Assessment of male reproductive dysfunction induced by oral subchronic exposure to heavy metals in Sprague Dawley rats.

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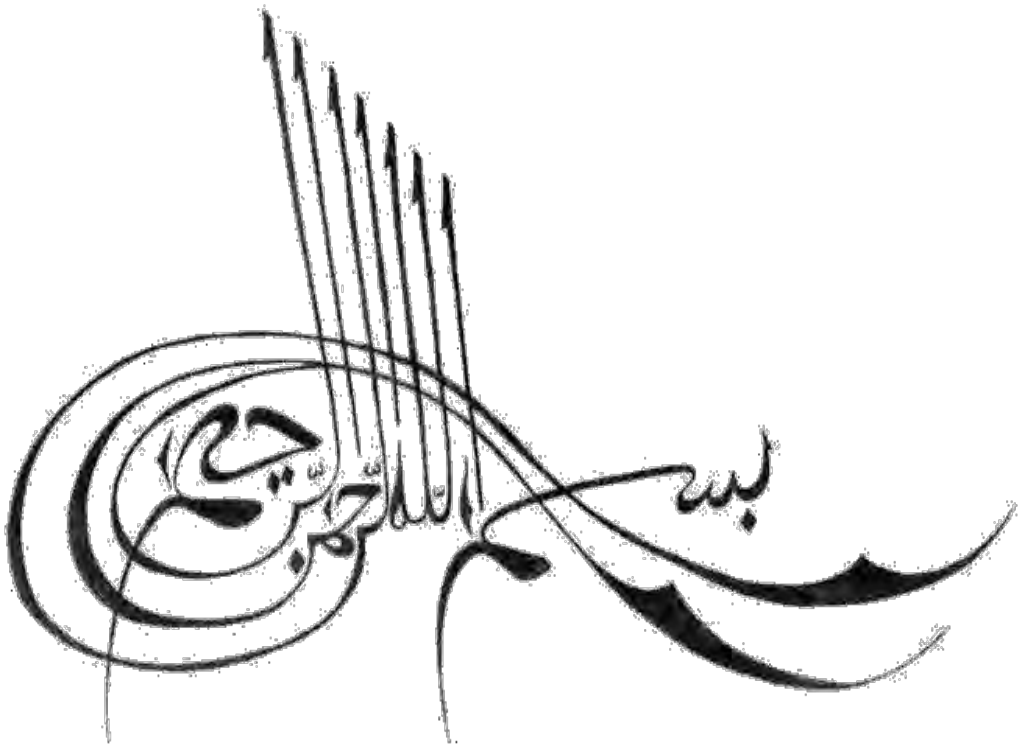
بسم الله الرحمن الرحيم

Dedication

When it comes to dedicate your work, it must always been the one who added much to your stamina and kept your faith and spirit high while working. I have tried my level best to produce and introduce a work that could be helpful to science and its learners, which obviously could not have been possible without my parents, siblings and teachers' support who believed in me and kept me high in spirits to produce this outcome. I would like to dedicate my work to my parents and teachers who bestowed me with their care and gave me the courage and awareness to follow the best possible route to produce a work which could enlighten the way for anyone who may need guidance in science. As I feel the need to give back for what has been taken, I dedicate this work of science to my teachers and my parents who are my first teachers.

Hafsa Yaseen

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Hafsa Yaseen

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List of abbreviations

BTB	Blood-testis barrier
CAT	Catalase
ELISA	Enzyme Linked Immunosorbent Assay
EIA	Enzyme immunoassay
HPG axis	Hypothalamic-pituitary-Gonadal axis
HPT axis	Hypothalamic-pituitary-testicular axis
HRP	Horse reddish peroxidase
OECD	Organisation for Economic Co-operation and Development
POD	Peroxidase
ROS	Reactive Oxygen Species
SOD	Super oxide dismutase
T	Testosterone
T-BARS	Thiobarbituric acid Reactive Species

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Abstract

Increase in male factor infertility is a rapidly emerging concern throughout the world, while in Asia alone, 37% of the couple infertility cases have been attributed to the male factor. Among the causes driving this reduction in male fertility are environmental pollutants, including heavy metals. Because of their extensive usage in many industrial productions, the environment is excessively exposed to heavy metals. The current study was designed to assess and compare reproductive toxicity of lead, cadmium, and arsenic in male rats, using doses simulating both environmental and occupational exposure. Stock solution of each metal, 2.25 and 250mg/kg of lead, 1 and 5mg/kg of cadmium, and 2.5 and 40mg/kg of arsenic were prepared. In the current study adult male Sprague Dawley rats (n= 35) were divided into seven groups. First group served as control and received distilled water by oral gavage. Second and third groups received low and high dose of lead acetate while the other four groups received contrasting doses of cadmium chloride and sodium arsenite for twenty eight days. Body weight and glucose level was checked at 1st, 14th and 28th day of the experiment. Animals were sacrificed at day 29 post treatment. Testicular tissue and blood samples were taken for histology and biochemical analysis. Lead acetate (250mg/kg), cadmium chloride (5mg/kg) and sodium arsenite (40mg/kg) caused significant decline in body weight, testicular and epididymal weight, while the animals exposed to low doses of lead acetate and cadmium chloride depicted less pronounced effect on body and reproductive organ weight when compared to control. Exposure to high doses of each metal led to significant decline in plasma and intra-testicular testosterone concentration, however this decline was less marked in animals treated with low doses of each metal respectively. Histomorphological studies showed significant degenerative alterations in rat testes in animals treated with high dose of each metal, but with more pronounced effect in cadmium (5mg/kg) treated rats as compared to low dose groups of each metal, and high dose groups of lead and arsenic. The results of current study indicate that exposure to high dose heavy metals caused pronounced reproductive toxicity in adult rats' testis as compared to low doses. Furthermore, exposure to cadmium produced more prominent effect on rats' testes, even at lower dose, as compared to damage induced by lead and arsenic. It can be preconcluded that this toxicity might be due to higher cellular toxicity and endocrine disrupting capability of cadmium, mediated through multiple molecular pathways.

Introduction

Endocrine disrupting chemicals and infertility

Endocrine disrupting chemicals (EDCs) include those exogenous chemicals which hamper any characteristic of hormonal activity, usually producing estrogen-like and/or anti-androgenic effects. These compounds can interrupt many regular processes related to hormones, such as stimulation, inhibition, or elimination. Such interference may lead to several diminished physiological processes such as homeostasis maintenance and regulation of normal development (Kavlock *et al*., 1996). There exist two main classes of the diverse range of EDCs in the environment: natural (acquired via foodstuff) and synthetic (frequently include solvents used in industries and their derivatives) (Kabir *et al.*, 2015). Humans are generally exposed to EDCs either through their occupation or through the environment. While the EDCs in the environment are normally found in low concentrations, and may produce a slight effect on general health, however, contact with various EDCs can possibly lead to synergistic effect and have an adverse impact on reproductive vigor (Krieg *et al.*, 2016). Reproductive fitness can be greatly influenced through exposure to compounds used in agriculture and industrial processes. Furthermore, newly developed packaging materials, which are both more durable and less costly, are another major source of EDCs. The EDCs present a diverse toxicology, ranging from affecting the gonads directly to modulation of hormones of the hypothalamus-pituitary-gonadal axis. When exposed to some of these chemicals, organisms have been rendered infertile, or, in some cases, pathology has been observed to be transferred to the next generation (Jenardhanan *et al.*, 2016). EDCs produce their toxic effect by hindering the regular homeostatic pathways of hormones which stimulate normal development of tissues. The typical mode of toxicity in case of reproduction includes EDCs' interference with hormone-receptor binding, particularly the androgen (AR) or the estrogen receptor (ER). After the EDC binds to a receptor, it can initiate two types of reactions: a hormonal action known as an agonistic effect, and/or an absence of hormonal action called an antagonistic action.

According to the definition of infertility, provided by the latest international glossary

on infertility and fertility care, it is a condition described as the inability to attain a clinical pregnancy after one year of consistent, unprotected sexual intercourse or due to a deficiency in one's capability to reproduce, either as an individual or with his/her spouse. Whereas, in a definition given by the WHO, infertility has been defined as a disease which engenders incapacity as a diminishing of reproductive function (Zegers-Hochschild *et al*., 2017). In Asia, 37% of the couple infertility cases are due to the male factor (Agarwal *et al.*, 2015). Administration of infertility induced by EDC exposure leads to an ever-increasing requirement of medical funds. In the European Union alone, costs of EDC-related infertility care sum up to approximately €15 billion yearly (Hauser *et al*., 2015). Whereas, in the USA, there exists no assessed cost of treatment of male infertility resulting from EDC exposure. Illness related to EDC is very expensive generally, totaling upto US $340 and US $217 billion in the USA and the European Union respectively (Attina *et al*., 2016).

Heavy metals and male infertility

Heavy metals are a wide range of compounds, universal in nature, which affect general and reproductive health in various aspects. Many industries extensively use heavy metals and their derivatives, allowing the environment to be exposed to these contaminants in diverse ways, making them one of the most commonly contacted categories of environmental toxicants. Metals can be released to the environment from agricultural activities, present in pesticides contain some of these elements, including arsenic (As), cadmium (Cd), lead (Pb), chromium (Cr), and nickel (Ni) (Gundert-Remy *et al*., 2015). In a review carried out by Rehman *et al*. (2018), the level of heavy metals in water used for drinking, is not below the tolerable perimeter and a multitude of health issues are linked with this problem, in different areas of Pakistan. Exposure to heavy metals occurs through two major routes, i.e. ingestion and inhalation.

A global decline in human male fertility has been noted in the recent years (He *et al*., 2016). According to Bas and Kalender (2016), amid the major factors contributing to this decline are environmental contaminants including heavy metals. Further explained by Rana (2014), heavy metals negatively influence male reproductive health by affecting the reproductive glands and their hormones, directly or indirectly.

It has been reported in both *in vivo* and *in vitro* studies, that a number of heavy metals directly bind with estrogen and androgen receptors, as they possess strong estrogenic and androgenic abilities, leading to reduction in sperm concentration and motility. In a study conducted by Carette *et al*. (2013), exposure to Cr resulted in oxidative stress, by increasing reactive oxygen species (ROS) generation, DNA fragmentation, and blood-testis barrier disruption, hence inducing sperm apoptosis. The harmful impact of various other heavy metals on male reproductive health has been demonstrated by malformed testicular ultrastructure, seminiferous tubules diminution, and changes in interstitium, attributed to increased reactive oxygen species production and consequent damage to DNA (Morales *et al*., 2016; Da Silva *et al*., 2016; Lamas *et al*., 2015; Mukhopadhyay *et al*., 2013; Predes *et al*., 2010; Pandey *et al*., 1999).

Lead

Lead (Pb) is one of the most extensively encountered environmental and industrial pollutants, having wide global distribution (Ramah *et al*., 2015). Lead together with its compounds is significant yet hazardous environmental chemicals (Mahaffey, 1990). Lead is used in numerous industrial and mining activities due to its characteristic chemical and physical properties such as ductility, malleability, poor conductibility and corrosion-resistance, rendering it vastly useful. However, the primary cause for the persistence of lead in the environment is its non-biodegradable property. Due to this nature, lead and its compounds accumulate in the environment thereby instigating a lot of irreversible hazards. Humans are commonly exposed to Pb and its compounds through occupations related to lead. Even though extensive use of lead has been not regular in many countries, some major activities such as battery production and recycling, car repair, refining and smelting, consider its usage unavoidable. Hence, human exposure to toxicities induced by inorganic Pb and its compounds (i.e. plumbism) continues to be inevitable (Parvez *et al*., 2006). This widespread use of Pb causes increase in human exposure to this metal resulting in a heavy disease burden, excessively affecting the developing countries, mainly owing to absence of strict regulations and strategies (Lu *et al*., 2015; Gillis *et al*., 2012; Gump *et al*., 2011). Pb has been known to induce an array of biochemical, physiological, histological, and developmental abnormalities in both humans and

animals, ranging from the nervous system (Flora *et al*., 2006), liver (Kasten-Jolly *et al*., 2010), kidneys (Rastogi, 2008), to the reproductive system (Flora *et al*., 2011; El-Sayed *et al*., 2015).

Reproductive toxicity of lead

Exposure to lead or its compounds in high dose, via environment or occupation for an extended period of time, can result in harmful impact on the male reproductive health. Increased blood lead levels (BLL) have been observed among infertile men when compared to fertile individuals in various epidemiological studies (Pant *et al*., 2003). Vigeh *et al.* (2011) reports that occupational lead exposure at various BLLs upsets male reproduction by diminishing spermatogenesis, sperm morphology, motility, and functional parameters, resulting in lowered sperm density per unit of seminal fluid, hypospermia, oligozoospermia, and even teratospermia and immature sperm. Other studies demonstrate that higher seminal lead levels lead to male infertility by adversely affecting sperm quality in addition to production of reactive oxygen species and DNA damage (Taha *et al*., 2013; Guzikowski *et al*., 2015). Batra *et al*. (2001), observed a dose-dependent decline in the activity of enzymes; alkaline phosphatase and sodium-potassium ATPase in both testes and epididymis, disrupting spermatogenesis in rat testis. Studies propose that lead exercises its neurotoxic action mainly at hypothalamus level (Sokol, 1987), and impairs mRNA expression of gonadotropin-releasing hormone (GnRH) after prolonged exposure to lead at low levels (Sokol *et al*., 2002). When the epidemiological studies are considered, the impact of lead exposure on serum testosterone concentrations can be termed as rather inconclusive. The serum testosterone levels, in occupationally Pb-exposed men, have been stated to be either decreased or increased with increasing lead exposure, without any detected relationship in other studies. Nonetheless, general population exposed to Pb through the environment presents positive correlation between blood lead levels and serum testosterone levels (Chen *et al*., 2016; Hosni *et al*., 2013; Haghighi *et al*., 2013; Yu *et al*., 2010). It has been further pronounced that chronic exposure to low levels of inorganic Pb could have greater damaging and permanent effects as compared to acute exposure to high Pb levels. While the exact pathogenic pathways have not been fully understood, it is evident that lead causes infertility by mediating

its toxicity via a pleiotropic, dose-dependent suppressing influence on the hypothalamic-pituitary-testicular (HPT) axis, sperm synthesis/maturation, and sperm function, eventually disrupting function at all three levels (Gandhi *et al*., 2017).

Cadmium

Another toxic heavy metal and a major pollutant of the environment is cadmium (Cd). According to WHO, (2011) and Agency for Toxic Substances and Disease Registry (ATSDR, 2008), Cd exposure of the general population occurs through Pb present in food and drinking water, while exposure through occupation usually takes place via mining activities and pigments/batteries industries that use cadmium. Cd is also discharged to the environment as cadmium sulfide, cadmium oxide, or cadmium chloride through manufacturing activities, including metal refining and smelting, and incineration for municipal waste management. ATSDR report (2008) reported that the average cadmium levels in the atmosphere and drinking water were observed to be ~0.04μg/m^3, which is less than 1μg/L, hence not alarming. On the other hand, an average individual acquires ~1μg cadmium/day via foodstuff, whereas an extra 1–3μg of cadmium is acquired by smoking a pack of cigarettes (20 cigarettes per day), leading to heavy smokers having more than twofold the cadmium load on the body (Waalkes *et al*., 2000). All organisms are exposed to an acute problem as heavy metal emissions to the environment continue to increase. Due to many unidentified biological functions, several sources and a long half-life, Cd induces toxicity of a wide range of organs (He *et al*., 2005; Shallari and Schwartz, 1998; Swarup *et al*., 2007; El-Sayed *et al*., 2016; Morais *et al*., 2012). Additionally, it has been verified that Cd induces carcinogenesis of many organs such as liver, kidney, pancreas, and prostate (Waalkes *et al*., 2000; Thompson and Bannigan, 2008; Goyer *et al*., 2004). Moreover, International Agency for Research on Cancer (IARC) recognized Cd as an identified human carcinogen in 1993. Cadmium is also ranked in the priority list of hazardous substances of the ATSDR (2007) as the 7th toxicant. In the recent years many efforts have been made by the governmental and international organizations to regulate and reduce the cadmium exposure to the general population. Nonetheless,

WHO (2011) reports that Cd accumulates in the body for an extended time period, predominantly in the liver and kidneys, due to its long biological half-life i.e. ~20–40 years in man. Thompson and Bannigan (2008) have further observed that the reproductive organs (ovary and testes of adults) and embryos are more sensitive to Cd toxicity and are severely damaged when exposed to Cd.

Reproductive toxicity of cadmium

Cd has toxic effects on several target tissues including the reproductive organs, and also possesses endocrine disrupting potentials (Satarug *et al*., 2003; Maciak *et al*., 2011; Abarikwu *et al*., 2013). Cd exposure has been linked to an extensive range of toxicities, and its ability to induce infertility and male reproductive function impairment is reported in mice and rats as animal models (Monsefi *et al*., 2010). Pandya *et al*. (2012) describes that Cd and lead co-exposure induces gross changes in the male reproductive system including reduced weights of reproductive organs, alterations in the histopathology of testis and accessory reproductive organs, altered semen quality parameters and hormonal imbalance as steroidogenesis is compromised. Cd-induced male infertility is also proposed to be indirectly mediated by oxidative stress, which is detected through reduced antioxidant enzymes' activity along with higher lipid peroxidation rate (Meeker *et al*., 2008; Monsefi *et al*., 2010). Furthermore, it was reported by Zhang *et al*. (2003) that Cd exposure leads to changes in multiple genes' expression via various signaling pathways leading to oxidative stress. Similar observations were reiterated by Adamkovicova *et al*. (2016) and Ansa *et al*. (2017). de Angelis *et al*. (2017) also defined multiple mechanisms involved in mediating cadmium-induced male reproductive toxicity, such as structural impairment of the testis vasculature and blood-testis barrier, Sertoli and Leydig cells cytotoxicity, inflammation, oxidative stress (chiefly via mimicry and interfering with essential ions), cell death, epigenetic regulation of genes that monitor reproductive function, interference with particular cell signaling pathways, and disruption of the Hypothalamus-Pituitary-Gonadal Axis (HPGA).

Arsenic

Arsenic (As) is another important toxic heavy metal and a natural pollutant (Jana *et*

al., 2006; Li *et al*., 2012). Numerous sources have been ascribed to increase As concentration, such as manufacturing of herbicides, agricultural pesticides, rodenticides, glass, food additives, wood preservatives, and metallurgical industries (Ferreira *et al*., 2012; Pant *et al*., 2001; Fouad *et al*., 2015; Baltaci *et al*., 2016; Zubair *et al*., 2016). Exposure to humans also occurs mainly via atmosphere, food and water. Inorganic As has been documented as a human toxin for a long time. It has been placed among the top hazardous elements by ATSDR (1999) and reported to be carcinogenic for a number of organs, including lungs, bladder, liver, skin, kidney, prostate and uterus (Tsai *et al*., 1998; Reddy *et al*., 2011; Ferreira *et al*., 2012; Jana *et al*., 2006; Sarkar *et al*., 2008). Moreover, As is associated with a number of metabolic illnesses including cardiovascular diseases, GIT ailments, and diabetes, and may lead to hypertension and hyperkeratosis (Jana *et al*., 2006; Sharma and Kumar, 2012). Furthermore, it has been associated with impairment of the respiratory, gastrointestinal, cardiovascular, and hemopoietic systems (Chen *et al*., 1996; Engel and Smith, 1994; Winski and Carter, 1998), reproductive dysfunction (Golub *et al*., 1998) and the skin diseases (Lin *et al*., 1998; Bernstam and Nriagu, 2010). A few recent studies have pointed out that As absorbed through drinking water leads to oxidative stress (Reddy *et al*., 2011; Sharma and Kumar, 2012; Sarkar *et al*., 2008). Amongst some 20 As species naturally present in the environment, five are catergorized as highly important, extensively studied, poisonous, and most common. The As species are arsenobetaine (AsB), dimethyl-arsenic (DMA), monomethyl-arsenic (MMA), arsenate (As^{5+}), and arsenite (As^{3+}), stated in order of increasing toxicity (Batista *et al*., 2011).

Reproductive toxicity of arsenic

As is another EDC which adversely effects the male reproductive health. Arsenic-induced male reproductive toxicity has been explained in many epidemiological researches, and linked with erectile dysfunction, altered semen quality parameters, and a positive association with the risk of unexplained male infertility (UMI) (Hsieh *et al*., 2008; Wang *et al*., 2016; Xu *et al*., 2012). Oxidative damage post short-term arsenic exposure has also been noted in the testes of rodent models (Guvvala *et al*., 2017; Das *et al*., 2009). The impairment of the male reproductive system due to As

has been proposed to occur via various mechanisms. Primarily, the metabolites of As amass in kidneys, liver, and blood and increase reactive oxygen species synthesis, causing toxicity at the systemic level, distressing the testes (Thomas *et al*., 2001). Secondarily, As might disturb the hypothalamic-pituitary-gonadal axis (HPG) and alter the plasma levels of leautinizing and follicle stimulating hormones, damaging the function of Leydig cells and diminishing steroidogenesis; and lastly, As can induce a direct inhibitory effect on the testes (Kim and Kim, 2015). Furthermore, multiple studies depict that arsenite exposure can impede normal sperm production and development. As is also linked with inhibiting androgen synthesis in the testes and therefore, reduce reproductive organs weight (Chang *et al*., 2007; Fouad *et al*., 2015; Ferreira *et al*., 2012; Li *et al*., 2012; Jana *et al*., 2006).

Purpose of the study

While aforementioned studies have concentrated on the consequences of heavy metal exposure on male reproductive health and spermatogenesis, data existing in literature is mostly partial and inconsistent. Various factors have been suggested that may interfere with results, including the type of heavy metal compound, its dosage, exposure period, dose administration method, animal model, and the age of animals. According to Nain and Smits (2012), rat has been valued model for examining the effects of heavy metals as their metabolism, drug distribution, and elimination functions comparable to those in man.

The present study aims to determine the possible consequences sub-chronic low and high doses (simulating environmental and occupational exposure) of cadmium chloride, lead acetate and sodium arsenite exposure via oral gavage on the reproductive parameters in male rats. The effects of lead, cadmium, and arsenic on male reproductive system of male rat is not yet entirely comprehended concerning its histology and stereology; therefore, it is essential to assess likely alterations in these factors under simulated environmental and occupational exposure conditions.

Aims and objectives

The main objective of the current research is to study the comparative effects of different doses of three heavy metals (lead, cadmium and arsenic) on male

reproductive system of rats and to determine the influence of heavy metals exposure on the oxidative stress markers (ROS and TBARS) and antioxidant enzyme status (SOD, POD and CAT) of testicular tissues of rats. Furthermore, the effect of the above-mentioned heavy metals on the synthesis and release of reproductive hormones of male rats was also evaluated. The comparative effect of heavy metals on the histopathology of male reproductive system was also checked.

Materials and methods

Current study was completed in the Laboratory of Reproductive Physiology, Animal Sciences department, Quaid-i-Azam University, Islamabad, Pakistan. Animal handling and all experimental procedures were assessed and permitted by the ethical committee of the department which is research specific in animal handling. The recommendations for the appropriate maintenance and usage of research lab animals were considered while performing all the processes in the study.

Experimental animals

Thirty-five male adult Sprague Dawley rats (*Rattus norvegicus*) of pubertal age (weight 100-150g) were acquired from the primate/rodent facility of Biological Sciences Faculty, Quaid-i-Azam University, Islamabad, to be used for the present research. Animals were separated randomly into seven groups having five animals each (n=7) and housed separately in stainless steel cages. A temperature of 22-25^{o}C and a 12-h light/dark cycle was kept in the rat house. Standard rodent feed and water were presented ad libitum. Before the start of the experiment, the rats were allowed to properly acclimatise to the environment for a minimum of 1 week.

Heavy metal salts

Salts of heavy metals viz., lead acetate (Pb), cadmium chloride (Cd), and sodium arsenite (As), were used for the experiment. These salts were selected for this study as they represent the most common form of the respective heavy metal to which humans are exposed in the environment. Lead acetate ($Pb(CH_3COO)_2$, mol. wt. 379.33 g/mol, 99.999%, CAS No. 6080-56-4), cadmium chloride ($CdCl_2$, mol. wt. 183.32 g/mol, 99.99, CAS No. 10108-64-2) and sodium arsenite ($NaAsO_2$, mol. wt. 129.9 g/mol ≥90%, CAS No. 7784-46-5). All three compounds were obtained from Sigma-Aldrich Chemical Co., USA.

Selection of heavy metal dosage

The dosage of heavy metal salts to be administered by oral gavage to the male rats corresponded to the lowest and highest doses of the heavy metals previously used for subchronic ex- posure studies in rat model. Low doses represent the environmental exposure and high doses represent occupational exposure. Following doses were chosen for assessment of low and high exposure to heavy metals;

Lead acetate:

The daily oral dose of 2.25mg/kg body weight of $Pb(CH_3COO)_2$ was selected for low level subchronic exposure to lead acetate (Godspower *et al*., 2015).

For high level subchronic exposure, a dose of 250mg/kg body weight $Pb(CH_3COO)_2$ was selected following Aliyu *et al*. (2017).

Cadmium chloride:

A dose of 1mg/kg body weight $CdCl_2$ was used for low level subchronic exposure to cadmium chloride, as suggested by Elblehi *et al*. (2019).

For high level subchronic exposure to cadmium chloride, a daily dose of 5mg/kg body weight $CdCl_2$ was chosen (El-Demerdash *et al*., 2004).

Sodium arsenite:

A daily oral dose of 2.5mg/kg $NaAsO_2$ was selected for low level arsenic exposure (Oyeronke, 2018).

For high level subchronic exposure to arsenic, a daily dose of 40mg/kg body weight $NaAsO_2$ was selected as used by Oyagbemi *et al*. (2017).

Preparation of heavy metal stock solutions

Solutions of heavy metal salts were prepared in distilled water according to the body weight of experimental animals. Following concentrations of heavy metal solutions were prepared to obtain the above-mentioned dosages:

Lead acetate (low dose):

$Pb(CH_3COO)_2$ (0.52g) was added to distilled water to make the final volume of 1000ml.

Lead acetate (high dose):

$Pb(CH_3COO)_2$ (14g) was added to distilled water and final volume was made to 500ml.

Cadmium chloride (low dose):

$CdCl_2$ (0.225g) was added in distilled water to make the final volume of 1000ml.

Cadmium chloride (high dose):

$CdCl_2$ (2.4g) was dissolved in water and final volume was made to1000ml.

Sodium arsenite (low dose):

$NaAsO_2$ (0.66g) was added in distilled water to make the final volume to 1000ml.

Sodium arsenite (high dose):

$NaAsO_2$ (5.52g) was added to distilled water and volume was set upto 1000ml.

The solutions were filtered thrice using Whatmann filter paper No 1 to eliminate any remaining impurities. Each solution was sonicated for 1 hour to make homogenous solutions of heavy metal salts and kept in refrigerator. In order to lower oxidation prior to usage, all solutions were prepared afresh every week using distilled water. Analytical grade chemicals were used throughout the experiment.

Experimental design

The current experiment aimed to examine the reproductive toxicity of three heavy metals (Pb, Cd, and As) on male rats. All animals used in this experiment were divided into seven groups with five male rats in a single group. The first group served as control while remaining groups were exposed to low and high doses of lead acetate, cadmium chloride, and sodium arsenite via a gastric feeding tube. All procedures were performed according to guidelines of OECD. The experimental design is presented in Figure 1.

The animal grouping was done as follows:

Group I: In the control group, animals were administered a daily dose of physiological saline (0.9% NaCl) by oral gavage throughout the experiment.

Group II: Animals were orally administered with low dose of lead acetate i.e. 2.25mg/kg $Pb(CH_3COO)_2$.

Group III: Rats received high oral dose of lead acetate i.e. 250mg/kg $Pb(CH_3COO)_2$.

Group IV: Animals were orally provided with low dose of cadmium chloride i.e. 1mg/kg $CdCl_2$ dissolved in distilled water.

Group V: Rats of this group were orally given high dose of cadmium chloride i.e. 5mg/kg $CdCl_2$ dissolved in distilled water.

Group VI: Animals of this group were given low dose of sodium arsenite i.e. 2.5mg/kg $NaAsO_2$ dissolved in distilled water, through oral gavage.

Group VII: Rats of this group orally received high dose of sodium arsenite i.e. 40mg/kg $NaAsO_2$ dissolved in distilled water.

All the doses were administered orally between 10-11am for 28 consecutive days. Body weight and glucose level of each rat was checked at day 1^{st}, 14^{th} and 28^{th} of experiment.

At the cessation of the treatment schedule, rats from control and experimental groups sacrificed on 29th day of the experiment by decapitation.

Determination of body weight

Rats were weighed on day 1st, 14th and 28th of the experiment using top loading Sartorius Digital Balance (Germany).

Determination of glucose level in blood

Glucose in blood drawn from rats was noted using glucometer on day 1st, 14th and 28th.

Collection and analysis of organs

Rats were killed by cervical dislocation at day 29 of the experiment and blood (upto 5ml) was drained into heparinised tubes via cardiac puncture. Blood was then centrifuged at 3000rpm for 15 min to obtain the plasma, and kept at -20°C until analyzed. Immediately after blood collection, reproductive organs, including, testes and epididymis, were separated, and weighed after removal of accessory fatty tissues. Half of the reproductive organs were kept in 10% PBS formalin solution (pH 7.4) for 48 hours for histology (fixation step), whereas, the other half were kept at -80°C to be used later during analysis of oxidant/antioxidant markers.

Tissue Histology

Histology of testis and epididymis was carried out to measure the reproductive toxicity of low and high concentrations of lead acetate, cadmium chloride, and sodium arsenite in the rats. Once testes and epididymis were collected after dissection of rats, the processes done subsequently are as follows;

Fixation

Testicular and epididymal tissues were fixed in PBS formalin (10%) for 24-48 hours.

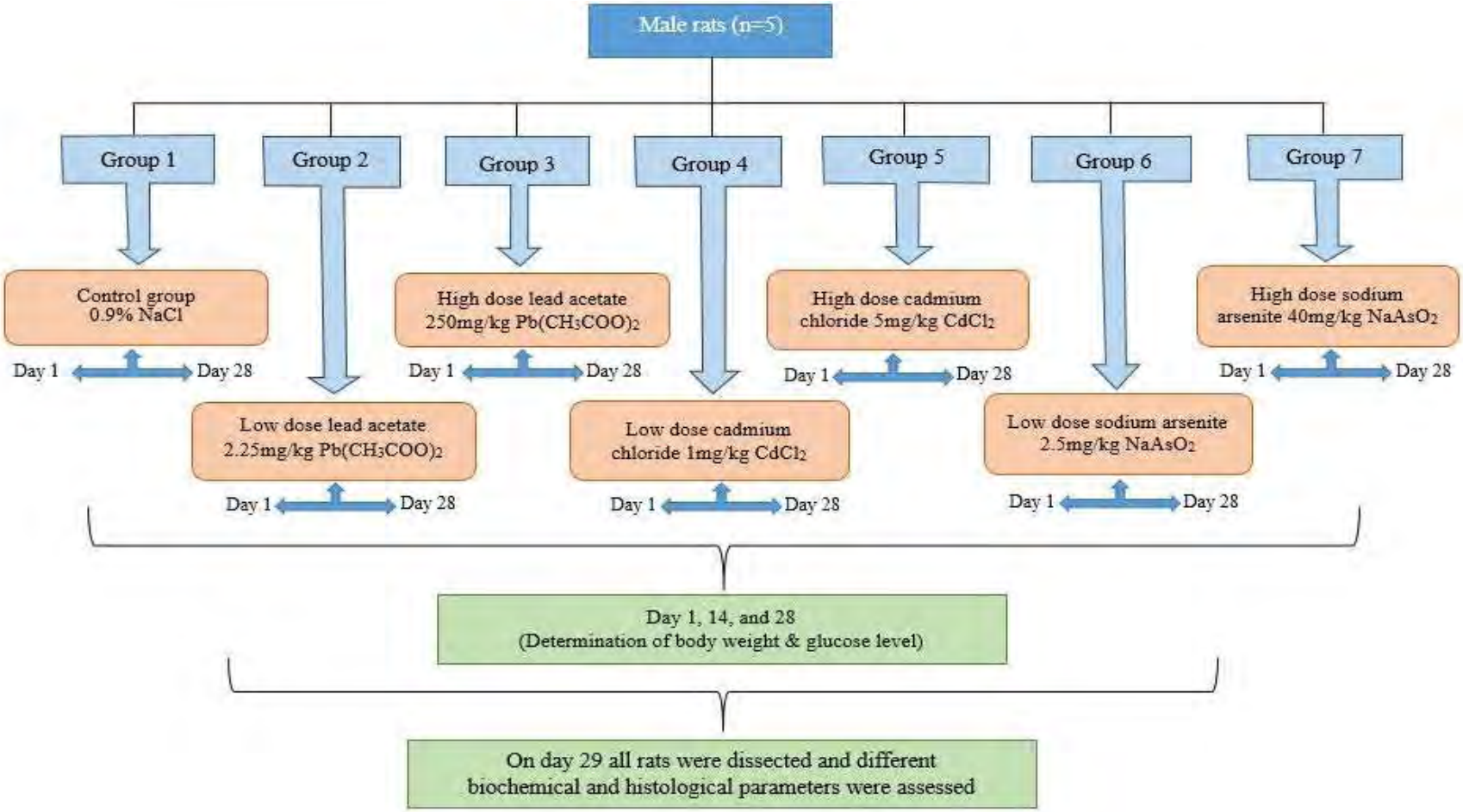

Figure 1: Schematic representation of oral sub chronic administration of different doses of lead acetate, cadmium chloride and sodium arsenite in adult male rats.

Dehydration

After fixing tissues in formalin, they were dehydrated in ascending grades of alcohol at room temperature

70% Ethanol ……120 min

80% Ethanol ……120 min

90% Ethanol ……120 min

100% Ethanol ……...3 changes (120 min each)

Embedding

The dehydrated tissues were then placed in xylene until they were cleared and fixed in paraffin as follows

Xylene I ---------- 60 min

Xylene I ---------- 60 min

Paraffin I (58 °C) ----------120 min

After embedding tissues were transferred to paper boats containing melted wax. After removing bubbles wax was left to solidify. Prior to mounting on wooden blocks for section cutting, a knife or scalpel was used for trimming the blocks of paraffin wax.

Preparing albumin slides

1. Albumin preparation
2. Two egg whites were added to 1200 mL deionized water.
3. Stirring on magnetic stirrer for 5 minutes.
4. Then, 4 mL concentrated ammonium hydroxide was added.
5. Again stirred for 5 minutes.
6. Filtering through a low grade filter (coffee filter).
7. Stored albumin in the dark, in a screw top glass bottle at 40 °C.

Coating slides

1. For coating slides, they were individually placed on a slide warmer at a low setting.
2. Using a clean small brush, a thin albumin layer was applied on each slide. The process was repeated at least thrice.
3. Slides were dried on the slide warmer.
4. Albumin coated slides were kept at room temperature, in the original packaging until needed.

Microtomy

Wooden blocks were fixed on microtome. 2-3 µm thin sections of tissues embedded

in paraffin were cut by microtome in which wooden blocks were placed (Shandon, Finesse 325, UK). The long ribbons of wax having tissues were stretched, following fixation on previously prepared albumenized glass slides. These slides were kept on Fischer slide warmer at 60 ^{o}C. Later, for full stretching, glass slides were kept in incubator for overnight.

Staining

For staining, following steps were carried out

Staining procedure

The slides were deparaffinized in xylene

1. Xylene I --- 3 min

2. Xylene II --- 3 min

Hydration

Sections were rehydrated in descending grades of alcohol.

3. 100% alcohol I --- 3 min

4. 100% alcohol II --- 3 min

5. 90% alcohol --- 3 min

6. 70% alcohol --- 3 min

7. Washed with water --- 3 min

Slides were stained in different grades as follows:

8. Hematoxylin --- 8 min

9. Washing --- 2 min

10. Acidified alcohol --- 1 min

11. Washing --- 2 min

12. Bluing solution (1mL NH4OH + 300mL water) --- 2 min

13. Washed with water --- 2 min

Dehydration

14. 90% alcohol --- 10 dips

15. Eosin ---2 min

16. Washed with water --- 2 min

17. 90% alcohol --- 5 min

18. Absolute alcohol I --- 5 min

19. Absolute alcohol II --- 5 min

20. Absolute alcohol III --- 5 min

21. Xylene I --- 5 min

22. Xylene II --- 5 min

After staining, 2-3 drops of canada bolsom were placed on the slides and concealed with xylene-dipped cover slips, and carefully positioned on the slides before placing them in incubator for one night.

Microscopy and Microphotography

Prepared slides were observed under light microscope (Leica LB Germany). 2-3 µm sections were observed at 10X, 20X, and 40X magnification. Following parameters were studied for histo-morphometric analysis, using image J 2x software package program; thickness of tunica albuginea, seminiferous tubular and luminal diameter, epithelial height of seminiferous tubules, and interstitial space of testes. Furthermore, epididymal tubular and luminal diameter, and epithelial height were also recorded. Leica LB microscope (Germany) paired with Canon digital camera (Japan) was used for microphotography of the sections.

Biochemical analysis

Analysis of antioxidant enzymes and cellular stress markers was done using testicular tissue of control and treated animals for the determination of oxidative stress. Frozen testicular tissue (90 mg) was thawed, homogenized in 3 ml of PBS (pH 7.4), and then centrifuged at 12000 rpm for half an hour at 4 °C. The resulting supernatant was separated for determination of antioxidant enzymes/oxidative stress markers in the tissue. This homogenate was utilized for assessment of CAT, POD, SOD, ROS and TBARS.

Catalase (CAT) activity

The method described by Aebi (1984) was used for the determination of catalase activity. This protocol is based on the principle that the rate of hydrogen peroxide breakdown by the catalase enzyme is directly proportional to the reduction of the absorbance at $\lambda = 240$ nm.

Procedure:

1. 0.1ml of testicular tissue homogenate.
2. 50mM PBS (2.5 ml) at pH 5.0.
3. 5.9mM H_2O_2 (0.4ml).
4. Thoroughly mix them and record their absorbance at 240nm at 0 second and 1 minute by using Smart Spec TM plus spectrophotometer.

5. One unit of CAT action is depicted by change in absorbance of 0.01 unit/minute.

Peroxidase (POD) activity

The spectrophotometric protocol initiallly described by Chance and Maehly (1955) was used to determine the Peroxidase (POD) activity.

Procedure:

1. 0.1ml of testicular tissue homogenate.
2. 0.1ml of freshly prepared (20mM) guaiacol.
3. 0.3ml of (40mM) H_2O_2.
4. 2.5ml of (50mM) phosphate buffer at a pH of 5.0
5. Note its absorbance at 0 second and after 1 minute at 470nm.
6. A change of 0.01 units per minute in absorbance was considered as one unit of POD activity. Its unit is mU/mg.

Superoxide dismutase (SOD) activity

Superoxide dismutase activity was examined in reference to the protocol established by Kakkar *et al.* (1984).

Procedure:

1. 0.3 ml homogenate.
2. 1.2 ml (0.052mM) sodium pyrophosphate buffer at pH of 7.0.
3. 0.1 ml (186µM) phenazine methosulphate.
4. 0.2 ml (780µM) NADH to start reaction.
5. 1.5 ml glacial acetic acid added after 1 minute to break the reaction.
6. Determination of absorbance at λ=560 nm.
7. Results stated as units/mg of protein.

Reactive Oxygen species (ROS) Assay

ROS were assessed in accordance with the method developed by Hayashi *et al.* (2007).

Procedure:

1. 4.1 g of Sodium acetate (CH_3COONa) were dissolved in 500 ml of distilled water to make sodium acetate (0.1M) buffer at pH 4.8.
2. 10 mg of N,N-Diethyl-p-phenylenediamine sulphate soil (DEPPD) was dissolved in CH_3COONa buffer (100 ml).
3. 50 mg of ferrous sulphate ($FeSO_4$) added to 10 ml of CH_3COONa buffer.

4. Both solutions taken in a ratio of 1:25, before incubation for 20 min in dark at normal room temperature.
5. 20 µl of the solution mixture, 1.2 ml of buffer and 20µl of homogenate were then dispensed them in cuvette.
6. Spectrophotometer was used to check the absorbance at λ=505 nm.
7. Take three consecutive readings for each sample then compute mean of them.

Thiobarbituric acid reactive substances (TBARS)

TBARS level was detected using the protocol developed by Wright *et al.* (1981) using the homogenate of control and treated animals' testes.

Procedure:

1. 0.02 ml (100mM), Ferric chloride ($FeCl_3$).
2. 0.2 ml (100mM) ascorbic acid.
3. 0.2 ml of homogenate sample.
4. 0.58 ml (0.1M) phosphate buffer with a pH value of 7.4.
5. The reaction mixture was kept in pulsating water bath for incubation at 37°C for 60 min.
6. 10% trichloroacetic acid (1 ml) used to halt the reaction.
7. 0.67% thiobarbituric acid (1 ml) added and placed in water bath at 95°C.
8. Transferred them to crushed ice bath to lower the temperature.
9. Centrifuged at 25000 rpm for 15 minutes.
10. The absorbance was noted at 535 nm and results were stated as µmol of TBARS/min/mg tissue at 37°C.

Protein Estimation Assay

Protein estimation kit, obtained from AMEDA Laboratory diagnostic GmbH (Krenngasse, Graz/Austria), was used to measure the protein content in testicular homogenate.

Principle of test:

Peptide bonds present in the protein form a chelate with the Cu^{2+} ions in alkaline solution which is gives a complex of violet color. The color intensity of the reation mixture is directly related to the quantity of protein existent in the sample.

Procedure

1. 40 µl of standard provided with kit was added with reagent (2.0 ml).
2. Six standards were prepared and 1 ml of each was taken and added to the reagent (1.0 ml).

3. To prepare standards of different concentrations, 1 ml of standard was mixed well in 1 ml of reagent (total volume 2.0 ml).
4. Only reagent was considered blank or 0 concentration.
5. Each standard had half the concentration of protein content present when compared to others.
6. Samples were prepared by adding 20µl of serum with reagent (1.0 ml).
7. Samples were incubated at 37°C for 10 minutes.
8. By using chemistry analyzer change in absorbance of standards and samples were noted at 546 nm.
9. The formula attained was used to calculate protein content as mg/mg of testicular tissue.
10. A linear graph was plotted with the absorbance of standards against the absorbance of samples to measure the protein contents of samples.

Hormonal analysis

Testosterone concentration in plasma was determined following the instructions provided by the manufacturers on the Enzyme Linked Immuno Sorbant Assay (ELISA) kit obtained from Amgenix, USA.

Test Principle

The testosterone enzyme immunoassay (EIA) is centered on the principle of competitive binding between the sample testosterone and testosterone-horseradish peroxidase (HRP) conjugate in the presence of a standard amount of rabbit anti-testosterone.

Procedure

1. 10µL of controls, standards and specimens were added to the required number of wells coated with goat anti-rabbit IgG antibody to determine testosterone concentration in the tissue.
2. Testosterone-HRP conjugate at a volume of 100 µL and rabbit anti-testosterone reagent (50 µL) were added in each well.
3. The plate was allowed to incubate at 37 °C for 90 min. This step is significant to allow the proper binding of sample testosterone and HRP-labelled testosterone with antibodies present in the well.
4. To remove unbound testosterone peroxidase conjugate all the wells were washed with distilled water (5 times) after incubation.
5. 100 µL of TMB-reagent was added into each well, allowed to mix for 10 min

and was kept in incubator for 20 min at normal room temperature. Afterwards stop solution (100 µL) was added to each well and mixed for 30 s. Microplate reader was used to read the absorbance at 450 nm.

6. The result was stated in ng/ml of serum and ng/g of testicular tissue.

Statistical analysis

Graph pad prism 5 Software was used to compare the values of control and experimental groups, applying one way analysis of variance (ANOVA). Dunnett's multiple comparison test was done post-ANOVA. Any value of $P<0.05$ was taken as statistically significant. Means ± standard errors of means (SEM) were calculated for all values.

Results

Effects on body weight

Mean ± SEM body weights of male rats treated with different concentrations of lead acetate, cadmium chloride and sodium arsenite are presented in Table 1. At day 14th of treatment, low dosage groups of lead acetate (2.25mg/kg), cadmium chloride (1mg/kg), and sodium arsenite (2.5mg/kg) showed no significant effect on body weight as compared to control. Whereas, high dosage groups of lead acetate (250mg/kg), cadmium chloride (5mg/kg), and sodium arsenite (40mg/kg) depicted significant effect ($P<0.05$) on body weight with reference to the control.

At day 28th of treatment, low doses of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) presented no significant effect on body weight as compared to control, but low dose group of sodium arsenite (2.5mg/kg) indicated significant decrease ($P<0.05$) in the rats' body weighs as compared to control. However, at day 28th, a significant decline ($P<0.001$) was observed in body weight of animals exposed to higher dose of sodium arsenite (40mg/kg) when compared to control. Similarly, a significant decrease ($P<0.01$) observed in the groups exposed to high doses of lead acetate (250mg/kg) and cadmium chloride (5mg/kg) when compared to control.

Effect on blood glucose level

The level of glucose in the blood of rats exposed to different concentrations of heavy metals (lead, cadmium and arsenic) is presented in Table 2. At day 14th of treatment, low doses of lead acetate (2.25mg/kg), cadmium chloride (1mg/kg), and sodium arsenite (2.5mg/kg) groups showed no significant difference in blood glucose level of the rats. However, significant diminution ($P<0.01$) was detected in blood glucose level of animals exposed to high dose of sodium arsenite (40mg/kg) as compared to control. Furthermore, significant decline ($P<0.05$) was noted in blood glucose level of animals exposed to high doses of lead acetate (250mg/kg) and cadmium chloride (5mg/kg) when compared to control. At 28th day of treatment low dose lead acetate (2.25mg/kg) exposure showed no significant difference in glucose level of male rats. However, low dose groups of cadmium chloride (1mg/kg) and sodium arsenite (2.5mg/kg) showed significant decline

($P<0.05$) in blood glucose level when compared to control.

At day 28^{th}, exposure to high dose (40mg/kg) of sodium arsenite showed significant rise ($P<0.001$) in glucose level of treated animals respectively. Similarly, at day 28^{th} high dose of lead acetate (250mg/kg) and cadmium chloride (5mg/kg) indicated significant decrease ($P<0.01$) in the blood glucose level of male rats as compared to control.

Table 1. Mean ± SEM body weight (g) at different days after administration of different doses of heavy metals (n=5 for all groups)

Treatment	Day 1	Day 14	Day 28
Control	117.8 ± 2.82	147.2 ± 4.10	174.4 ± 3.12
$Pb(CH3COO)_2$			
2.25mg/kg	116.2 ± 2.52	143.4 ± 3.11	169.2 ± 1.56
250mg/kg	115.8 ± 4.47	134.2 ± 1.93*	165.6 ± 1.03**
$CdCl_2$			
1mg/kg	116.8 ± 3.02	142.4 ± 3.31	168.8 ± 1.71
5mg/kg	117.2 ± 2.31	135.2 ± 1.77*	165 ± 1.30**
$NaAsO_2$			
2.5mg/kg	116.6 ± 2.34	139.2 ± 2.75	167.6 ± 0.81*
40mg/kg	116.2 ± 2.58	134.6 ± 1.57*	159.2 ± 0.97 ***

Values are expressed as Mean ± SEM.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Table 2. Blood glucose level (mg/dl) (Mean ± SEM) at different days after oral administration of sub-chronic doses of heavy metals (n=5 for all groups).

Treatments	Day 1	Day 14	Day 28
Control	118.8 ± 6.41	139.2 ± 2.97	143.8 ± 2.63
$Pb(CH3COO)_2$			
2.25mg/kg	115.6 ± 4.37	133.8 ± 1.88	138.4 ± 2.98
250mg/kg	116.4 ± 3.19	121.6 ± 2.32*	134 ± 2.10**
$CdCl_2$			
1mg/kg	119 ± 7.01	134.6 ± 3.08	135 ± 1.30*
5mg/kg $CdCl_2$	120.2 ± 5.69	124 ± 2.77*	133 ± 4.66**
$NaAsO_2$			
2.5mg/kg	119.6 ± 5.70	122.4 ± 4.23	137.0 ± 4.14*
40mg/kg	117.2 ± 4.33	120.2 ± 3.98**	119.6 ± 3.50***

Values are expressed as Mean ± SEM.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Effect of different doses of lead acetate, cadmium chloride, and sodium arsenite on antioxidant enzyme (CAT, POD, & SOD) activity, and oxidative stress markers (ROS & TBARS) in testicular tissues of rats

Mean ± SEM of the antioxidant enzymes activity and level of oxidative stress markers in testicular tissue of male rats treated with different doses of lead acetate, cadmium chloride, and sodium arsenite are presented in Table 3. No significant decrease was noted in the activity of CAT, POD and SOD in testicular tissues of rats exposed to low doses of lead acetate (2.25mg/kg) and sodium arsenite (2.5mg/kg). However, a significant decline ($P<0.05$) in CAT, POD and SOD activity was observed in testicular tissue of animals exposed to low dose (1mg/kg) of cadmium chloride.

After exposure to high doses of lead acetate, cadmium chloride, and sodium arsenite, a significant decrease was noted in activity of antioxidant enzymes and subsequent rise in ROS and LPO levels in the testes of treated rats. The activity of CAT, POD and SOD significantly declined ($P<0.001$) in testicular homogenate of animals exposed to high dose (5mg/kg) of cadmium chloride. Similarly, activity of CAT, POD and SOD was significantly reduced ($P<0.01$) in rats treated with high dose lead acetate (250mg/kg) and sodium arsenite (40mg/kg).

A significant increase ($P<0.01$) was detected in ROS in the testicular tissue of animals exposed to high concentrations of lead acetate (250mg/kg), cadmium chloride (5mg/kg), and sodium arsenite (40mg/kg). Similarly, activity of ROS in testicular tissue of animals exposed to low concentration (1mg/kg) of cadmium chloride indicated significant increase ($P<0.05$). Correspondingly, there was significant increase ($P<0.01$) in LPO in the testicular tissue of animals exposed to high concentrations of lead acetate (250mg/kg), cadmium chloride (5mg/kg), and sodium arsenite (40mg/kg). Likewise, significant increase ($P<0.05$) was noted in the testicular tissue of animals exposed to low concentration (1mg/kg) of cadmium chloride.

Effect of different doses of lead acetate, cadmium chloride, and sodium arsenite on weight of testis, epididymis, prostate, seminal vesicle, liver, kidneys, and heart

Testicular and accessory organs weight of male rats exposed to different doses of lead acetate, cadmium chloride, and sodium arsenite in Table 4. The weight of other body

organs i.e. liver, paired kidneys, and heart are presented in Table 5. Right testicular weight of animals treated with low dose of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) showed no significant difference. However, right testicular weight of animals treated with low dose of sodium arsenite (2.5mg/kg) reduced significantly ($P<0.05$). On the other hand, the right testicular weight of animals treated with high doses of lead acetate (250mg/kg), cadmium chloride (5mg/kg), and sodium arsenite (40mg/kg) was reduced significantly ($P<0.01$) when compared to control.

Left testicular weight in animals exposed to low dose of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) showed no significant effect of the treatment. However, left testicular weight in animals treated with low dose of sodium arsenite (2.5mg/kg) presented significant decrease ($P<0.05$) as compared to control. Corresponding to right testicular weight, significant decline ($P<0.01$) was also observed in left testicular weight of the animals treated with high doses of lead acetate (250mg/kg), cadmium chloride (5mg/kg), and sodium arsenite (40mg/kg) as compared to control.

Change in epididymis weight observed in animals exposed to low dose lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) was non-significant. However, animals treated with low dose sodium arsenite (2.5mg/kg) displayed significant decrease ($P<0.05$) in epididymis weight as compared to control. Likewise, significant decline ($P<0.001$) in epididymis weight was noted in animals exposed to high dose of sodium arsenite (40mg/kg). Similarly, Epididymis weight in rats treated with high dose lead acetate (250mg/kg) and cadmium chloride (5mg/kg) also showed significant decrease ($P<0.01$).

Weight of prostate and seminal vesicle in animals treated with low dose of lead acetate (2.25mg/kg), cadmium chloride (1mg/kg), and sodium arsenite (2.5mg/kg) showed no significant difference. However, weight of prostate and seminal vesicle in animals treated with high dose (40mg/kg) of sodium arsenite was significantly decreased ($P<0.01$). Significant decline ($P<0.05$) was also observed in weight of prostate and seminal vesicle of animals treated with high dose lead acetate (250mg/kg) and cadmium chloride (5mg/kg).

Liver, kidneys, and heart weight showed no significant difference in animals treated with low doses of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg). However, animals treated with low dose sodium arsenite (2.5mg/kg) indicated significant increase

(P<0.05) in liver weight, but no significant effect on kidney and heart weight. A significant increase (P<0.001) in liver and kidney weight was observed in animals treated with high dose of lead acetate (250mg/kg). Similarly, a significant elevation (P<0.05) in heart weight was also noted in animals treated with high dose lead acetate. Similarly, a significant increase in liver, kidney, and heart weight was indicated in rats treated with high dose (5mg/kg) of cadmium chloride (P<0.05) and (40mg/kg) sodium arsenite (P<0.01).

Effect of different concentrations of heavy metals (lead acetate, cadmium chloride, amd sodium arsenite) on plasma and intra testicular testosterone

Mean ± SEM of plasma and intra testicular testosterone levels (ng/ml and ng/g tissue) are presented in Table 6. Both serum and intra-testicular testosterone levels in animals exposed to low dose of lead acetate (2.25mg/kg), cadmium chloride (1mg/kg) and sodium arsenite (2.5mg/kg) were reduced significantly (P<0.05) when compared to control group. Whereas, serum and intra-testicular testosterone concentrations in animals exposed to high dose (40mg/kg) of sodium arsenite were reduced significantly (P<0.001) in reference to control. Likewise, a significant (P<0.01) reduction was observed in serum and intra testicular testosterone concentrations of male rats treated with high dose lead acetate (250mg/kg) and cadmium chloride (5mg/kg).

Table 3. Effects of oral administration of sub-chronic doses of heavy metals (lead, cadmium and arsenic) on CAT, POD, SOD, total ROS and TBARS level in male rats (n=5 for all groups).

Treatments	CAT (U/mg protein)	POD (U/mg protein)	SOD (U/mg protein)	ROS (U/g tissue)	TBARS (min/mg tissue)
Control	7.49± 0.30	9.13 ± 0.19	5.44 ± 0.22	1.57 ± 0.04	1.38 ± 0.02
$Pb(CH_3COO)_2$					
2.25mg/kg	7.14 ± 0.19	9.02 ± 0.16	5.02 ± 0.21	1.60 ± 0.03	1.39 ± 0.02
250mg/kg	6.54± 0.11**	8.41 ± 0.09**	4.25 ± 0.22**	1.71 ± 0.01**	1.47 ± 0.02**
$CdCl_2$					
1mg/kg	6.82± 0.12*	8.58 ± 0.09*	4.57 ± 0.20*	1.67 ± 0.01*	1.45 ± 0.02*
5mg/kg	6.25± 0.07***	8.20 ± 0.11***	4.06 ± 0.20***	1.70 ± 0.02**	1.46 ± 0.01**
$NaAsO_2$					
2.5mg/kg	7.11± 0.17	8.98 ± 0.16	4.82 ± 0.24	1.63 ± 0.04	1.39 ± 0.01
40mg/kg	6.55± 0.12**	8.46 ± 0.08**	4.31 ± 0.23**	1.72 ± 0.01**	1.47 ±0.01**

Values are expressed as Mean ± SEM.

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Histomorphometric analysis of testis

Effect of different doses lead, cadmium, and arsenic on morphology of rat testis and epididymis was examined through histological study (Table 7). Treatment with different doses caused degenerative alterations in seminiferous tubules of testis. Cross section of testicular tissue suggests shrinkage of seminiferous tubules producing large intersititium. Similar vacuolation of epithelial layer of testicular tissue was noted. However, no such histological changes were noted in control group.

Different parts of the testis sections were measured by image J software. Significant decline ($P<0.001$) was observed in tubular area of seminiferous tubules of animals exposed to high dose (5mg/kg) of cadmium chloride. Similarly, significant decrease ($P<0.01$) in seminiferous tubule area was observed in high dose group of lead acetate (250mg/kg) and both groups of sodium arsenite. Furthermore, low dose groups of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) also showed significant ($P<0.05$) decrease in seminiferous tubule area. However, interstitial space area increased significantly ($P<0.01$) in animals exposed to high dose of lead acetate (250mg/kg) and both doses of sodium arsenite. Likewise, there was significant increase ($P<0.001$) in interstitial space area in animals treated with high dose (5mg/kg) of cadmium chloride. Moreover, low dose groups of lead acetate (2.25mg/kg) and cadmium chloride (1mg/kg) also indicated significant ($P<0.05$) increase in interstitial space area.

Area enclosed by lumen of seminiferous tubule was significantly ($P<0.01$) high in animals treated with high dose of lead acetate (250mg/kg) and both doses of sodium arsenite. Similarly, area enclosed by lumen was significantly high ($P<0.001$) in animals exposed to high dose (5mg/kg) of cadmium chloride as compared to control. Likewise, area of epithelium and tubular diameter in animals exposed to high dose of lead acetate (250mg/kg) and both doses of sodium arsenite was reduced significantly ($P<0.01$). Similarly, area of epithelium and tubular diameter in high dose (5mg/kg) group of cadmium chloride also decreased significantly ($P<0.001$) in comparison to the control group.

Histomorphometric analysis of epididymis

Parameters studied in epididymal histology are given in Table 8. Exposure to different doses of lead, cadmium and arsenic produced changes in various variables of epididymal morphology. Furthermore, histology revealed decrease in concentration of sperm in the epididymal lumen of treated groups in comparison to the control group. Different parts of the epididymis sections were examined by image J software. Significant decrease ($P<0.01$) in ductular diameter of caput and cauda epididymis was observed in animals exposed to high doses of cadmium chloride (5mg/kg) and sodium arsenite (40mg/kg). Caput and cauda ductular diameter also revealed significant reduction ($P<0.05$) in high dose lead acetate-treated rats. However, low dose groups of cadmium chloride (1mg/kg) and sodium arsenite (2.5mg/kg) indicated significant ($P<0.05$) diminution only in caput ductular diameter.

Alternatively, significant reduction ($P<0.01$) was observed in lumen diameter of caput epididymis in animals treated with high doses of cadmium chloride (5mg/kg) and sodium arsenite (40mg/kg). Similarly, significant decrease ($P<0.05$) in caput and cauda lumen diameter was noted in high dosage group of lead acetate (250mg/kg). However, low dose groups of cadmium chloride (1mg/kg) and sodium arsenite (2.5mg/kg) indicated significant ($P<0.05$) reduction only in caput lumen diameter. Epithelial cell height of caput epididymis was observed to be reduced significantly in animals treated with high dose of cadmium chloride ($P<0.01$) and high dose groups of lead acetate and sodium arsenite ($P<0.05$). Similarly, epithelial cell height in cauda epididymis also reduced significantly ($P<0.05$) in animals treated with high (5mg/kg) of cadmium chloride as compared to control.

Table 4. Effect of oral administration of different doses of heavy metals (lead, cadmium, and arsenic) on reproductive organs weight.

	Reproductive organs weight					
Treatments (n=5)	**Right Testis (g)**	**Left Testis (g)**	**Paired Testes (g)**	**Epididymis (g)**	**Prostate (g)**	**Seminal Vesicle (g)**
Control	1.29 ± 0.02	1.29 ± 0.01	2.57 ± 0.03	0.74 ± 0.01	0.80 ± 0.01	1.04 ± 0.02
2.25mg/kg $Pb(CH3COO)_2$	1.26 ± 0.01	1.27 ± 0.01	2.52 ± 0.01*	0.71 ± 0.01	0.78 ± 0.01	1.02 ± 0.02
250mg/kg $Pb(CH3COO)_2$	1.24 ± 0.01**	1.24 ± 0.01**	2.47 ± 0.01***	0.69 ± 0.01**	0.76 ± 0.01*	0.97 ± 0.01*
1mg/kg $CdCl_2$	1.26 ± 0.01	1.27 ± 0.01	2.51 ± 0.01*	0.71 ± 0.01	0.79 ± 0.01	1.01 ± 0.02
5mg/kg $CdCl_2$	1.24 ± 0.01**	1.24 ± 0.01**	2.46 ± 0.01***	0.68 ± 0.01**	0.75 ± 0.01*	0.97 ± 0.01*
2.5mg/kg $NaAsO_2$	1.25 ± 0.01*	1.26 ± 0.01*	2.50 ± 0.01**	0.70± 0.01*	0.79 ± 0.01	1.01 ± 0.01
40mg/kg $NaAsO_2$	1.24 ± 0.01**	1.25 ± 0.01**	2.45 ± 0.01***	0.66 ± 0.01***	0.74 ± 0.01**	0.95 ± 0.01**

Values are expressed as Mean ± SEM.

*,**,*** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Table 5. Effect of oral administration of different doses of heavy metals (lead, cadmium, and arsenic) on body organs weight.

	Body organs weight		
Treatments (n=5)	**Liver (g)**	**Paired kidneys (g)**	**Heart (g)**
Control	8.51 ± 0.22	1.57 ± 0.05	0.80 ± 0.01
2.25mg/kg $Pb(CH_3COO)_2$	8.66 ± 0.11	1.50 ± 0.03	0.78 ± 0.01
250mg/kg $Pb(CH_3COO)_2$	9.84 ± 0.25***	1.38 ± 0.01***	0.75 ± 0.01*
1mg/kg $CdCl_2$	8.79 ± 0.18	1.54 ± 0.03	0.78 ± 0.01
5mg/kg $CdCl_2$	9.36 ± 0.21*	1.44 ± 0.01*	0.75 ± 0.01*
2.5mg/kg $NaAsO_2$	9.41 ± 0.21*	1.56 ± 0.03	0.79 ± 0.01
40mg/kg $NaAsO_2$	9.68 ± 0.24**	1.43 ± 0.01**	0.74 ± 0.01**

Values are expressed as Mean ± SEM.

*,**,*** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Table 6: Mean ± SEM of plasma testosterone level (ng/ml) and intra-testicular testosterone (ng/g) concentration in control and other group after 28 days of treatment with different doses of heavy metals (lead, cadmium, and arsenic).

Treatments (n=5)	Plasma testosterone (ng/ml)	Intra testicular testosterone (ng/g tissue)
Control	0.76 ± 0.04	51.83± 1.14
2.25mg/kg $Pb(CH3COO)_2$	0.65 ± 0.02*	44.55± 1.53*
250mg/kg $Pb(CH3COO)_2$	0.63 ± 0.02**	42.41± 2.02**
1mg/kg $CdCl_2$	0.65 ± 0.01*	43.72± 1.79*
5mg/kg $CdCl_2$	0.62 ± 0.02**	42.19± 1.14**
2.5mg/kg $NaAsO_2$	0.66 ± 0.03*	43.31± 2.69*
40mg/kg $NaAsO_2$	0.60 ± 0.02***	40.89± 1.71***

*,**,*** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Table 7: Mean ± SEM of histomorphological results of seminiferous tubular area, area of interstitium, area of lumen, area of epithelium and tubular diameter of testis in control and other groups after 28 days of treatment with different doses of heavy metals (n=5 for all groups).

Treatments	Seminiferous tubule area (μm^2)	Area of Interstitium (μm^2)	Area of Lumen (μm^2)	Area of Epithelium (μm)	Tubular Diameter (μm)
Control	73.60± 0.44	40.71 ± 0.81	7.10± 0.21	100.47 ± 0.73	363.99 ± 1.58
2.25mg/kg $Pb(CH_3COO)_2$	70.02± 0.18*	40.79 ± 0.43*	7.34± 0.12*	98.56 ± 0.42*	358.78 ± 1.27*
250mg/kg $Pb(CH_3COO)_2$	69.18± 0.07**	40.94 ± 0.50**	7.89± 0.08**	98.04 ± 0.28**	357.59 ± 0.67**
1mg/kg $CdCl_2$	70.04± 0.21*	40.75 ± 0.28*	7.47± 0.14*	98.60 ± 0.26*	358.82 ± 0.44*
5mg/kg $CdCl_2$	66. 00± 0.55***	42.13 ± 0.20***	8.73± 0.03***	93.02 ± 0.44***	348.32 ± 1.86***
2.5mg/kg $NaAsO_2$	69.50± 0.54**	40.88 ± 0.41**	7.92± 0.24**	98.10 ± 0.31**	357.37 ± 0.65**
40mg/kg $NaAsO_2$	69.27± 0.35**	41.00 ± 0.63**	7.96± 0.08**	97.97 ± 0.31**	357.16 ± 0.66**

*,**,*** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Table 8. Mean ± SEM (µm) of ductular diameter, luminal diameter and epithelial cell height of epididymis in control and treated groups after 28 days of treatment with heavy metals.

Treatments (n=5)	Ductular diameter		Lumen diameter		Epithelial height	
	Caput	Cauda	Caput	Cauda	Caput	Cauda
Control	678.32 ± 1.29	452.13 ± 1.13	422.66± 0.87	295.54± 0.98	86.05 ± 0.73	59.65 ± 0.96
2.25mg/kg $Pb(CH_3COO)_2$	676.43 ± 0.73	450.62 ± 0.45	422.36± 0.53	294.94± 0.65	84.90 ± 0.76	58.11 ± 0.60
250mg/kg $Pb(CH_3COO)_2$	673.64 ± 0.84*	448.57 ± 0.85*	420.14± 0.86*	292.02± 0.77*	82.85 ± 0.91*	58.08 ± 0.61
1mg/kg $CdCl_2$	673.65 ± 1.07*	450.74 ± 0.85	420.17± 0.57*	294.27± 0.62	85.16 ± 0.73	57.92 ± 0.40
5mg/kg $CdCl_2$	672.03 ± 1.38**	447.43 ± 0.83**	419.15± 0.23**	291.76±0.80*	81.37 ± 0.57**	56.76 ±0.66*
2.5mg/kg $NaAsO_2$	673.11 ± 1.25*	451.43 ± 1.16	420.18± 0.24*	294.16±0.80	84.99 ± 0.82	57.94 ± 0.41
40mg/kg $NaAsO_2$	672.53 ± 1.27**	447.59 ± 0.45**	419.19± 0.74**	292.03± 0.95*	82.90 ± 0.90*	57.80 ± 0.53

*, **, *** indicate significant difference at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control.

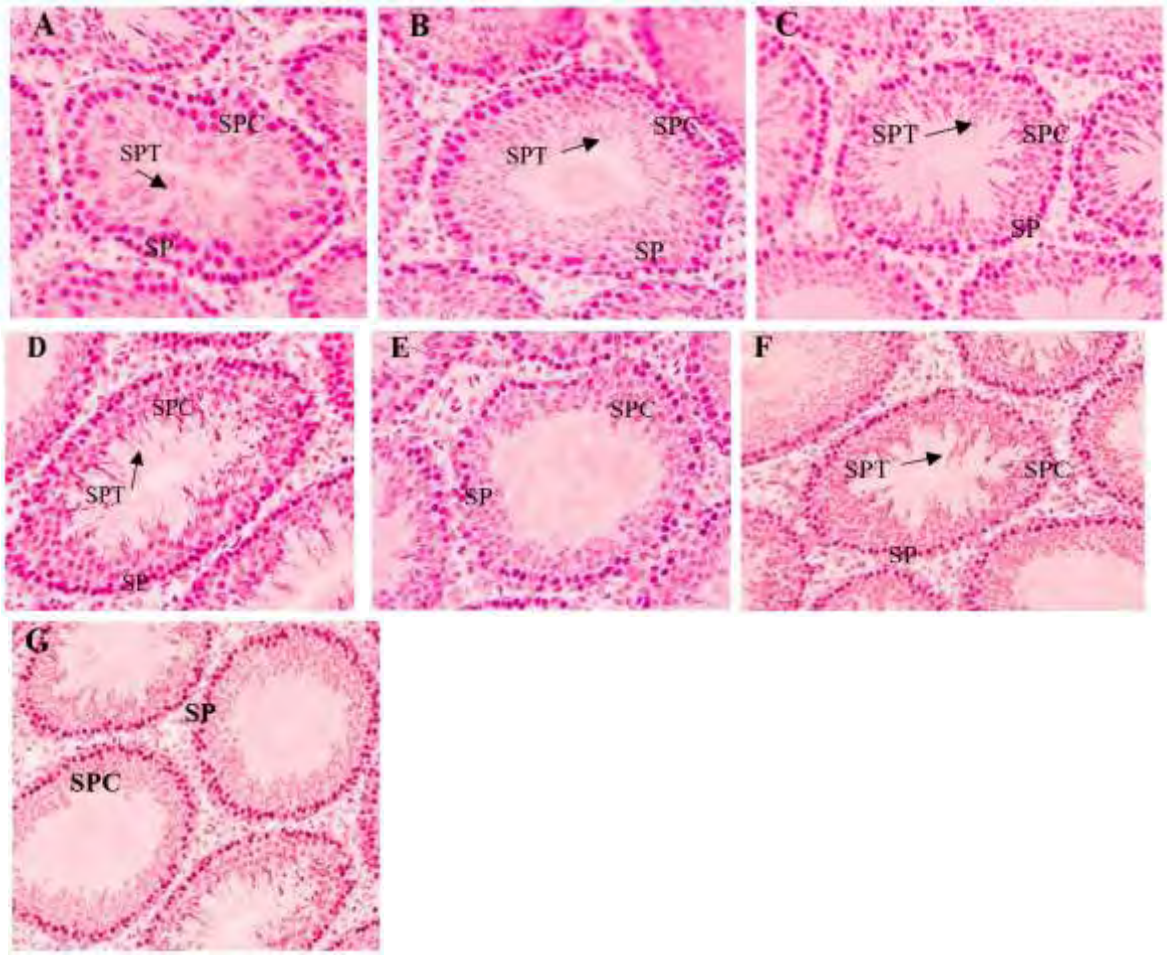

Figure 2. Photomicrograph of testis of adult male rats treated with different doses of heavy metals. (A) Control; displaying compact organization of seminiferous tubules, thick epithelium and lumen filled with spermatids, (B) 2.25mg/kg $Pb(CH_3CHOO)_2$ showing normal tubules with thick epithelium and sperm filled lumen, (C) 250mg/kg of $Pb(CH_3CHOO)_2$ showing epithelial sloughing and wider lumen and less number of spermatids, (D and F) represent testis of animals treated with low dose of $CdCl_2$ (1mg/g) and $NaAsO_2$ (2.5mg/kg), both presenting less vacuolation and filled lumen, (E and G) represent testis of animals treated with high dose of $CdCl_2$ (5mg/kg) and $NaAsO_2$ (40mg/kg) showing empty lumen with less number of spermatids. Spermatogonia (SP), Spermatocytes (SPC), Spermatids (SPT). (40X magnification)

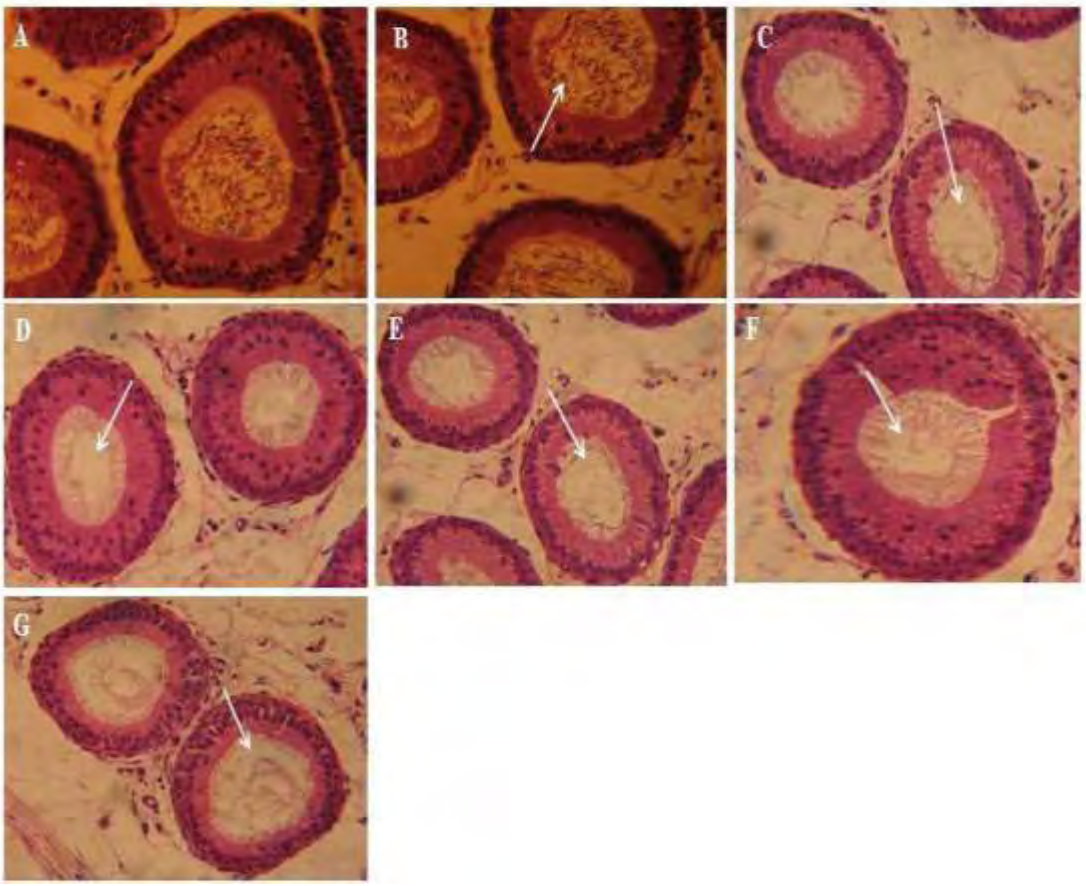

Figure 3. Photomicrograph of caput epididymis of male rats receiving different doses of heavy metals. (A) Control group; with normal morphology of caput epididymis with thick epithelium and compactly arranged tubules, lumen filled with sperm (arrow), (B, D and F) treated with low dose of $Pb(CH_3CHOO)_2$ (2.25mg/kg), $CdCl_2$ (1mg/kg), and $NaAsO_2$ (2.5mg/kg) presenting normal caput tubules, thick epithelium and sperm filled lumen (arrow), (E) $CdCl_2$ (5mg/kg) showing very little concentration of sperm in the lumen as compared to (C and G) groups treated with high dose of $Pb(CH_3CHOO)_2$ (250mg/kg) and $NaAsO_2$ (40mg/kg) (arrow). (40X Magnification)

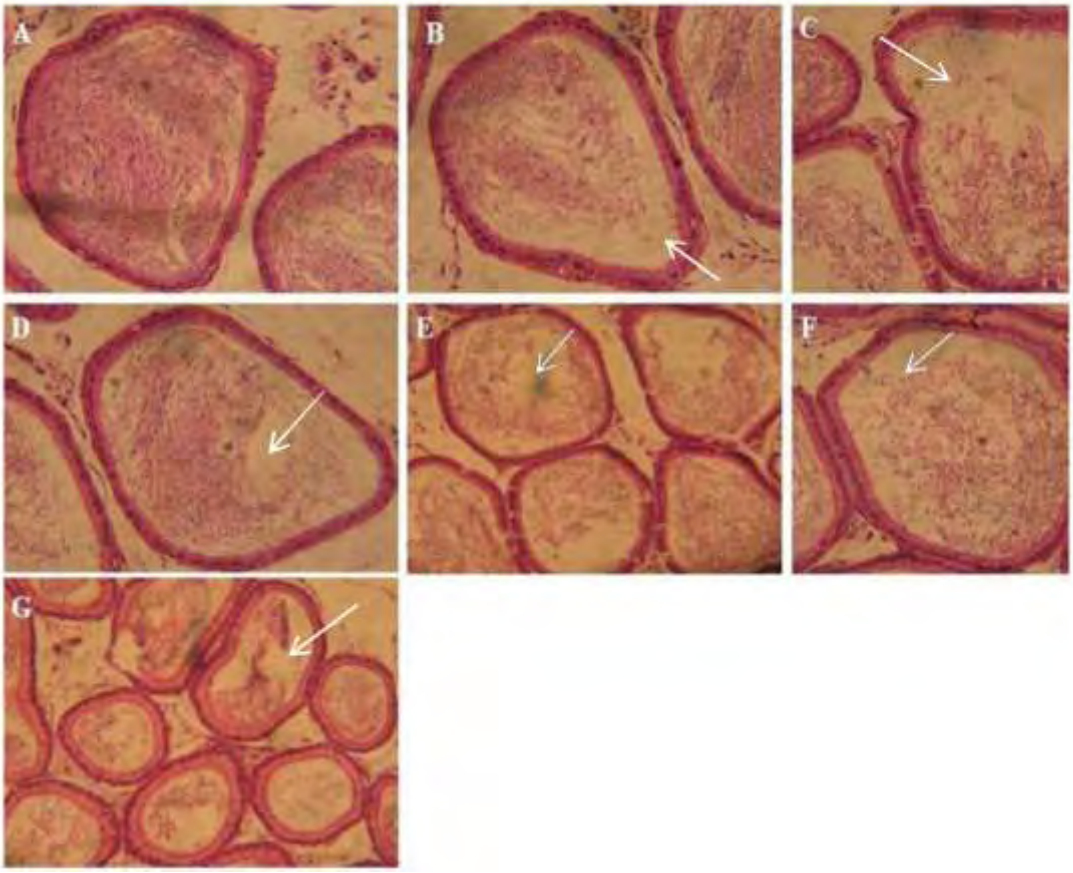

Figure 4. Photomicrograph of cross section of cauda epididymis of adult male rats receiving different doses of heavy metals showing (A) Control; showing normal morphology of cauda epididymis with tubules and thick epithelium, lumen filled with sperm (arrow), (B, D and F) treated with low dose $Pb(CH_3CHOO)_2$ (2.25mg/kg), $CdCl_2$ (1mg/kg), and $NaAsO_2$ (2.5mg/kg) presenting normal cauda tubules with epithelium and sperm filled lumen (arrow), (E) treated with high dose (5mg/kg) of $CdCl_2$ presenting cauda tubules with less number of sperm in the lumen as compared to (C and G) treated with high dose of $Pb(CH_3CHOO)_2$ (250mg/kg) and $NaAsO_2$ (40mg/kg) (arrow). 40X Magnification.

Discussion

A continuous debate regarding the potential decline in human male fertility has been emerging during the last few decades. Variations in living standards and occupational contact to several environmental contaminants have been mentioned as the possible causes for the rise in male factor infertility (Anjum *et al.*, 2011). Lead, cadmium and arsenic are the most common heavy metals and their significant chemical characteristic is high density ($45g/cm^3$). These metals are present in nature in geological formations of the earth's crust and are exposed by natural forces and anthropological actions. Exposure to these heavy metals usually occurs coincidently, in specific professions, via consumption of contaminated food and water, or inhalation of polluted air. While heavy metals are not crucial for human health and are not purposeful part of the usual diet, a major cause of worldwide exposure to heavy metals is food consumption (Wirth and Mijal, 2010). The use of physical and chemical procedures that are frequently considered as conventional techniques are subjected to the toxic chemicals usage that have high risk of triggering environmental toxicity and carcinogenicity.

The statistical analysis of current study to find out the effect of heavy metals (lead, cadmium, and arsenic) on the body weight revealed decrease in body weight gain of experimental rats at higher doses in comparison to the control. These results are in accordance with the previous studies by Hamadouche *et al.* (2013) {10 and 15mg/kg $Pb(CH_3COO)_2$ for 20 days}, Ibrahim *et al.* (2018) (1.8mg/kg bw/day $CdCl_2$ for 28 days), and Schulz *et al.* 2002 (26.6 mg/kg $NaAsO_2$ for 28 days), all reporting a decrease in body weight gain. Cezard and Haguenoer (1992) propose that the weight loss may be due to loss of appetite and gastrointestinal disturbances; as well as to disturbance in absorption and general metabolism of essential food nutrients. Furthermore, this decrease in body weight gain may be attributed to toxic effect of heavy metals on cellular metabolism, and not to underfed or undernourished conditions (Wapnir *et al.*, 1977). However, Li *et al.* (2018) {0.1-1.0% $Pb(CH_3COO)_2$ for six weeks}, Mouro *et al.* (2019) (4.29 mg/kg/day $CdCl_2$ for 1 week) and Ramos *et al.* (2017) (5 mg/kg $NaAsO_2$ for 8 weeks) reported no such changes in body weight gain as compared to control. Our results also revealed effective decrease in glucose

level of male rats, which might be due to antidiabetic property of heavy metals, as reported by Pandya *et al.* (2012), who noted a decline in glucose 6 phosphate dehydrogenase (G6PDH) activity, after exposing male rats to 0.025mg/kg each of lead and cadmium, decreasing glucose metabolism and consequently increasing serum glucose (Pandya *et al*., 2012).

The cellular antioxidant enzymes can be categorized into superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) that form a vital part of the cell's defense mechanism to combat oxidative stress in the body (Stahl *et al.*, 1998). Significant decrease in testicular SOD activity, revealed in the current study, is in accordance with the findings of Hassan *et al.* 2019, who presented remarkable decrease in enzymatic activity of SOD, which can be due to the direct inhibiting influence of lead on the level or expression of antioxidant enzymes. Furthermore, Hassan *et al.* (2019) also reported increase in LPO demonstrated as TBARS, which indicates amplified cellular ROS production, causing injury to spermatozoa and structures of cytoplasmic organelle membranes through proteins, lipids, and nucleotide peroxidation, leading to altered sperm motility. Similar increase in TBARS was also noted in the current study, which also corresponds to a study of Marchlewicz *et al.* (2007), who reported elevated lipid peroxidation, as an additional effect of ROS that damages testicular and epididymal cell function, after chronic exposure to lead acetate to rats (Marchlewicz *et al.*, 2007). Reproductive toxicity of lead was induced by initiation of oxidative stress and apoptosis in the testes, and disturbed hormonal balance in the HPT (Hassan *et al.*, 2019). Furthermore, results reported by Li *et al.* (2018) also portray that exposure to lead stimulates ROS production in semen, with rise of ROS generation in sperm cells can cause damage to sperm DNA structure, generating high DNA fragmentation in spermatozoa (Li *et al.*, 2018).

Likewise reduction in antioxidant enzymes was also noted in cadmium treated groups of the current study, corroborating the results of other studies where Cd increased ROS and oxidative stress, lipid peroxidation, and inactivated the antioxidant defensive enzymes, hence causing oxidative damage (Haouem *et al.*, 2013; Chater *et al*., 2009; Acharya *et al.*, 2008). A number of hypotheses have been proposed to describe the

mechanism of cadmium-induced testicular toxicity. Firstly, cadmium has been reported to cause oxidative stress in the testes, reducing antioxidant enzymes levels, enhancing ROS generation, and increasing LPO levels. According to Ola-Mudathir *et al.* (2008), cadmium does not initiate direct production of reactive oxygen species, instead cadmium causes generation of hydroxyl free ions (OH^-), hydrogen peroxide (H_2O_2), and superoxide anions (O^{-2}) (Ola-Mudathir *et al.*, 2008). On the other hand, human spermatozoa have been described to have elevated levels of polyunsaturated fatty acids in the cell membrane, and therefore would be more prone to peroxidative harm due to their substantial capability to produce hydrogen peroxide and superoxide anion. Hence, predisposition of sperm to oxidative stress and reactive oxygen species after cadmium exposure, consequently causes decline in semen quality. Similarly, El-Demerdash *et al.* (2004) and Oliveira *et al.* (2009) reported that elevated testicular ROS generation can result in additional harm to essential constituents of the cell, comprising of proteins, RNA, and DNA, leading to production of less and abnormal spermatozoa (El-Demerdash *et al.*, 2004; Oliveira *et al.*, 2009). Secondly, Siu *et al.* (2009) provided the mechanism of cadmium toxicity through various cell signaling pathways including mitogen-activated protein kinase (MAPK), PI3K/c-Src/FAK, and c-Jun N-terminal kinases (c-JNK) signal transduction pathways, and subsequently disturbs blood-testis barrier (BTB) and cellular junctions in the seminiferous epithelium (Siu *et al.*, 2009). Additionally Al-Azemi *et al.* (2010) informed that cadmium triggers a cascade of inflammatory responses and amplified generation of pro-inflammatory cytokines, predominantly tumor necrosis factor α (TNFα) which produces reactive oxygen species, and subsequently results in lipid peroxidation, initiating further testicular tissue damage (Al-Azemi *et al.*, 2010).

The animals treated with $NaAsO_2$ in the present study also revealed reduction in antioxidant enzymes and increase in oxidative stress markers (ROS and TBARS), verifying the reduced SOD and CAT activity and enhanced testicular intracellular ROS generation, reported by Zeng *et al.* (2018), Ince *et al.* (2016) and Das *et al.* (2009). Results of the present study indicates significant diminution in weight of testes in group treated with high doses of $Pb(CH_3COO)_2$, $CdCl_2$, and $NaAsO_2$ in comparison to control. Conversely, reduction in testes weight was less pronounced in

the animals treated with low dose groups of all three metals. The current study found degenerative alterations in animals exposed to high dose of lead acetate, which are in parallel with previous studies that showed that lead particles can cross the blood-testis barrier and subsequently damage the spermatogenic cells, spermatids, and sperms (Naha and Manna, 2007; Vigeh *et al.*, 2011; Hassan *et al.* 2019). In a study carried out by Batra *et al.* (2001), exposure of male rats to different doses of lead acetate (10-200mg/kg bw) for 3 months, resulted in disturbed spermatogenesis causing deposition of immature spermatozoa in the epididymis, with complete arrest of spermatogenesis at higher doses of lead treatment. Batra *et al.* (2001) also described dose-dependent decline in the activities of alkaline phosphatase and sodium-potassium ATPase enzymes, both in the testes and epididymis of lead-treated animals. On the other hand, the basement membrane plus the caput and corpus epididymis epithelium were also damaged in addition to the vacuolization of caput and cauda epididymis cells, with the increase in lead levels (Batra *et al.*, 2001).

The significant decrease in body and testis weight noted in the high dose cadmium treated groups, confirmed and elucidated the findings of Babaknejad *et al* (2018) who reported similar results after 3 weeks of Cd exposure (Babaknejad *et al.*, 2018). This reduction in testes weight can be attributed to inhibition of growth and protein synthesis induced by cadmium (Waalkes and Diwan, 1999). There exists a positive correlation between the testicular weight and germ cell number, and loss of testes weight can be due to cellular injury to the differentiated spermatogenic cells, as previously suggested by Schlappack *et al.* (1988). The results of the present histomorphometric study are in agreement with these findings. Moreover, the histomorphometric examination delivered more thorough information on the mechanisms of toxicity induced by Cadmium in the testes. Cadmium brought about degenerative alterations in the seminiferous tubules and caused disruption of spermatogenesis. Additionally, the pathological injury to seminiferous epithelium could initiate the damage to sertoli cells and spermatocytes, eventually resulting in decreased spermatogenesis (De Souza Predes *et al.*, 2010). Degenerative histological alterations in the tubular epithelium of the testes and epididymis of high dose cadmium-treated rats can be attributed to distortion of the blood-testis and blood-

epididymis barrier with consequent loss of germ cells, testicular atrophy, vacuolation, decline in spermatogenesis, edema, and haemorrhage (Adamkovicova *et al.*, 2014). Cadmium exposure also inflicted damage to the testicular interstitium and reduced tubular diameter of seminiferous tubules, similar to the findings reported by Adamkovicova *et al.* (2014). Likewise, the histomorphometry of testes in the present study also demonstrated that the tubules have numerous vacuolar spaces, declined epithelium height and reduced diameter of the seminiferous tubules, and these changes might have led to the decreased organ weights, corroborating the findings of Jahan *et al.* (2014). Adamkovicova *et al.* (2014) also reported the development of vacuolar spaces instead of germ cells, in sertoli cells and the nearly complete deficiency of sperms, indicating a loss of spermatogenesis, similar to the results of the current study. Furthermore, Cheng *et al.* (2011) described that the testicular vasculature after cadmium exposure became dilated and congested, which elucidates that testicular necrosis induced by cadmium led to disruption of the blood-testis barrier integrity, cell-to-cell endothelial and epithelial junctions. Moreover, the deficits in the epididymis weight after cadmium exposure depict the vulnerability of the epididymis to cadmium toxicity. The epididymis of control rats possess compactly arranged tubules, with their lumen occupied by spermatozoa, and enclosed by connective tissue without any observable signs of pathological modifications. Conversely, after $CdCl_2$ treatment, the interstitial spaces were noticed to be enlarged and displayed intense staining, indicative of damage to the vasculature, verifying the effects of cadmium exposure reported by Marettova *et al.* (2015). It has been previously established that the main target of cadmium induced toxicity on epididymis are disruption of cell-cell junctions and the blood-epididymis barrier, resulting in poor sperm maturation and motility (Dube and Cyr, 2013). In the present study, high dose cadmium treatment also initiated distension of the tubular lumen, with reduced number of spermatozoa, deteriorated epithelium, and thick walls. These findings correspond to the study of Abarikwu *et al.* (2018) who reported that cadmium exposure led to generation of vacuolar spaces within pseudo-stratified epithelium of the epididymis tubules, and also revealed the presence of degenerated spermatozoa within them (Abarikwu *et al.*, 2018).

A prominent decrease in the testes weight was noted in both low and high dose sodium arsenite-treated animals of the current study, which elucidates the findings of Ramos *et al.* (2017), who reported a similar significant reduction in testes weight after exposing rats to 5mg/kg sodium arsenite (Ramos *et al.* 2017). Moreover, exposure to sodium arsenite in the current study decreased the tubular diameter and the epithelial height of testes. Such changes lead to the decrease in the epithelial volumetric proportion in both groups that received arsenic treatment, validating the results established in other studies (Ferreira *et al.* 2012; Sanghamitra *et al.* 2008; Altoe *et al.* 2016). In a recent study carried out by Zeng *et al.* (2019), who treated rats with 0.82 and4.82mg arsenic/kg, and reported a significant rise in the apoptosis rate of seminiferous epithelium, that could be linked with diminished spermatogenic cells and spermatozoa observed in the testicular histology (Zeng *et al.*, 2019). The more pronounced effect of high dose lead exposure to the epididymis weight and structure and reduced spermatozoa number in the epididymis lumen, noted in the histology of epididymis carried out in the current study, can be attributed to deposition of lead in smooth myocytes, epithelium and lumen of epididymis, as suggested by Marchlewicz (1994), which leads to reduction in epididymal spermatozoa. Furthermore, Marchlewicz (1994) also reported that the blood-epididymis barrier fails to protect epididymis form lead intoxication as compared to relatively better ability of protection of seminiferous epithelium provided by blood−testes barrier (Marchlewicz, 1994).

The results of present study have revealed a prominent and remarkable diminution in serum and intra-testicular testosterone levels predominantly in high dose lead acetate {250mg/kg $Pb(CH_3COO)_2$}, cadmium chloride (5mg/kg $CdCl_2$), and sodium arsenite (40mg/kg $NaAsO_2$) treated rats, which elucidate the findings of a number of previous studies (Hamadouche *et al.,* 2013;Wani *et al.*, 2015; djuric *et al.*, 2015; Gandhi *et al.*, 2017; Nna *et al.*, 2017; Jana *et al.*, 2006). It has been proposed that this reduction in testosterone concentrations in rats treated with lead, could be attributed to apoptosis of leydig cells, LH and GnRH inhibition, and reduced HPT axis activity (Hassan *et al.*, 2019; He *et al.*, 2017; Klein *et al.*, 1994; Doumouchtsis *et al.*, 2009;). Similarly, Djuric *et al.* (2015) exposed male rats to 1 mg $CdCl_2$/kg/day for 21 days through intra-peritoneal pathway, corresponding to low dose cadmium chloride concentration used

in the current study, and reported a decline in testicular testosterone level, and linked this decline to its reduced production by Leydig cells placed in interstitial spaces, which was edematous and disrupted by cadmium (Djuric *et al.*, 2015). Siu *et al.* (2009) suggests that testosterone reduction caused by cadmium exposure is possibly multi-factorial, brought about through its influence on Leydig cells and the HPT axis. (Siu *et al.*, 2009). A dose-dependent decline in serum and intra-testicular testosterone levels has been formerly reported by Jana *et al.* (2006) after chronic exposure to sodium arsenite, and this decrease was attributed to the disruption of the testicular androgenic enzymes activities, as their activity regulates testosterone biosynthesis. Furthermore, the interruption of testicular androgenic enzymes in rats after arsenic exposure could be due to low serum LH levels as this is a primary regulator of testosterone synthesis pathway (Jana *et al.*, 2006).

Conclusion

Current study has validated that heavy metals (lead, cadmium, and arsenic) have cytotoxic effect on testicular tissue in a dose dependent manner. All doses (low and high) caused reduction in testis weight, seminiferous tubule diameter, epithelial height, and Leydig cell number in a dose dependent manner. The reduction in Leydig cell number led to decline in testosterone concentration. The current study results further show that exposure of male rats to different concentrations of lead, cadmium, and arsenic led to oxidative stress in the reproductive tissues by disturbing antioxidant enzyme status. However, the results of current comparative study also revealed that toxicity level was high in group exposed to high dose of cadmium chloride as compared to sodium arsenite and lead acetate treated groups. This might be due to higher cellular toxicity and endocrine disrupting capacity of cadmium. However, further investigations are needed to understand the exact cellular and molecular mechanisms mediating the reproductive toxicity of lead, cadmium, and arsenic. Moreover, the understanding of how these heavy metals penetrate into the reproductive organs also needs to be elaborated and will be useful in understanding the current problems of male infertility.

References

Abarikwu, S.O., Iserhienrhien, B.O., and Badejo, T.A. 2013. Rutin and selenium attenuated cadmium-induced testicular pathophysiology in rats. Human and Experimental Toxicology. **32**: 395–406.

Abarikwu, S. O., Oruitemeka, S., Uwadileke, I. A., Omeodu, S. I., Okoye, N. F., Mgbudom-Okah, C. J., and Ohanador, R. 2018. Oral administration of cadmium depletes intratesticular and epididymal iron levels and inhibits lipid peroxidation in the testis and epididymis of adult rats. Journal of Trace Elements in Medicine and Biology. **48**: 213–223.

Acharya, U.R., Mishra, M., Patro, J., and Panda, M.K. 2008. Effect of vitamin C and E on spermatogenesis in mice exposed to Cadmium. Reproductive Toxicology. **25**: 84–88.

Adamkovicova, M., Toman, R., Cabaj, M., Massanyi, P., Martiniakova, M., Omelka, R., Krajcovicova, V., and Duranova, H. 2014. Effects of subchronic exposure to cadmium and diazinon on testis and epididymis in rats. Science World Journal. **2014.**

Adamkovicova, M., Toman, R., Martiniakova, M., Omelka, R., Babosova, R., Krajcovicova, V., Grosskopf, B., and Massanyi, P. 2016. Sperm motility and morphology changes in rats exposed to cadmium and diazinon. Reproductive Biology and Endocrinology. **14**.

Aebi, H. 1984. Catalase in vitro. Methods in Enzymology. **105**(1984): 121-126.

Ansa, A.A., Akpere, O., and Imasuen, J.A. 2017. Semen traits, testicular morphometry and histopathology of cadmium-exposed rabbit bucks administered methanolic extract of *Phoenix dactylifera* fruit. Acta Scientiarum. Animal Sciences. b**39**(2): 207–215.

Agarwal, A., Mulgund, A., Hamada, A., and Chyatte, M.R. 2015. A unique view on male infertility around the globe. Reproductive Biology and Endocrinology. **13**(1): 1–9.

Al-Azemi M., Omu, F.E., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Omu, A.E. 2010. Lithium protects against toxic effects of cadmium in the rat testes. Journal of

Assisted Reproduction and Genetics. **27**(8): 469–476.

Aliyu, U.D., Daku, A.B. and Ibrahim, S.A. 2017. Ameliorative effect of vitamin c in lead poisoning on some hematological parameters in adult wistar rats. Bayero Journal of Pure and Applied Sciences. **2**(2): 45–52.

Altoe´, L.S., Reis, I.B., Gomes, M.L.M., Dolder, H., and Pirovani, J.C.M. 2016. Could vitamin C and zinc chloride protect the germ cells against sodium arsenite? Human and Experimental Toxicology. **36**(10): 1–10.

Anjum, M. R., Sainath, S. B., Suneetha, Y., and Reddy, P. S. 2011. Lead acetate induced reproductive and paternal mediated developmental toxicity in rats. Ecotoxicology and Environmental Safety. **74**: 793–799.

Attina, T.M., Hauser, R., Sathyanarayana, S., Hunt, P.A., Bourguignon, J.P., Myers, J.P., Gangi, J.D., Zoeller, R.T., and Trasande, L. 2016. Exposure to endocrine-disrupting chemicals in the USA: a population-based disease burden and cost analysis. Lancet Diabetes Endocrinol. **4**(12): 996–1003.

Baltaci, B.B., Uygur, R., Caglar, V., Aktas, C., Aydin, M., and Ozen, O.A. 2016. Protective effects of quercetin against arsenic-induced testicular damage in rats. International journal of andrology. **48**(10): 1–12.

Bas, H., and Kalender, S. 2016. Antioxidant Status, lipid peroxidation and testis-histoarchitecture induced by lead nitrate and mercury chloride in male rats. Brazilian Archives of Biology and Technology. **59**: 1–9.

Batista, B.L., Souza, J.M.O., Souza, S.S., and Junior, F.B. 2011. Speciation of arsenic in rice and estimation of daily intake of different arsenic species by Brazilians through rice consumption. Journal of Hazardous Materials. **191**: 342–348.

Batra, N., Nehru, B., and Bansal, M.P. 2001. Influence of lead and zinc on rat male reproduction at “biochemical and histopathological levels.” Journal of Applied Toxicology. **21**(6): 507–512.

Bernstam, L., and Nriagu, J. 2010. Molecular aspects of arsenic stress. Journal of Toxicology and Environmental Health Biology. **3**: 293–322.

Carette, D., Perrard, M.H., Prisant, N., Gilleron, J., Pointis, G., and Segretain, D. 2013.

Hexavalent chromium at low concentration alters Sertoli cell barrier and connexin 43 gap junction but not claudin-11 and N cadherin in the rat seminiferous tubule culture model. Toxicology and Applied Pharmacology. **268**(1): 27–36.

Cezard, C. and Haguenoer, J.M. 1992. Toxicology of lead. Technique and documentation. Lavoisier, Paris, France. **1992**: 172-173.

Chance, B., and Maehly, A.C. Assay of catalases and peroxidases. Methods in Enzymology. **2**(1955): 764-775.

Chater, S., Douki, T., Fvier, A., Sakly, M., and Abdelmelek H. 2009. Changes in antioxidant status and biochemical parameters after orally Cd administration in females rats. Acta biologica Hungarica. **60**(2009): 79–88.

Chen, C.J., Chiou, H.Y., Chiang, M.H., Lin, L.J., and Tai, T.Y. 1996. Dose-response relationship between ischemic heart disease mortality and long-term arsenic exposure. Arteriosclerosis, Thrombosis, and Vascular Biology. **16**: 504–510.

Chen, C., Wang, N., Zhai, H., Nie, X., Sun, H., Han, B., Li, Q., Chen, Y., Cheng, J., Xia, F., Zhao, L., Zheng, Y., Shen, Z., and Lu, Y. 2016. Associations of blood lead levels with reproductive hormone levels in men and postmenopausal women: results from the SPECT-China study. Scientific Reports. **6**(1): 1-9.

Cheng, C.Y., Wong, E.W.P., Lie, P.P., Li, M.W., Su, L., Siu, E.R., Yan, H.H., Mannu, J., Mathur, P.P., Bonanomi, M., Silvestrini, B., and Mruk, D.D. 2011. Environmental toxicants and male reproductive function. Spermatogenesis. **1**(2011): 2–13.

Das, J., Ghosh, J., Manna, P., Sinha, M., Sil, P.C. 2009. Taurine protects rat testes against $NaAsO_2$-induced oxidative stress and apoptosis via mitochondrial dependent and independent pathways. Toxicology letters. **187**: 201–210.

da Silva, R.F., Borges C.D.S., Silva, P.V., Missassi, G., Kiguti, L.R.A., Pupo, A. S., Barbosa, F.J., Anselmo-Franci, J.A., and Kempinas, W.D.G. 2016. The coadministration of N-acetylcysteine ameliorates the effects of arsenic trioxide on the male mouse genital system. Oxidative Medicine and Cellular Longevity. **2016**:1–11.

de Angelis, C., Galdiero, M., Pivonello, C., Salzano, C., Gianfrilli, D., Piscitelli, P.,

Lenzi, A., Colao, A., and Pivonello, R. 2017. The environment and male reproduction: the effect of cadmium exposure on reproductive function and its implication in fertility. Reproductive Toxicology. **73**: 105–27.

De Souza Predes, F., Diamante, M.A., and Dolder, H. 2010. Testis response to low doses of cadmium in Wistar rats. International Journal of Experimental Pathology **91**(2): 125–131.

Djuric, A., Begic, A., Gobeljic, B., Stevanovic, I., Pantelic, A., Zebic, G., Prokic, V., Dejanovic, B., Ninkovic, M., Stojanovic, I., Pavlica, M., Djukic, D., Saso, L., Djurdjevic, D., Pavlovic, M., Topic, A., Vujanovic, D., Stanojevic, I., Vojvodic, D., and Djukic, M. 2015. Oxidative Stress, Bioelements and Androgen Status in Testes of Rats Subacutely Exposed to Cadmium, Food and Chemical Toxicology. **2015**.

Doumouchtsis, K., Doumouchtsis, S., Doumouchtsis, E., and Perrea, D. 2009. The effect of lead intoxication on endocrine functions. Journal of Endocrinological Investigation. **32**: 175–183.

Dube, E., and Cyr, D.G. 2013. The blood-epididymis barrier and human male fertility, Advances in Experimental Medicine and Biology. **763**: 218–236.

Elblehi, S.S., El Euony, O.I., and El-Nahas, A.F. 2019. Partial ameliorative effect of Moringa leaf ethanolic extract on the reproductive toxicity and the expression of steroidogenic genes induced by subchronic cadmium in male rats. Environmental Science and Pollution Research. **26**(23): 23306–23318.

El-Demerdash, F.M., Yousef, M.I., Kedwany, F.S., and Baghdadi, H.H. 2004. Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene. Food and Chemical Toxicology. **42**(10): 1563–1571.

El-Sayed, M.F., Abdel-Ghafar, S.K., Adly, M.A., Salim, A.A., Abdel-Samei, W.M., 2015. The ameliorative effects of DMSA and some vitamins against toxicity induced by lead in the testes of albino rats II. Journal of Basic and Applied Zoology. **71:** 60–65.

El-Sayed, Y.S., El-Gazzar, A.M., El-Nahas, A.F., and Ashry, K.M. 2016. Vitamin C

modulates cadmium-induced hepatic antioxidants' gene transcripts and toxicopathic changes in Nile tilapia, Oreochromis niloticus. Environmental Science and Pollution Research. **23**(2): 1664–1670.

Engel, R.R., and Smith, A.H. 1994. Arsenic in drinking water and mortality from vascular disease: An ecologic analysis in 30 counties in the United States. Archives of Environmental & occupational Health. **49**: 418–427.

Ferreira, M., Matos, R.C., Oliveira, H., Nunes, B., and Pereira, M.L. 2012. Impairment of mice spermatogenesis by sodium arsenite. Human and Experimental Toxicology. **31**(3): 290–302.

Flora, S.J., Flora, G., and Saxena, G. 2006. Environmental occurrence, health effects and management of lead poisoning. Lead Chemistry, Analytical Aspects, Environmental Impact and Health Effects. **2006**: 158–228.

Flora, S.J., Pachauri, V., and Saxena, G. 2011. Arsenic, cadmium and lead. Reproductive and Developmental Toxicology. **2011**: 415–438.

Fouad, A.A., Albuali, W.H., Al-Mulhim, A.S., and Jresat, L. 2015. Protective effect of telmisartan treatment against arsenic-induced testicular toxicity in rats. Journal for Nature Research. **70**(7–8): 175–181.

Gandhi, J., Hernandez, R.J., Chen, A., Smith, N.L., Sheynkin, Y.R., Joshi, G., and Khan, S.A. 2017. Impaired hypothalamic-pituitary-testicular axis activity, spermatogenesis, and sperm function promote infertility in males with lead poisoning. Zygote. **25**(2): 103–110.

Gillis, B.S., Arbieva, Z., and Gavin, I.M. 2012. Analysis of lead toxicity in human cells. BMC Genomics. **2012**(13): 344.

Godspower, O., Williams, N., and Elochukwu, N. 2015. Ameliorative Potential of Methanolic Extract of *Citrullus lanatus* (Watermelon) Seeds on the sperm parameters, testosterone level and testicular cyto-architecture of male albino rats induced with lead acetate. British Journal of Pharmaceutical Research. **6**(1): 35–43.

Goyer, R.A., Liu, J., and Waalkes, M.P. 2004. Cadmium and cancer of prostate and testis. Biometals. **17**(5): 555–558.

\

Gump, B.B., MacKenzie, J.A., Bendinskas, K., Morgan, R., Dumas, A.K., Palmer, C.D., and Parsons, P.J. 2011. Low level Pb and cardiovascular responses to acute stress in children: the role of cardiac autonomic regulation. Neurotoxicology and Teratology. **33**(2): 212–219.

Gundert-Remy, U., Damm, G., Foth, H., Freyberger, A., Gebel, T., Golka, K., Röhl, C., Schupp, T., Wollin, K.M., and Hengstler, J.G. 2015. High exposure to inorganic arsenic by food: the need for risk reduction. Archives of toxicology. **89**(12): 2219-2227.

Guvvala, P.R., Ravindra, J.P., Rajani, C.V., Sivaram, M., Selvaraju, S. 2017. Protective role of epigallocatechin-3-gallate on arsenic induced testicular toxicity in Swiss albino mice. Biomedicine and Pharmacotherapy. **96**: 685–694.

Guzikowski, W., Szynkowska, M.I., Pochrzęst, H.M., Pawlaczyk, A., and Sypniewski, S. 2015. Trace elements in seminal plasma of men from infertile couples. Arch. Med. Sci. **11**(3): 591–598.

Hamadouche, N., Nesrine, S., and Abdelkeder, A. 2013. Lead Toxicity and the Hypothalamic-Pituitary-Testicular Axis. J. Not. Sci. Biol. **5**(1): 1-6.

Haghighi, S.K., Aminian, O., Chavoshi, F., Bahaedini, L.S., Soltani, S., and Najarkolaei, F.R. 2013. Relationship between blood lead level and male reproductive hormones in male lead exposed workers of a battery factory: a cross-sectional study. Iran Journal of Reproductive Medicine. **11**(8): 673–676.

Haouem, S., and El Hani, A. 2013. Effect of cadmium on lipid peroxidation and on some antioxidants in the liver, kidney and testes of rats given diet containing cadmium polluted radish bulbs. Journal of Toxicology and Pathology. **26**(4): 359–364.

Hassan, E., Kahilo, K., Kamal, T., El-Neweshy, M., and Hassan, M. 2019. Protective effect of diallyl sulfide against lead-mediated oxidative damage, apoptosis and down-regulation of CYP19 gene expression in rat testes. Life Sciences. **226**: 193–201.

Hauser, R., Skakkebaek, N.E., Hass, U., Toppari, J., Juul, A., Andersson, A.M., Kortenkamp, A., Heindel, J.J., and Trasande, L. 2015. Male reproductive disorders,

diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. Journal of Clinical Endocrinology & Metabolism. **100**(4): 1267–77.

Hayashi, I., Morishita, Y., Imai, K., Nakamura, M., Nakachi, K., and Hayashi, T. 2007. High throughput spectrophotometric assay of reactive oxygen species in serum. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. **631**(1): 55-61.

He, X., Wu, J., Yuan, L., Lin, F., Yi, J., Li, J., Yuan, H., Shi, J., Yuan, T., Zhang, S., Fan, Y., and Zhao, Z. 2017. Lead induces apoptosis in mouse TM3 Leydig cells through the Fas/FasL death receptor pathway. Environmental Toxicology and Pharmacology. **56**(2017): 99–105.

He, Y.Q., Zou, Q.X., Chen, H., Weng, S.Q., Luo, T., and Zeng, X.H. 2016. Lead inhibits human sperm functions by reducing the levels of intracellular calcium, cAMP, and tyrosine phosphorylation. Tohoku Journal of Experimental Medicine. **238**(4): 295–303.

He, Z.L., Yang, X.E., and Stoffella, P.J. 2005. Trace elements in agroecosystems and impacts on the environment. Journal of Trace elements in Medicine and Biology. **19**(2– 3): 125–140.

Hosni, H., Selim, O., Abbas, M., and Fathy, A. 2013. Semen quality and reproductive endocrinal function related to blood lead levels in infertile painters. Andrologia. **45**(2): 120–127.

Hsieh, F.I., Hwang, T.S., Hsieh, Y.C., Lo, H.C., Su, C.T., Hsu, H.S., Chiou, H.Y., and Chen, C.J. 2008. Risk of erectile dysfunction induced by arsenic exposure through well water consumption in Taiwan. Environmental health perspectives. **116**: 532–536.

Ibrahim, M.A., Almaeen, A.H., El Moneim, M.A., Tammam, H.G., Khalifa, A.M., and Nasibe, M.N. 2018. Cadmium-induced hematological, renal, and hepatic toxicity: The amelioration by S*pirulina platensis*. Saudi Journal of Forensic Sciences. **1**: 5-13.

Ince, S., Avdatek, F., Demirel, H.H., Arslan-Acaroz, D., Goksel. E., and Kucukkurt, I.

2016. Ameliorative effect of polydatin on oxidative stress-mediated testicular damage by chronic arsenic exposure in rats. Andrologia **48**: 518–524.

International Agency for Research on Cancer. 1993. Cadmium and Cadmium Compounds. IARC Summary and Evaluation. **58**(1993).

Jahan, S., Zahra, A., Irum, U., Iftikhar, N., and Ullah, H. 2014. Protective effects of different antioxidants against cadmium induced oxidative damage in rat testis and prostate tissues. Systems biology in reproductive medicine. **60**(2014): 199–205.

Jana, K., Jana, S., and Samanta, P.K. 2006. Effects of chronic exposure to sodium arsenite on hypothalamo-pituitary-testicular activities in adult rats: possible an estrogenic mode of action. Reproductive Biology and Endocrinology. **4**: 9–22.

Jenardhanan, P., Panneerselvam, M., and Mathur, P.P. 2016. Effect of environmental contaminants on spermatogenesis. Seminars in Cell and Developmental Biology. **59**: 126–40.

Kabir, E.R., Rahman, M.S. and Rahman, I. 2015. A review on endocrine disruptors and their possible impacts on human health. Environmental Toxicology and Pharmacology. **40**(1): 241–58.

Kakkar, P., Das, B., and Viswanathan, P.N. 1984. A Modified Spectrophotometric Assay of Superoxide Dismutase. Indian Journal of Biochemistry and Biophysics. **21**(2): 130-132.

Kasten-Jolly, J., Heo, Y., and Lawrence, D.A. 2010. Impact of lead exposure on splenic factors. Toxicology and Applied Pharmacology. **247**(2): 105–115.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., and Tilson, H.A. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environmental Health Perspectives. **104** (Suppl 4): 715–40.

Kim, Y.J., and Kim, J.M. 2015. Arsenic toxicity in male reproduction and development. Development & Reproduction. **19**: 167–180.

Klein, D., Okuda, H., Sokol, R.Z., Kamyab, S., and Wan, Y.J.Y. 1994. Effects of toxic levels of lead on gene regulation in the male axis: increase in messenger ribonucleic acids and intracellular stores of gonadotrophs within the central nervous system. Biology of Reproduction. **50**(1994): 802–811.

Krieg, S.A., Shahine, L.K., and Lathi, R.B. 2016. Environmental exposure to endocrine-disrupting chemicals and miscarriage. Fertility and Sterility. **106**(4): 941–7.

Lamas, C.A., Gollucke, A.P., and Dolder, H. 2015. Grape juice concentrate (G8000®) intake mitigates testicular morphological and ultrastructural damage following cadmium intoxication. International Journal of Experimental Pathology. **96**:301–310.

Li, C., Zhao, K., Zhang, H., Liu, L., Xiong, F., Wang, K., and Chen, B. 2018. Lead exposure reduces sperm quality and DNA integrity in mice. Environmental Toxicology. **33:** 594–602.

Li, Y., Wang, M., Piao, F., and Wang, X. 2012. Subchronic exposure to arsenic inhibits spermatogenesis and downregulates the expression of Ddx3y in testis and epididymis of mice. Toxicological Sciences. **128**(2): 482–489.

Lin, H. T., Huang, Y. L., and Wang, M. Y. 1998. Arsenic species in drinking water, hair, fingernails, and urine of patients with blackfoot disease. Journal of Toxicology and Environmental Health A. **53**: 85–93.

Lu, C.F., Yuan, X.Y., Li, L.Z., Zhou, W., hao, J., Wang, Y.M., and Peng, S.Q. 2015. Combined exposure to nano-silica and lead induced potentiation of oxidative stress and DNA damage in human lung epithelial cells. Ecotoxicology and Environmental Safety. **122**(2015): 537–544.

Maciak, S., Włostowski, T., Salińska, A., and Bonda-Ostaszewska, E. Tissue cadmium accumulation is associated with basal metabolic rate in mice. Biology of Trace Elements in Research. **144**(2011): 944–950.

Mahaffay, K.K. 1990. Introduction: Advances in lead research: Implications for environmental health. Environmental Health Perpectives. **89**: 95-100.

Marchlewicz, M. 1994. Effectiveness of blood-testis and blood-epididymis barriers for lead. Annales Academiae Medicae Stetinensis. **40**: 37–51.

Marchlewicz, M., Wiszniewska, B., Gonet, B., Bosiacka, I.B., Safranow, K., Kolasa, A., Głabowski, W., Kurzawa, R., Jakubowska, K., and Rac, M.E. 2007. Increased lipid peroxidation and ascorbic acid utilization in testis and epididymis of rats chronically exposed to lead. Biometals. **20**: 13–9.

Marettova, E., Maretta, M., and Legath, J. Toxic effects of cadmium on testis of birds and mammals: a review. Animal Reproductive Sciences. **155**(2015): 1–10.

Meeker, J.D., Rossano, M.G., Protas, B., Diamond, M.P., Puscheck, E., Daly, D., Paneth, N., and Wirth, J.J. 2008. Cadmium, lead, and other metals in relation to semen quality: human evidence for molybdenum as a male reproductive toxicant. Environmental Health Perspectives. **116**: 1473–1479.

Monsefi, M., Alaee, S., Moradshahi, A., and Rohani, L. 2010. Cadmium-induced infertility in male mice. Environmental Toxicology. **25**(1): 94–102.

Morais, S., Costa, F.G., and Pereira, M.L. 2012. Heavy metals and human health. Environ. Health – Emerging Issues Practice. **2012**: 227–246.

Morales, M.E., Derbes, R.S., Ade, C.M., Ortego, J.C., Stark, J., Deininger, P.L., and Engel A.M.R. 2016. Heavy metal expo- sure influences double strand break DNA repair outcomes. PLoS ONE **11**(3): e0151367.

Mouro, G.S.V., Martins, A.L.P., Silva, J., Menezes, T.P., Gomes, M.L.M., Oliveira, J.A., Fabiana, C.S.A., and Matta, S.L.P. 2019. Subacute Testicular Toxicity to Cadmium Exposure Intraperitoneally and Orally. Oxidative Medicine and Cellular Longevity. **2019**.

Mukhopadhyay, P.K., Dey, A., Mukherjee, S., and Pradhan, N.K. 2013. The effect of coadministration of alpha- tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. Journal of Basic and Clinical Physiology and Pharmacology. **24**: 245–253.

Naha, N., and Manna, B. 2007. Mechanism of lead induced effects on human spermatozoa after occupational exposure. Kathmandu University Medical Journal.

5(2007): 85–94.

Nnaa, V.U., Ujaha, G.A., Mohamed, M., Etima, K.B., Igbaa, B.O., Augustinea, E.R., and Osima, E.E. 2017. Cadmium chloride–induced testicular toxicity in male wistar rats; prophylactic effect of quercetin, and assessment of testicular recovery following cadmium chloride withdrawal. Biomedicine & Pharmacotherapy. **94**(2017): 109-123

Ola-Mudathir, K.F., Suru, S.M., Fafunso, M.A., Obioha, U.E., and Faremi, T.Y. 2008. Protective roles of onion and garlic extracts on cadmium induced changes in sperm characteristics and testicular oxidative damage in rats. Food and Chemical Toxicology. **46**(12): 3604–3611.

Oliveira, H., Spano, M., Santos, C., and Pereira. M.L. 2009. Adverse effects of cadmium exposure on mouse sperm. Reproductive Toxicology. **28**(4): 550–555.

Oyagbemi, A.A., Omobowale, T.O., Asenuga, E.R., Ochigbo, G.O., Adejumobi, A.O., Adedapo, A.A., and Yakubu, M.A. 2017. Sodium arsenite-induced cardiovascular and renal dysfunction in rat via oxidative stress and protein kinase B (Akt/PKB) signaling pathway. Redox Report. **22**(6): 467–477.

Oyeronke, A.O. 2018. Co-exposure of Lead Acetate and Sodium Arsenite Causes Alteration in the Markers of Liver and Kidney Functions in Male Wistar Rats. Journal of Chemical, Environmental and Biological Engineering. **2**(1): 32.

Pandey, R., Kumar, R., Singh, S.P., Saxena, D.K., and Srivastava, S.P. 1999. Male reproductive effect of nickel sulphate in mice. Biometals. **12**: 339–346.

Pandya, C., Pillai, P., Nampoothiri, L.P., Bhatt, N., Gupta, S., and Gupta, S. 2012. Effect of lead and cadmium co-exposure on testicular steroid metabolism and antioxidant system of adult male rats. Andrologia. **44**(Suppl.1): 813–822.

Pant, N., Kumar, R., Murthy, R.C., and Srivastava, S.P. 2001. Male reproductive effect of arsenic in mice. Biometals **14**: 113–117.

Pant, N., Upadhyay, G., Pandey, S., Mathur, N., Saxena, D.K., and Srivastava, S.P. 2003. Lead and cadmium concentration in the seminal plasma of men in the general population: correlation with sperm quality. Reproductive Toxicology. **17**(4): 447–450.

Parvez, F., Chen, Y., Argos, M., Hussain, A.Z., Momotaj, H., Dhar, R., Geen, A., Graziano, J.H., and Ahsan, H. 2006. Prevalence of arsenic exposure from drinking water and awareness of its health risks in a Bangladeshi population; results from a large population-based study. Environmental Health Perspectives. **114**(3): 355–359.

Ramah, A., EL-shwarby, R.M., Nabila, M.A., and El-shewey, E.A. 2015. The effect of lead toxicity on male albino rats' reproduction with ameliorate by vitamin E and pumpkin seeds oil. Benha Veterinary Medical Journal. **28**(1): 43–52.

Ramos, A.T.A., Diamante, M.A.S., Lamas, C.A., Dolder, H., and de Souza Predes, F. 2017. Morphological and morphometrical changes on adult Wistar rat testis caused by chronic sodium arsenite exposure. Environmental Science and Pollution Research. **24**(2017): 27905–27912.

Rana, S.V.S. 2014. Perspectives in endocrine toxicity of heavy metals—a review. Biology of Trace Elements in Research. **160**(1): 1–14.

Rastogi, S.K. 2008. Renal effects of environmental and occupational lead exposure. Indian Journal of Occupational and Environmental Medicine. **12**(3): 103–106.

Reddy, P.S., Rani, G.P., Sainath, S.B., Meena, R., and Supriya, C.H. 2011. Protective effects of N-acetylcysteine against arsenic-induced oxidative stress and reprotoxicity in male mice. Journal of Trace Elements in Medicine and Biology. **25**: 247–253.

Rehman, K., Fatima, F., Waheed, I., and Akash, M.S.H. 2018. Prevalence of exposure of heavy metals and their impact on health consequences. Journal of Cellular Biochemistry. **119**(1): 157–184.

Sanghamitra, S., Hazra, J., Upadhyay, S.N., Singh, R.K., and Chowdhury, A.R. 2008. Arsenic induced toxicity on testicular tissue of mice. Indian Journal of Physiology and Pharmacology. **52**(1): 84–90.

Sarkar, S., Hazra, J., Upadhyay, S.N., Singh, R.K., and Chowdhury, A.R. 2008. Arsenic induced toxicity on testicular tissue of mice. Indian Journal of Physiology and Pharmacology. **52**: 84–90.

Satarug, S., Baker, J.R., Urbenjapol, S., Haswell-Elkins M., Reilly, P.E., Williams, D.J., and Moore, M.R. 2003. A global perspective on cadmium pollution and toxicity in

non-occupationally exposed population, Toxicological Letters. **137**(2003): 65–83.

Schlappack, O.K., Delic, J.I., Harwood, J.R., and Stanley, J.A. 1988. Protection from radiation-induced damage to spermatogenesis in the androgen pretreated rat. Radiotherapy and Oncology. **12**(3): 219–224.

Schulz, H., Nagymajtényi, L., Institoris, L., Papp, A., and Siroki, O. 2002. A study on behavioral, neurotoxicological, and immunotoxicological effects of subchronic arsenic treatment in rats. Journal of Toxicology and Environmental Health. **65**(16): 1181-1193.

Shallari, S., Schwartz, C., Hasko, A., and Morel, J.L. 1998. Heavy metals in soils and plants of serpentine and industrial sites of Albania. Science of the Total Environment. **209**(1998): 133–142.

Sharma, G., Kumar, M. 2012. Antioxidant and modulatory role of *Chlorophytum borivilianum* against arsenic induced testicular impairment. Journal of Environmental Science. **24**(12): 2159–2165.

Siu, E.R., Mruk, D.D., Porto, C.S., and Cheng, C.Y. 2009. Cadmium-induced testicular injury. Toxicology and Applied Pharmacology. **238**(3): 240–249.

Sokol, R.Z. 1987. Hormonal Effects of Lead Acetate in the Male Rat: Mechanism of Action1. Biology of Reproduction. **37**(5): 1135–1138.

Sokol, R.Z., Wang, S., Wan, Y.J.Y., Stanczyk, F.Z., Gentzschein, E., and Chapin, R.E. 2002. Long-term, low-dose lead exposure alters the gonadotropin-releasing hormone system in the male rat. Environmental Health Perspectives. **110**(9): 871–874.

Stahl, W., Junghans, A., de Boer, B., Driomina, E.S., Briviba, K., and Sies, H. 1998. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. Federation of European Biochemical Societies Letters. **427**(2): 305–308.

Swarup, D., Naresh, R., Vaeshney, V.P., Balagangaththh, A.M., Kumar, P., Nandi, D., and Patra, R.C. 2007. Changes in plasma hormones profile and liver function in cows naturally exposed to lead and Cd around different industrial areas. Research in

Veterinary Science. **82**(1): 16–21.

Taha, E.A., Sayed, S.K., Ghandour, N.M., Mahran, A.M., Saleh, M.A., Amin, M.M. and Shamloul, R. 2013. Correlation between seminal lead and cadmium and seminal parameters in idiopathic oligoasthenozoospermic males. Central European Journal of Urology. **66**(1): 84–92.

Thomas, D.J., Styblo, M., and Lin, S. 2001. The cellular metabolism and systemic toxicity of arsenic. Toxicology and Applied Pharmacology. **176**: 127–144.

Tsai, S.M., Wang, T.N., and Ko, Y.C. 1998. Cancer mortality trends in a blackfoot disease endemic community of Taiwan following water source replacement. Journal of Toxicology and Environmental Health A. **55**: 389–404.

Waalkes, M.P., and Diwan, B.A. 1999. Cadmium-induced inhibition of the growth and metastasis of human lung carcinoma xenografts: role of apoptosis. Carcinogenesis. **20**(1): 65–70.

Waalkes, M.P., Rehm, S., and Cherian, M.G. 2000. Repeated cadmium exposures enhance the malignant progression of ensuing tumors in rats. Toxicological Sciences. **54**(1): 110–120.

Wang, X., Zhang, J., Xu, W., Huang, Q., Liu, L., Tian, M., Xia, Y., Zhang, W., and Shen, H. 2016. Low-level environmental arsenic exposure correlates with unexplained male infertility risk. Science of the Total Environment. **15**: 307–313.

Wani, A.L., Ara, A., and Usmani, J.A. Lead toxicity: a review. Interdisciplinary toxicology. **8**(2015): 55–64.

Wapnir, R.A., Exeni, R.S., Mc Viar, M., and Lifschitz, E. 1977. Experimental lead poisoning and intestinal transport of glucose, amino acids and sodium. Pediatric Research. **11**: 153-157.

Vigeh, M., Smith, D.R., and Hsu, P.C. 2011. How does lead induce male infertility? Iran Journal of Reproductive Medicine. **9**(2011): 1–8.

Wirth, J.J., and Mijal, R.S. Adverse Effects of Low Level Heavy Metal Exposure on Male Reproductive Function. Systems Biology in Reproductive Medicine. **56**(2010):147-167.

Winski, S.L., and Carter, D.E. 1998. Arsenate toxicity in human erythrocytes: Characterization of morphologic changes and determination of the mechanism of damage. Journal of Toxicology and Environmental Health A. **53**: 345–355.

World Health Organization. 2011. Cadmium in Drinking-water Background document for development of WHO Guidelines for Drinking-water Quality.

Wright, J., Colby, H., and Miles, P. 1981. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Archives of Biochemistry and Biophysics. **206**(2): 296-304.

Xu, W., Bao, H., Liu, F., Liu, L., Zhu, Y.G., She, J., Dong, S., Cai, M., Li, L., Li, C., and Shen, H. 2012. Environmental exposure to arsenic may reduce human semen quality: associations derived from a Chinese cross-sectional study. Environment and Health. **11**(2012): 46.

Yu, T., Li, Z., Wang, X., Niu, K., Xiao, J., and Li, B. Effect of lead exposure on male sexual hormone. Journal of Hygiene Research. **39**(4): 413–415.

Zeng, Q., Yi, H., Huang, L., An, Q., and Wang, H. 2019. Long-term arsenite exposure induces testicular toxicity by redox imbalance, G2/M cell arrest and apoptosis in mice. Toxicology. **411**(2019): 122-132.

Zhang, B., Georgiev, O., Hagmann, M., Günes, C., Cramer, M., Faller, P., Vasák, M., and Schaffner, W. 2003. Activity of metal responsive transcription factor 1 by toxic heavy metals and H2O2 in vitro is modulated by metallothionein. Molecular Cell Biology. **23**: 8471–8485.

Zubair, M., Ahmad, M., Jamil, H., and Deeba, F. 2016. Toxic effects of arsenic on semen and hormonal profile and their amelioration with vitamin E in Teddy goat bucks. Andrologia. **48**(10): 1120–1228.