin structure assay (SCSA) and the single cell gel electrophoresis (SCGE) assay or comet assay. In situ nick translation utilizes DNA polymerase 1 to incorporate labeled nucleotides in a template specific manner (Manicardi et al., 1995). The TdTA or TUNEL detects DNA strand breaks where 3'OH groups are available (in ss and ds DNA breaks). The SCSA indirectly measures DNA stability via the relative amount of acridine orange fluorescence indicating ss DNA (Evenson et al., 1980). The SCSA has been previously correlated with fertility in an ART setting (Evenson et al., 1999; Larson et al., 2000; Evenson et al., 2002). The comet assay (SCGE) also detects ss and ds DNA strand breaks (Singh et al., 1989; Hughes et al., 1996; McKelvey Martin et al., 1997). The comet assay has been previously used to correlate sperm DNA damage with implantation success after ICSI (Donnelly et al., 2000; Morris et al., 2002). Aravindan and co-workers (1997) have compared the SCSA with a version of the neutral comet assay (NCA) and TUNEL assay and found the correlation between SCSA and comet assay to superior to that between SCSA and TUNEL assay.

The advantages of comet assay compared to other genotoxicity include: (1) its demonstrated sensitivity for dectecting low levels of DNA damage; (2) the requirement of small number of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) relatively small amounts of a test substance to conduct studies; and (7) relatively short time period required to complete an experiment (Tice et al., 2000). General reviews on comet assay have been published, include Tice et al (1991), McKelvey-Martin et al (1993), Tice (1995), Fairbrain et al (1995), Anderson et al (1998), Rojas et al (1999), and Speit and Hartmann (1999).

The comet assay was first introduced by Östling and Johanson (1984) as a technique which allowed the quantification of DNA damage in an electric field. Singh et al (1988) and Olive et al (1990) independently modified the method by developing alkaline version of this assay using pH >13 and pH ~12.3, respectively. Generally, DNA is denatured and

unwound at pH above 12.0 because of the disruption of hydrogen bonds between double-stranded DNA (Kohn, 1991). Since the introduction of alkaline comet assay the breadth of applications and the number of investigations using this technique have increased almost exponentially. This assay has been widely used in genetic toxicology (Tice et al., 2000; Saleha et al., 2001), environmental biomonitoring (Plappert et al., 1997; Hartmann et al., 1998; Mitchelmore et al., 1998; Garaj-Vrhovac et al.,2000; Gichner et al., 2000), clinical investigations (McCurdy et al., 1997; Sarkaria et al., 1998; Morris et al., 1999; Blasiak et al., 2000; Zhang et al., 2000), molecular epidemiology (de Restrepo et al., 2000) and human epidemiology (Berwick and Vineis, 2000).

The single cell gel electrophoresis technique (the comet assay) measures DNA damage, including double-strand and single-strand breaks, in somatic cells after a variety of genotoxic insults (Tice, 2000). This assay has been applied to both animal and human spermatozoa (Haines et al., 1998; Singh and Stephens, 1998). Comet assay was used for the detection of DNA damage in lymphocytes (Benitez-Bribiesca et al., 2001), in islolated cells from cardiac muscle (Wojewodzka et al., 2002) in skeletal muscle cells (Yasuhara et al., 2003), in eosinophils (Malm-Erjefalt et al., 2004), in sperm cells from semen samples (Ahmad et al., 2007) and in isolated cumulus cells (Sinko et al., (2005). In the present study comet assay was used to examine the effect of sodium arsenite on epididymal sperm DNA damage in growing age Sprague Dawley rats.

One of the principles of the comet assay is that double stranded DNA remains in the comet head and short fragments of double and single stranded DNA move into the tail area (Klaude et al., 1996). Therefore, spermatozoa with high levels of DNA strand breaks show more comet tail fluorescent intensity (Hughes et al., 1996) and tail length (Singh and Stephen, 1998). In comet measurement, tail related parameters such as comet length, tail length, comet tail percentage of DNA, tail moment and olive moment, reflects the sperm DNA damage whereas, comet head DNA percentage and intact DNA number represents the sperm DNA integrity. Tail and head DNA percentage have the equal prognostic ability to discriminate the abnormal chromatin structure of sperm (Ahmed, 2007). The concept of tail moment (= a measure of tail length × a measure of DNA in the tail) as a metric for DNA migration was introduced by Olive et al (1990).

Most of the work related to arsenic toxicity has been done in the adult animals. There is lack of literature about the reproductive toxicity of sodium arsenite in growing age (from 4 – 8 weeks of age) male Sprague Dawley rats. In current study the treatment was initiated at 28 days age and continued upto 56 days of age to know the adverse effects of different doses (50ppm, 100ppm and 200ppm) of sodium arsenite on structure and function of testes, epididymes, vas deferens and steroidogenic activity and DNA damage in epididymal sperm of male rats.

MATERIALS AND METHODS

Materials and Methods

ANIMAL SELECTION AND CARE:

Adult male and female Sprague Dawley rats were procured from National Institute of Health (Islamabad, Pakistan) and were maintained in a 12 h light and 12 h dark cycle in the animal house of Quaid-I-Azam university with free access to standard laboratory food (formulated and prepared in our laboratory) and water ad libitum. After mating female rats were allowed to give birth. The resultant offspring at 28 days age were used in present study. The animals were weighed before the initiation of experiment, their body weight ranged from 75-80 gm.

EXPERIMENTAL DESIGN AND CHEMICAL TREATMENT:

The chemical used as toxicant in the current study was sodium arsenite (Merck) dissolved in drinking water.

All the animals were randomly divided in 4 groups of 8 animals (n=8) each. Animals of each group were treated as follows.

Group-1 control was given drinking water.

Group-2 was given 50ppm sodium arsenite in drinking water.

Group-3 was given 100ppm sodium arsenite in drinking water.

Group-4 was given 200ppm sodium arsenite in drinking water.

This treatment was continued for 28 days from 4^{th} week to 8^{th} week of age. Body weight was recorded weekly during the experiment.

ANIMAL SACRIFICE, COLLECTION OF BLOOD AND REPRODUCTIVE ORGANS:

After 28 days treatment of sodium arsenite body weight was recorded and all the animals were sacrificed under chloroform anesthesia within 8:00 and 10:00 h to avoid any diurnal fluctuation in the concentrations of hormones. Blood was collected from each animal from dorsal aorta using hepranized syringe (21-guage needle). From the collected blood 0.5ml was stored at -70 °C for the estimation of arsenic deposition by flame atomic

absorption spectrophotometry equipped with Hydroid Generator. From the rest of blood plasma was separated by centrifugation at 4000 rpm for 20 minutes. Plasma samples were stored at -20 °C until hormonal estimations were performed. The paired testes, epididymes, and vas deferens were dissected out quickly and washed in 0.9% (w/v) normal saline, pat dried and the wet weight taken in Seritorious electrical balance. The size of the organs was recorded by digital vernier caliper. Half of the right testis, epididymis and vas deferens from control and all the three treated groups were used for routine histology and the other half of the testes from all groups were used for estimation of daily sperm production and epididymes for comet assay. The tissues of the remaining animals were stored at -70 °C for atomic absorption spectrophotometry.

HISTOLOGICAL PROCEDURE:

The tissues were immersed in fixative sera (60% alcohol + 30% formaldehyde + 10% acetic acid) for 4-5 hours and dehydrated in graded ethanol series (80-100%) in subsequent steps. Cedar wood oil was used for the clearing of tissues. After embedding, the tissues were mounted on wooden blocks and were sectioned with rotatory microtome (Shandon, Finesse 325) at a thickness of 5 µm. The sections were affixed to precleaned albuminized glass slides. After deparafinizing by xylene, the slides were passed through different grades of alcohol (hydration and dehydration). The sections were then stained by hematoxylene and eosin and mounted with Canada balsam (Kalsoom et al., 2005; Akram et al., 2002). Microscopic examination was carried out under Nikon Optiphot research microscope with ocular micrometer at different magnifications. Microphotography was done by microscope (Leica LB) equipped with Canon digital camera.

Morphometric study of the following organs was done;

Testis:

Seminiferous tubule diameter, its epithelial height, nuclear diameter of Leydig cell, Sertoli cell, spermatogonia, primary spermatocyte, secondary spermatocyte and spermtids.

Tubular diameter and epithelial height of the three parts (caput, corpus and cauda) of epididymis.

Vas deferens:

Vas deferens diameter, muscular thickness and epithelial height.

HORMONAL ANALYSIS:

Hormones analysis was carried out for plasma Testosterone, FSH and LH in 56 days old Sprague Dawley rats. For the estimation of Testosterone EIA kit was purchased from Biochek, Inc, for FSH, ELISA and for LH, RIA kits were purchased from Biocode Hycel.

EIA for the quantitative determination of Testosterone concentration in plasma:

The principle of assay was based on the competitive binding of Testosterone in the test sample with the Testosterone-HRP conjugate for a constant amount of rabbit anti-Testosterone.

- Desired number of coated wells was secured in the holder.
- Standards, specimen and controls (10 µl) were dispensed into the appropriate wells.
- Testosterone-HRP conjugate reagent (100 µl) was dispensed into each well.
- Rabbit anti-Testosterone reagent (50 µl) was dispensed to each well.
- The content of the microwells was mixed thoroughly for 30 seconds.
- The microwell plate was incubated at 37 °C for 90 minutes. A fixed amount of HRP-labelled Testosterone competes with endogenous Testosterone in the standard, sample or quality specific Testosterone antibody during incubation. Thus the amount of Testosterone peroxidase conjugate immunological bound to the well progressively decreases as the concentration of Testosterone in the specimen increases
- Unbound Testosterone peroxidase conjugate is then removed by washing microwells 5 times with the distilled or deionized water.
- TMB reagent (100 µl) was dispensed into each well and was mixed gently for 10 seconds, resulted in blue colour.

- The microwell plate was incubated at room temperature (18-25 °C) for 20 minutes.
- The reaction was stopped by adding 100 µl of stop solution to each well.
- The content of microwells was mixed gently for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
- The absorbance read at 450 nm with a microtiter well reader within 15 minutes.
- The results were expressed in ng/ml.

ELISA for the Quantitative Determination of FSH in Plasma:

The principal of assay is based on the use of high affinity antibody and specifically for two different epitopes on rat FSH. A first mouse anti-rFSH monoclonal antibody bound to polysterene well will capture the rFSH of sample in the presence of a second horseradish peroxidase conjugated mouse anti-rFSH monoclonal antibody. Following the incubation and one step formation of solid phase-rFSH-conjugated monoclonal antibody, the chromogen/substrate is added, which turns from pink to blue proportionally to the rFSH concentration in the sample. Addition of stop solution turns the color to yellow. The intensity of the yellow color is measured using a spectrophotometer with a 450nm filter. The protocol with the kit was followed.

- Each calibrator (25 µl) was dispensed into the appropriate wells.
- Samples and controls (25 µl) were dispensed into appropriate wells.
- Conjugate (200 µl) was added into each well.
- The microwells plate was incubated for 180 minutes at room temperature (25 °C) without shaking.
- The plate was aspirated with an automated plate washer.
- The wells were washed 7 times with an automated system set to 250 µl per well.
- Chromogen substrate (SUBS TMB) solution (200 µl) was dispensed into each well, ensuring that it is initially pale colored.
- The microwells plate was incubated for 30 minutes at controlled room temperature (25 °C) without shaking.
- The reaction was stopped by adding 50 µl of stop solution (SOLN STOP) to each well.

- The plate was swirled gently to mix contents.
- The absorbance was measured at 450 nm on a 96 well microplate reader.
- Results were expressed in ng/ml.

RIA for the Quantitative Determination of LH in Plasma:

The principle of LH RIA assay is based on the competitive binding of LH in rat sample with ^{125}I-labelled rat LH tracer to the highly specific rabbit polyclonal antibody (Ab). As the concentration of ^{125}I-LH and Ab are constant, the advancing state of equation depends on the concentration of rat LH in the sample. After incubation, separation of bound from free is achieved by a second antibody (mouse monoclonal anti rabbit IgG antibody) coupled to magnetic particles. The separated particles are counted in gamma counter. The reagents provided in kits were calibrators, rabbit polyclonal antibody, ^{125}I- labeled rat LH tracer, SORB Ab Fc magnetic particles and the magnetic plates. The protocol with the kit was followed.

- The tubes for T ("Total Count"), NSB, calibrators, control and samples were labeled.
- For NSB, 25 µl of calibrator and 100 µl of distilled water was pipetted into corresponding tubes.
- Calibrator (25 µl) was dispensed into the corresponding tubes.
- Sample and control (25 µl each) was dispensed into the corresponding tubes.
- Antiserum (Ab Rat LH) 100 µl was added to each tube except NSB and "Total Count" tubes.
- All the tubes were mixed with a vortex mixer.
- The tubes were incubated for 3 hours at 37 °C.
- Rat LH ^{125}I tracer (100 µl) was added to each tube. "Total Count" tubes did not participate to the following steps.
- The tubes were incubated over night at room temperature.
- The magnetic particles (SORB Ab Fc) were mixed with a vortex mixer and 200 µl was added to each tube except "Total Count" tubes.
- All tubes were mixed with a vortex mixer and incubated for 1 hour at room temperature without further mixing.

- All tubes were arranged on a magnetic plate and the magnetic particles were allowed to sediment at the bottom of the tubes for 15 minutes. Then the contents were decanted of the tubes by inverting the magnetic plate over a waste container. The liquid was then allowed to decant on an absorbent layer for 5 minutes.
- The radioactivity precipitated in each tube was counted for at least 60 seconds.
- Rat sample concentrations were read from the calibration curve.
- Results were expressed in ng/ml.

ESTIMATION OF DAILY SPERM PRODUCTION:

A modified protocol was used to estimate DSP of each rat as described by Yucra et al (2008). Testis was weighed, decapsulated and parenchyma weighed. Testis without tunica was homogenized for 10 minutes in homogenizer at 5000 rpm in 25 ml physiological saline solution (0.05% (vol/vol) Triton X-100 and 0.25 mg sodium azide). To count the spermatids, each 0.2 ml sample of homogenate was diluted with 0.8 ml of saline solution containing 1% trypan blue (Sigma Chemical Company, St Louis, USA). Trypan blue stains the spermatids and facilitates counting. A drop of sample (50 µl) was placed on Harwel chamber to count the number of homogenization-resistant elongated spermatids under microscope at 40x magnification. Average number of spermatids per sample was determined by recording three readings and obtained the total number of spermatids per testis and this number was then divided by the testis weight to give spermatids per gram of testes.

Formula:

$$Y = X / 10 \times 100 \times 50 \times 5 \times 1000$$

(X= number of spermatids counted in Harvel chamber, 10= number of squares observed in one reading, 100= total number of squares in chamber, 5 = dilution with physiological saline, 50=µl used for loading the chamber and 1000= to change µl into ml).

Daily sperm production (DSP) = Y/ 6.3

Efficiency of sperm production (spermatids per gram testes) = DSP/ Weight of decapsulated testis.

In the Sprague Dawley rat, developing spermatids spend approximately 6.3 days in steps 17-19 during spermatogenesis (Clermont and Harvey., 1995; Amann etal., 1976).

EPIDIDYMAL SPERM COUNT:

Right epididymes of five animals were minced by using fine forceps in 1 ml of Medium 199 (Cat # M7528, Sigma, USA). From each epididymis suspension 50 µl sample was used to estimate the number of sperms using Harwel Chamber. Sperms were counted three times in 10 squares of Harwel Chamber. Three readings for each sample were recorded and the average of these was taken. The average sperm count of each rat was multiplied by 0.06 (sperm x 10^6 / ml). These reading were used to refer number of sperms per epididymis (Yucra et al; 2007). Fresh epididymal suspension was further processed for comet assay.

ATOMIC ABSORPTION SPECTROPHOTOMETRY:

Digestion:

Deposition of arsenic in testes, epididymes, vas deferens and blood of control and sodium arsenite treatment groups was measured by atomic absorption spectrophotometry. Frozen left testes, epididymes, vas deferens and blood was digested in 3 ml concentrated HNO_3 (Merck, Germany) using a microwave digestion system (MARS, CEM, USA). The maximum temperature set was 210 °C, and the power was 1200 watts. Samples were run for 10 minutes. Samples were brought to a constant (10 ml) volume with 7 ml deionized water and read at this stage for atomic absorption spectrophotometry to determine arsenic concentration present in testes, epididymes, vas deferens and blood.

Determination of metal concentration:

Digest from each tissue and blood was subjected to flame atomic absorption spectrophotometer equipped with Hydroid Generator (varian, AA240 FS, USA) at wave length 193.7 nm, slit (nm) 0.5, relative intensity 50, relative sensitivity 1, and current was 9 mA. The results were expressed as µg/mg of tissue or µg/ml of blood.

COMET ASSAY:

Single cell gel electrophoresis technique, the comet assay is equally important for obtaining reproducible and reliable results. In general, best results are obtained if sample processing, solution preparation and usage, equipment utilization and maintenance are conducted using the strict quality control (QC) criteria considered appropriate for techniques in molecular biology. The protocol as used by (Haines et al., 2002) was adapted with some modifications, the basic steps of the assay included

1. Preparation of microscope slides layered with cells in agarose.
2. Lysis of cells to liberate DNA.
3. Exposure to alkali (pH. 13) to obtain single-stranded DNA and to express AlS as SSB.
4. Electrophoresis under alkaline (pH. 13) conditions.
5. Neutralization of alkali.
6. DNA staining and comet visualization.
7. Comet scoring.

The entire procedure is conducted under low indirect incandescent light (60 watts) in order to minimize light induced DNA damage to sperms.

Sperm collection:

Epididymal sperms were collected by removing the excessive fats and then minced with forceps in Medium 199 for comet assay. The sperms were diluted in PBS solution by taking 1 ml of concentrate in 10 ml of PBS. Dilutions were made until we got 10,000 sperms in 20 µl.

Embedding of spermatozoa in agarose gel:

The ultimate goal of slide preparation is to obtain uniform gels sufficiently stable to survive through to data collection, as well as to ensure easily visualized comets with minimal background noise.

Slide preparation:

A number of different techniques have been used to prepare comet slides. Generally, but not exclusively, microscope slides are used with each slide containing one or two independent gels. In the current study we used the three-layered procedure.

For 1st layer:

Prepared 1% (w/v) low melting point (LMP) agarose (sigma) 500 mg/50 ml Ca and Mg free PBS. The solution was heated until near boiling point approximately 95 °C and the agarose was dissolved. While agarose was hot, microscopic glass slides (Sail brand 25.4×76.2 mm) were dipped up to half and gently removed. Underside of each slide was wiped to remove agarose and laid the slides on the icepacks for quick solidification of agarose. The slides were dried at room temperature and stored until use.

For 2nd layer:

20 µl of sperm suspension and 70 µl of 0.5% (w/v) LMP Agarose were applied on coated slides to make second layer with the help of large cover slips (24×50 mm) and allowed to set on ice packs for 5 minutes.

For 3rd layer:

Third layer of 70 µl of same concentration of LMP agarose was added to fill in any residual holes in the second layer and to increase the distance between the cells and the gel surface. Coverslips were placed on slides and placed on icepacks until the agarose layer hardens.

The optimal number of cells (at least for image analysis) should not be more than a few per visual field. Higher cell densities can result in a significant proportion of overlapping comets especially at higher levels of DNA migration. Higher agarose concentration can affect the extent of DNA migration as well as the accessibility of the DNA to other manipulations.

Lysing of cells and decondensation of DNA:

Coverslips were removed and the slides were immersed in a jar containing freshly prepared cold lysing solution containing ingredients 2.5 M sodium chloride, 100 mM disodium ethylene diamine tetra acetic acid, 10 mM Tris, 1% triton X-100, 10% DMSO, 40 mM dithiothrietol (the purpose of the DMSO in the lysing solution is to scavenge

radicals generated by the iron released from hemoglobin when blood or animal tissues are used. It is not needed for other situations or where the slides will be dipped in lysing solution for brief time only). These ingredients were added to about 90 ml distilled water and the mixture was stirred properly. Sodium hydroxide pellets were added to dissolve mixture. pH was adjusted to about 10 using concentrated HCl or NaOH. The solution was brought upto 99 ml with distilled water and 1% Triton X-100 was added just prior to use. The solution was refrigerated for 30 minutes prior to slide addition. The lysing solution was chilled prior to use, primarily to maintain the stability of the agarose gel. Slides were immersed in cold lysing solution for 3 hours at room temperature. Then slides were dipped in lysing solution containing proteinase K (10 μg/ml) and for additional lysis over night. This removes protamines that otherwise impede DNA migration through gel.

Unwinding of DNA:

Slides were removed from protienase K solution and were washed thrice with deionized water at 20 minutes interval to remove salts and detergent. A horizontal gel electrophoresis tank was filled with fresh alkaline electrophoresis solution (TBE buffer). Ingredients of electrophoresis solution were 10 mM Tris, 0.08M boric acid, 0.5 M EDTA. These ingredients were dissolved in 950 ml of distilled water and pH was adjusted to 8.2 by adding NaOH pellets and concentrated HCl. The solution was brought upto 1000 ml by adding distilled water. The slides were placed side to side in electrophoresis tank. Bubbles were avoided over the agarose. The slides were left in this high buffer for 30 minutes at 4 °C to allow DNA in the cells to unwind.

Separation of DNA fragments by electrophosesis:

After alkali unwinding, single stranded DNA in the gel was electrophoresed under alkaline conditions to separate the fragmented DNA.

Power supply was turned on to 25 volts and current was adjusted to 300 mA for 20 minutes. The power was turned off and slides were gently lifted from the buffer. Variability was reduced by ensuring that coded replicate slides were scored for each sample and that the coded slides were randomly distributed in a gel box in a fully balanced design. A fully balanced design means that to the extent possible, each

electrophoresis run should contain one slide from each sample within an experiment. Fresh electrophoresis buffer was used through out the study.

Neutralization:

After electrophoresis the slides were rinsed with absolute ethanol for a brief time and then dried at room temperature and stored in a slide box.

Staining the slides:

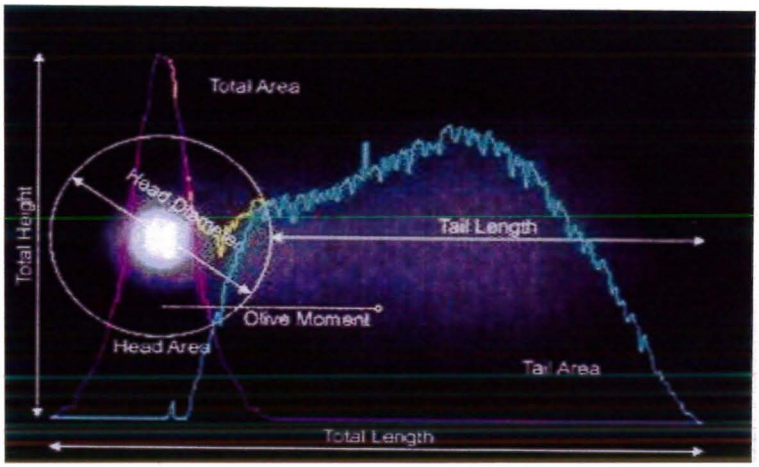
Acridine orange (ICN Biomedicals) was used as DNA specific fluorescent dye in this experiment. Acridine was dissolved in distilled water at 1 % (w/v) concentration. 500 µl of Acridine Orange solution was flooded over the slides and left for 5 minutes in the dark. Before image analysis the slides were rinsed with double distilled water and cover slips were applied.

Comet scoring:

The slides were observed under flourescent microscope (Leica) at 40X magnification and photographed by cannon digital camera. Comets were scored in photographs by using TRITEK software and observations were made. Randomly 50 sperm nuclei were analyzed per slide and five slides for each sample were evaluated. Cells with intact DNA and cells having comets were counted. Comet parameters e.g comet length, comet height, comet head diameter, percentage DNA in comet head, comet tail length, percentage DNA in comet tail, comet tail movement and olive movement were calculated using TRITEK comet score software. Tail length is the distance of DNA migration from the body of the nuclear core and it is used to evaluate the extent of DNA damage. Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).

Head % DNA= (head. Optical intensity / (head. Optical intensity + tail. Optical intensity)) × 100.

Tail % DNA= 100 - head % DNA.



Photograph with diagrammatic explanation of different comet measurements including comet length, comet height, comet head diameter, comet tail length and olive tail moment used in this study.

STATISTICAL ANALYSIS:

Results of the experiment were expressed as Mean ± SEM. The difference between the mean values of all the experimental groups was evaluated by Tukey's test. As there were marked dose dependent variations between control and sodium arsenite treated (50ppm, 100ppm and 200ppm) groups so the results were analyzed using linear regression of variance. The value for $p<0.05$ was considered significant. Accordingly a statistical software Graph pad prism version 5 was used.

RESULTS

RESULTS

GENERAL OBSERVATIONS:

Treatment of sodium arsenite (50ppm, 100ppm and 200ppm) for 28 days to young male Sprague dawley rats during the growing age caused a dose dependent decrease in feed consumption, water uptake and body weight. After 28 days (at the age of 56 days), rats treated with 200ppm sodium arsenite showed the highest decrease in feed consumption, water uptake, body weight and movement compared to control and low dose (50ppm and 100ppm) treated groups. The treated rats were weak and anemic. There was no body fats in 200ppm sodium arsenite treatment group.

BODY WEIGHT:

Weekly body weight:

Mean weekly body weight of control and treated groups (50ppm, 100ppm and 200ppm) is shown in Fig. 2.

Treatment of sodium arsenite was initiated at 4th week (28 days) of age. There was no significant ($P>0.05$) difference in mean body weight of rats in different treatment groups at the start of experiment.

At 5th week (35 days) of age mean body weight of 50ppm group decreased non-significantly ($P>0.05$) compared to controls. Body weight of rats treated with 100ppm and 200ppm sodium arsenite showed highly significant ($P<0.001$) reduction compared to controls. There was significant ($P<0.05$) reduction in body weight of 35 days old 100ppm sodium arsenite treated rats compared to 50ppm treatment group. Rats treated with 200ppm showed significant ($P<0.05$) reduction compared to 100ppm and highly significant ($P<0.001$) reduction in mean body weight compared 50ppm treated rats.

At 6th week (42 days) of age mean body weight of sodium arsenite treated (50pm, 100ppm and 200ppm) rats showed highly significant ($P<0.001$) reduction compared to controls. The comparison of the three sodium arsenite treatment (50ppm Vs 100ppm, 50ppm Vs 200ppm and 100ppm Vs 200ppm) also showed highly significant ($P<0.001$)

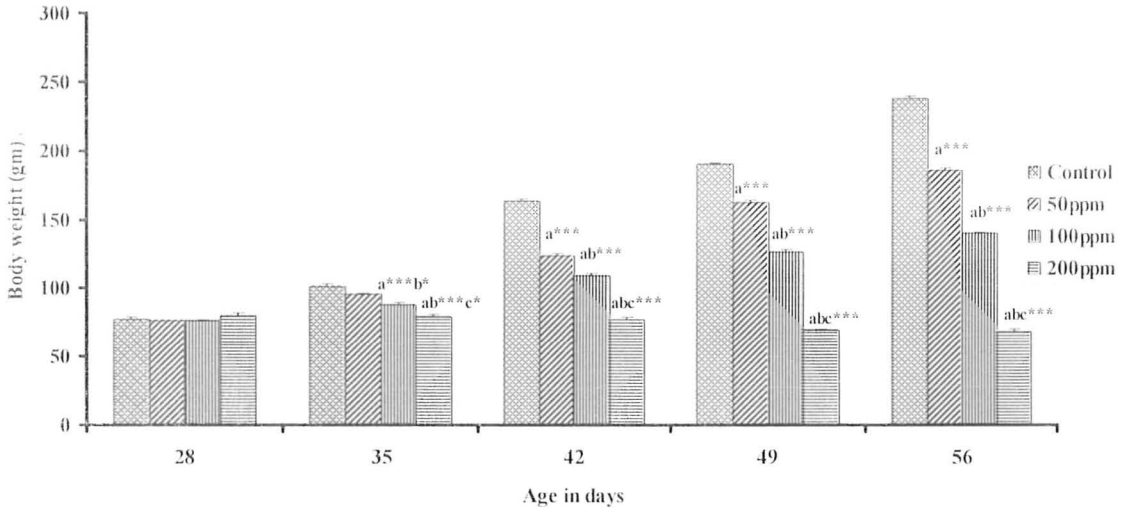


Fig. 2. Mean body weight of control and treated (50ppm, 100ppm and 200ppm) groups at the start of experiment (28 days of age), 35 days, 42 days, 49 days and 56 days of age.

a=control VS treated groups, b=50ppm VS 100ppm and 200ppm, c=100ppm VS 200ppm. P<0.05*, P<0.001***
Values=SEM

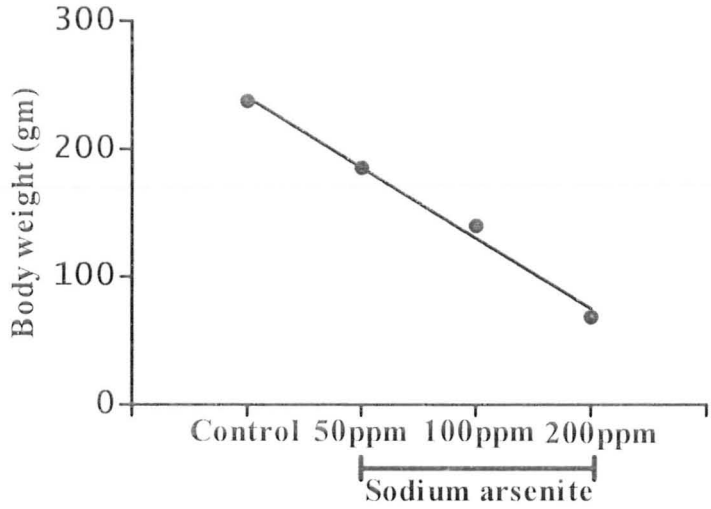


Fig. 3. Regression line showing a significant dose dependent decrease in mean body weight of rats at 56 days age against increased doses of sodium arsenite.

difference in body weight. The decrease in mean body weight of 6 weeks old rats was highest in 200ppm treatment group.

At 7th week (49 days of age) there was highly significant decrease in body weight ($P<0.001$) in 50ppm, 100ppm and 200ppm treatment groups compared to the controls. Seven weeks old rats treated with 100ppm also showed very high significant ($P<0.001$) decrease in mean body weight compared to 50ppm. The reduction in mean body weight of 200ppm treatment group was highly significant ($P<0.001$) compared to 50ppm and 100ppm groups.

Mean body weight of 8 weeks old 50ppm, 100ppm and 200ppm sodium arsenite treated rats showed highly significant ($P<0.001$) reduction compared to the control. There was also very highly significant ($P<0.001$) dose dependent body weight reduction in between all the three sodium arsenite treated (50ppm, 100ppm and 200ppm) groups at 8th week (56 days) of age.

It was also observed that in control, 50ppm and 100ppm treatment groups there was gradual increase in the body weight compared to their initial weight (at 28 days). However, in 200ppm treatment group a significant decrease in body weight compared to the initial body weight was observed.

Mean body weight:

Regression analysis of variance showed (Fig. 3) a dose dependent highly significant decrease in mean body weight of 56 days old male Sprague dawley rats against 50ppm, 100ppm and 200ppm sodium arsenite treatment ($b=-55.29\pm3.835$; $F_{(1, 2)}=207.8$; $P=0.004$).

TESTES:

Mean testicular weight, length and width of control and sodium arsenite treated (50ppm, 100ppm and 200ppm) groups is shown in Table 1.

The mean testicular weight of rats treated with 50ppm sodium arsenite showed a significant ($P<0.05$) decrease compared to the controls. Further highly significant decrease in testicular weight was observed in 100ppm ($P<0.01$) and 200ppm ($P<0.001$) treatment groups compared controls. The decrease in mean testicular weight was more

Table. 1. Effect of various doses of sodium arsenite for 28 days duration on the testicular weight, length and width of male Sprague dawley rats at 56 days age.

	Testes		
Groups	**Weight (mg)**	**Length (mm)**	**Width (mm)**
Control	1071.54± 0.03	17.13± 0.27	10.00± 0.08
50ppm	930.83± 0.02^{a*}	16.70± 0.12	9.76± 0.15
100ppm	899.16± 0.02^{a**}	16.31± 0.17	9.51± 0.11
200ppm	726.30± 0.04^{ab***c**}	14.50± 0.31^{abc**}	8.39± 0.22^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppm VS 200ppm

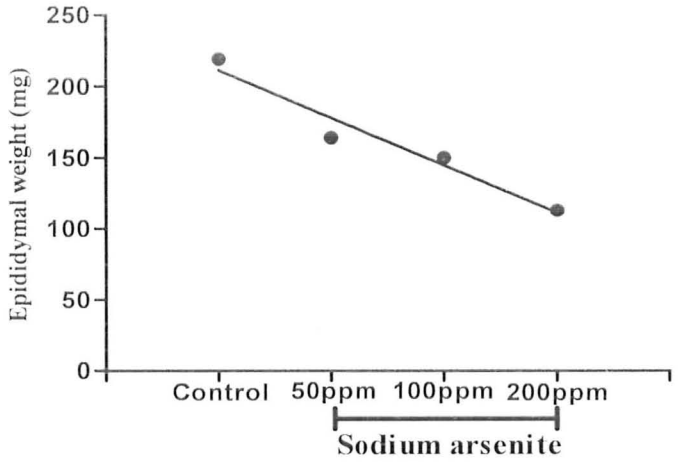
P<0.05*, P<0.01P<0.001*****

Values±SEM

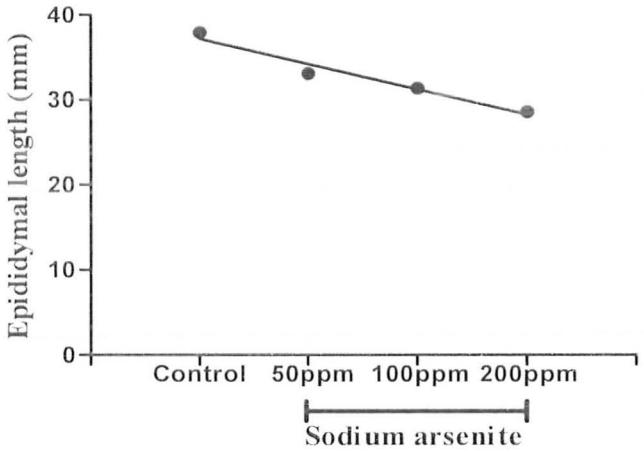
Table. 2. Effect of various doses of sodium arsenite for 28 days on weight and length of epididymes and vas deferens of male Sprague dawley rats at 56 days age.

Groups	Epididymes		Vas deference	
	Weight (mg)	Length (mm)	Weight (mg)	Length (mm)
Control	219.35 ± 0.009	37.90 ± 0.602	65.68 ± 0.002	33.76 ± 0.667
50ppm	164.34 ± 0.004^{a***}	33.07 ± 0.330^{a***}	50.78 ± 0.002^{a***}	30.85 ± 0.648^{a**}
100ppm	150.39 ± 0.003^{a***}	31.35 ± 0.482^{a***}	44.88 ± 0.002^{a***}	29.60 ± 0.314^{a***}
200ppm	113.49 ± $0.007^{ab***c**}$	28.58 ± $0.547^{ab***c**}$	29.88 ± 0.003^{abc***}	24.80 ± 0.597^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.01, P<0.001*****
Values±SEM



(a).



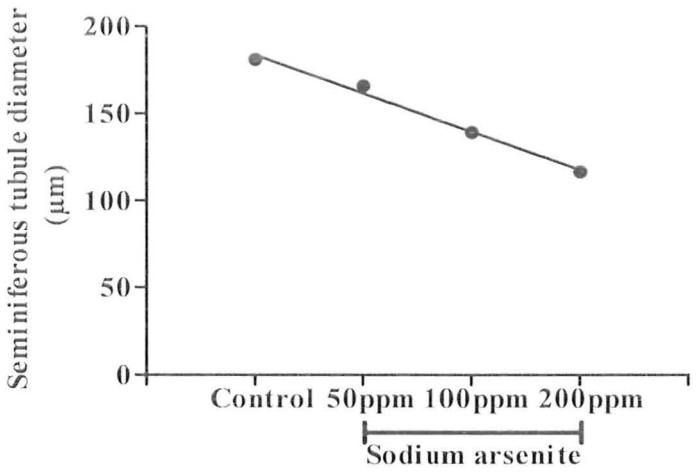
(b).

Fig. 5. Regression line showing a significant decrease in mean epididymal (a) weight and (b) length in rats at 56 days age against increased doses of sodium arsenite.

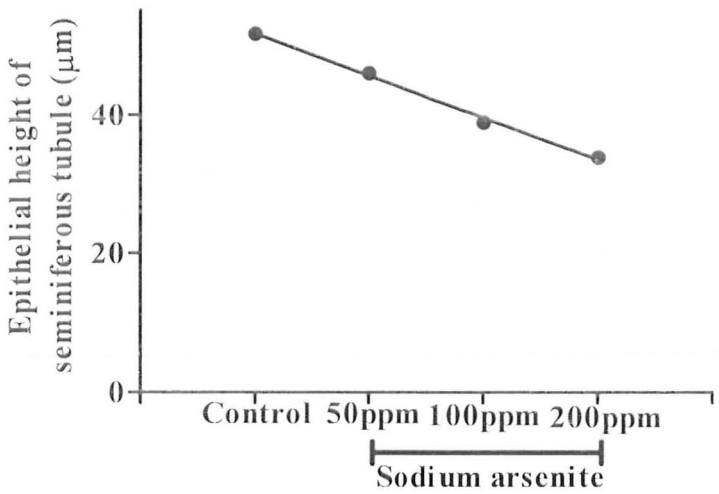
Table. 3. Effect of various doses of sodium arsenite for 28 days duration on testicular seminiferous tubule diameter, epithelial height, nuclear diameter of Leydig cell and Sertoli cell of male Sprague dawley rats.

Groups	Tubule Diameter (µm)	Epithelial height (µm)	Nuclear diameter (µm)	
			Leydig cell	Sertoli cell
Control	180.92 ± 0.42	51.64 ± 1.17	6.34 ± 0.03	7.53 ± 0.02
50ppm	165.70 ± 0.28^{a***}	45.95 ± 0.74^{a***}	5.72 ± 0.03^{a***}	6.80 ± 0.03^{a***}
100ppm	139.14 ± 0.27^{ab***}	38.86 ± 0.53^{ab***}	4.86 ± 0.02^{ab***}	6.10 ± 0.02^{ab***}
200ppm	116.48 ± 0:29^{abc***}	33.80 ± 0.67^{abc***}	3.51 ± 0.04^{abc***}	4.03 ± 0.07^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.001***
Values±SEM



(a).



(b).

Fig. 7. Regression line showing a significant decrease in mean (a) seminiferous tubule diameter and (b) its epithelial height in rats at 56 days age against increased doses of sodium arsenite.

Table. 4. Effect of various doses of sodium arsenite for 28 days on testicular spermatogonia, primary spermatocyte, secondary spermatocyte and spermatid diameter of male Sprague dawley rats at 56 days age.

Groups	Spermatogonia (µm)	Pr.Spermatocyte (µm)	Sec.Spermatocyte (µm)	Spermatid (µm)
Control	5.72 ± 0.037	8.44 ± 0.025	5.99 ± 0.034	2.13 ± 0.02
50ppm	5.43 ± 0.028^{a***}	7.85 ± 0.02^{a***}	5.30 ± 0.03^{a***}	1.86 ± 0.02^{a***}
100ppm	4.71 ± 0.02^{ab***}	6.52 ± 0.03^{ab***}	4.62 ± 0.03^{ab***}	1.72 ± 0.02^{ab***}
200ppm	3.33 ± 0.03^{abc***}	4.90 ± 0.04^{abc***}	3.31 ± 0.02^{abc***}	1.34 ± 0.02^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.001***
Values±SEM

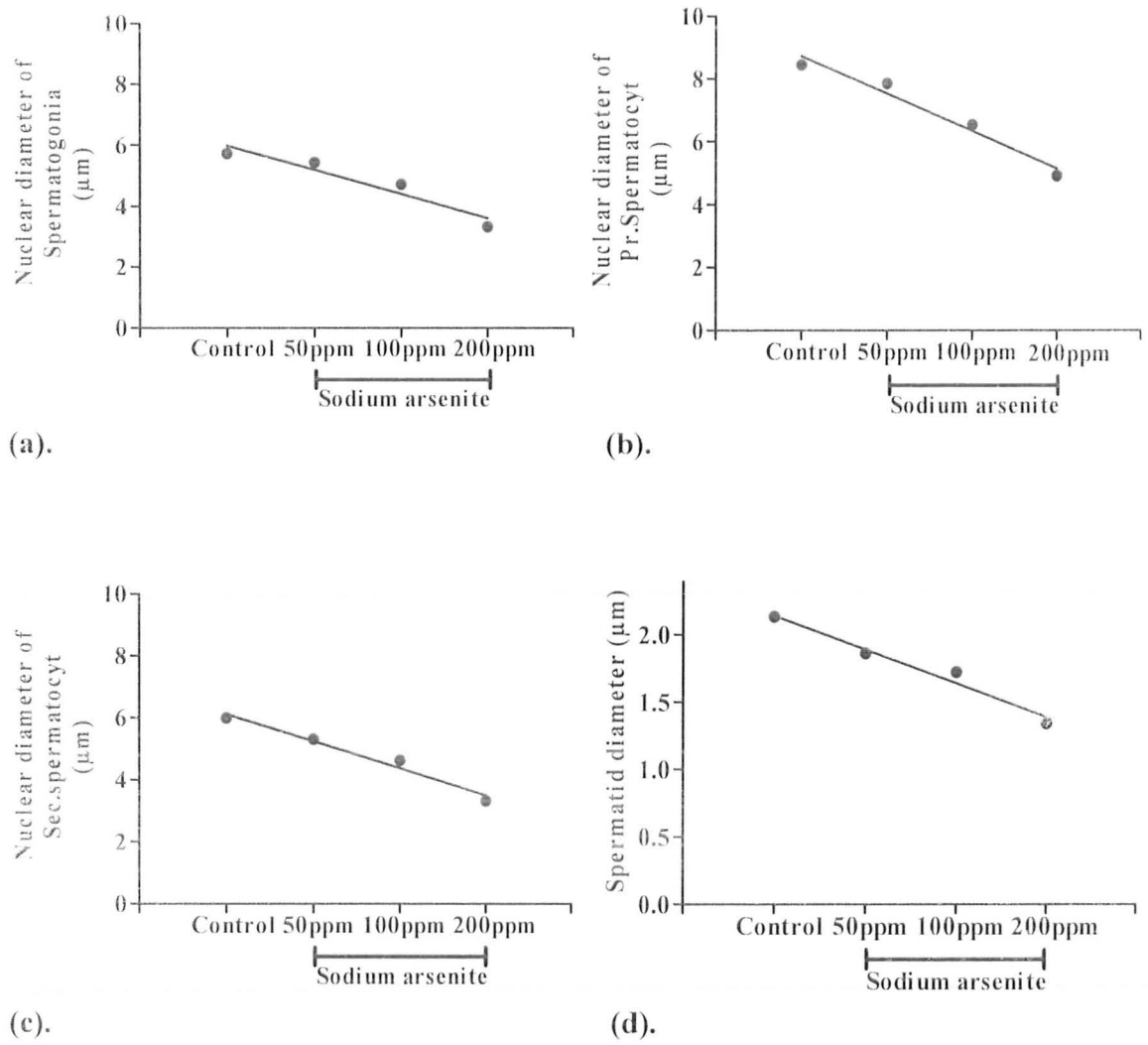


Fig. 9. Regression line showing significant decrease in mean nuclear diameter of (a) spermatogonia, (b) primary spermatocytes, (c) secondary spermatocyte and (d) spermatids in rats at 56 days age against increased doses of sodium arsenite.

Highly Significant ($P<0.001$) reduction in mean nuclear diameter of secondary spermatocytes of 50ppm, 100ppm and 200ppm treatment groups was noticed compared to controls. Comparison of all the three sodium arsenite treated (50ppm Vs 100ppm, 50ppm Vs 200ppm and 100ppm Vs 200ppm) groups also showed very highly significant ($P<0.001$) dose dependent decrease in mean nuclear secondary spermatocyte diameter. The regression analysis of variance showed (fig. 9c) significant decrease in mean secondary spermatocyte nuclear diameter of rats ($b=-0.87\pm0.11$; $F_{(1, 2)}=65.22$; $P=0.01$) against increased doses of sodium arsenite.

Mean spermatid diameter in 50ppm, 100ppm and 200ppm treatment groups decreased highly significantly ($P<0.001$) compared to controls. Rats treated with 100ppm showed very highly significant ($P<0.001$) decrease in mean spermatid diameter compared to 50ppm treatment group. Further highly significant ($P<0.001$) decrease in mean spermatid diameter was noticed in 200ppm treatment compared to 50ppm and 100ppm treatment groups. The regression analysis of variance showed (fig. 9d) significant decrease in mean spermatid diameter in 56 days old Sprague dawley rats ($b=-0.25\pm0.03$; $F_{(1, 2)}=63.83$; $P=0.01$) against increased doses of sodium arsenite.

EPIDIDYMES:

Mean epididymal caput, corpus and cauda tubule diameter and its epithelial height of controls and treatment (50ppm, 100ppm and 200ppm) groups is given in Tables. 5.

CAPUT:

The highest mean caput tubular diameter and its epithelial height was noticed in controls. There was highly significant ($P<0.001$) decrease in mean tubular diameter and its epithelial height in 50ppm, 100ppm and 200ppm treatment groups compared to controls. Comparison of the three treatment groups also showed dose dependent significant ($P<0.001$) decrease in mean tubular diameter and its epithelial height. The regression analysis of variance showed significant dose dependent decrease in mean epididymal caput (fig. 10a) tubular diameter ($b=-15.92\pm1.417$; $F_{(1, 2)}=126.4$; $P=0.007$) and its

Table. 5. Effect of various doses of sodium arsenite for 28 days duration on tubular diameter and epithelial height of epididymal caput, corpus and cauda in growing age (28-56 days) male rats.

Groups	Tubular diameter of epididymal (μm)			Epithelial height (μm)		
	Caput	Corpus	Cauda	Caput	Corpus	Cauda
Control	191.79 ± 0.46	237.36 ± 0.51	285.87 ± 0.66	31.49 ± 0.12	25.11 ± 0.16	23.86 ± 0.17
50ppm	172.71 ± 0.51^{a***}	176.95 ± 0.55^{a***}	242.67 ± 0.43^{a***}	26.30 ± 0.12^{a***}	21.00 ± 0.16^{a***}	21.56 ± 0.17^{a***}
100ppm	155.08 ± 0.73^{ab***}	154.01 ± 0.46^{ab***}	221.62 ± 0.49^{ab***}	22.82 ± 0.09^{ab***}	19.70 ± 0.14^{ab***}	17.60 ± 0.19^{ab***}
200ppm	144.59 ± 0.61^{abc***}	135.37 ± 0.38^{abc***}	172.78 ± 0.68^{abc***}	15.82 ± 0.12^{abc***}	18.30 ± 0.1^{abc***}	15.01 ± 0.11^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm,
c= 100ppmVS 200ppm
P<0.001***
Values±SEM

epithelial height (fig. 10b) height (b=-5.05±0.47; $F_{(1, 2)}$=116.6; P=0.008) with the increase in the dose of sodium arsenite.

CORPUS:

There was highly significant (P<0.001) decrease in mean corpus tubular diameter and its epithelial height in 50ppm, 100ppm and 200ppm sodium arsenite treatment groups compared to controls. Rats treated with 100ppm sodium arsenite showed very highly significant (P<0.001) decrease in mean corpus tubular diameter and its epithelial height compared to 50ppm group. The highest decrease in mean corpus tubular diameter and epithelial height was noticed in 200ppm treatment group compared to the other two treatment (50ppm and 100ppm) groups. The regression analysis of variance showed significant decrease in mean corpus (fig. 10c) tubular diameter (b=-32.89±7.009; $F_{(1, 2)}$ 22.02; P=0.042) and its epithelial height (fig. 10d) height (b=-2.17±0.48; $F_{(1, 2)}$=20.90; P 0.04) against increased doses of sodium arsenite.

CAUDA:

The highest mean tubular diameter and epithelial height was seen in controls. Very highly significant (P<0.001) reduction in mean cauda tubular diameter and its epithelial height was noticed in 50ppm, 100ppm and 200ppm treatment groups compared to control group. Medium (100ppm) dose treatment caused very highly significant (P<0.001) decrease in mean tubular diameter and epithelial height of cauda compared to 50ppm sodium arsenite treatment. High dose (200ppm) treatment exhibited the highest reduction (P<0.001) in mean tubular diameter and epithelial height of cauda compared to 50ppm and 100ppm sodium arsenite treatment groups. The regression analysis of variance showed significant decrease in mean tubular diameter (fig. 10e) and epithelial height (fig. 10f) of cauda (b=-36.03±3.642; $F_{(1, 2)}$=97.87; P=0.01) (b=-3.05±0.219; $F_{(1, 2)}$=193.9; P=0.005) respectively with increase in the dose of sodium arsenite.

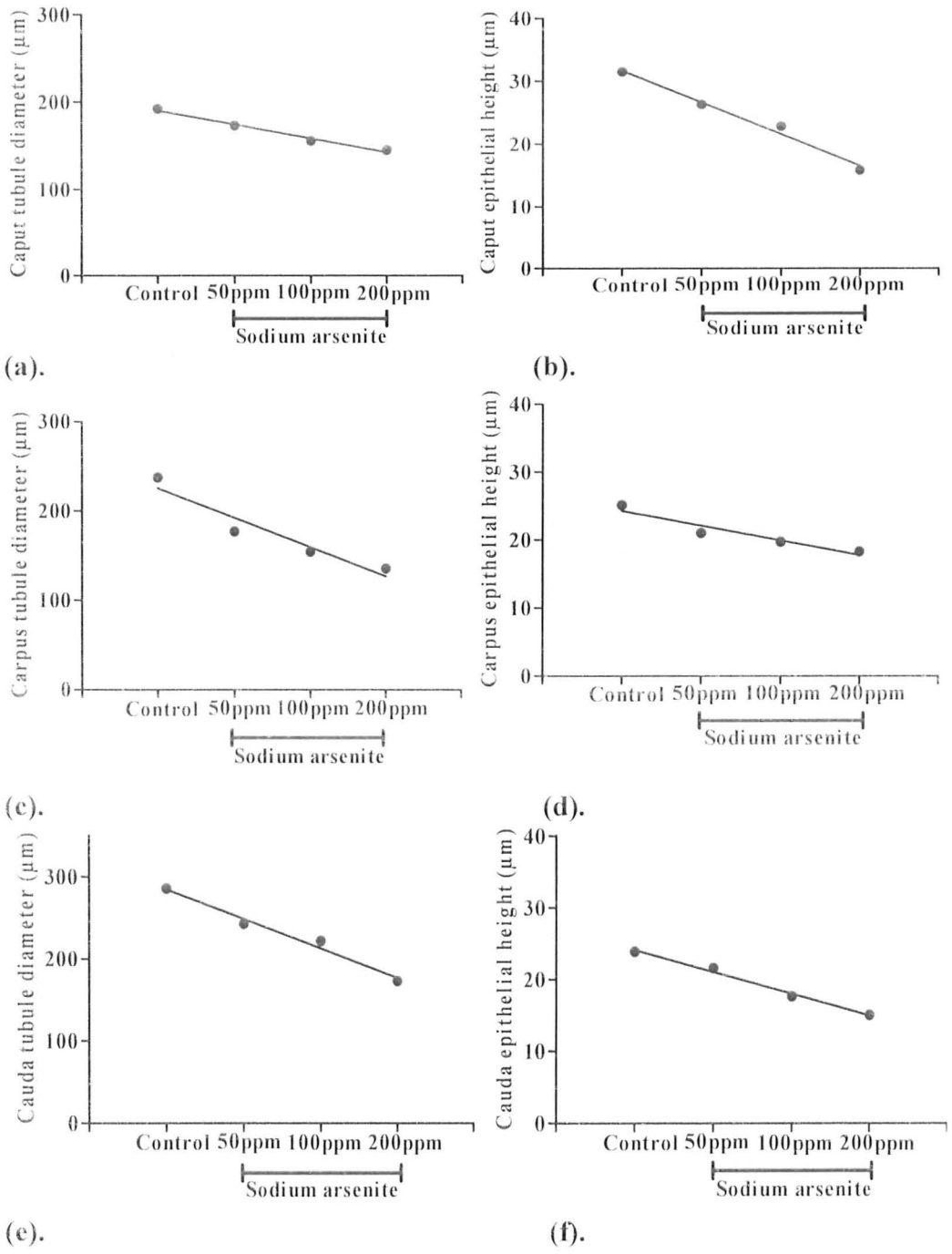


Fig. 10. Regression line showing significant decrease in mean tubular diameter of epididymal (a) caput, (c) carpus (e) cauda and their mean epithelial height (b, d, f respectively) in rats at 56 days age against increased doses of sodium arsenite.

VAS DEFERENS:

Mean diameter of vas deferens, its muscular thickness and epithelial height in controls and treated (50ppm, 100ppm and 200ppm) groups is given in Table. 6.

Mean vas deferens diameter, its muscular thickness and epithelial height decreased highly significantly ($P<0.001$) in 50ppm, 100ppm and 200ppm sodium arsenite treated groups compared to controls. Significant ($P,<0.001$) decrease in mean vas deferens diameter, its muscular thickness and epithelial height was noticed in comparisons 50ppm Vs 100ppm, 50ppm Vs 200ppm and 100ppm Vs 200ppm treatment group.

The regression analysis showed non-significant dose dependent decrease in mean vas deferens diameter (fig. 11a), its muscular thickness (fig. 11b) and epithelial height (fig. 11c) in treated rats ($b=-250.4\pm60.85$; $F_{(1, 2)}=16.94$; $P=0.05$), ($b=-107.6\pm33.4$; $F_{(1, 2)}=10.35$; $P=0.08$) and ($b=-4.18\pm1.085$; $F_{(1, 2)}=14.87$; $P=0.06$) respectively against increased doses of sodium arsenite.

HORMONE ANALYSIS:

Mean plasma testosterone, FSH and LH concentration of control and treated (50ppm, 100ppm and 200ppm) groups is given in Table. 7.

TESTOSTERONE:

Rats treated with 50ppm, 100ppm and 200ppm sodium arsenite showed very highly significant ($P<0.001$) decrease in mean plasma testosterone concentration compared to controls. Medium (100ppm) dose treatment caused non-significant ($P>0.05$) difference in mean plasma testosterone concentration of rats compared to 50ppm treatment group. Rats treated with 200ppm sodium arsenite showed significant ($P<0.05$) decrease in plasma testosterone concentration compared to rats treated with 50ppm sodium arsenite. High dose (200ppm) treatment decreased plasma testosterone concentration non-significantly ($P>0.05$) compared to 100ppm treatment group. The regression analysis showed (fig. 12a) significant decrease in plasma testosterone concentration with increase in dose of sodium arsenite ($b=-107.0\pm18.39$; $F_{(1, 2)}=33.86$; $P=0.03$).

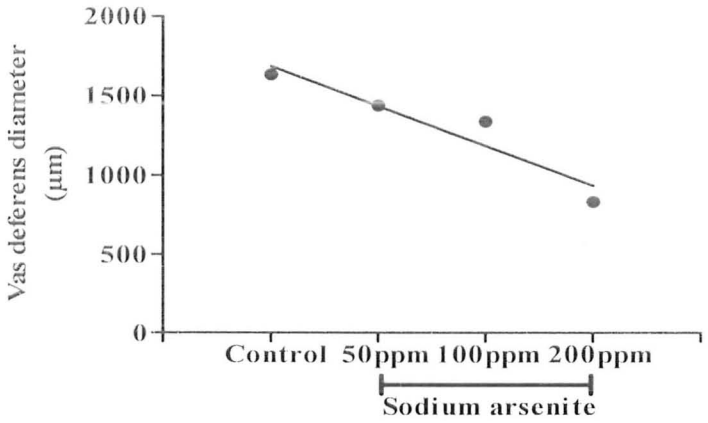
Table. 6. Effect of sodium arsenite on vas deferens diameter, muscular thickness and epithelial height of rat at 56 days of age.

Groups	VAS DEFERENS (µm)		
	Diameter (whole)	Muscular thickness	Epithelial height
Control	1634.00 ± 1.50	532.69 ± 0.82	46.37 ± 0.27
50ppm	1439.38 ± 1.23^{a***}	455.18 ± 0.78^{a***}	44.03 ± 0.27^{a***}
100ppm	1339.61 ± 1.20^{ab***}	430.89 ± 0.48^{ab***}	41.76 ± 0.28^{ab***}
200ppm	832.50 ± 1.05^{abc***}	182.19 ± 0.42^{abc***}	33.18 ± 0.33^{abc***}

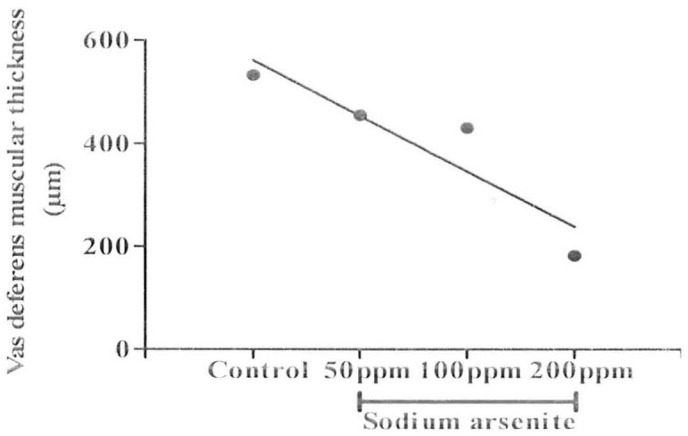
a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm

P<0.001***

Values±SEM



(a).



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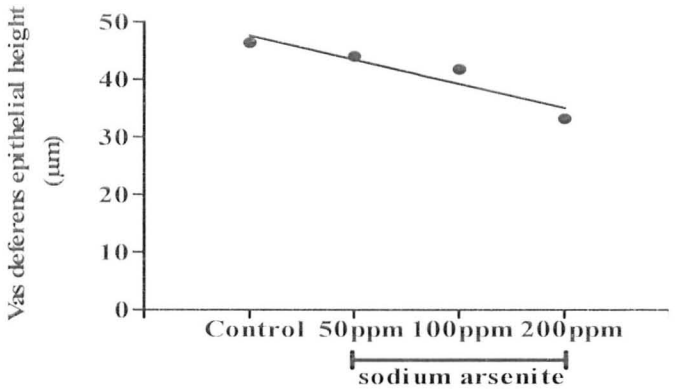


(c).

Fig. 11. Regression line showing a significant decrease in mean (a) vas deferens diameter, (b) its muscular thickness and (c) epithelial height in rats at 56 days age against increased doses of sodium arsenite.

FOLLICLE STIMULATING HORMONE (FSH):

Sodium arsenite treatment (50ppm, 100ppm and 200ppm) caused very highly significant ($P<0.001$) decrease in mean plasma FSH concentration compared to the control and this reduction was highest in 200ppm group. Rats treated with 100ppm sodium arsenite showed reduction in mean plasma FSH concentration compared to 50ppm treatment group but this reduction was non-significant ($P>0.05$). High dose (200ppm) treatment caused very highly significant ($P<0.001$) decrease compared to 50ppm treatment group and highly significant ($P<0.01$) decrease in mean plasma FSH concentration of rats compared to 100ppm treatment group. The regression analysis showed (fig. 12b) dose dependent decrease in mean plasma FSH concentration ($b=-3.73\pm0.83$; $F_{(1, 2)}=20.26$; $P=0.04$) against increased doses of sodium arsenite.

LUTEINIZING HORMONE (LH):

Mean plasma LH concentration of rats treated with 50ppm and 100ppm sodium arsenite exhibited significant ($P<0.05$) reduction while 200ppm group showed very highly significant reduction ($P<0.001$) in mean plasma LH concentration compared to controls. Comparisons of treated (50ppm VS 100ppm, 50ppm VS 200ppm and 100ppm VS 200ppm) groups showed non-significant ($P>0.05$) difference in mean plasma LH concentration in rats. The regression analysis of variance showed (fig. 12c) significant decrease in plasma Luteinizing hormone concentration against increased doses of sodium arsenite ($b=-0.05\pm0.012$; $F_{(1, 2)}=18.77$; $P=0.04$).

DAILY SPERM PRODUCTION, ITS EFFICIENCY AND EPIDIDYMAL SPERM COUNT:

Daily sperm production, its efficiency and epididymal sperm count of control and treated (50ppm, 100ppm and 200ppm) groups is given in Table 8.

DAILY SPERM PRODUCTION:

Sodium arsenite treatment (50ppm, 100ppm and 200ppm) caused very highly significant ($P<0.001$) decrease in mean DSP of rats compared to controls. Both 100ppm and 200ppm treatment groups showed highly significant ($P<0.001$) decrease in mean DSP of rats

Table. 7. Effect of sodium arsenite on serum testosterone, follicle stimulating hormone (FSH) and Luteinizing hormone (LH) of rat at 56 days age.

Groups	Testosterone (ng/ml)	FSH (ng/ml)	LH (ng/ml)
Control	3.58 ± 0.31	15.58 ± 0.29	0.41 ± 0.11
50ppm	1.85 ± 0.32^{a***}	8.36 ± 0.57^{a***}	0.37 ± 0.01^{a*}
100ppm	1.32 ± 0.16^{a***}	6.69 ± 0.42^{a***}	0.34 ± 0.06^{a*}
200ppm	0.67 ± 0.12^{a***b*}	3.72 ± 0.45^{ab***c**}	0.24 ± 0.01^{a***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm,
c= 100ppmVS 200ppm
P<0.05*, P<0.01**, P<0.001***
Values±SEM

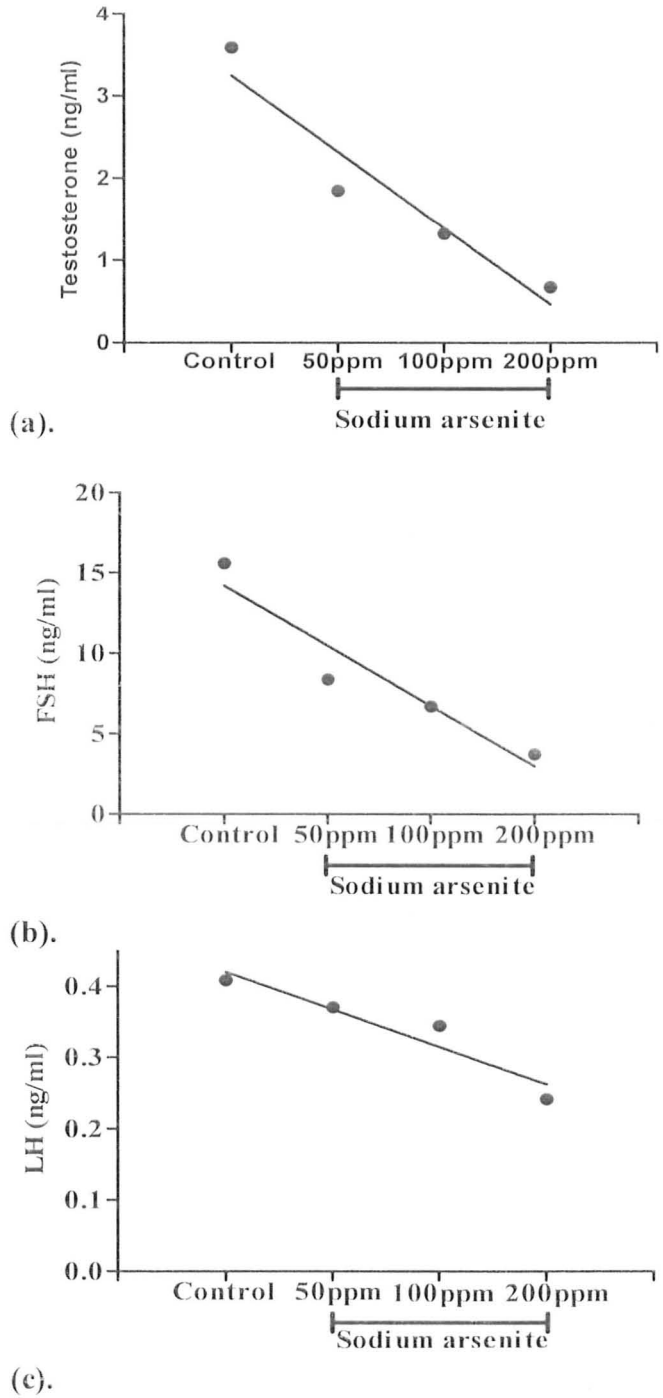


Fig. 12. Regression line showing a significant decrease in plasma (a) testosterone, (b) follicle stimulating hormone (FSH), (c) leutinizing hormone (LH) concentration in rats at 56 days age against increased doses of sodium arsenite.

compared to 50ppm treatment group. Mean DSP in 200ppm treatment group decreased non-significantly (P>0.05) compared to 100ppm treatment group. The regression analysis of variance showed (fig. 13a) dose dependent decrease in mean DSP of rats (b = -277800±25770; $F_{(1, 2)}$ =116.1; P=0.008) against increased doses of sodium arsenite.

EFFICIENCY OF DSP:

Low dose (50ppm) treatment caused reduction in efficiency of DSP compared to controls but this reduction was non-significant (P>0.05). Rats treated with 100ppm and 200ppm sodium arsenite showed very highly significant (P<0.001) decrease in efficiency of DSP compared to controls and 50ppm treatment group. The reduction in efficiency of DSP caused by 200ppm sodium arsenite treatment was non-significant (P>0.05) compared to 100ppm treatment. The regression analysis of variance showed (fig. 13b) significant decrease in DSP efficiency of the sodium arsenite treated rats (b=-1.92±0.27; $F_{(1, 2)}$ =48.99; P=0.01) against increased doses of sodium arsenite.

EPIDIDYMAL SPERM COUNT:

Mean epididymal sperm count in 50ppm treatment group decreased highly significantly (P<0.01), while in 100ppm and 200ppm treatment groups decreased very highly significantly (P<0.001) compared to controls. Rats treated with 100ppm sodium arsenite showed significant (P<0.05) decrease in mean epididymal sperm count compared to 50ppm treatment group. Mean epididymal sperm count in 200ppm treatment group decreased very highly significantly (P<0.001) compared to 50ppm and 100ppm treatment groups. The regression analysis of variance showed (fig. 13c) significant decrease in epididymal sperm count against increased doses of sodium arsenite (b=-4.94±0.601; $F_{(1, 2)}$ =67.56; P=0.01) in sodium arsenite treated rats.

ARSENIC DEPOSITION:

Mean deposition of arsenic in testes, epididymes, vas deferens and blood of control and sodium arsenite treated (50ppm, 100ppm and 200ppm) groups is given in Table 9.

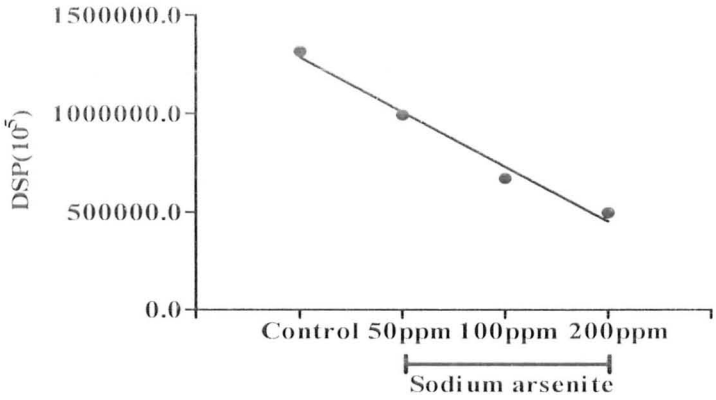
Table. 8. Effect of sodium arsenite on daily sperm production (DSP), its efficiency (DSP/g) and epididymal sperm count.

Groups	DSP of single testis	DSP/g Testis	Epididymal sperm count (10^6/ml)
Control	13.14 ± 0.47	12.03 ± 0.44	28.87 ± 1.01
50ppm	9.92 ± 0.51^{a***}	10.50 ± 0.57	24.87 ± 0.64^{a**}
100ppm	6.69 ± 0.47^{ab***}	7.51 ± 0.51^{ab***}	21.07 ± 0.90^{a***b*}
200ppm	4.96 ± 0.44^{ab***}	6.64 ± 0.56^{ab***}	13.67 ± 0.71^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppm VS 200ppm

P<0.05*, P<0.01**,P<0.001***

Values±SEM



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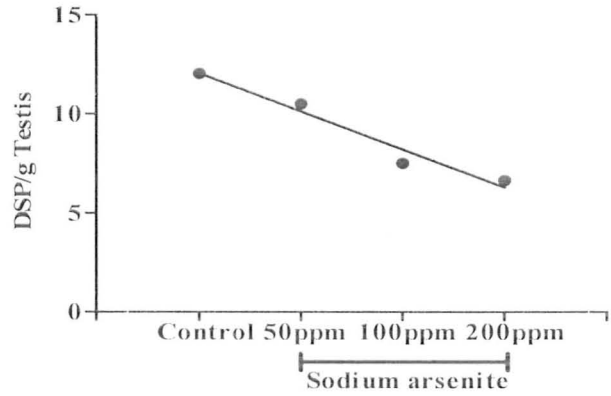

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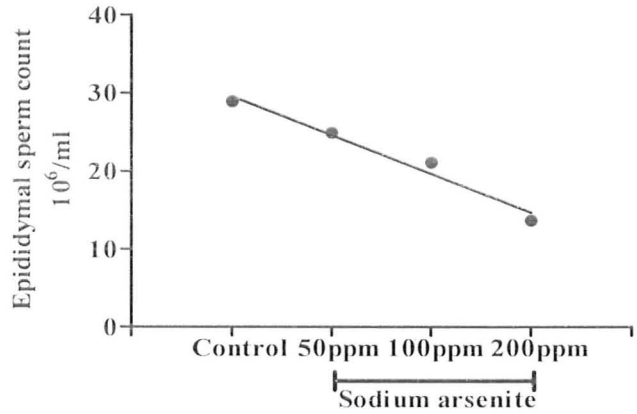


(c).

Fig. 13. Regression line showing a significant decrease in mean (a) testicular daily sperm production (DSP), (b) efficiency of DSP and (c) epididymal sperm count in rats at 56 days age against increased doses of sodium arsenite.

TESTES:

Highly significant increase mean testicular arsenic deposition was observed in 50ppm ($P<0.01$), 100ppm ($P<0.001$) and 200ppm ($P<0.01$) sodium arsenite treated groups compared to controls. Sodium arsenite treatment of 100ppm caused significant ($P<0.05$) increase in testicular arsenic deposition compared to 50ppm treatment group. High dose (200ppm) treated group exhibited highly significant ($P<0.001$) increase in mean testicular deposition of arsenic compared to 50ppm and 100ppm treatment groups. The regression analysis of variance showed (fig. 14a) dose dependent increase in mean testicular arsenic deposition ($b=0.009 \pm 0.001$; $F_{(1, 2)}=81.96$; $P=0.01$) against increased doses of sodium arsenite.

EPIDIDYMES:

Treatment of 50ppm sodium arsenite caused significant ($P<0.05$) increase while 100ppm and 200ppm caused very highly significant ($P<0.001$) increase in mean deposition of arsenic in epididymes compared to the control group. Treatment of 100ppm sodium arsenite caused non-significant ($P>0.05$) increase in mean epididymal arsenic deposition compared to 50ppm treatment group. High dose (200ppm) treatment caused very highly significant ($P<0.001$) increase in mean epididymal arsenic deposition compared to 50ppm and 100ppm sodium arsenite treatment groups. The regression analysis of variance showed (fig. 14b) significant increase in epididymal arsenic deposition in treated rats ($b=0.076 \pm 0.009$; $F_{(1, 2)}=66.27$; $P=0.01$) against increased doses of sodium arsenite.

VAS DEFERENS:

Rats treated with 50ppm sodium arsenite showed non-significant ($P<0.05$) increase, while 100ppm treatment group showed significant ($P<0.05$) and 200ppm treatment group showed very highly significant ($P<0.001$) increase in mean arsenic deposition in vas deferens compared to the control. The highest increase was observed in 200ppm treatment group. Rats treated with 100ppm sodium arsenite exhibited non-significant ($P>0.05$) increase in mean arsenic deposition in vas deferens compared to the rats treated with 50ppm sodium arsenite. Rats treated with 200ppm sodium arsenite showed very highly significant ($P<0.001$) increase in mean deposition of arsenic in vas deferens

Table. 9. Deposition of arsenic in testes, epididymes, vas deferens and blood of 56 days old Sprague dawley rats, measured by atomic absorption spectrophotometry.

Groups	Testes (µg/mg)	Epididymes (µg/mg)	Vas deferens (µg/mg)	Blood (µg/ml)
Control	0.0180 ± 0.0009	0.1308 ± 0.0127	0.4006 ± 0.0432	94.48 ± 2.577
50ppm	0.0255 ± 0.0012^{a**}	0.2089 ± 0.0133^{a*}	0.7559 ± 0.0330	145.58 ± 2.633^{a***}
100ppm	0.0319 ± 0.0013^{a***b*}	0.2536 ± 0.0146^{a***}	0.9825 ± 0.0541^{a*}	246.16 ± 4.531^{ab***}
200ppm	0.0433 ± 0.0016^{abc***}	0.3681 ± 0.0206^{abc***}	1.7101 ± 0.2675^{ab***c**}	289.20 ± 3.390^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.05*, P<0.01,P<0.001*****
Values±SEM

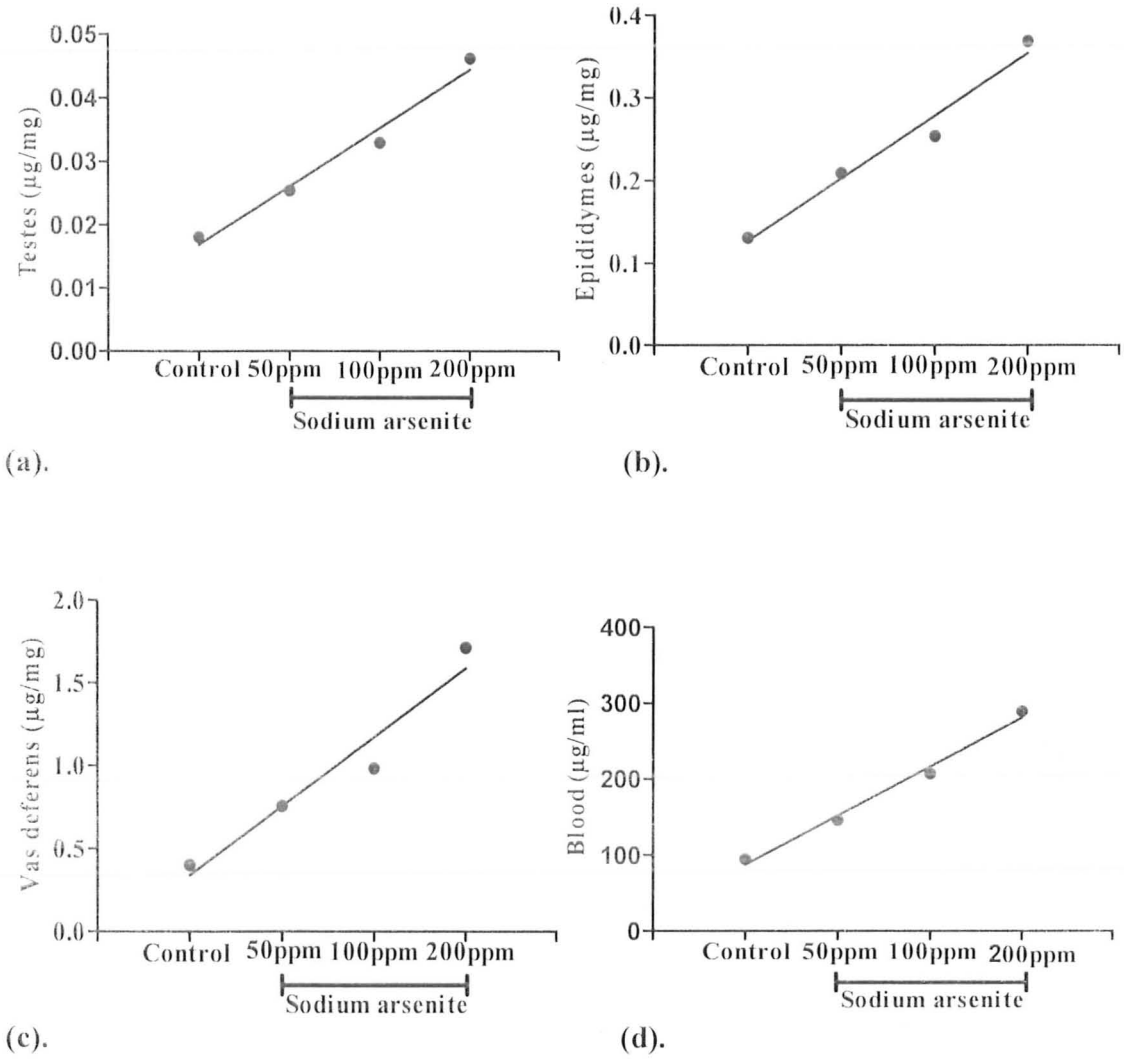


Fig. 14. Regression line showing significant increased arsenic deposition in (a) testes, (b) epididymes, (c) vas deferens and (d) blood in rats at 56 days age against increased doses of sodium arsenite (treatment for 28 days duration).

compared to 50ppm treatment group while this increase was highly significant ($P<0.01$) compared to 100ppm treatment group. The regression analysis of variance showed (fig. 14c) highly significant increase in mean deposition of arsenic in vas deferens against increased doses of sodium arsenite ($b=0.42 \pm 0.074$; $F_{(1, 2)}=31.69$; $P=0.03$).

BLOOD:

Rats treated with 50ppm, 100ppm and 200ppm sodium arsenite showed very highly significant ($P<0.001$) increase in mean blood arsenic deposition compared to the control. Comparisons of the treated groups (50ppm Vs 100ppm, 50ppm Vs 200ppm and 100ppm Vs 200pp) also showed very highly significant ($P<0.001$) increase in mean deposition of arsenic in blood. The regression analysis of variance showed (fig. 14d) significant dose dependent increase in mean deposition of arsenic in blood ($b=64.53 \pm 5.02$; $F_{(1, 2)}=165.4$; $P=0.006$) of treated rats against increased doses of sodium arsenite.

ESTIMATION OF EPIDIDYMAL SPERM DNA DAMAGE BY COMET ASSAY:

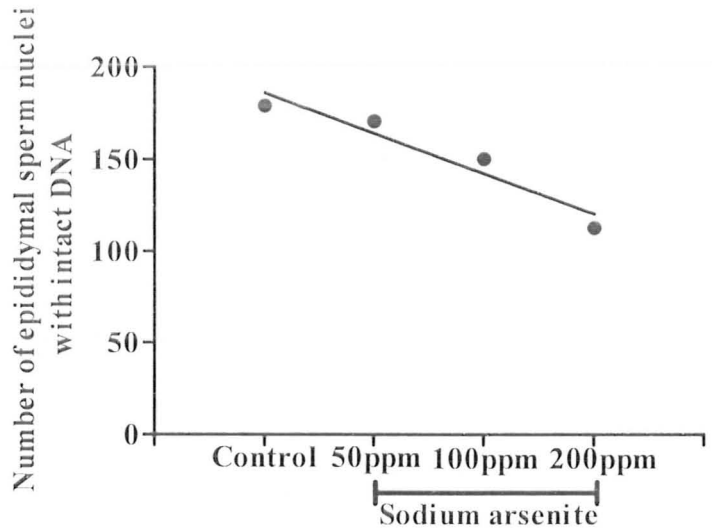
Sperm nuclei with intact DNA:

Mean number of epididymal sperm nuclei with intact DNA in control and treated (50ppm, 100ppm and 200ppm) groups is shown in Table 10. Sodium arsenite treatment (50ppm, 100ppm and 200ppm) caused very highly significant ($P<0.001$) decrease in mean number of epididymal sperm nuclei with intact DNA compared to the control, indicating damage to the sperm DNA with the increased doses of sodium arsenite. Treatment of 100pm and 200ppm sodium arsenite resulted in very highly significant ($P<0.001$) decrease in number of sperm nuclei with intact DNA compared to 50ppm treatment group. High dose (200ppm) treatment caused very highly significant ($P<0.001$) decrease in mean number of sperm nuclei with intact DNA compared to 100ppm treatment group. The regression analysis of variance showed (fig. 15a) significant decrease in mean number of the sperm nuclei with intact DNA in treated rats with the increase in dose of sodium arsenite ($b=-21.90 \pm 4.63$; $F_{(1, 2)}=22.37$; $P=0.04$).

Table. 10. Effect of sodium arsenite on number of epididymal sperm nuclei having intact DNA and comets in 56 days old Sprague dawley rats.

Groups	Epididymal sperm nuclei	
	Intact DNA number	Comet number
Control	179.0 ± 0.75	21.0 ± 0.75
50ppm	170.7 ± 0.98^{a***}	29.3 ± 0.98^{a***}
100ppm	150.3 ± 1.07^{ab***}	49.7 ± 1.07^{ab***}
200ppm	112.8 ± 1.62^{abc***}	87.2 ± 1.62^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.05*, P<0.01**,P<0.001***
Values±SEM



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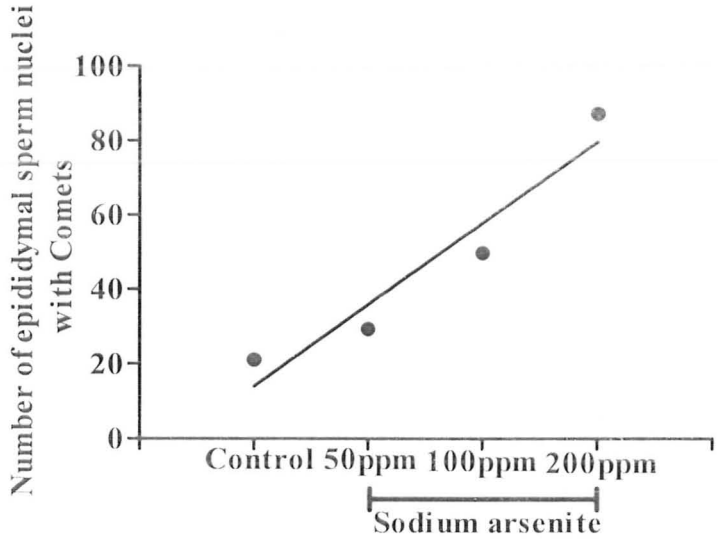


(b).

Fig. 15. Regression line showing a significant (a) decrease in epididymal sperm nuclei with intact DNA and (b) increase in comet number in rat at 56 days age against increased doses of sodium arsenite.

Comet number:

Mean number of comets of control and treated (50ppm, 100ppm and 200ppm) groups is shown in Table 10. Mean comet number increased very highly significantly ($P<0.001$) in 50ppm, 100ppm and 200ppm treatment groups compared to controls. The comparisons of various treatment groups (50ppm VS 100ppm, 50ppm VS 200ppm and 100ppm VS 200ppm) showed very highly significant (0.001) increase in mean comet number. The highest increase was shown by 200ppm treatment group. Regression analysis of variance showed (fig. 15b) significant dose dependent increase in comet number ($b=21.90 \pm 4.63$; $F_{(1, 2)}=22.37$; $P=0.04$) with the increase in the dose of sodium arsenite indicating highly damage to the sperm DNA.

Comet length:

Mean epididymal sperm comet length of control and treated (50ppm, 100ppm and 200ppm) groups is given in Table 11. Mean sperm comet length increased very highly significantly ($P<0.001$) in sodium arsenite treated (50ppm, 100ppm and 200ppm) groups compared to controls. There was no significant ($P>0.05$) difference in mean comet length in 100ppm treatment group compared to 50ppm treatment group. Very highly significant ($P<0.001$) increase in mean comet length was noticed in 200ppm treatment group compared to 50ppm and 100ppm ($P<0.01$) treatment groups. The regression analysis of variance showed (fig. 16a) non-significant increase in comet length against increased doses of sodium arsenite ($b=4.989\pm1.51$; $F_{(1,2)}=10.95$; $P=0.08$).

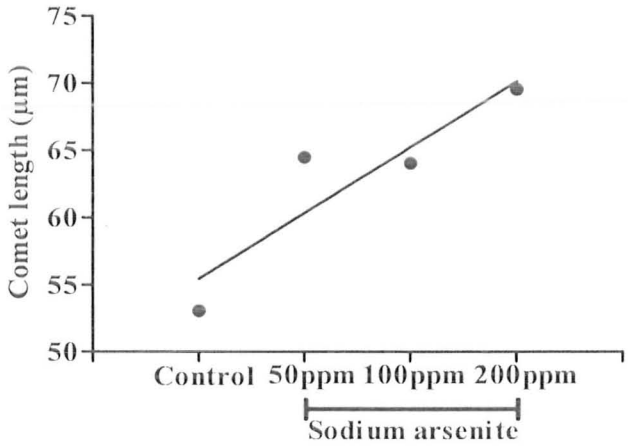
Comet height:

Mean sperm comet height of control and treated (50ppm, 100ppm and 200ppm) groups is shown in Table 11. Mean sperm comet height of sodium arsenite treated (50ppm, 100ppm and 200ppm) groups increased very highly significantly ($P<0.001$) compared to controls. Mean comet height in100ppm treatment group increased significantly ($P<0.05$) compared to 50ppm treatment group. High dose (200ppm) treatment caused very highly significant ($P>0.001$) increase in mean comet height compared to low dose (50ppm) treatment but non-significant ($P>0.05$) increase compared to 100ppm treatment group. The regression analysis of variance showed (fig. 16b) significant increase in epididymal

Table. 11. Effect of sodium arsenite on comet length, height and head diameter of rat epididymal sperm measured by comet assay at 56 days age.

Groups	Comet Length (µm)	Comet Height (µm)	Head Diameter (µm)
control	53.08 ± 0.73	49.42 ± 0.58	49.42 ± 0.58
50ppm	64.46 ± 0.95^{a***}	54.22 ± 0.74^{a***}	54.22 ± 0.74^{a***}
100ppm	64.04 ± 0.96^{a***}	57.01 ± 0.87^{a***b*}	57.01 ± 0.87^{a***b*}
200ppm	69.53 ± 0.99^{abc***}	59.67 ± 0.69^{ab***}	59.67 ± 0.69^{ab***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm,
c= 100ppmVS 200ppm
P<0.05*, P<0.001***
Values±SEM



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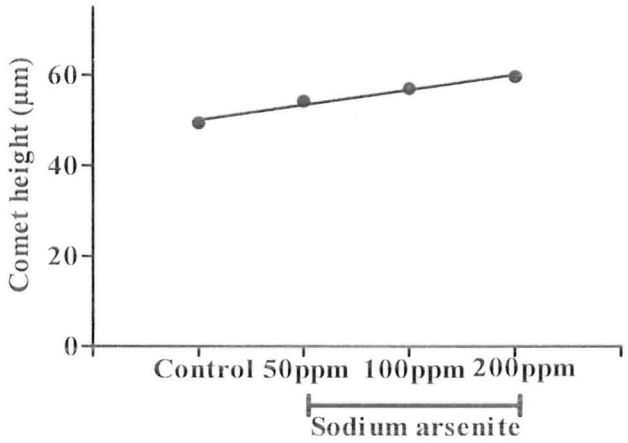

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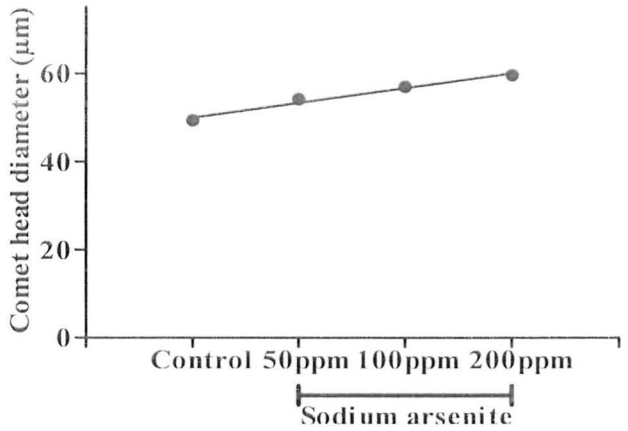


(c)

Fig. 16. Regression line showing (a) non-significant increase in mean comet length, (b) significant increase in mean comet height, (c) and head diameter in rat epididymal sperm at 56 days age against increased doses of sodium arsenite.

sperm comet height with the increase in the dose of sodium arsenite (b=3.35±0.36; $F_{(1,2)}$=85.12; P=0.01).

Comet head diameter:

Mean sperm comet head diameter of control and treated (50ppm, 100ppm and 200ppm) groups is shown in Table 11. All the three (50ppm, 10ppm and 200ppm) treatments caused very highly significant ($P<0.001$) increase in mean sperm comet head diameter compared to controls. Rats treated with 100ppm sodium arsenite exhibited significant ($P<0.05$) increase in mean sperm comet head diameter compared to 50ppm treatment group. Rats treated with 200ppm sodium arsenite showed very highly significant ($P<0.001$) increase in mean sperm comet head diameter compared to 50ppm treatment group but this increase was non-significant ($P>0.05$) compared to 100ppm treatment group. Regression analysis of variance showed (fig. 16c) significant increase in comet head diameter of epididymal sperm against increased doses of sodium arsenite (b=3.35±0.36; $F_{(1,2)}$=85.12; P=0.01).

Comet head DNA percentage:

Mean epididymal sperm comet head percentage in control and treated (50ppm, 100ppm and 200ppm) groups is given in Table 12. Sodium arsenite (50ppm, 100ppm and 200pp) treatment caused significant ($P<0.01$) decrease in mean sperm comet head DNA percentage compared to controls. No significant ($P>0.05$) difference in mean comet head DNA percentage was noticed in 100ppm treatment group compared to 50ppm treatment group. A highly significant ($P<0.001$) decrease in mean comet head DNA percentage was noticed in 200ppm treatment group compared to 50ppm and 100ppm treatment groups. Regression analysis of variance showed (fig. 17a) non-significant dose dependent decrease in mean comet head DNA percentage in sodium arsenite treated rats (b=-0.48±0.115; $F_{(1,2)}$=17.62; P=0.05).

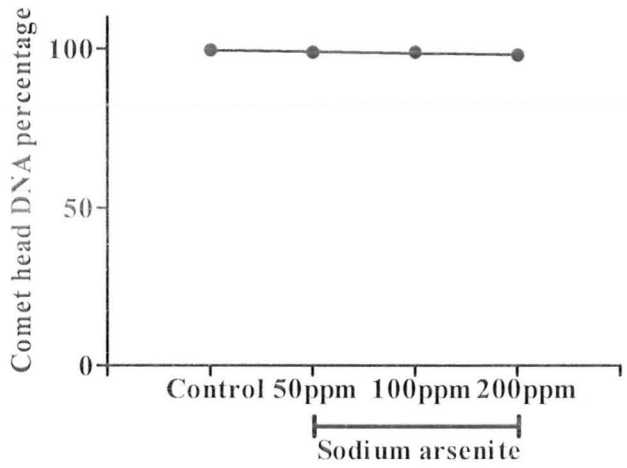
Comet tail length:

Mean epididymal sperm comet tail length of control and treated rats is given in Table 12. Mean comet tail length increased non-significantly ($P>0.05$) in 50ppm sodium arsenite

Table. 12. Effect of sodium arsenite on comet head DNA percentage, tail length and comet tail DNA percentage in 56 days old rat epididymal sperm.

Groups	%DNA in Head	Tail Length (μm)	%DNA in Tail
control	99.47 ± 0.04	6.38 ± 0.46	0.57 ± 0.05
50ppm	98.76 ± 0.15^{a**}	8.27 ± 0.55	0.78 ± 0.05
100ppm	98.75 ± 0.11^{a**}	10.40 ± 0.70^{a***}	1.25 ± 0.11^{a***b*}
200ppm	97.86 ± 0.18^{abc***}	12.35 ± 0.80^{ab***}	1.91 ± 0.18^{abc***}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm,
c= 100ppmVS 200ppm
P<0.05*, P<0.01**,P<0.001***
Values±SEM



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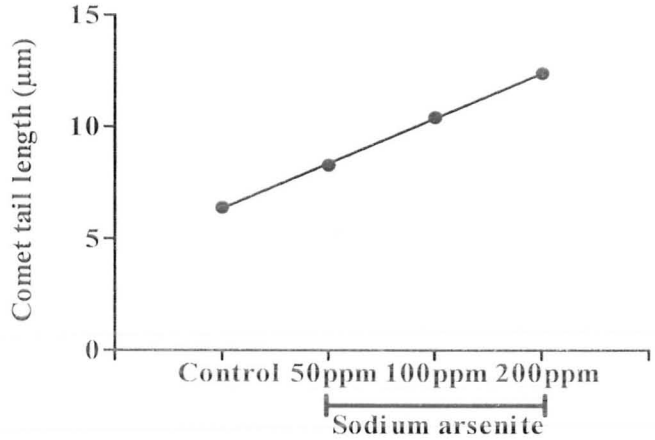

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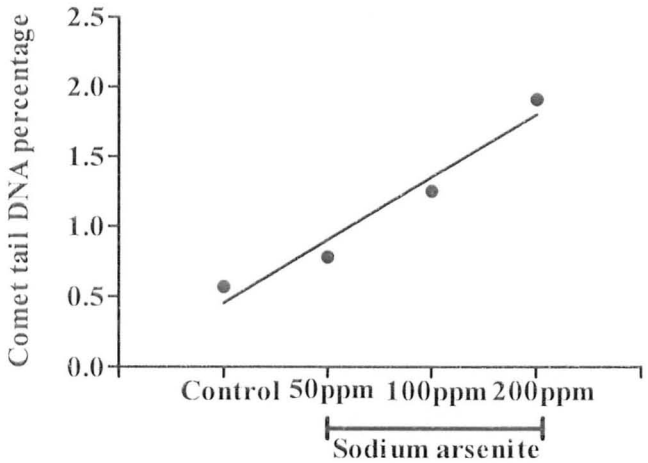


(c)

Fig. 17. Regression line showing (a) non-significant decrease in comet head DNA percentage, (b) significant increase in mean comet tail length and (c) comet tail DNA percentage in rat epididymal sperm at 56 days age against increased doses of sodium arsenite.

treated rats and highly significantly ($P<0.001$) in 100ppm and 200ppm treatment groups compared to controls. There was no significant ($P>0.05$) increase in mean comet length in 100ppm treatment group compared to 50ppm treatment group. Mean comet tail length in 200ppm sodium arsenite treated rats increased highly significantly ($P<0.001$) compared to 50ppm treatment group and non-significantly ($P>0.05$) compared to 100ppm treatment group. The regression analysis of variance showed (fig. 17b) highly significant increase in comet tail length with the increase of the dose of sodium arsenite ($b=2.004\pm0.03$; $F_{(1,2)}=4132$; $P=0.0002$).

Comet tail DNA percentage:

Mean epididymal sperm comet tail DNA percentage in the control and treated (50ppm, 100ppm and 200ppm) groups is given in Table 12. A non-significant ($P>0.05$) increase in mean comet tail DNA percentage in 50ppm treatment group was noticed compared to controls, while in other two treatment groups (100ppm and 200ppm) mean comet tail DNA percentage increased highly significantly ($P<0.001$) compared to controls. Significant ($P>0.05$) increase in mean comet tail DNA percentage was observed in 100ppm treatment group compared to 50ppm treatment group. Mean comet tail DNA percentage increased highly significantly ($P<0.001$) in 200ppm treatment group compared to 50ppm and 100ppm treatment groups. The regression analysis of variance showed (fig. 17c) a significant increase in mean comet tail DNA percentage against increased doses of sodium arsenite ($b=0.449\pm0.07$; $F_{(1,2)}=39.63$; $P=0.02$).

Comet tail moment:

Mean epididymal sperm comet tail moment of control and sodium arsenite treated (50ppm, 100ppm and 200ppm) groups is given in Table 13. Mean comet tail moment in 100ppm sodium arsenite treated group increased significantly ($P<0.05$), 200ppm treatment group increased highly significantly ($P<0.001$) and non-significantly ($P>0.05$) in 50ppm treatment group compared to controls. Mean comet tail moment increased non-significantly ($P>0.05$) in 100ppm treatment group compared to 50ppm treatment group. Highly significant ($P<0.001$) increase in mean comet tail moment was noticed in 200ppm treatment group compared to 50ppm and 100ppm treatment groups. The regression

Table. 13. Effect of sodium arsenite on comet tail and Olive moment in epididymal sperm of 56 days old rats measured by comet assay.

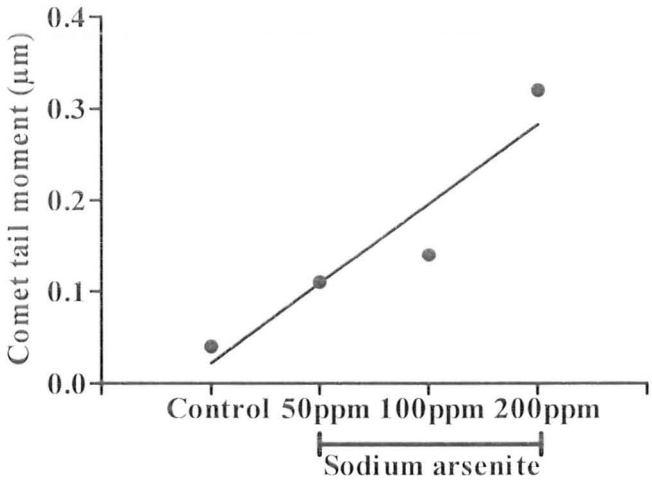
Groups	Tail Moment	Olive Moment
control	0.04 ± 0.01	0.09 ± 0.01
50ppm	0.11 ± 0.01	0.13 ± 0.01
100ppm	0.14 ± 0.02^{a*}	0.22 ± 0.02^{a***b*}
200ppm	0.32 ± 0.04^{abc***}	0.34 ± 0.04^{ab***c**}

a= Control VS treated groups, b= 50pp VS 100ppm and 200ppm, c= 100ppmVS 200ppm
P<0.05*, P<0.01,P<0.001*****
Values±SEM

analysis of variance showed (fig. 18a) non-significant increase in mean comet tail moment with the increase of the dose of sodium arsenite ($b=0.087\pm0.02$; $F_{(1,2)}=15.67$; $P=0.05$).

Olive moment:

Mean comet Olive moment of control and treated (50ppm, 100pm and 200ppm) groups is given in Table 13. Mean epididymal sperm comet Olive moment increased non-significantly ($P>0.05$) in 50ppm treatment group but highly significantly ($P<0.001$) in 100pp and 200ppm groups compared to controls. Mean Olive moment in 100ppm treatment group increased significantly ($P<0.05$) compared to 50ppm treatment group. The highest increase in mean comet Olive moment was noticed in 200ppm treatment group compared to 50ppm ($P<0.001$) and 100ppm ($P<0.01$) treatment groups. The regression analysis of variance showed (fig. 18b) significant dose dependent increase in mean comet Olive moment ($b=0.084\pm0.013$; $F_{(1,2)}=43.56$; $P=0.02$).



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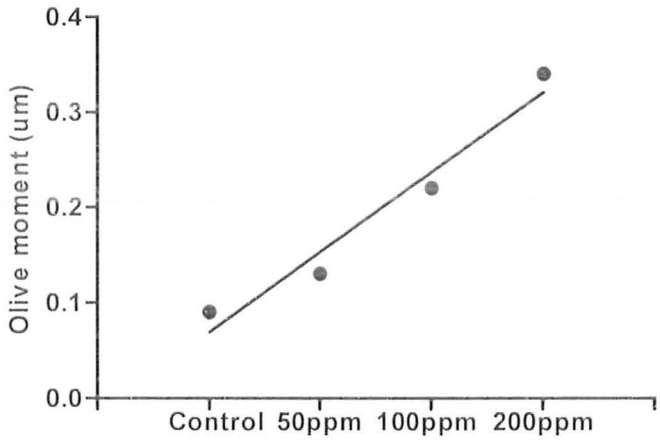

(b)

Fig. 18. Regression line showing (a) non significant increase in comet tail moment and (b)significant increase in comet Olive moment in rat epididymal sperm at 56 days of age against increased doses of sodium arsenite.

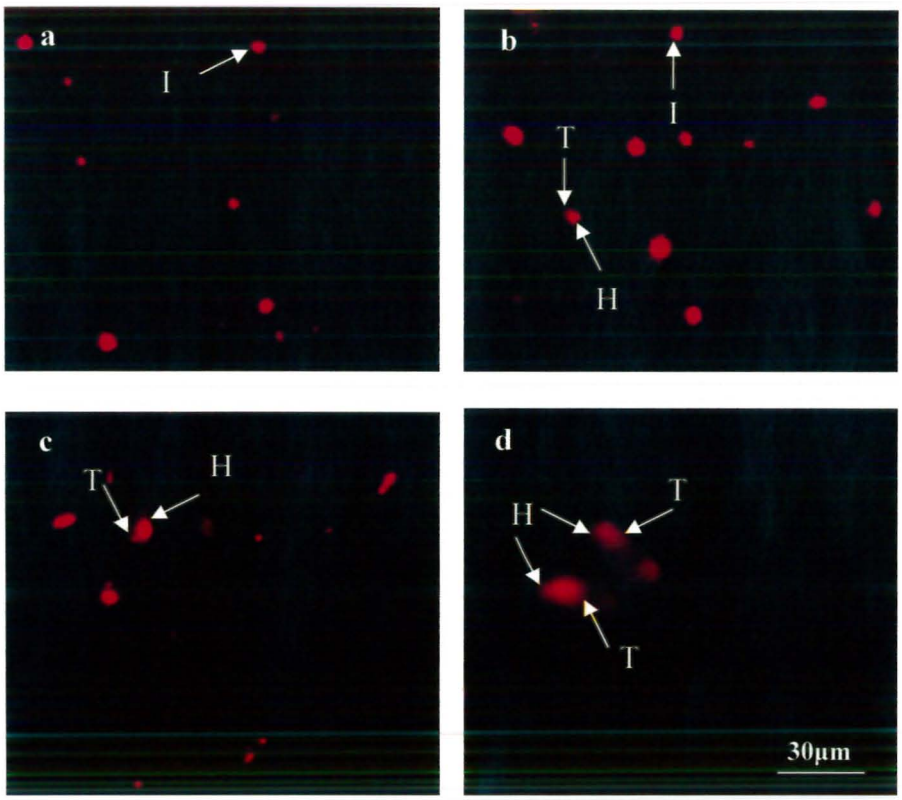

Fig. 19. Florescence photograph (X400) of epididymal sperm nuclei showing (a) control having most of the epididymal sperms with intact DNA (b) 50ppm sodium arsenite treated sperms with intact DNA and also comets with moderate DNA damage, (c) 100ppm sodium arsenite treated sperms with some intact DNA and more comets with increased DNA damage and (d) 200ppm sodium arsenite treated sperms showing the highest number of comets with fragmented DNA. I= Intact DNA, H= Comet head, T= Comet tail.

HISTOMORPHOLOGY

Testes:

The testis was covered by testicular capsule consisting of three layers (fig. 23a). In the testicular parenchyma several round, elongated and oval seminiferous tubules were scattered. Tubules were compactly arranged in the peripheral region compared to the central portion of testis. The interstitial spaces in between the seminiferous tubules contained blood vessels, connective tissues and Leydig cells. The Leydig cells were round or oval in shape. Each cell had prominent central round nucleus (fig. 22a). Outside each seminiferous tubule a thin layer of peritubular cells was present. These cells were spindle shaped with elongated nuclei in centre. Sertoli cells extended from the basement membrane towards the lumen of seminiferous tubule. Prominent Sertoli cells nuclei were lying on the basement membrane. The nuclei were irregular mostly oval or elongated in controls (fig. 21e). Various spermatogenic cells were seen at different stages. The spermatogenic cells were compactly arranged in seminiferous tubule. Some tubules with packed lumen were also seen (fig. 20a). Round spermatids as well as mature spermatozoa were found in the lumen of seminiferous tubules (fig. 21a)

Sprague dawley rats treated with 50ppm sodium arsenite showed significant reduction in the size of Leydig cells, seminiferous tubules, seminiferous epithelilium, Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. The thickness of testicular capsule decreased and the cells were mostly oval shaped compared to controls (fig. 23b). The tubules were loosely arranged inside testicular parenchyma compared to the control (fig. 20b). Number of Leydig and Sertoli cells reduced compared to control. Leydig cells with both round and elongated nuclei were present in the testes of 50ppm sodium arsenite treated rats (fig. 22b). The nuclei of Sertoli cells were of variable shapes (fig. 21f). The spermatogenic cells were compactly arranged in seminiferous tubule like controls. All stages of spermatogenesis as in control were observed in the testis of 50ppm treated testes (fig. 21b). The interstitial spaces enlarged compared to the control.

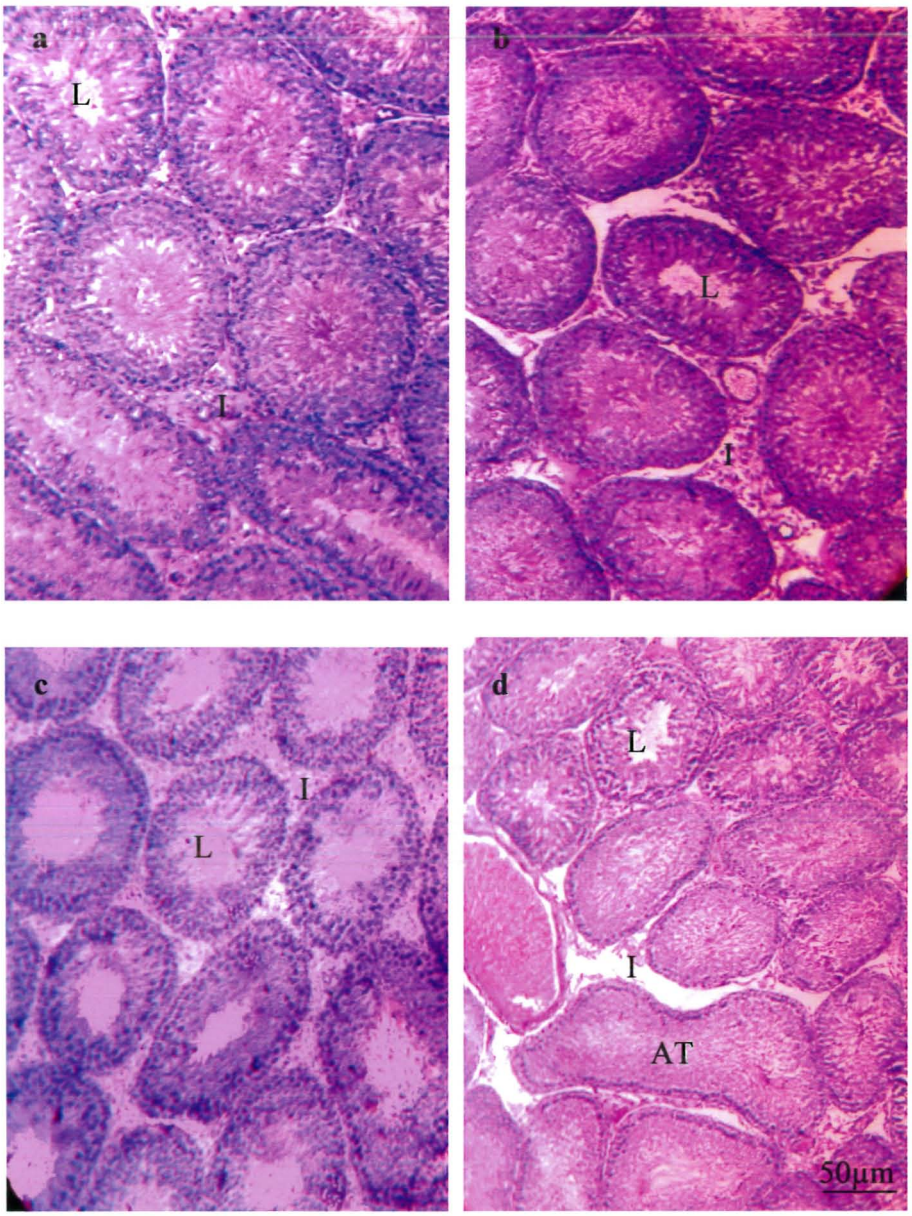

Fig. 20. Photomicrograph of seminiferous tubules in testis of control and sodium arsenite treated rats at 56 days age, (a) Control showing compact arrangement of tubules having lumen (L) packed with sperms (S), low interstitial (I) spaces are also observed. Reduction in tubular diameter and sperms is observed in a dose dependent manner. (b) 50ppm showing some loosening in tubular arrangement with extended lumen and interstitial spaces, (c) 100ppm and (d) 200ppm further extension in lumen and interstitial spaces is observed which is more prominent in high dose group. Some atretic or regressed tubules (AT) are noticed in 200ppm group. H & E.

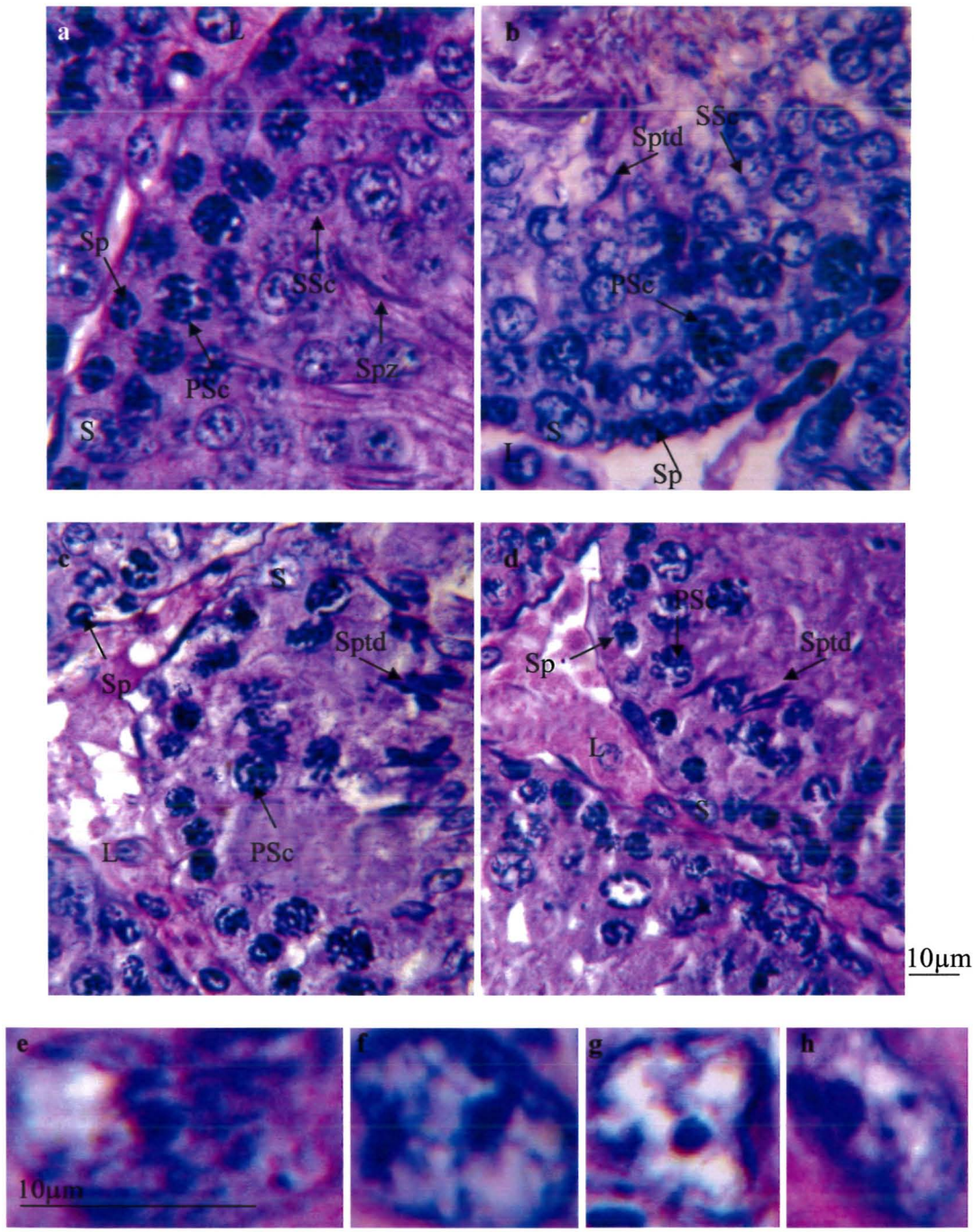

Fig. 21. Photomicrograph of seminiferous tubule section showing spermatogenesis in control and sodium arsenite treated rats. (a) Control showing Leydig cell (L), nuclei of Sertoli cell (S), spermatogonia (Sp), primary spermatocyte (PSc), secondary spermatocyte (SSc) and spermatozoa (Spz). The number and size of nuclei of Sertoli cell, and all spermatogenic cells decreased in a dose dependent manner. (b) 50ppm showing spermatogenic cells like control, (c) 100ppm showing X stage of spermatogenesis with fragmented primary spermatocyte, (d) 200ppm showing XII stage of spermatogenensis with fragmented and necrotic primary spermatocytes and very low number of elongated spermatids.(e, f, g, h) showing variable shapes of Sertoli cell nucleus in control, 50ppm, 100ppm and 200ppm respectively. H& E.

Sodium arsenite treatment of 100ppm also caused significant reduction in diameter of seminiferous tubule and its epithelial height. Significant decrease in nuclear diameter of Leydig cell, Sertoli cells, spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids, was found compared to controls and 50ppm group. Sodium arseninte of 100ppm caused decrease in thickness of testicular capsule (fig. 23c). The Leydig cells had mostly round nuclei (fig. 22c). The nuclei of Sertoli cells were of irregular shapes and shrinked compared to low dose and controls (fig. 21g). The number of Leydig and Sertoli cells decreased with the treatment of 100ppm sodium arsenite. The peritubular membrane became thin with reduced number of cells. The spermatogenic cells were loosely arranged compared to compactly arranged spermartogenic cells in 50ppm treatment group and controls. There were found IX, X and XI stages of spermatogenesis most abundantly. Fragmentation was observed in primary spermatocytes. The number of primary spermatocytes, round and elongated spermatids decreased in these stages (fig. 21c). Interstitial spaces also enlarged compared to controls and low dose treatment (fig. 20c).

Rats treated with 200ppm sodium arsenite showed highly significant reduction in nuclear diameter of Leydig cells, Sertoli cells, and various spermatogenic cells. Seminiferous tubule diameter and epithelial height of the tubule decreased significantly. High dose treatment caused the highest decrease in thickness of testicular capsule. The cells were small and mostly round compared to the elongated cells in the capsule of control testis (fig. 23d). The seminiferous tubules were of variable shapes and were more loosely arranged compared to the control and low dose treatments (fig. 20d). The number of Leydig and Sertoli cells showed the highest reduction compared to the other groups. The shape of Leydig cells were mostly elongated compared to the round and oval Leydig cells in controls and low dose treated groups (fig. 22d). The nuclei of Sertoli cells were also irregular (fig. 21h). The peritubular membrane around the seminiferous tubule was thin and at certain points detached from the germinal epithelium of seminiferous tubule. The cells of this layer became thin and decreased in number compared to the controls, 50ppm and 100ppm sodium arsenite. Spermatogenic cells were loosely arranged. The over all picture of spermatogenesis showed XII, XIII and XIV stages most abundantly. There was depletion of primary spermatocytes and elongated spermatids in these stages. The

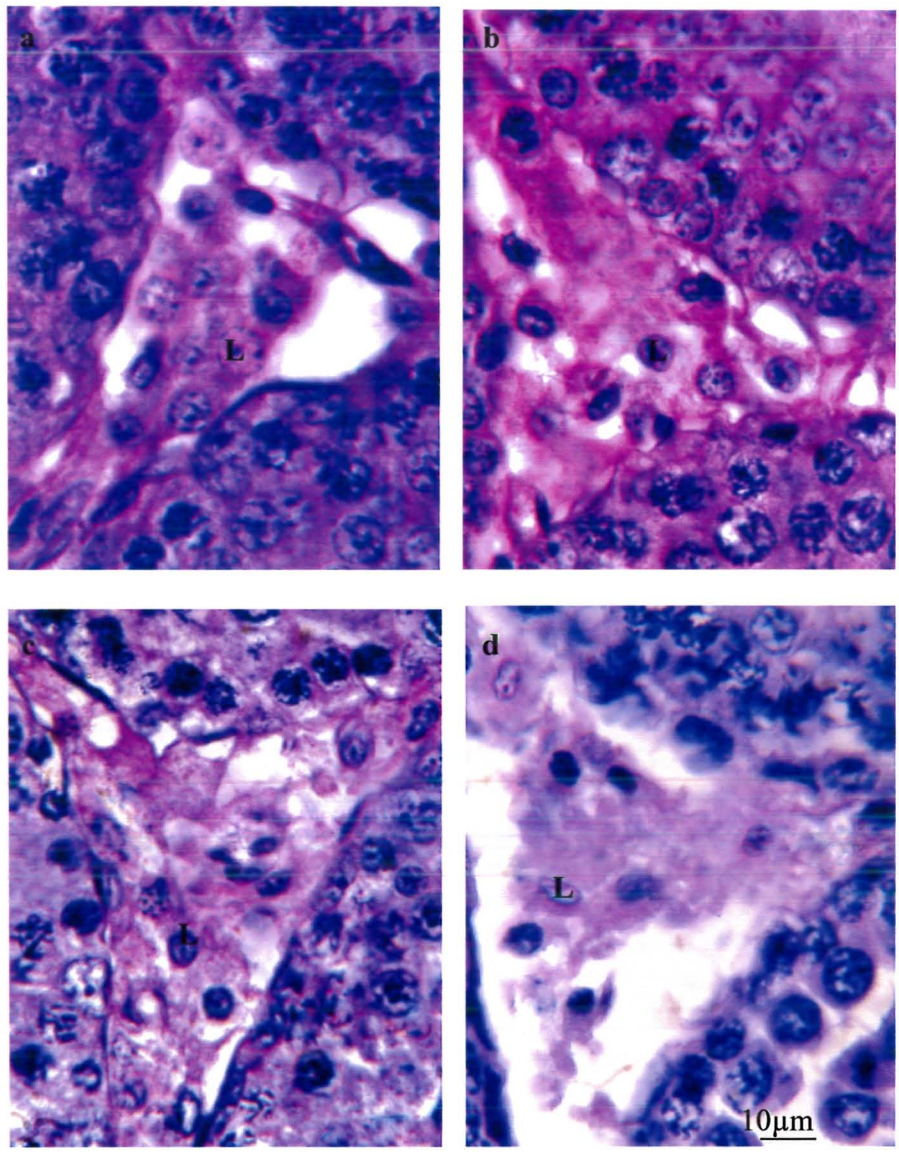

Fig. 22. Photomicrograph of testicular interstitial spaces showing Leydig cells in control and sodium arsenite treated rats at 56 days age. (a) Control showing large number of Leydig cells (L) with round nuclei in the interstitial spaces. The number and size of Leydig cell decrease in a dose dependent manner. (b) 50ppm showing some Leydig cells with round and some with elongated nuclei, (c) 100ppm showing Leydig cell with small and round nuclei, (d) 200ppm showing few Leydig cells with elongated nuclei only. Interstitial spaces are more extended in high dose groups. H & E.

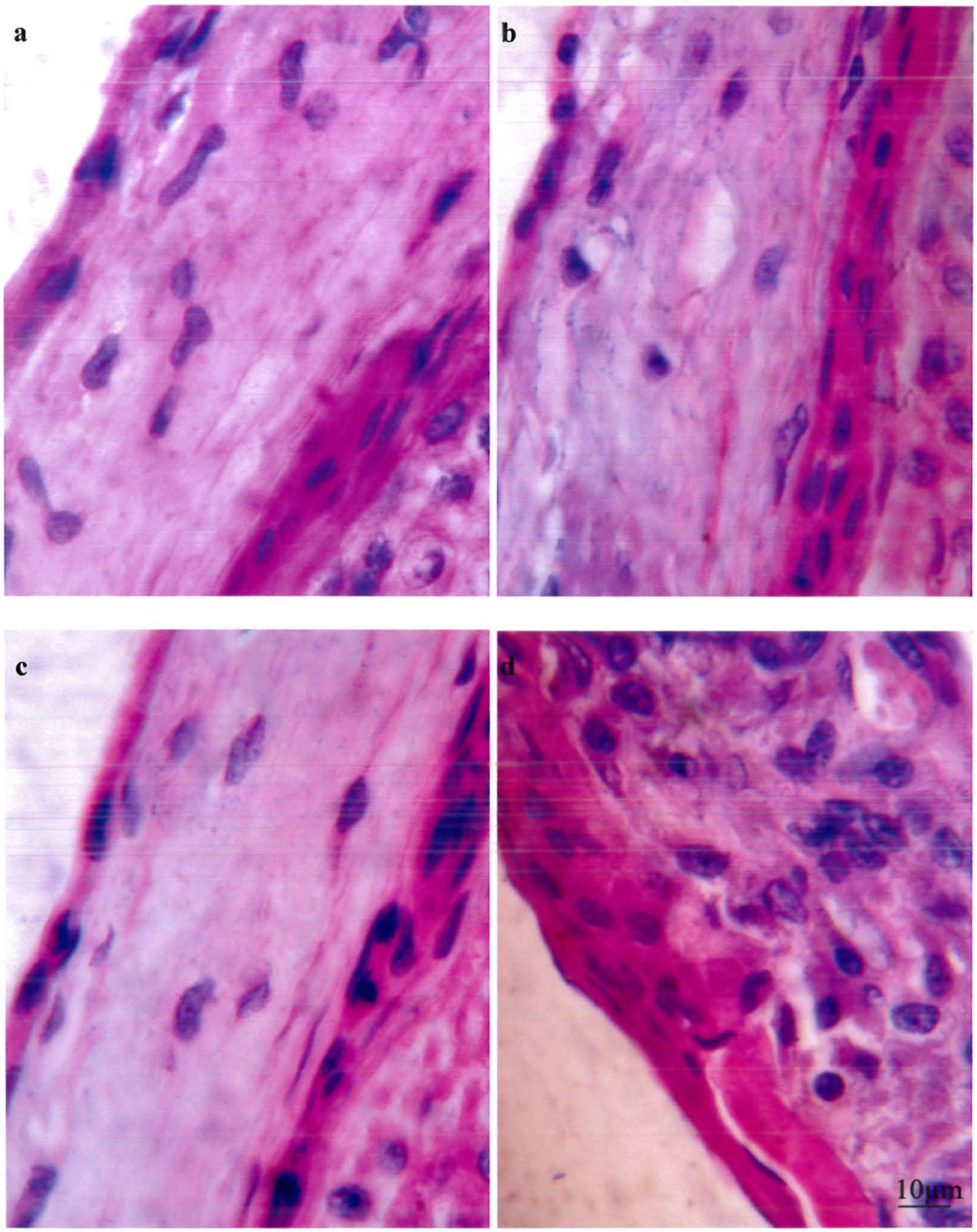

Fig. 23. Photomicrograph of testicular capsule of control and sodium arsenite treated rats showing (a) Control with dense fibers and compact cellular arrangement. Decrease in thickness and loose cellular arrangement is observed in a dose dependent manner. (b) 50ppm treatment group reduced number of cells is noticed, (c) 100ppm further reduction in cell number is observed, (d) 200ppm group profound decrease in number of cells and thickness. H & E.

chromatin of some primary spermatocytes appeared fragmented and few showed necrotic changes with thoroughly damaged chromatin (fig. 21d). Atretic or atrophic seminiferous tubules were also found in 200ppm group (fig. 20d).

EPIDIDYMIS:

Three parts of epididymis caput, corpus and cauda were used for histological observations.

CAPUT:

Epididymal caput of control group showed lumen packed with sperms (fig. 24a). The epithelium was composed mainly of principal cells and basal cells. Principal cells were tall columnar cells with elongated nuclei. Numerous cilia extended from the luminal surface of the principal cells. The basal cells were short and rest on the basal lamina. One or two muscle layers surrounded tubule segments externally (fig. 25a). The interstitial spaces between the tubule were packed with connective tissues and blood vessels (fig. 24a).

Low dose (50ppm) treated rats showed reduction in tubular diameter and epithelial height of caput compared to the control group. The principal cells were columnar with elongated nuclei but small in height compared to the controls. The cilia present at the tips of principal cells were also short compared to the control. The basal cells at the base of principal cells also reduced in size (fig. 25b). The 50ppm sodium arsenite treated rats showed enlarged interstitial areas compared to control (fig. 24b).

Rats treated with 100ppm sodium arsenite also showed reduction in tubular diameter and epithelial height of caput compared to control and 50ppm group. The nuclei of the principal cells were irregular in shape compared to the elongated nuclei of control and 50ppm group. The cilia were short and rare compared to control and low dose treated group. The basal cells were very small and hardly distinguishable from the cells of muscle layer (fig. 25c). The intertubular areas enlarged compared to the control and 50ppm group. Few atretic tubules were present in the epididymal caput of 100ppm sodium arsenite treated rats (fig. 24c).

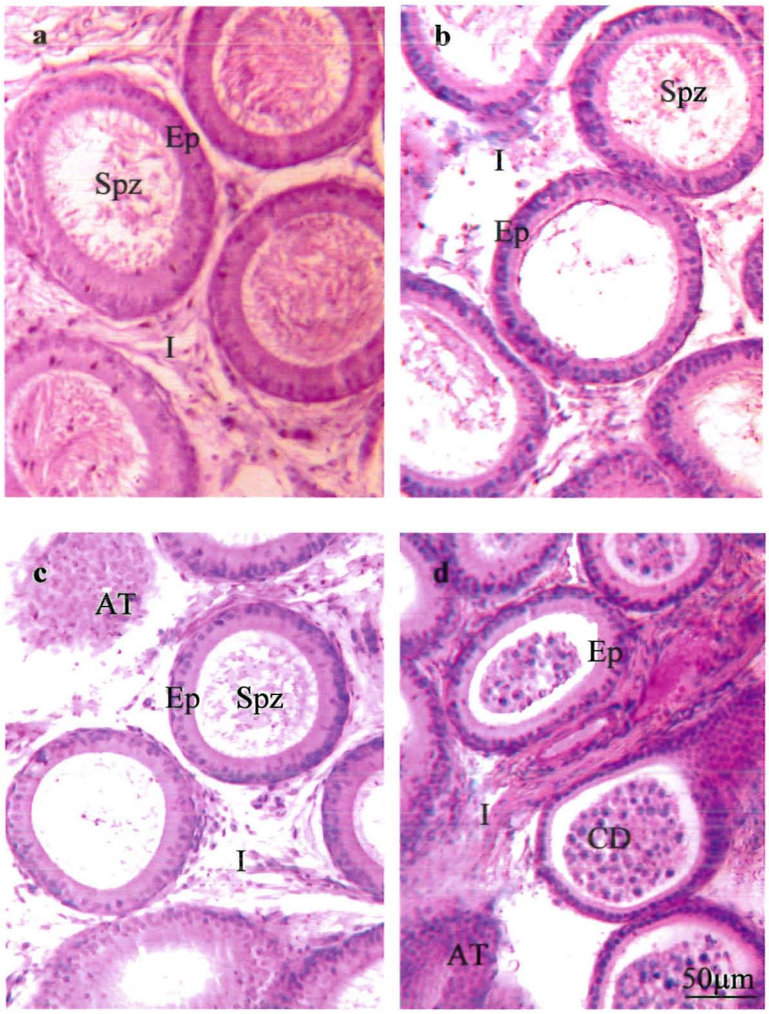

Fig. 24. Photomicrograph of epididymal caput showing tubules in control and sodium arsenite treated rats at 56 days age. (a) control showing tubules with prominent epithelium (Ep), large number of spermatozoa (Spz) and small interstitial spaces (I). Decrease in tubular diameter and extended interstitium is observed in a dose dependent manner. (b) 50ppm showing decrease in epithelium and spermatozoa, (c) 100pm and (d) 200ppm showing further reduction in epithelium and some atretic or regressed tubules (AT) are noticed. Cellular debris (CD) is present in the lumen of high dose epididymal caput. H & E.

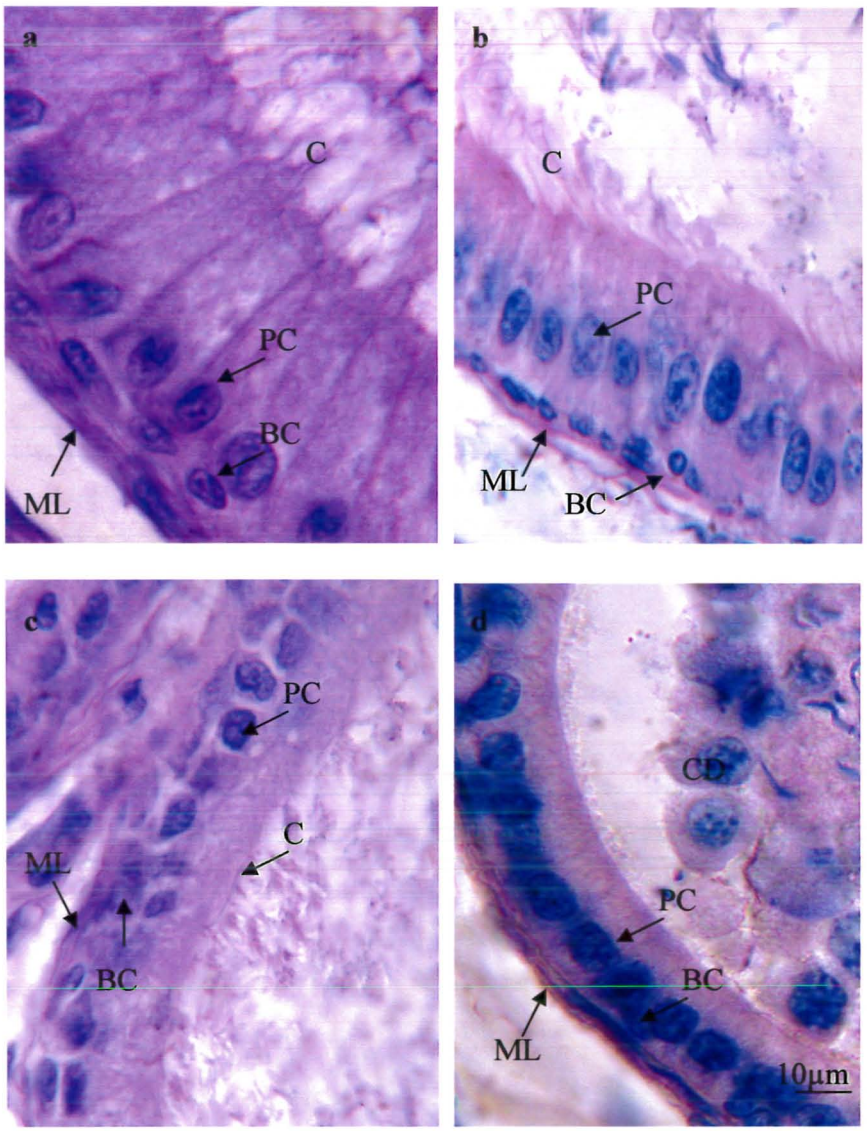

Fig. 25. Photomicrograph of epididymal caput showing cellular arrangement in epithelium in control and sodium arsenite treated rats at 56 days age. (a) Control showing epithelium having outer muscle layer (ML), columnar principal (PC) and small basal cells (BC) prominent nuclei and luminal cilia (C). The size and number of ciliated cells and basal cells nuclei decrease dose dependently. (b) 50ppm showing epithelium with elongated nuclei of principal and small round basal cells. The surrounding muscle layer is not very prominent, (c) 100ppm showing disarrangement and irregular nuclei of principal and basal cells. The muscle layer is not intact at some places, (d) 200ppm showing epithelium with round nuclei of principal and flattened nuclei of basal cell. The basal cells are indistinguishable from surrounding muscle layer. H & E.

The tubular size and epithelial height of caput showed the highest reduction in rats treated with 200ppm sodium arsenite compared to control 50ppm and 100ppm group. The epithelium became cuboidal compared to the columnar epithelium of controls. The size of principal cells was small with almost rounded nuclei compared to control and treated groups. The cilia were absent. The size of basal cells showed highest reduction with round nuclei. There was also observed cellular debris in lumen. The debris comprised of mostly mitotic cells, some sperms and secretions (fig. 25d). The intertubular areas also extended. Some atretic tubules were also observed (fig. 24d).

CORPUS:

Corpus showed increased lumen compared to that of caput (fig. 26a). Columnar cells of the epithelium were tall with somewhat elongated nuclei. The basal cells were short with small rounded nuclei resting on the lining of epithelium, surrounded by muscle layer (fig. 27a).

Rats treated with 50ppm sodium arsenite showed reduction in tubular diameter and epithelial height compared to controls. The size of the principal cells decreased with somewhat irregular nuclei. The basal cells were also short with irregular nuclei compared to control. The tubules were surrounded by muscle layer like that of control (fig. 27b). The interstitium became extended compared to controls (fig. 26b).

Epididymal corpus of 100ppm group showed decreased tubular diameter and epithelial height compared to the control and 50ppm group. The size of the principal cells was short with flattened nuclei. Rare or no cilia present at the tips of principal cells towards lumen. The basal cells were short with elongated nuclei, few lying close to the muscle layer (fig. 27c). There was observed an extension in interstitial spaces compared to controls and 50ppm sodium arsenite treated group (fig. 26c).

Corpus of 200ppm sodium arsenite treated rats showed highest decrease in tubule diameter and epithelial height. The size of principal cells showed highest reduction with round nuclei. There was observed disarrangement of epithelial cells. No cilia were present. The size of basal also reduced, not distinguishable from muscle layer. Cellular debris was found in the lumen of corpus (fig. 27d). The extension in interstitial spaces was

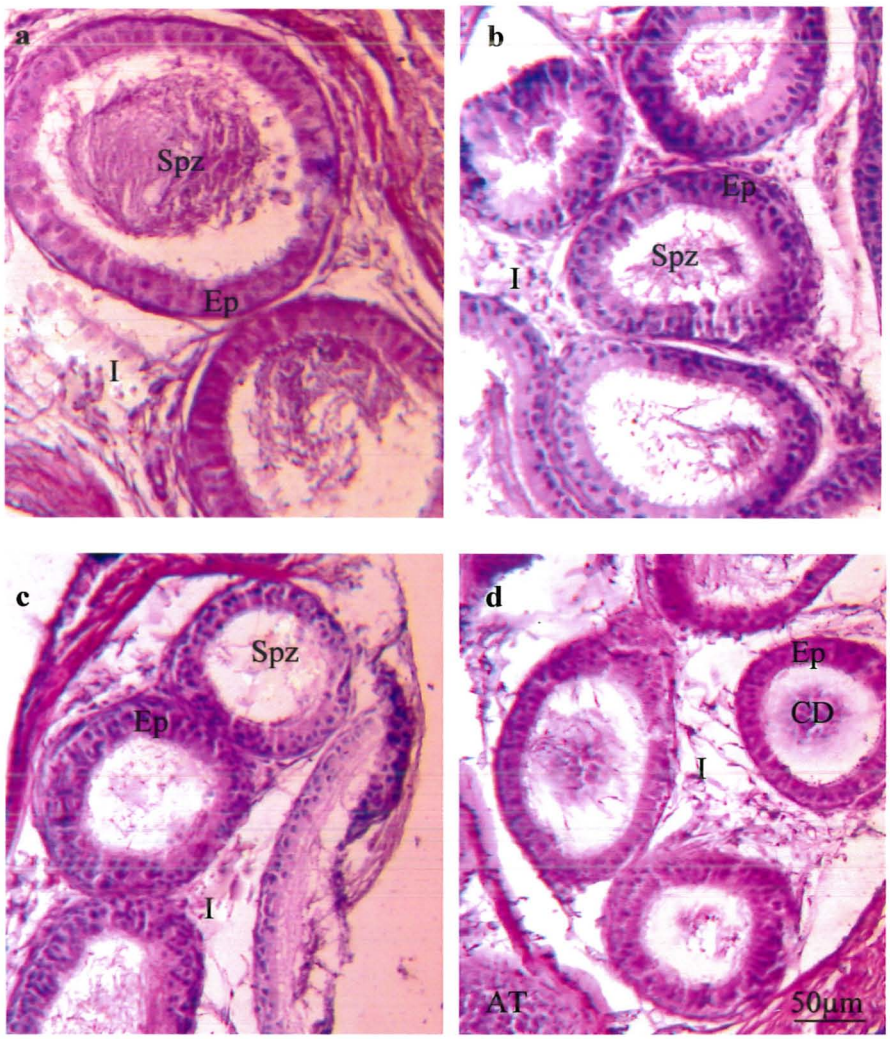

Fig. 26. Photomicrograph of epididymal corpus showing tubules in control and sodium arsenite treated rats at 56 days age. (a) Control showing tubules with prominent epithelium (Ep), large number of spermatozoa (Spz) and small interstitial spaces (I). Decrease in tubular diameter and extended interstitium is observed in a dose dependent manner. (b) 50ppm showing decrease in epithelium and spermatozoa, (c) 100pm and (d) 200ppm showing further reduction in epithelium. Some atretic or regressed tubules (AT) and cellular debris (CD) are noticed in high dose treated group. H & E.

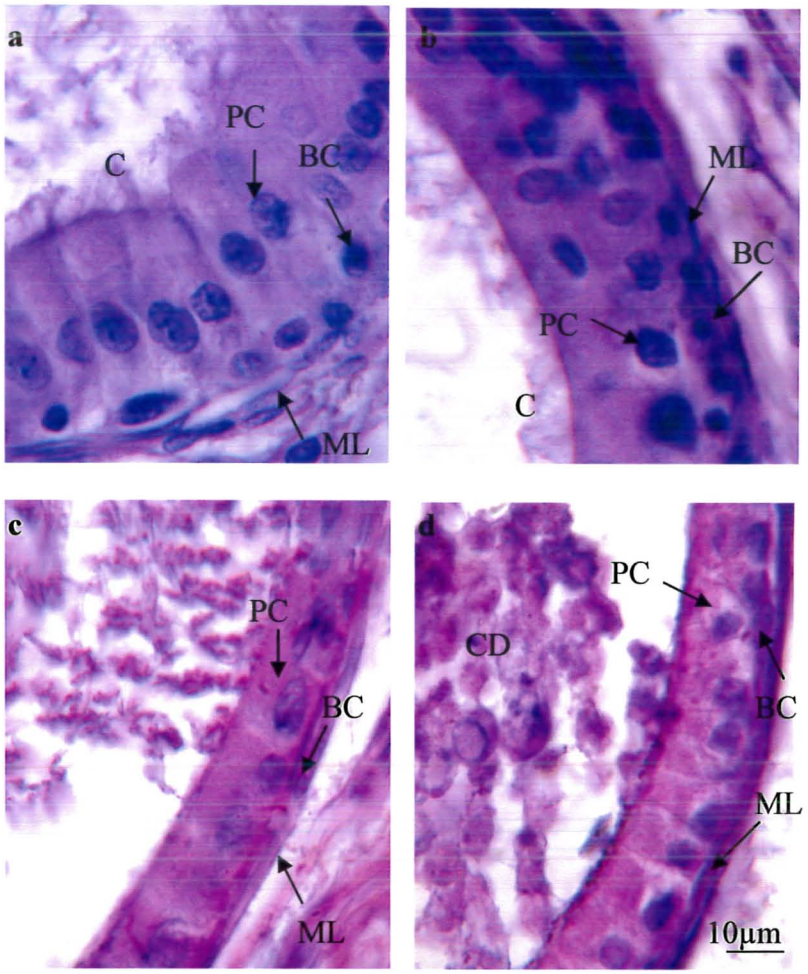

Fig. 27. Photomicrograph of epididymal corpus showing cellular arrangement in epithelium in control and sodium arsenite treated rats at 56 days age. (a) Control showing epithelium having outer muscle layer (ML), columnar principal (PC) and small basal cells (BC) prominent nuclei and luminal cilia (C). The size and number of ciliated cells and size of principal and basal cells nuclei decrease dose dependently. (b) 50ppm showing epithelium with irregular nuclei of principal cells, (c) 100ppm with flattened nuclei of principal and small rare basal cells, (d) 200ppm showing epithelium with irregular shaped nuclei of principal cells and no distinction in basal and surrounding muscle layer. H & E.

more pronounced compared to control and low dose groups. Some atretic tubules were also found in the corpus of high dose (200ppm) treated rats (fig. 26d).

CAUDA:

The size of cauda lumen was observed to be greater than caput and corpus (fig. 28a). Epididymal cauda showed long columnar principal cells having elongated nuclei with luminal cilia. The basal cells were small, present at the base of the principal cells. The epithelium was surrounded by muscle layer (fig. 29a).

Epididymal cauda of low dose treatment showed decreased epithelial height. The size of principal cells was small. The nuclei of principal cells were irregular (cuboidal, rounded) compared to the elongated nuclei of control epididymal cauda. The cilia were short compared to control group. The nuclei of the basal cells were also of various shapes like principal cells nuclei. The epithelium was surrounded by muscle layer like control group (fig. 29b). The interstitial spaces enlarged compared to controls (fig. 28b).

Rats treated with 100ppm sodium arsenite showed reduction in epithelial height and principal cells compared to controls and 50ppm treatment group. The nuclei of principal cells were round and that of basal cells were elongated. Rarely any cilia were present in the cauda of 100ppm sodium arsenite treated rats. The epithelium was surrounded by muscle layer (fig. 29c). The interstitial spaces in cauda of 100ppm treatment group extended compared to controls and 50ppm treatment group. Some atretic tubules were also observed in 100ppm treatment group (fig. 28c).

High dose (200ppm) treatment caused the highest reduction in epithelial height and size of principal cells compared to control, 50ppm and 100ppm treatment. Rats treated with 200ppm sodium arsenite showed highly stratified epithelium with no cilia compared to columnar epithilium of control group. The nuclei of principal cells were rounded. The muscle layer was not distinguishable compared to control, 50ppm and 100ppm treatment group (fig. 29d). The interstitial spaces showed the most profound extension compared to controls and low dose groups. Some atretic tubules were found in epididymal cauda of this group (fig. 28d).

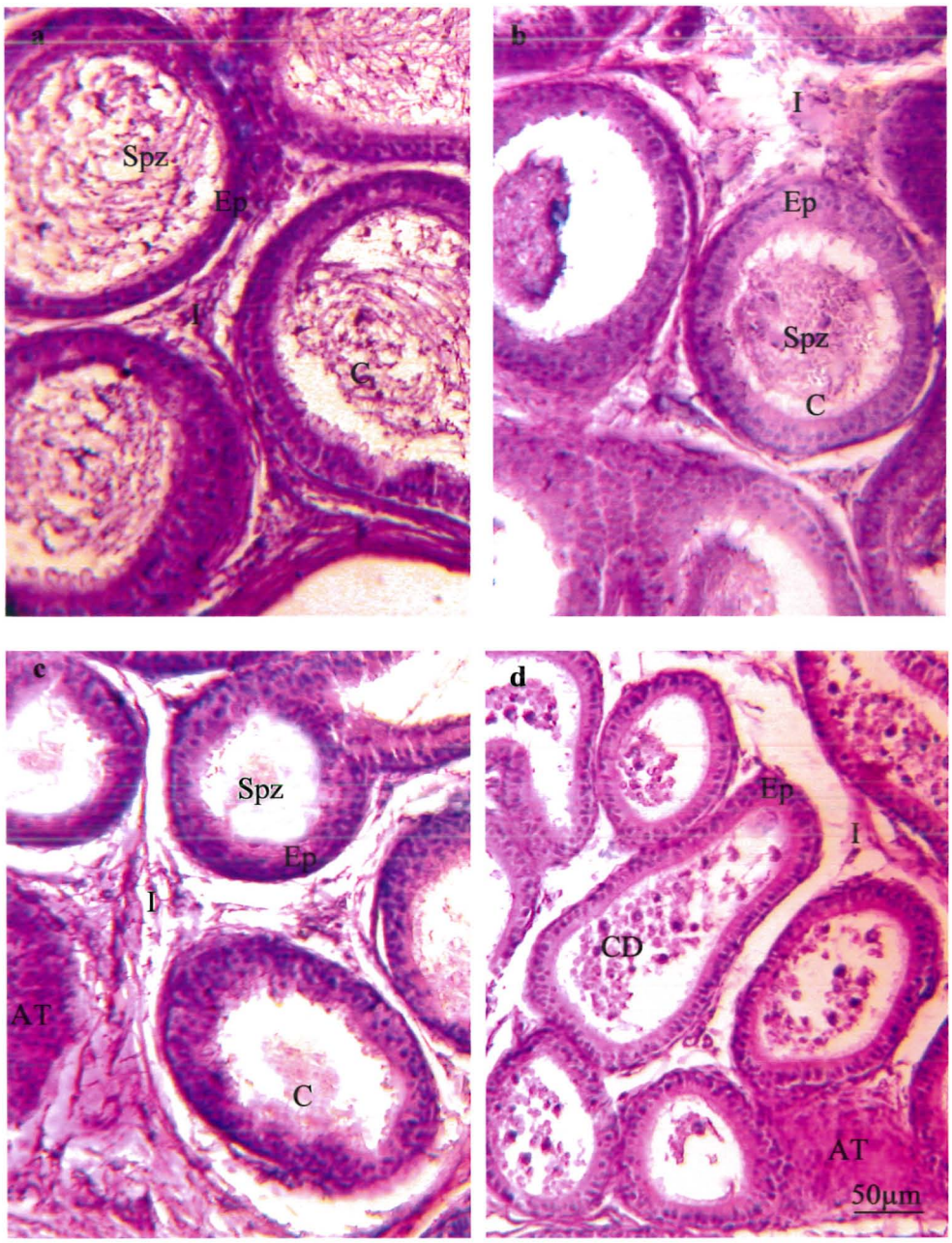

Fig. 28. Photomicrograph of epididymal cauda showing tubules in control and sodium arsenite treated rats at 56 days age. (a) Control showing tubules with prominent epithelium (Ep), large number of spermatozoa (Spz) and small interstitial spaces (I). Decrease in tubular diameter and extended interstitium is observed in a dose dependent manner. (b) 50ppm showing decrease in epithelium and spermatozoa, (c) 100pm and (d) 200ppm showing further reduction in epithelium and some atretic or regressed tubules (AT). Cellular debris (CD) is present in the lumen of high dose epididymal cauda. H & E.

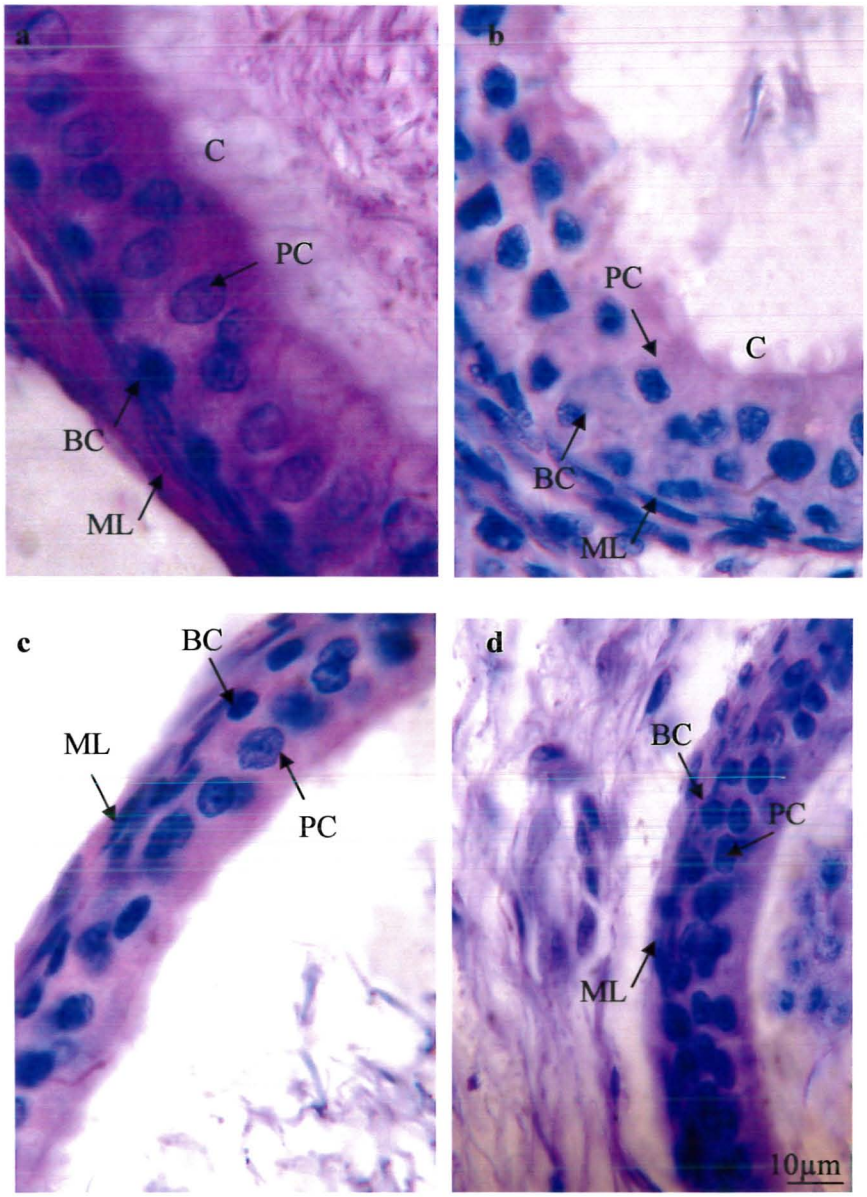

Fig. 29. Photomicrograph of epididymal cauda showing cellular arrangement in epithelium in control and sodium arsenite treated rats at 56 days age. (a) Control showing epithelium having outer muscle layer (ML), columnar principal (PC) and small basal cells (BC) prominent nuclei and luminal cilia (C). The size and number of ciliated cells and size of principal and basal cells nuclei decrease dose dependently. (b) 50ppm showing epithelium with irregular nuclei of principal and basal cells, (c) 100ppm with flattened nuclei of principal and basal cells, (d) 200ppm showing stratified epithelium. H & E.

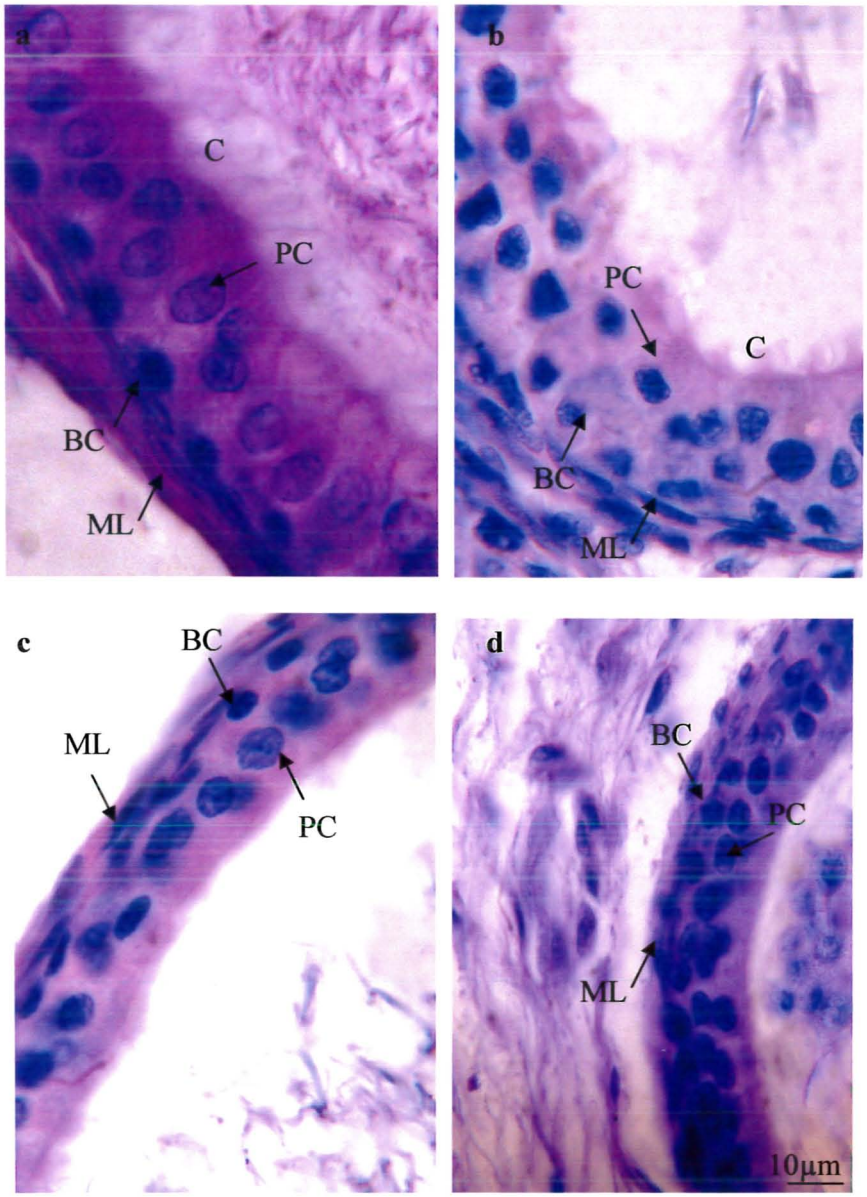

Fig. 29. Photomicrograph of epididymal cauda showing cellular arrangement in epithelium in control and sodium arsenite treated rats at 56 days age. (a) Control showing epithelium having outer muscle layer (ML), columnar principal (PC) and small basal cells (BC) prominent nuclei and luminal cilia (C). The size and number of ciliated cells and size of principal and basal cells nuclei decrease dose dependently. (b) 50ppm showing epithelium with irregular nuclei of principal and basal cells, (c) 100ppm with flattened nuclei of principal and basal cells, (d) 200ppm showing stratified epithelium. H & E.

VAS DEFERENS:

The mucosa of control vas deferens showed prominent pseudostratified columnar epithelium mainly composed of principal cells. The nuclei of principal cells were luminal in position compared to that of epididymis. Well developed, fine, and regular cilia were present at the free ends of the principal cells. The basal cells were also present at the base of principal cells. The epithelium was bounded externally by a thin lamina propria. The mucosa was thrown into folds. The vas deferens exhibited a packed mass of luminal sperms (fig. 31a). The muscularis of the control vas deferens showed normal inner longitudinal, prominent middle circular and well-defined outer longitudinal layers. The muscularis was intact with increased number of round and elongated nuclei (fig. 32a). The muscularis was externally bounded by adventitia with connective tissues and blood vessels (fig. 30).

Rats treated with 50ppm sodium arsenite showed reduction in epithelium height, organ (fig. 30b), principal and basal cells size compared to control. The muscularis was loosely arranged compared to controls with elongated and round nuclei (fig. 32b). The size of nuclei of principal and basal cells decreased. Few of the nuclei of principal cells were displaced towards the basal cells compared to the normal luminal principal cells nuclei. The cilia were also short compared to controls (fig. 31b).

Treatment of 100ppm sodium arsenite caused further significant reduction in vas deferens size and epithelium compared to controls and 50ppm treatment group (fig. 30c). There was also loosening of muscularis with some round and mostly elongated nuclei in 100ppm sodium arsenite treated group (fig. 32c). Some principal cell's nuclei were elongated but most of the principal cells and all basal cells nuclei were irregular in shape. The cilia were sparsely arranged compared to the densely arranged cilia of controls and 50ppm arsenic treated group (fig. 31c).

High dose (200ppm) treated rats showed highly significant reduction in vas deferens size and epithelium compared to the control, 50 and 100ppm sodium arsenite treated rats (fig. 30d). The loosening of muscularis was more prominent with cylindrical nuclei compared to the round nuclei in control and low dose treated groups (fig. 32d). The epithelial cells were undifferentiated. The nuclei of principal cells were rounded and basal cells were triangular and polygonal in shape compared to the elongated principal cells and rounded

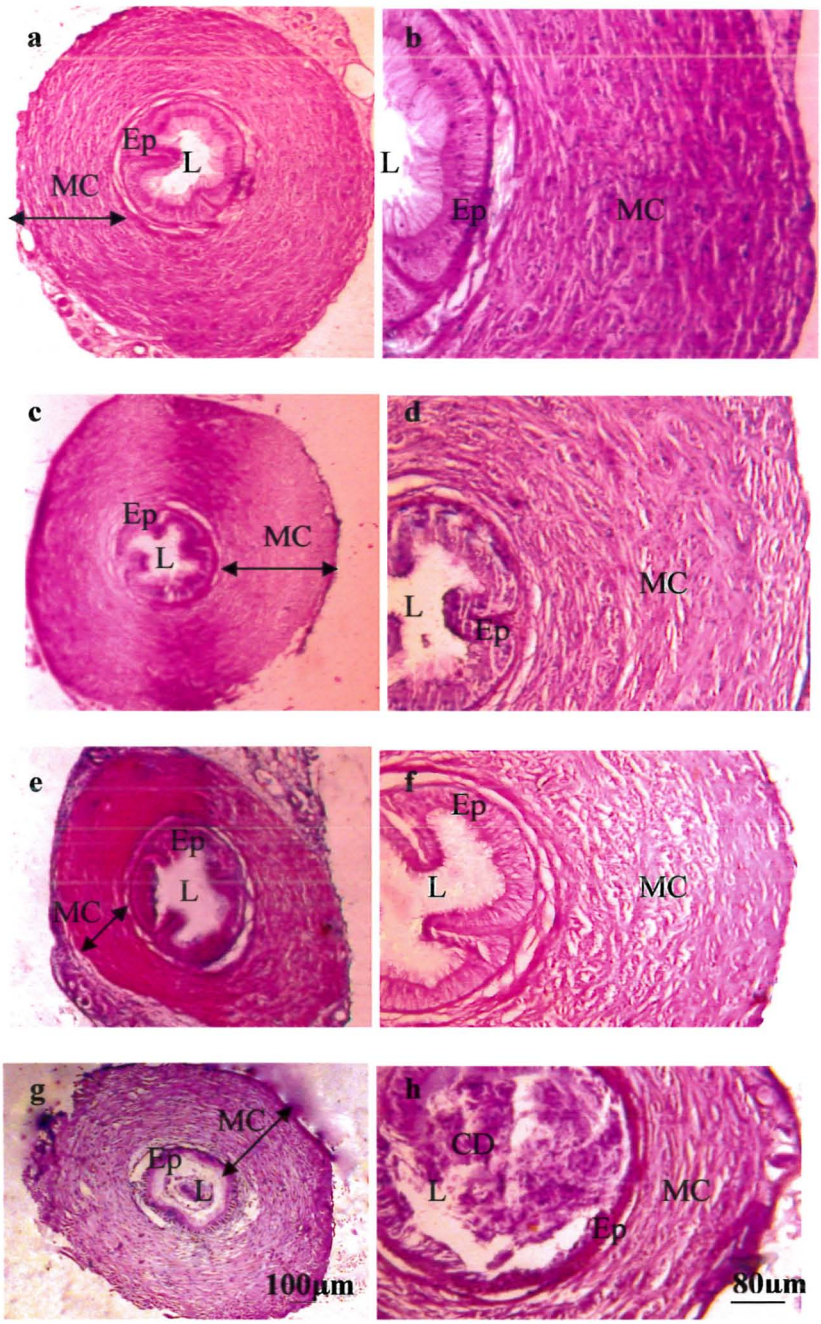

Fig. 30. Photomicrograph showing vas deferens in controls and sodium arsenite treated rats at 56 days age. (a, b) Control showing thick muscular coat (MC) and epithelium (Ep) with small lumen (L). The diameter of vas deferens decreases dose dependently. (c, d) 50ppm with loose muscular coat and thin epithelium, (e, f) 100ppm and 200ppm (g, h) further loosening in muscular coat and thinner epithelium. Cellular debris (CD) is noticed in the lumen of high dose treatment group. H & E.

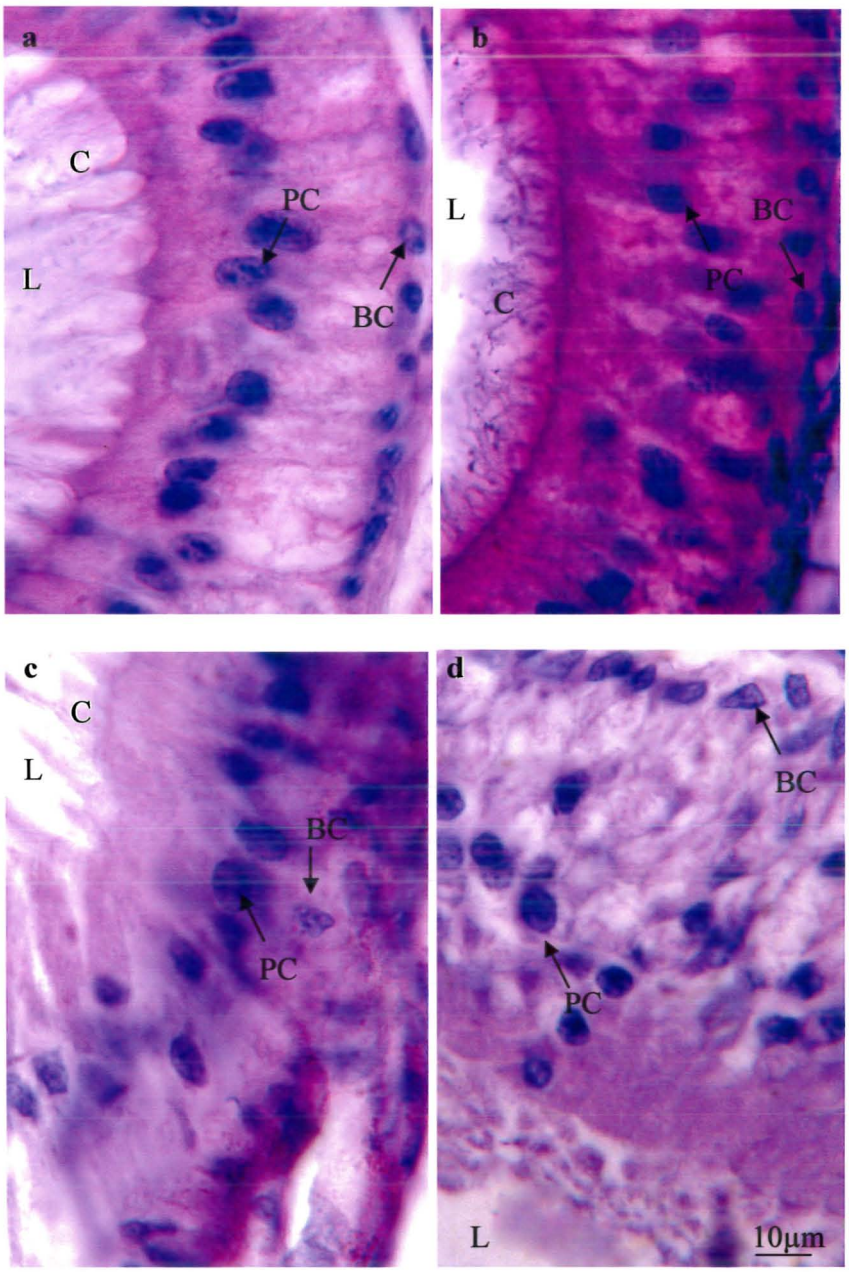

Fig. 30. Photomicrograph of vas deferens showing cellular arrangement in epithelium in control and sodium arsenite treated rats at 56 days age. (a) Control showing epithelium with columnar principal (PC) and small basal cells (BC) prominent nuclei and luminal cilia (C). The size and number of ciliated cells and size of principal and basal cells nuclei decrease dose dependently. (b) 50ppm showing epithelium with displaced nuclei of principal and basal cells, (c) 100ppm with elongated nuclei of principal and irregular basal cells, (d) 200ppm showing complete disarrangement of principal and basal cells with round nuclei of principal and variable shaped basal cells. H & E.

basal cells of control. Cellular debris was found in the lumen of high dose treated vas deferens. The lamina propria was not distinguishable in the vas deferens of rats treated with 200ppm sodium arsenite. The lumen of vas deferens had rare or no cilia in 200ppm sodium arsenite treated rats (fig. 31d).

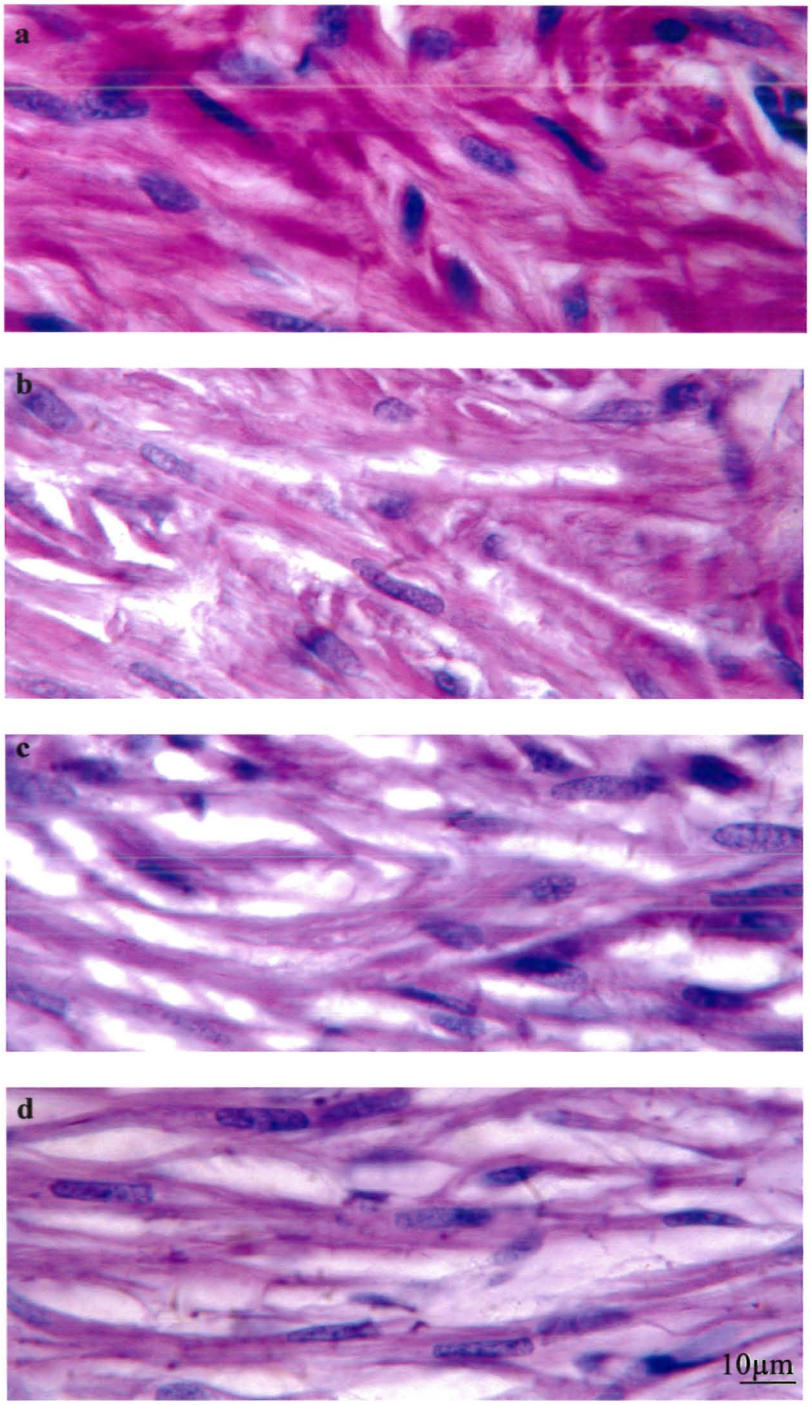

Fig. 32. Photomicrograph of vas deferens showing muscle arrangement in control and sodium arsenite treated rats. (a) Control with compactly arranged muscularis and increased number of elongated and round nuclei. Loosening in muscularis is observed in a dose dependent manner. (b) 50ppm showing muscularis with round and elongated nuclei, (c) 100ppm group showing some small round and mostly elongated nuclei, (d) 200ppm showing cylindrical and small number of elongated nuclei and rarely any round nuclei. H & E.

DISCUSSION

DISCUSSION

Arsenic, as trivalent arsenite and pentavalent arsenate is naturally occurring and ubiquitously present in the environment. Arsenic compounds are environmental toxins with multiple effects in animal and humans (Liu et al., 2001; Waalkes et al., 2003). The main source of environmental arsenic exposure is drinking water in which inorganic form of arsenic predominates (Bates et al., 1992; Pott et al., 2001). The risks of, exposure to inorganic arsenic in water supplies is a key issue facing the scientific community. High level of arsenic in the drinking water can be found in areas within many countries including Taiwan, China, Chile, Mexico, Agentina, Thailand, Finland, Hungary, Bungladesh, Pakistan and India (Kaltreider et al., 2001; Pott et al., 2001; NRC, 1999).

The effect of sodium arsenite on male reproduction is not well established, although there are some reports in which arsenic intoxication is associated with spermatotoxicity (Pant et al., 2004), inhibition of testicular androgenesis and reduction of the weight of the testes and accessory sex organs (Sarkar et al., 2003) in adult experimental animals. However the actual molecular events resulting in male reproductive toxicity from exposure of inorganic arsenic remain unclear. There is also lack of literature about the reproductive toxicity of sodium arsenite in growing age (from 4 – 8 weeks of age) male Sprague dawley rats. The current study has been specially designed for this purpose.

The present study was carried out for 28 days to observe the adverse effects of sodium arsenite on structure and function of testes and accessory ducts (epididymis and vas deferens) of treated (50ppm, 100ppm and 200ppm) male Sprague dawley rats at 56th day of age.

Rats treated with different doses of sodium arsenite (50ppm, 100ppm and 200ppm) in the current investigation were inactive, slow movement, smaller in size, weak, and showed inattention to general environment in a dose dependent manner. The effect on these aspects was highest in 200ppm group. Similar results have been reported by Rodriguez et al (2001) who observed reduced locomotor activity in a dose dependent manner after exposing adult rats for 2 to 4 weeks with a dose of 5, 10 and 20 mg/kg arsenite.

exposing adult rats for 2 to 4 weeks with a dose of 5, 10 and 20 m g/kg arsenite. Accordingly Schulzh et al (2002) observed hypoactivity and depressed rates of grooming in male Wistar rats treated with 3.33, 6.66, 13.3 or 26.6 mg/kg of sodium arsenite for 4, 8 and 12 weeks. Arsenic is a well-known neurotoxicant to affect CNS and some studies had reported that the combined exposure to arsenic and lead might impair neuropsychological development in the children (Calderon et al, 2001). Tsai et al (2003) reported a dose response relationship between cumulative arsenic exposure and neurobehavioral performance in children living in Taiwan. Wasserman et al (2004) reported reduced intellectual level and lower performance in children living in high arsenic (>50 μg/L) contaminated area compared to children who lived in low arsenic (<5.5 μg/L) contaminated area. Present investigation also indicated similar changes in general activity of growing age rats (28-56 days) depending upon the dose of sodium arsenite.

In the current study sodium arsenite caused profound effect on body weight throughout the experiment. At the start of experiment there was no difference in body weight of the three treatment groups and controls. After treatment of sodium arsenite there was gradual (weekly) and dose dependent decrease in body weight gain of the treatment groups compared to controls. The effect was more profound at high dose level (200ppm) compared to low dose treated groups and controls. Jana et al (2006) observed no significant difference in body weight of adult male albino Sprague dawley rats after treatment with 5 mg/kg body weight sodium arsenite per day for 4 weeks. Pant et al (2004, 2001) observed no significant difference in body weight of adult male Swiss albino mice after treatment with sodium arsenite. There are many studies in which decrease in body weight has been reported. Nagaraja and Desiraju, (1993, 1994) reported a decrease in body weight of male and female Wistar rats when treated with 0 or 5 mg/kg sodium arsenate from age 2 to 60 days. Decrease in body weight was observed by Nandi et al (2006) in adult rats after exposure to 10ppm of arsenic through drinking water ad libitum for 12 weeks. Sprando et al (2007) also found a dose related decrease in body weight in male Sprague dawley rats after administering single oral dose of sodium arsenite (0.41, 4.1, 41.0 and 410.0 mg/kg) in half and half cream. Decrease in body mass was noticed by Carol et al (2000), when injected adult male Wistar rats with $CdCl_2$ (2 mg Cd/kg body mass/day) for 14 days. Similarly using other chemicals like propyl paraben, a

significant decrease in body weight was observed by Oishi (2002) in male Wistar rats (19-21 days of age) treated with 0.01, 0.10 and 1.00 propyl paraben given in slightly modified AIN93G diet. Results of present and previous work suggested the adverse effects of arsenic on growth of exposed animals particularly at high dose level. The decrease in body weight may be due to reduced food consumption. The adverse effect of arsenic on central nervous system may also be a reason for the decreased body weight gain in treated rats. Reduction of organs weight along with internal aberrations may also be one of the causes.

Mean testicular weight decreased significantly compared to control in all the treatment groups. This decrease in testicular weight was more pronounced in high dose (200ppm) treated group. There was no significant effect on the length and width of testes with low dose but significant decrease was seen with high dose of 200ppm of sodium arsenite. Even low dose of 5ppm sodium arsenite administered for 28 days duration decreased paired testicular weight in Sprague dawley (90 days old) rats (Jana et al., 2006). Sarkar et al (2003) in Wistar rats (4 months old), Pant et al (2004) in Swiss male albino mice (8-10 weeks old), Healy et al (1998) in mice, reported decrease in testicular weight with sodium arsenite treatment. A significant reduction in testicular weight of Swiss albino male mice (25-30 g) was observed by Sarkar et al (2008), from 45 days onward when administered 30 mg/L Sodium-meta-arsenite. But more conspicuous decrease in testicular weight was noted at dose level of 40 mg/L sodium meta-arsenite after 30 days and significant diminution was found on day 60. the decrease in weight of testes and accessory sex organs have been reported in experimental animals following exposure to other chemicals such as carbofuran (Pant et al., 1995), sodium selenite (Nebbia et al., 1987) and lead (Koris and Badger, 1996) which are known to exert testicular and spermatotoxic effects. Testicular size and weight are normally regulated by fluid secretion from Sertoli cell (Waites and Gladwell, 1982) and the production of sperm (Aman, 1970) in the seminiferous tubule. The reduced testicular weight and size in the current study is suggested due to the adverse effect of sodium arsenite on Sertoli cell and sperm production as well as spermatogenic cells. Another key factor is the reduction in testosterone level, which causes weight loss in testis (Chattopadhyay et al., 1999; Chang et al., 2007). Probably the deleterious effect of sodium arsenite on the testicular weight in

present study was mainly due to the loss of body weight gain or might be the testicular hormones and gonadotropins responsible for the regulation of testicular weight.

Some information is available about the effect of arsenic on the structure and function of epididymis but there is lack of literature about the adverse effect of high dose of sodium arsenite on rat epididymis, the present study was designed to achieve this goal. In the current study mean epididymal weight and length of growing age sodium arsenite treated rats decreased significantly compared to controls. The epididymal weight and length showed the lowest value at the dose of 200ppm sodium arsenite. Other chemical like carbofuran also showed the same results. Pant et al (1995), administered carbofuran orally to adult male rats at dose levels of 0.1, 0.2, 0.4, and 0.8 mg/kg body weight, 5 days a week, for 60 days. A significant dose dependent decrease in epididymal weight was observed at all test doses except the lowest dose. No significant difference in epididymal weight of 8-10 weeks old Swiss albino mice was noticed after treating with 4ppm sodium arsenite for 365 days (Pant et al., 2004) and 53.39, 133.47, 266.95 and 533.90μmol sodium arsenite for 35 days (Pant et al., 2001). The epididymal weight also remained unaffected when adult male cotton rats (104±4 g) were treated with 5ppm and 10ppm sodium arsenite in drinking water for 6 weeks (Savabieasfahani et al., 1998). This means very low dose of sodium arsenite does not cause any adverse effect on epididymis structure and function. In the present study high doses of sodium arsenite (50ppm, 100ppm and 200ppm) were used, thus causing high damage to the epididymis. The epididymis is dependent upon androgen stimulation, therefore any compound that decreases Leydig cell function will decrease androgenic concentration in blood and rete-testis fluid, which will have subsequent effects on the epididymis, sperm maturation and fertility (Hess, 1998). The observed loss in weight and size of epididymis may be due to decrease in sperm count and histomorphological changes.

In the present study 50ppm, 100ppm and 200ppm sodium arsenite treatment caused highly significant dose dependent reduction in mean vas deferens weight and length of growing age male Sprague dawley rats. High dose (200ppm) showed more adverse effect on vas deferens of 56 days old male rats. The present study is in accordance with the study of Mandava et al (2001); they observed a significant decrease in vas deferens weight of adult male Swiss albino mice (35-40 g) following 1.25 mg/kg mercuric

chloride treatment for 45 days. Estradiol may also have same effect like arsenic. Chinoy et al (1984) investigated the effect of estradiol benzoate (50 µg/day/rat for 15 days) in intact male albino rats. They observed significant reduction in androgen dependent parameters and histology of androgen dependent tissues. This led to alteration in milieu of epididymis and vas deferens rendering them hostile for sperm maturation, metabolism and survival, as evident by reduced sperm motility, alteration in their morphology, which resulted in loss of fertility. As vas deferens is also androgen dependent like epididymis so the reduction in androgen caused an adverse effect on vas deferens structure and function under high doses (50ppm, 100ppm and 200ppm) of sodium arsenite in the growing age (28-56 days of age) male Sprague dawley rats.

In current study mean plasma testosterone concentration of 50, 100ppm and 200ppm groups decreased significantly in a dose dependent manner in growing age male Sprague dawley rats. The same dose dependent decrease in testosterone level was reported by Sarkar et al (2003) who administered sodium arsenite intraperitoneally to 4 months old male Wistar rats at doses of 4, 5 or 6 mg/kg for 26 days. Plasma testosterone significantly decreased in rats of 5 and 6 mg/kg group, more prominent in latter group but showed no significant decrease in low dose group. A similar dose dependent decrease in plasma and intratesticular concentrations of testosterone was observed by Jana et al (2006) in 90 days old male albino rats (200-250 g body weight) treated with 5 mg/kg body weight sodium arsenite for four weeks. In the present study high doses of 50ppm, 100ppm and 200ppm sodium arsenite were given in drinking water to growing age male rats, thus reducing testosterone concentration even in growing age rats. It is known that biological activity of steroid hormones is regulated by several enzymes including 17-beta hydroxysteroid dehydrogenases (17β-HSD). This enzyme plays a very important role in the biosynthesis and metabolism of steroid hormones (Puranen et al., 1997). Probably in present study the arsenic treatment altered the activity of testicular enzymes (HSD) which ultimately suppressed the plasma levels of testosterone. This possible correlation of arsenic treatment with the inhibition of delta 5, 3 beta hydroxysteroid dehydrogenase (Δ5-3β-HSD) and 17β-HSD activity is strongly supported by the study of Jana et al (2006). The result of present study suggests that decreased levels of LH and FSH may decrease the activity of testicular enzymes, which in turn diminishes the plasma concentration of

testosterone in arsenic treated rats. Reduction in testosterone level causes a weight loss in testis and also inhibits testicular enzymes (Chattopadhyay et al., 1999; Chang et al., 2007).

The decrease in plasma testosterone is caused by the decrease in plasma LH concentrations. In male rats circulating LH is required for maintaining normal plasma testosterone concentrations (Ellis and Desjardins, 1982). Arsenic alters steroidogenesis by acting as an endocrine disruptor. Arsenic as a potent endocrine disruptor (Sakurai and Himeno, 2006), altering gene regulation by the closely related receptors of glucocorticoids (GR), mineralocorticoids (MR), progesterone (PR), androgens (AR), and estrogens (ER) at concentration as low as 0.01 μM or 0.7ppb (Davey et al., 2007). The loss in testicular weight in arsenite treated rats (50ppm, 100ppm, 200ppm) could be due the low levels of gonadotropins and steroid hormones indicating a direct relationship of testicular weight with pituitary-hypogonadal axis.

LH stimulates Leydig cells to produce testosterone within the testis. Intratesticular testosterone is an absolute prerequisite for normal spermatogenesis (Matsumoto and Bremner, 1989). In experimental circumstances in which the intratesticular testosterone concentration is reduced a significant germ cell death is seen (Kim et al., 2001).

The present study showed that mean FSH and LH concentrations decreased significantly in growing (28-56 days) age male Sprague dawley rats when treated with 50, 100 and 200ppm sodium arsenite via drinking water. This reduction was highest in 200ppm treated rats. A similar significant reduction in plasma FSH and LH was observed by Jana et al (2006) in adult male rats when treated with 5ppm sodium arsenite for four weeks. Sarkar et al (2003) also found a dose dependent decrease in plasma FSH and LH levels in adult male wistar rats treated with 5 and 6ppm sodium arsenite. Like the present study results of Sarkar et al (2003) also showed adverse effect at high dose level.

In the current study nuclear diameter of Leydig cell and Sertoli cell showed a highly significant dose dependent decrease in all the three treated (50ppm, 100ppm and 200ppm) groups. The decrease in nuclear diameter was highest in 200ppm sodium arsenite treated group. Sertoli cell is under the control of FSH. So the decrease in the size of Sertoli cell may be due to FSH diminution in the current study. Testicular size and weight are normally regulated by fluid secretion from Sertoli cell (Waites and Gladwell,

1982). Thus the reduced testicular weight and size in the current study may be due to the adverse effect of sodium arsenite on Sertoli cell. The nuclear diameter of Leydig cell was studied by Sarkar et al (2008) in adult Swiss albino mice. They observed the effect of sodium-meta-arsenite on mice (25±30 g) at dose level of 30 mg/L and 40 mg/L through drinking water for 30, 45 and 60 days. They observed marked variation in Leydig cell nuclear diameter in both of the treated groups. The nuclear diameter significantly increased in both the treated groups initially in 30 days, but followed a slow and gradual decrease over 60 days. The exogenous exposure of male rats to sodium arsenite may cause a chemical stress on the cellular function. So the changes in nuclear size of Leydig cell may be as a result of this chemical stress. As the Leydig cell play an important role in testosterone production under LH regulation (Sharpe et al., 1992), so these changes in Leydig cell intern decreased androgen concentration in sodium arsenite treated male Sprague dawley rats in current study.

Seminiferous tubule diameter and its epithelial height decreased highly significantly at all the dose levels. The high (200ppm) dose showed more pronounced effect on these parameters. The present study is in line with the finding of Sarkar et al (2008) in adult Swiss albino mice. They found a significant dose and duration dependent reduction in seminiferous tubule diameter of 30 mg/L and 40 mg/L sodium-meta-arsenite treated mice over a period of 60 days. The reduction in diameter of seminiferous epithelium is due to the disintegration and reduction in number and size of spermatogenic cells. Mehranjani and Hemadi, (2007) also reported a significant decrease in seminiferous tubule diameter and germinal epithelium thickness of vesectomised adult male Wistar rats (200±20 g) treated with 8 mg/kg sodium arsenite for 8 weeks. Since FSH inhibits normal degeneration of germinal cells (Chattopadhyay et al., 1999), and reduction of testosterone level lead to the separation of germinal cells from the epithelium of the seminiferous tubules (Jarow et al., 1985). So the reduction of testosterone and FSH in the current study could be a reason for the significant reduction of seminiferous tubule diameter and thickness of germinal epithelium.

In current study the diameter of spermatogonia, primary spermatocytes, secondary spermatocytes and spermatid decreased highly significantly in all the three sodium arsenite treated groups compared to controls. The adverse effect was more pronounced in

high dose group. The number of Leydig, Sertoli and spermatogenic cells was observed to be decreased. Thickness of tunica albuginea reduced with the increase in the dose of sodium arsenite. The interstitial spaces were extended and spermatogenesis was disrupted in a dose dependent manner. In 200ppm sodium arsenite treated rats there was degeneration at XII to XIV stages of spermatogenesis. Few elongated spermatids were present. The chromatin of some primary spermatocytes were fragmented (in 100ppm and 200ppm sodium arsenite treated groups) and few exhibited necrotic changes (200ppm treatment group). Few atretic seminiferous tubules were also observed in high dose treated group. Aruldhas et al (2005) also observed fragmentation and necrotic changes in primary spermatocytes of adult monkey (Macaca radiate) treated with chromium (100, 200 and 400ppm) for six months. Spermatogenesis in the rat is a highly ordered process occurring in 14 stages, each corresponding to a characteristic cellular association (Leblond and Clermont, 1952). The germ cells in the adluminal compartment are held in position by the characteristic inter-Sertoli cell junctions and the Sertoli cell-germ cell junctions (Griswold, 1995; Bardin et al., 1994). These junctional complexes are maintained by cytoskeltal elements and the ectoplasmic specialization of the Sertoli cell cytoplasm (Bardin et al., 1994). FSH and testosterone both regulate the formation of ectoplasmic specialization (Sluka et al, 2006). As in the current study both FSH and testosterone decreased with the increase in the dose of sodium arsenite, which may be a reason for the disturbance of these complexes in testes of treated rats.

Spontaneous germ cell death during spermatogenesis is an important event that takes place mostly at some specific stages of the spermatogenic cycle, mainly stages XIV and XII and, to a lesser degree, at stages I, VII, and VIII (Huckins and Oakberg, 1978; Kerr, 1992; Blanco-Rodriguez and Martinez-Garcia, 1996b). The occurrence of increased germ cell apoptosis under several circumstances, such as hormone deprivation particularly testosterone and gonadotropins (Tapananinen et al, 1993; Henriksen et al, 1995a, 1995b; Sinha-Hikim et al, 1995; Blanco_Rodriguez and Martinez-Garcia, 1996a; 1997a), experimental chryptorchidism (Shikone et al., 1994), and mild hypothermia (Blanco-Rodriguez and Martinez-Garcia, 1997b), have also been reported. The relevance of increased apoptosis in the pathogenesis of a number of human diseases has been emphasized (Thompson, 1995). Probably as many as one in three of all cases of human

male infertility cannot be explained by reasons such as chromosome abnormalities or endocrine dysfunction (Wong, 1973). In these idiopathic male infertilities, the male often produces a low number of spermatozoa, suggesting an imbalance between cell proliferation and cell death. This seems to be the case with male infertilities caused by endocrine dysfunction or cryptorchidism, since gonadotropins and testosterone withdrawal (Tapanainen et al, 1993; Billig et al, 1995; Henridsen et al, 1995 Sinha-Hikim et al, 1995) and experimental cryptorchidism (Allan et al, 1987; Shikone et al, 1994) lead to a dramatic increase in germ cell apoptosis. In the present study the degeneration at the particular (XII-XIV) stages in the treated rat testes may be due to the increased apoptosis as a result of decreased testosterone and FSH concentration.

Throughout their development from spermatogonia into fully differentiated spermatozoa, the germ cells are nursed by the enveloping Sertoli cells. From Sertoli cell reconstruction studies, it has been calculated that about 50 germ cells of four different cell types are embedded in the surface of each Sertoli cell (Weber et al., 1983). Therefore, since Sertoli cells are the main target of FSH and testosterone, they should be suspected as being the primary cell type affected by the depletion of these hormones, with germ cell apoptosis and detachment from the seminiferous epithelium being simply a consequence of effects on the Sertoli cells. In the current study the germinal epithelium became thin with loosely arranged spermatogenic cells. This damage may also be due to the alteration in the junctional complexes and the cytoskeletal framework of the Sertoli cells. Sertoli cells itself becoming a target of the toxicant cannot be ruled out. LH and FSH are required for the initiation and maintenance of spermatogenesis in prepubertal and pubertal rats (Chowdhury, 1979) and for quantitatively normal spermatogenesis in pubertal rats (Russell et al., 1987). Besides this the germ cell degeneration by arsenic may be due to low intratesticular testosterone concentration, as high level of testosterone is required for the maintenance of structural morphology and normal physiology of seminiferous tubule (Sharpe et al., 1988; Sharpe et al., 1992; Blanco-Rodriguez and Martinez-Garcia, 1998; Perez et al., 2006). Testosterone is needed for the continued production and maintenance of different generation of germinal cells in the seminiferous tubules (Santen, 1999; Weinbauer et al., 2001), therefore reduction of testosterone level may lead to the separation of germinal cells from the epithelium of the seminiferous tubules (Jarow et al.,

1985). It may be suggested that the decrease in Leydig cell nuclear diameter and its shape variation in current study would have resulted in decreased syntheses of testosterone, which in turn disturb the process of spermatogenesis. It has already been established that Leydig cell plays important role in the structural and functional integrity of seminiferous tubules and testosterone synthesis, which is the main component of regulation of spermatogenesis (Bartke, 1991; Steinberger and Duckett, 1965; Sharpe et al., 1990). Leydig cells along with germ cells have been identified as the main targets of metal cytotoxicity leading to a reduced steroidogenesis and, thereby, disruption of spermatogenesis (Laskey and Phelps, 1991). Leydig cells are under the control of LH. Thus reduction of FSH and LH and a consequent reduction in testosterone production may, therefore, be held responsible for these arsenite-induced changes in spermatogenesis.

Germ cells at different stages of development require specific nutrients, such as lactate, pyruvate, faty acids and regulatory peptides (de Krestser and Kerr, 1994). In the present study arsenic toxicity probably affects the supply of such specific nutrients and/ or regulatory substances required by meiotic/ post meiotic germ cells, resulting in their premature death.

In the current study the tubule diameter and epithelial height of epididymal caput, corpus and cauda decreased significantly compared to controls. Sodium arsenite also caused histomorphological changes in the three parts of epididymis. The size of principal and basal cells decreased and intertubular spaces enlarged in the epithelial caput, corpus and cauda of all treated groups compared to controls. The epithelium of caput in 50ppm sodium arsenite treated group was like controls except that the size of cilia became short. The caput of 100ppm sodium arsenite treated group had irregular nuclei of principal and basal cells, short and rare cilia. In 200ppm treatment group the epithelium of caput became columnar with round nuclei of principal and basal cells. Cilia were absent and the lumen had cellular debris. Some atretic tubules were also observed. The corpus in low dose (50ppm) group had irregular nuclei of principal cells and short cilia. There was disarrangement of Principal cell's nuclei. The corpus of 100ppm sodium arsenite treated rats showed flattened nuclei of principal cells, round or fibrous nuclei of basal cells almost indistinguishable from surrounding muscle layer and rare or no cilia. The

epithelium became cuboidal compared to controls and low dose treated group. The corpus of high dose (200ppm) treated group became cuboidal and the nuclei of principal and basal cells were round. The nuclei of principal cells were displaced towards the basal cells. There were no cilia. The lumen of high dose treated group had cellular debris and some atretic tubules were also observed. The last portion (cauda) of epithelium in low dose (50ppm) group had irregular nuclei of principal cells, triangular nuclei of basal cells and short cilia. In cauda of 100ppm sodium arsenite treated group round nuclei of principal cells and elongated nuclei of basal cells were present. Rarely any cilia were present. In high dose (200ppm) treated group there was observed hypertrophy in the epididymal cauda. The nuclei of principal and basal cells were round and cellular debris was observed in the lumen. No cilia were present. Some atretic tubules were also present in 200ppm treatment group. Similarly Murakami et al (1976) studied the epididymis of castrated Japanese monkeys (Macacus fuscatus). They reported that epithelium decreased in height, cilia reduced in length and in number and the principal cells lining the epithelium became cuboidal. Admininistration of testosterone to these castrated monkeys resulted in almost complete recovery of epididymal epithelium as well as regeneration of cilia. Other chemical like diethylstilboestrol (DES) also showed the same effects. Atanassova et al (2005) observed cellular debris in the epididymal lumen of 10 µg DES treated neonatal (days 2-12) Wistar rats. Batran et al (2001) reported damage of the epididymal basement membrane and disorganization of epithelium in adult rats treated with higher doses of lead (10, 50 and 200 mg/kg body weight). Similarly Pandey et al (1999) observed no change in caput but degeneration of epithelial cells in epididymal cauda of nickel sulphate (5 and 10 mg/kg body weight) treated adult Swiss albino mice (for 35 days duration). The situation in the present case may be due to a hormonal imbalance consequent of sodium arsenite treatment, as epididymis is an androgen-dependent organ (Robaire and Hermo, 1988). Lei et al (2003) observed morphological phenotype in knockout animals as: decrease in luminal diameter of the proximal and distal caput and cauda, the decrease in the height of principal cells and the number of cells containing cilia, a decrease in cilia length and change from basal to central location of nuclei in the principal cell, the biochemical phonotype as: a decrease in periodic acid-Schiff reaction product, reflecting glycogen and glycoprotein synthesis and secretion, and

decrease in androgen receptors. They reported that after 21 days testosterone replacement therapy in 30 days old knockout animals reversed some, but not all (luminal diameter, the percentage of ciliated principal cells in caput epididymis and nuclear androgen receptors localization) phenotypes, indicating although androgens are important for normal epididymal morphology and function, but LH could also be required for certain facets of epididymal morphology and/or function. In the epididymis, the presence of microvilli on the apical surface of principal cells allows for the proper transport of ions, solutes, and water across the epididymal epithelium as these structures express various pumps, cannels and transporters and in this way maintain the luminal microenvironment (Wong et al., 2002; Turner, 2002; Hermo et al, 2005) in a manner that is essential for the maturation spermatozoa (Hermo et al., 2000; Hermo and Robaire, 2002; Breton, 2003). Microvilli composed of parallel actin bundles (Bartles, 2000). Espin is a component of microvilli (Bartles, 2000) and is regulated by high doses of testosterone (Primiani et al., 2007) because testosterone levels in the lumen of the epididymis are approximately 10 times higher than in the blood (Turner, 2002). Thus the decrease in testosterone and gonadotropins with the increased doses of sodium arsenite affected the structure and function of epididymis. The reduction in the viable number of epididymal sperm in the present situation also reflected this damage.

The pseudostratified epithelium of vas deferens consists mainly of small hemispherical basal cells and columnar principal cells with tall microvilli (Popovic et al., 1973; Hoffer, 1976, Regadera et al., 1997). The principal cells possess the machinery for endocytosis and merocrine secretion. Electron-dense tracers injected into the lumen of the rat vas deferens were found in coated pits and vesicles, endosomes, multivesicular bodies and lysosomes, which together with the presence of endocytic receptors on the principal cell surface, suggest that these cells are involved in the endocytosis of substances from the lumen (Friend and Farquhar, 1967; Hermo and de Melo, 1987; Andonian and Hermo, 1999c). Immunocytochemical and radioautographic studies indicated that principal cells synthesize and secrete glycoproteins into the lumen via the classical merocrine manner involving secretory granules (Westrom and Hamilton, 1984; Burkett et al., 1987; Pailhoux et al., 1990; Andonian and Hermo, 1999c). The vas deferens of rat also synthesizes different subunits of glutathione S-transferases to protect spermatozoa while

they are stored and/or transported through the vas deferens (Andonian and Hermo, 1999b). The current investigation showed a significant decrease in vas deferens diameter, muscular thickness and epithelial height. The highest reduction was observed in 200ppm treatment group. Histomorphological study showed loosening in the cells of muscularis and decrease in size of principal and basal cells nuclei. In 50ppm sodium arsenite treated group the principal's cells nuclei were towards the basal cells compared to the luminal principal cells nuclei in controls. The cilia were dense but short. In 100ppm treatment group some nuclei of principal cells were elongated but most nuclei of principal and basal cells were irregular in shape. The cilia were sparsely arranged. In high dose treatment group the epithelial cells were disorganized with scattered nuclei of principal and basal cells. The nuclei of principal cells were round and basal cells were triangular and polygonal. There were rare or no cilia observed in the lumen of high dose treated group. Wright and Hamidinia (1983) reported shortening of principal cells, along with a decreased number and size of microvilli in the vas deferens proximal to the vasectomy site. The synthesis and secretion of proteins by the vas deferens have been demonstrated to be androgen dependent (Pailhoux et al., 1990). Moreover luminal fluid is essential for the normal integrity of the epithelium (Hermo and Morales, 1984). In the current study morphological changes in vas deferens may be due to the decrease in concentration of testosterone as vas deferens is testosterone dependent.

Daily sperm production and its efficiency in the present study decreased significantly in sodium arsenite treated rats. The decrease in daily sperm production and its efficiency in male Sprague dawley rats being more profound at the highest dose. Other chemicals like propyl paraben also has the same effect. Oishi (2002) observed a significant decrease in daily sperm production and its efficiency in testes of three weeks old male Wister rats after administering propyl paraben for four weeks. Exposure to arsenic can produce dramatic effects on reproduction, resulting in decrease in fertility, which may be associated with a drop in sperm production and an increase in dominant lethal mutations in offspring, predisposing future generations to risks such as cancer (Lord, 1999). The reduction in sperm count is correlated with decrease in weight, atrophy of testis and arrest of spermatogenesis (Chinoy et al., 1984). In addition a decrease in testosterone level can also account for decreased DSP in treated rats. Androgen receptors are ligand-activated

transcription factors that regulate genes involved in development of the male urogenital tract, secondary sexual characteristics, and sperm production. Since the androgen receptors play a critical role in male fertility it may be a target of arsenic toxicity in the urogenital tract and thus one reason of reduction in sperm production.

Sodium arsenite treatment in the present study also caused highly significant reduction in epididymal sperm count and this reduction was highest in 200ppm sodium arsenite treated group. Pant et al (2001) also observed dose dependent effect in mice. They documented that sodium arsenite treatment for 35 days at low doses (53.39, 133.47, 266.95 µmol/L) caused no significant difference in epididymal sperm count of 35 days old mice but at high dose level (533.90 µmol/L) caused a significant decrease in epididymal sperm count and motility along with increase in abnormal sperm. However in another study Pant et al (2004) observed that chronic administration (for 365 days) of low dose (53.39 µmol/L) sodium arsenite to 8-10 weeks old male Swiss albino mice caused a significant decrease in epididymal sperm count and sperm motility. Sarkar et al (2003) also found that epididymal sperm count significantly reduced in 5 and 6ppm sodium arsenite treated male Wistar rats but not in 4ppm treated rats. Pandey et al (1999) found reduction in epididymal sperm count in adult Swiss male albino mice when treated with 5 and 10 mg/kg body weight nickel sulphate. The sperm count also decreased significantly when 90 days old (200-250 g) Sprague dawley rats were treated with 5 mg/kg body weight/day sodium arsenite for 4 weeks via drinking water (Jana et al., 2006). Inter-Sertoli tight junctions may be affected by metals with consequent exfoliation of immature cells into the lumen of seminiferous tubules and reduction of viable sperm count in the epididymis (McClusky, 2008). The diminution in the epididymal sperm count in arsenic treated animals may be due to the lower concentrations of testosterone as the sperm production in testis and maturation in epididymis is under the control of testosterone (Sharpe et al., 1992).

Arsenic was present in appreciable quantity in the male reproductive organs even in control animals and it became clear from the current study that 50ppm, 100ppm and 200ppm sodium arsenite administration in drinking water to 28 days old rats for 4 weeks resulted in significant amount of arsenic deposition in testes, epididymes, vas deferens and blood. The accumulation of arsenic was highest in 200ppm sodium arsenite treated

rats. The same results were found by Pant et al (2004), when treated mice with 4ppm sodium arsenite for 365 days. Pant et al (2001) noticed arsenic accumulation at 133.44, 266.95 and 533.90 µmol/L dose levels; however such an effect was not observed at low dose level (53.39 µmol/L). Accordingly Pandey et al (1999) reported an increase in testicular and epididymal nickel accumulation of adult Swiss albino male mice (25±5 g) after administering nickel sulphate at dose of 5 and 10 mg/kg body weight. In the blood arsenic accumulates preferentially in RBC's after being transformed to DMA in liver and other organs/tissues (Shiobar et al., 2001). Similarly in the current study higher concentration of arsenic was reported in the blood compared to that of reproductive organs. Accumulation of arsenic in rats blood is greater than human due to comparable its long half life (60 days) in rats. The degree of arsenic accumulation in the male reproductive organs of treated animals was observed as; Vas deferns > Epididymis > Testis, suggesting organ and dose dependent role of arsenic. The presence of arsenic in tissue of normal animals may be due to the consumption of food, water and air contaminated with the traces of this universally distributed metal in the environment (Krishnamurthy and Vishwanathan, 1990). The accumulation of arsenic in the current study in testes, epididymes and vas deferens, caused cellular disorganization, necrosis, atrophy and other morphological changes in these organs as reported by Dixon (1986).

The comet assay is a sensitive measure in individual sperm nuclei, to determine whether or not all cells within a population show the same degree of damage. Only few studies are available in which comet assay has been used to assess genotoxic damage to male germ cells after in vivo exposure to testicular genotoxicant (Anderson et al., 1996; Haines et al., 2001, 2002).

Commonly used parameters to measure the extent of DNA damage are tail length, relative fluorescence intensity of head and tail expressed as % DNA in tail and tail moment (Collins, 2004).). In the current study different parameters including comet length and height, head diameter, comet head and tail DNA percentage, tail length, tail moment and olive moment were measured. All these parameters exhibited dose dependent response in treated groups particularly in high dose groups compared to control and low dose groups. Only one or two parameters of comet assay were studied with regard to arsenic treatment in reported literature.

In the present study 50ppm sodium arsenite treatment resulted in a non significant increase in tail length, % DNA in tail and tail moment while 100ppm and 200ppm sodium arsenite caused highly significant increase in DNA damage with reference to all the three parameters i.e. Tail length, %DNA in tail and tail moment. In case of comet length, comet height and comet head diameter, all the three doses caused highly significant increase compared to controls. The observed effect was more adverse at high dose (200ppm) level. Haploid and euploid male germ cells can be differentiated by head diameter (Morgan et al., 1998). Morris et al (2002) used the length of tail to detect the effect of in vitro irradiation and chemotherapy upon DNA damage in sperms (Haines et al., 1998; Singh and Stephens, 1998; Chatterjee et al., 2000). Irvine et al (2000) measured tail length, percentage DNA and moment but documented results only from percentage tail DNA, presumably because this measure gave better discrimination in their analysis. Tail moment is a product of both, percentage DNA in the tail and tail length and so there is the possibility that induced effects may be masked. For example, if percentage DNA decreases and tail length increases, this would result in stable tail moment. Therefore, when using tail moment as a parameter of DNA damage, data on tail length and percentage DNA in the tail should also be provided (Tice et al., 2000).

Banu et al (2001) administered mice orally 0, 0.13, 0.27, 0.54, 1.08, 2.15, 4.3 and 6.45 mg/kg body weight of arsenic trioxide and collected blood samples at 24, 48, 72h, first and second week post treatment. Arsenic trioxide from 0.13-2.15 mg/kg body weight induced significant increase in comet tail length at 24h post treatment and a dose dependent decrease in higher doses (4.3-6.45 mg/kg b.wt). They observed that all doses caused an increased in comet tail length at 48h post treatment and a gradual decrease in this parameter for all doses at 72h post treatment, indicating a non-linear dose and time response relationship between DNA damage and different doses of arsenic trioxide at different time intervals. In the present study significant increase in comet tail length was seen in arsenite treatment group particularly at high dose level. Current investigation suggested the relationship between arsenite treatment and DNA damage in epididymal sperms.

In the current study Sodium arsenite (50ppm, 100ppm and 200pp) treatment caused significant decrease in mean comet head DNA percentage in rats compared to control.

Mean comet head DNA percentage showed highest decrease in sperms of rats treated with 200ppm sodium arsenite. Olive moment increased non-significantly in 50ppm group while this increase was highly significant in 100 and 200ppm groups, being highest in 200ppm sodium arsenite treated rat sperm. Ahmed et al., 2007 noticed that tail related parameters, such as, comet length, tail length, comet tail DNA percentage, tail moment and olive tail moment, reflected the sperm DNA damage whereas, comet head DNA percentage and intact DNA number represented the sperm DNA integrity.

Arsenic promotes DNA damage by inhibiting the enzymes involved in DNA repair, thus leading to the alteration of DNA replication and repair mechanism (Snow, 1992). Trivalent arsenic metabolites are capable of interacting with cellular targets inducing DNA damage including double strand breaks (Kitchen and Ahmad, 2003; Wanibuchi et al., 2004).

DNA single strand breaks in the male germ cell could be detected only in the presence of proteinase K. It has been suggested that DNA damage may arise from abortive apoptosis within the germ cells of the testis (Sakkas et al., 1999), or this is a post testicular effect of free radicals generated within the reproductive tract (Aitken, 1999; Yamanaka et al., 2002). In the current investigation the DNA damage caused by sodium arsenite may be due to:

(i) Inhibition of various enzymes involved in DNA repair and expression,

(ii) and possibly by; induction of reactive oxygen species (ROS) capable of inflicting DNA damage.

Oxidative stress effects the integrity of sperm chromatin and causes high frequencies of single and double stranded DNA breaks(Aitken and Krausz, 2001; Saleh et al., 2003). There is significant positive correlation between sperm DNA fragmentation and the level of reactive oxygen species in testicular tissue (Rajesh et al., 2002) and in semen (Barroso et al., 2000; Henkel et al., 2003). Arsenic cause chromosomal damage but unable to cause direct mutations, led to the concept that arsenic promotes DNA damage by inhibiting DNA repair. Snow (1992) reported that arsenic inhibited the enzymes involved in repair and thus led to the alteration of DNA replication and repair mechanism. Inorganic arsenic induces gene expression of a number of stress proteins such as ubiquitin which resulted in altering the DNA repair mechanism causing DNA damage (Bond and Schlesinger, 1985;

Parag et al., 1987). Ascorbic and folic acid provide a major role against metal induced oxidative stress sperm damages (Acharya et al., 2002; Yousef et al., 2006). Glutathione (GSH) also provides major protection against metal toxicity (Uckun et al., 2002). Depletion of glutathione below a critical value increases lipid peroxidation evoked by endogenous substances. It results in oxidative stress leading to induction of ROS that play a key role in damaging DNA. Inorganic arsenic is reported to deplete cellular glutathione levels and induce oxidative stress (Matsaui et al., 1999). According to Hei et al (1998); Nordenson and Beckman (1991) inorganic arsenic is responsible for DNA damage by inducing ROS in mammalian cells. The DNA damage in the present case may also be due to the depletion of glutathione and with resultant induction of oxidative stress.

From the present study it may be suggested that sodium arsenite treatment impairs male reproductive system. It is concluded that sodium arsenite adversely affected the body and organ (testes, epididymes and vas deferens) weight, size and their structural and functional variations.

CONCLUSION:

The body weight gain decreased dose dependently in treated animals through out the study period. Sodium arsenite treatment impairs the structure and function of male reproductive organs (testis, epididymis and vas deferens). Daily sperm production and epididymal sperm count also affected badly by sodium arsenite treatment. With the increased doses of sodium arsenite, there was increase in deposition of arsenic in testes, epididymes, vas deferens and blood of treated rats. DNA damage in the epididymal sperms was noticed by comet assay. The effect was more pronounced at high dose level.

All of the above parameters are possible factors causing infertility so arsenic was proved to cause infertility not only in animals but also in human population as well. Thus arsenic in drinking water is a huge threat to growing age organisms. Arsenic is toxic at high dose level as well as low dose levels. It cuases adverse effects on general health as well as reproductive health of humans.

Current investigation suggested arsenic as a major reproductive toxicant and an endocrine disruptor. The current study was designed to notice the reproductive toxicity of arsenic in

Current investigation suggested arsenic as a major reproductive toxicant and an endocrine disruptor. The current study was designed to notice the reproductive toxicity of arsenic in growing age animals so arsenic is a huge threat for the growing age animals as well as humans.

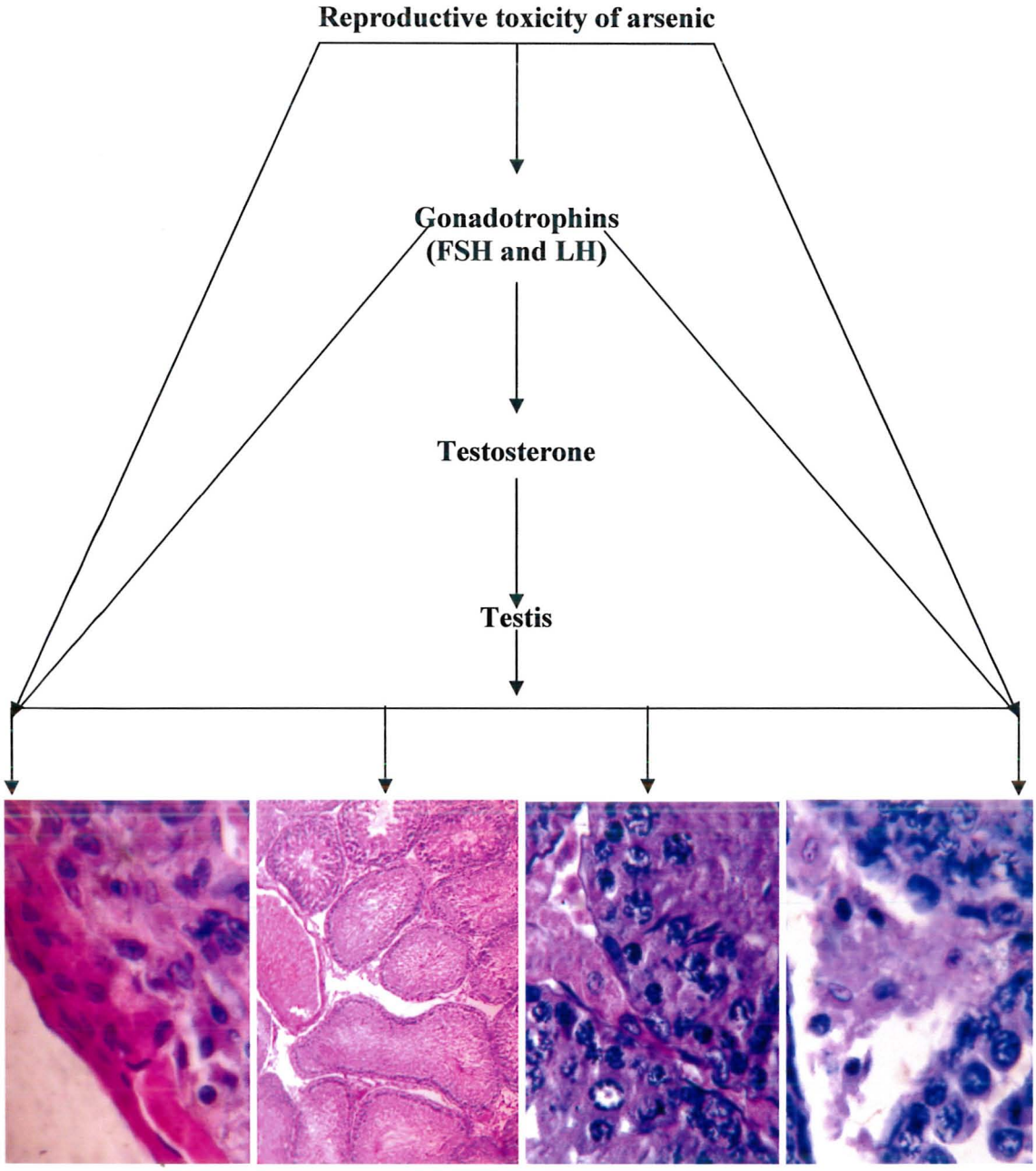

Fig. 33. Reproductive toxicity of arsenic causes reduction in gonadotrophins which in turn decreases testosterone secretion. Testicular histology is affected by testosterone as well as gonadotrophins directly. Arsenic treatment causes decrease in testicular capsule thickness, seminiferous tubules diameter, impair spermatogenesis causing decrease in spermatogenic, Leydig and Sertoli cells and fragmented primary spermatocytes. The inter tubular areas also enlarged and some atretic tubules are observed in high dose treated group.

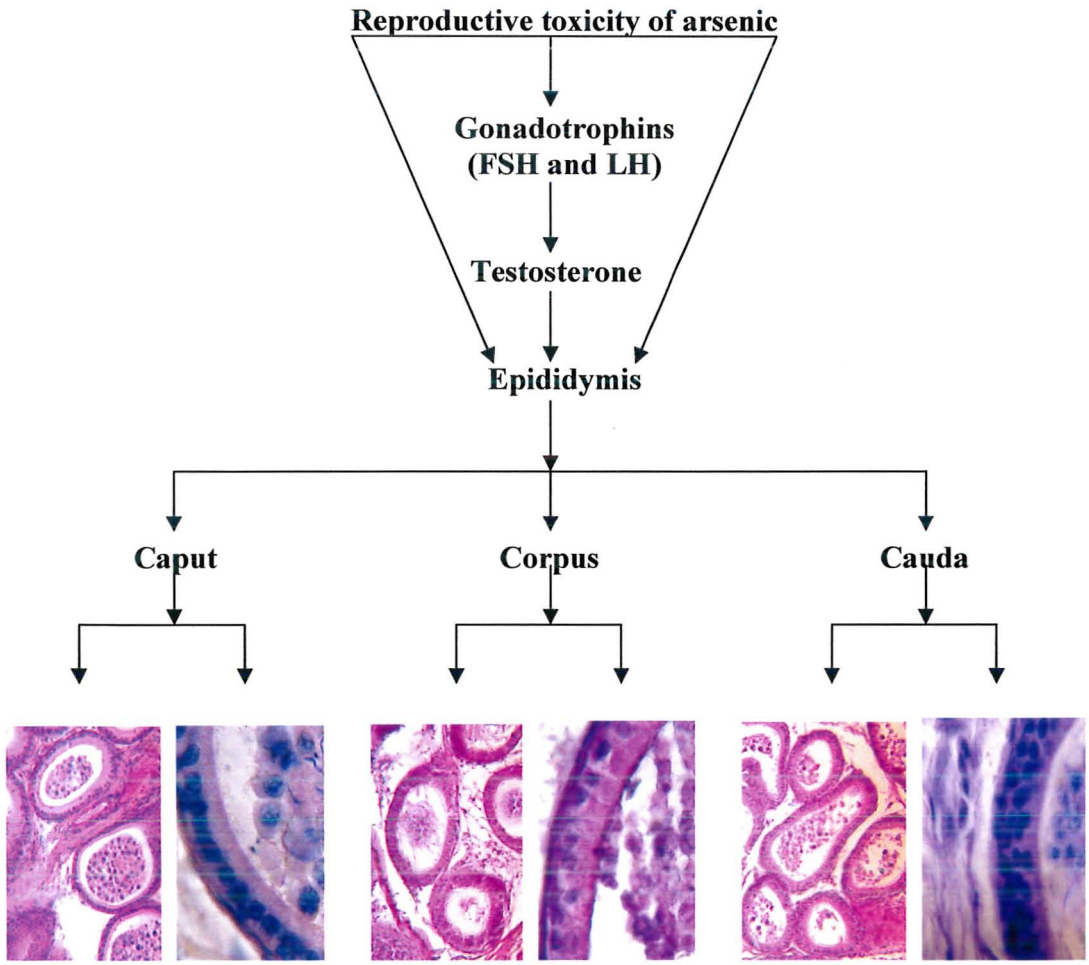

Fig. 34. Reproductive toxicity of arsenic causes reduction in gonadotrophins which in turn decreases testosterone secretion. Epididymis is an androgen dependent organ. Arsenic treatment causes decrease in tubule diameter and extension in inter tubular spaces. There is also observed disarrangement of epithelial cells. The size and number of cilia also reduced. Some atretic tubules and cellular debris in tubular lumen of the three parts of epididymis are observed in high dose treated group.

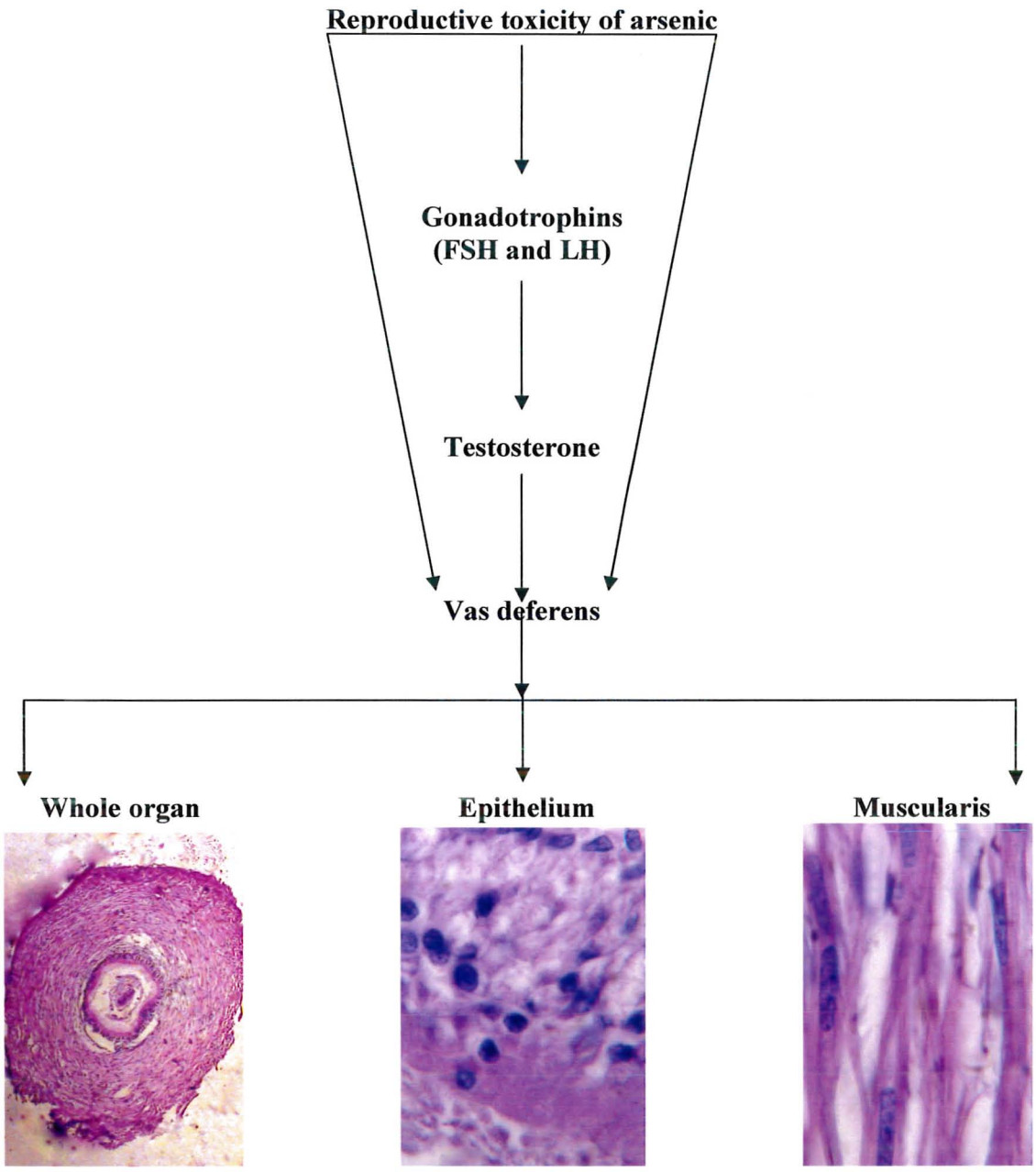

Fig. 35. Reproductive toxicity of arsenic causes decrease in gonadotrophins (LH, FSH) which in turn reduces testosterone secretion. Vas deferens is an androgen dependent organ so arsenic causes reduction in size, epithelium and loosening of muscularis. The arrangement, shape and size of nuclei of principal and basal cells are also effected by arsenic treatment particularly at high dose level.

FUTURE PERSPECTIVES:

Arsenic toxicity has been proved in the present investigation, however there are several parameters which are not clear and may be elucidated in future as:

Arsenic adversely affects steroid hormones as well as gonadotropins so the actual mechanism should be investigated. The proteins and enzymes involved in control of these hormones should also be studied. Arsenic cause oxidative stress at cellular level, its exact mechanism, biochemical study as well as means of its detoxification should be investigated. Molecular level investigation of arsenic should be done. The electron microscopy of the targeted organs should be done at different age levels. Arsenic alters gene expression so genomic analysis should be done.

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