A biochemical and histological approach to investigate comparative reprotoxic effects of Lead, Cadmium and Arsenic in female adult Sprague Dawley rats.

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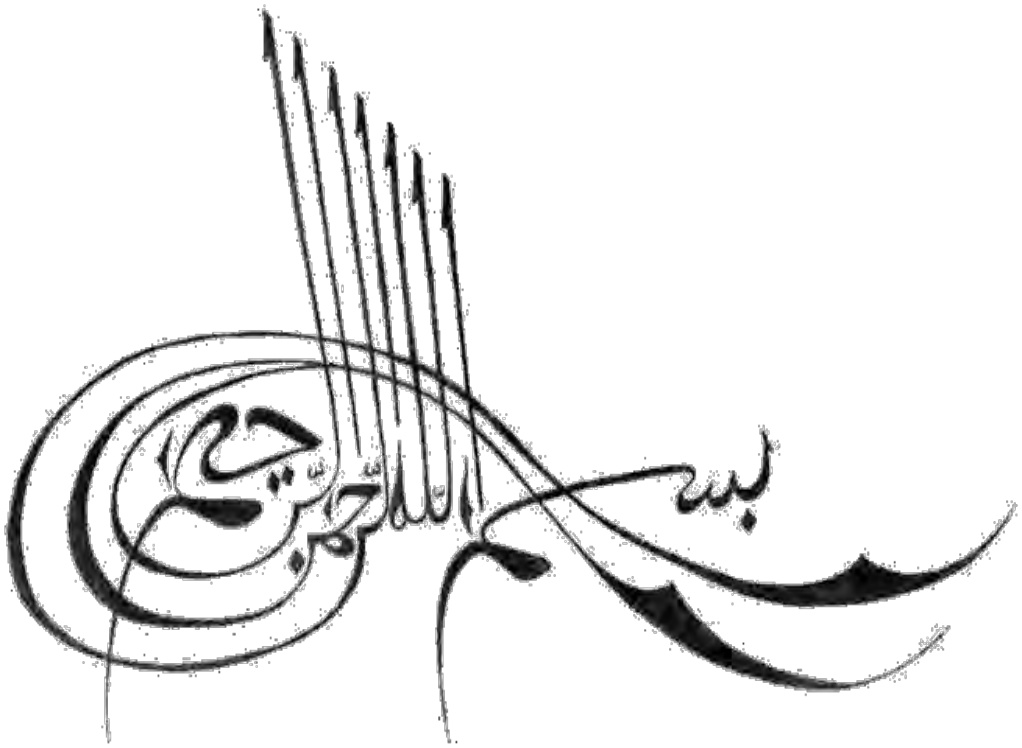
بسم الله الرحمن الرحيم

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.

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List of abbreviations

ATSDR	Agency for Toxic Substances and Disease Registry
BLL	Blood Lead Level
CAT	Catalase
E2	Estradiol
ER	Estrogen Receptor
EDCs	Endocrine Disrupting Chemicals
EFSA	European Food Safety Authority
FD-Pase	Fructose-1,6-diphosphatase
G-6-Pase	Glucose-6-Phosphatase
HPA Axis	Hypothalamic-pituitary-Adrenal axis
HPG axis	Hypothalamic-pituitary-Gonadal axis
HPT axis	Hypothalamic-pituitary-Thyroid axis

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IARC	International Agency for Research on Cancer
MDA	Malonaldehyde
OECD	Organisation for Economic Co-operation and Development
POD	Peroxidase
PEPCK	Phosphoenolpyruvate Carboxy Kinase
ROS	Reactive Oxygen Species
SOD	Super oxide dismutase
T-BARS	Thiobarbituric acid Reactive Species

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Abstract

Infertility is a rapidly emerging concern throughout the world, with female infertility accounting for 37% of couples' infertility in developed countries. Quality of environment is very important for human health status. Female infertility is mainly caused by exposure to environmental pollutants, including heavy metals. Metals can be released to the environment from different branches of industry, hence in the environment, the amount of heavy metals rises. The current study was designed to evaluate and compare repro-toxic effects of lead, cadmium, and arsenic for 28 days in female Sprague Dawley rats. Stock solution of lead (1.5 and 8mg/kg), cadmium (0.09 and 5mg/kg), and arsenic (1.6 and 24.6mg/kg) were prepared. First group served as control, received normal saline by oral gavage. Second and third group received low and high dose of lead acetate respectively. Fourth and fifth group received contrasting dose of cadmium chloride. Sixth and seventh group received low and high dose of sodium arsenite. Body weight and glucose level was checked at 1st, 14th, and 28th day of the experiment. Animals were dissected at day 29. Vaginal smear was collected every 7th day during a period of 28 days for determination of changes in estrous cyclicity. Ovarian and uterine tissues were collected and stored at -80ºC until analysed. Lead acetate exposure caused significant decline in body weight, while the animal treated with low and high doses of cadmium chloride and sodium arsenite showed normal weight gain when compared to control. Low and high doses of these three heavy metals caused significant decline in reproductive organ weight when compared to control. Heavy metals (Pb, Cd, As) disturbed regularity of estrous cyclicity of treated rats. Lead acetate treatment increased ovarian CAT and decreased ovarian SOD and POD. Both doses of lead acetate also increased ovarian reactive oxygen species (ROS) and malonaldehyde (MDA) levels. Low and high doses of cadmium chloride and sodium arsenite decreased ovarian CAT, SOD, and POD and increased ROS and MDA levels. Histomorphological studies showed changes in the ovaries including reduction in ovarian follicles number, elevation in atretic follicles number, follicular cell dispersion, optical empty spaces, degenerated corpus luteum, corpus luteum cyst, ovarian follicles necrosis, damaged oocytes and follicular cell dispersion. These alterations reduced ovarian tissue function in female rats. In conclusion, the present study demonstrated reprotoxic effect of heavy metals by disrupting antioxidant defence mechanism, ovarian histomorphology, and estrous cyclicity, leading to infertility.

Introduction

Endocrine disruptors

Endocrine disruptors are exogenous chemicals that disrupt natural hormones synthesis, elimination, stimulation, binding, transportation and excretion, leading to disturbance in physiological processes such as homeostatic mechanisms, regulation of normal development and reproduction. These chemical agents are discharged through human activities and undergo physical and chemical reactions in the air. These chemicals interact with endocrine glands by exerting agonist or antagonist actions at hormone receptors. Even nanogram levels of EDCs can cause infertility in humans, early onset of puberty, diabetes, obesity and cancer as reported by Annamalai and Namasivayam (2015). There are two main categories of EDCs in the environment; one is natural which is acquired by foodstuff and another one is synthetic which includes solvents used in industries (Kabir *et al*., 2015). EDCs hamper or disrupt normal action of natural hormones (Beronius and Vandenberg, 2015). Exposure of humans to EDCs occur through environment or through their occupation. US Food and Drug Administration reported that minimum thousand chemicals may be regarded as endocrine disruptors because of their endocrine disrupting characteristics (Beronius and Vandenberg, 2015). These chemicals are incorporated into various consumer products e.g. food contact material, medical equipments, fabrics, cosmetics, as well as in different pesticides (Molander and Ruden, 2012) and considerable exposure of the environment to these chemicals is due to discharge from everyday products. EDCs are capable of altering nuclear receptor signaling, producing estrogen-like and/or anti-androgenic effect, altering the endocrine and reproductive systems (Schug *et al*., 2011). Mechanism of direct and epigenetic action of EDCs on genes is under study (Moral *et al*., 2008; Anway and Skinner, 2008).

Infertility

Infertility is a disorder in which a couple fails to attain pregnency after a year of regular sexual intercourse without any practice of contraception. About 20 to 30% of infertile couples remain childless after routine checkup without detectible causes and suffer from idiopathic infertility (Esteves *et al*., 2015). About 5 to 10% infertile women may have underlying genetic abnormalities e.g. chromosomal anomalies, gene mutations, or polymorphisms. Infertility is mainly caused by exposure to endocrine disrupting substances, environmental pollutants and hormonal imbalances (Tarin *et al*., 2015; Hanson *et al*., 2017). Anovulation, endometriosis, uterine tube disease, pelvic adhesions and idiopathic infertility are major causes of female infertility (Barbieri, 2019; Unuane

et al., 2011). In developed countries, 37% of the couple infertility cases are due to female factor, 8% due to male factor and 35% due to both male and female factor (Unuane *et al*., 2011).

Heavy metals

Elements having a specific density higher than 5 g/cm3 are characterized as heavy metals (Duffus, 2002). They represent pollutants, which affect general and reproductive health in various aspects causing serious health problems (Rzymski *et al*., 2015). These metals bioaccumulate in the food chains and are not decomposed under natural conditions (Jarup, 2003; Rzymski *et al*., 2014).World health organization reported that quality of the environment is very important for human health status. One fourth of worldwide disease and over one-third of disease amongst teenagers might be present due to change in environmental quality (Pruss and Corvalan, 2006). Living organisms require some heavy metals in minute amount but when these metals are present in large amounts they are harmful for living organisms. Metals can be released to the environment from various industries, transport, waste treatment, fertilizers and waste used to fertilize soil (Szyczewski *et al*., 2009; Reeder *et al*., 2006). Exposure of humans to heavy metals occurs mainly through contaminated air, food and/or water.

Female reproductive toxicity of heavy metals

Heavy metals may affect the ovary either directly or indirectly. Primary ovarian dysfunction might be produced by direct alteration in cell signaling pathway of ovarian cells by heavy metals. Secondary ovarian dysfunction involves problem in hypothalamus-pituitary axis (Rana, 2014). Massanyi *et al*. (2007) describes that ovary of rats exposed to cadmium exhibited reduction in developing follicles volume and increase in atretic follicles amount and stroma. Heavy metals exhibit endocrine disrupting properties with either agonist or antagonist endocrine effects in human and animals. Metals such as, cadmium, and nickel can replace zinc atom from estrogen receptor zinc finger, and when these metals replaced zinc, singling mechanism of estrogen receptor affected. Arsenic has the ability to bind or interact with the glucocorticoid receptor and disturb biological processes controlled by this receptor. Mercury (Hg) may hinder steroid hormones biosynthesis, disrupt HPT axis or HPA axis, inducing alterations in female fertility (Georgescu *et al*., 2011). Two factors which mainly affect health status of an individual are phenotypic and genotypic factors, environment is important phenotypic effect that can affect genotype also (Bhargava *et al*., 2017). Exposure to heavy metal can result in oxidative damage and adverse health effects by increasing reactive oxygen species (ROS) generation. High mortality and morbidity rates all over

the world are reported by using water polluted with heavy metals (Rehman *et al*., 2018). Sometimes due to their chemical coordination and oxidation-reduction characteristics, they can counter various control operations like homeostasis, transportation, and compartmentalization (Jaishankar *et al*., 2014). Heavy metals have ability to bind with binding sites of original metals by replacing them, causing cells malfunctioning and poisonousness. Earlier studies showed that when heavy metals bind with genetic components of the cell, biological macromolecules undergo oxidative decay (Flora *et al*., 2008).

Lead

Lead is abundantly distributed, dangerous chemical in environment (Mahaffay, 1990). Lead (Pb) is present as a bluish gray metal in earth crust. Lead has many agricultural and industrial applications (Rehman *et al*., 2018). The amount of lead utilized in the last century surpasses the amount of lead used in all earlier times (Phillips *et al*., 2003). Lead exposure increased by a number of activities such as mining, and fossil fuel consumption. It is also used in manufacturing of lead-acid batteries (Gabby, 2006). Important lead properties are ductility, malleability, softness, poor conductivity, resistance to corrosion, which make lead use extensive. Non-biodegradable property of lead and its extensive use increased lead concentration to hazardous level in the environment. Human are commonly exposed through various lead related occupations (Ara and Usmani, 2015). Humans are also exposed to lead through household paints, cosmetics and medicines (Potula and Kaye, 2005). 35 to 50% of lead absorption occurs through drinking water. Lead is absorbed in large amount in kidneys than in liver and other tissues of human body (Flora *et al*., 2006). Lead can prompt a number of functional, biochemical, and social deformities in man and various fauna affecting nervous system (Chakraborti *et al*., 1999), heamopoietic system (De Silva, 1981), and reproductive system of both male and female (Rom, 1980; Lancranjan *et al*., 1975).

Female reproductive toxicity of lead

Lead is a broadly studied industrial and ecological toxicant. Even in minute amount, lead is considered toxic (Uchewa and Ezugworie, 2019). Lead has negative impact on reproduction (Zheng *et al*., 2003). Toxic effects of lead is caused by increased blood lead levels (BLL). Female reproductive toxicity includes reduced fertility (Ara and Usmani, 2015), reduced pregnancy, low birth weight, spontaneous abortions, miscarriages (Park *et al*., 2008), impede folliculogenesis, damage ovaries (Fortune, 2003). Animal studies indicate that lead treatment has adverse effects on HPG axis of female (Doumouchtsis *et al*., 2009). For ovarian function, cellular integrity is

important because gonadotropins bind to receptors, which lie on ovarian granulosa cell membrane. Gonadotropin hormones are important for initiation of steroidogenic process, that regulates ovulation and estrous cycle (Nampoothiri *et al*., 2007). Lead exposure reduces binding ability of LH and FSH with gonadotropin receptors (Wiebe *et al*., 1988; Priya *et al*., 2004) which decreases secretion of steroid hormones E_2 and P (Paksy *et al*., 1997 and 2001; Nampoothiri and Gupta, 2006; Wiebe *et al*., 1988; Sleven *et al*., 2003). Structural changes in ovary can result by accumulation of lead in granulosa cells (Junaid *et al*., 1997). Furthermore, ovaian cysts were also observed after high dose lead exposure (Junaid *et al*., 1997). Lead acetate treated rats may show irregularity in estrous cyclicity, prolonged diestrus phase duration (Shubina and Dudenkova, 2014). In ovarian tissues, antioxidant enzymes play part in sustaining balance of free oxygen radicals (Qanungo *et al*., 1999), however, reactive oxygen species amount may be increased by lead(El-Maraghy *et al*., 2001). When concentration of free oxygen radicals increases, they convert fat molecules into lipid peroxidation and then disturb cells oxidant/antioxidant balance (Stohs *et al*., 2001). Lead produce oxidative stress by interfering with cell boundary, DNA and antioxidant shielding method of cell (Hsu and Guo, 2002). Zinc is required for normal functioning of superoxide dismutase activity. Lead exposure displaces zinc ions from active site and thus inhibit SOD activity in granulosa cells of ovary. Similar observation was reported by Ariza *et al*. (1998).

Cadmium

Cadmium is an vital industrial and environmental contaminant. Cadmium has been placed at seventh position on the list of hazardous substances due to their known toxicity (Nasiadek *et al*., 2019). Humans exposed to Cd through occupations related to cadmium e.g. electroplating, welding, mining, smelting, Ni-Cd batteries, plastic making (Li *et al*., 2018; Nordberg *et al*., 2018). Cd exposure of the general population occurs mainly through food, water and tobacco smoking (Nasiadek *et al*., 2018; Jarup and Akesson, 2009). Cd incorporates in the food chains due to modern civilization processes. It has an extended biological half-life and causes chronic problems and has adverse health effects when accumulated in the body. Humans absorb about 3–5% of cadmium from food, which is reabsorbed in kidneys, and bio-accumulated for about 10 to 30 years (Satarug *et al*., 2020). In non-smoking general population, the main source of Cd exposure is diet. Monthly intake of cadmium about 25mg/kg body weight is consider as provisionally tolerable according to WHO (Ferrari *et al*., 2013). 2-4µg of cadmium is acquired by smoking a pack of cigarettes. Average dietary exposure of cadmium range from 20-74µg/day per seventy kg person has been

estimated by the European Food Safety Authority (EFSA) (Winiarska –Mieczan *et al*., 2015). Due to low concentration of iron, cadmium storage is higher in women than in males (Uetani *et al*., 2006). Cadmium also accumulates in organs related to reproduction in addition to kidney and liver (Rzymski *et al*., 2016). Ovary and uterus are more sensitive to Cd toxicity and are severely damaged when exposed to Cd (Wang *et al*., 2015). Cadmium mimics estrogen action and has ability to bind with estrogen receptor (ER), hence cadmium is also known as metalloestrogen (Stoica *et al*., 2000).

Female reproductive toxicity of cadmium

Structural and functional disruption of ovaries by cadmium was first described by Kar (1959). In vivo studies showed that cadmium have toxic effects on reproductive organs of female mainly endocrine system (Nasiadek *et al*., 2018). Cadmium exposure causes female reproductive function impairment by disrupting HPG axis signaling, reported in rats as animal model (Arciszewski and Zacharko-Siembida, 2007; Varga *et al*., 1993; Rzymski *et al*., 2016). LH and FSH are essential for production steroid hormones {estradiol (E_2) and progesterone (P)}, which are important for female reproductive functions. Cadmium can increase and decrease the secretion of steroid hormones and the effect of cadmium depends on the dose, route of administration and stage of estrous cycle (Zhang *et al*., 2008). Cadmium poisoning decreases the secretion of LH and FSH. Cadmium can behave as agonist (Höfer *et al*., 2009) as well as antagonist (Rider *et al*., 2009). Cd imitates estrogen action and possess endocrine disrupting properties (Garcia-Morales *et al*., 1994; Byrne *et al*., 2009). In experimental animals, cadmium effects vary depending upon the levels of sex hormones (E_2 and P). Toxicity induced by cadmium exposure in ovaries may result in decreased follicular growth, increased follicular atresia, degenerated corpus luteum, change in sexual cyclicity, distorted Graafian follicle, and decreased follicle number (Massanyi *et al*., 2020; Nna *et al*., 2017). In ovariectomized rats, Cd exposure caused uterine hyperplasia, and increased mammary glands growth (Johnson *et al*., 2003). Histopathological alterations in the ovary includes degenerated corpus luteum, damaged oocytes and degenerated granulosa cells that resulted after cadmium administration. The effects of cadmium toxicity on uterus are rise in tallness of lumen epithelium and increase in width of endometrium (Wang *et al*., 2015). Cadmium exposure may disturb estrous cyclicity. Low dose of cadmium may cause irregularity of estrous cycle by prolongation of estrus phase and high dose may increase diestrus phase duration and decrease proestrus phase length (Nasiadek *et al*., 2019). Cadmium induce oxidative stress in ovaries of

cadmium treated rats by decreasing the levels of antioxidant enzymes and increasing the levels of oxidative stress marker in ovaries of rats (Ruslee *et al*., 2020). Cadmium has age-specific effect, and it may alter anti-mullerian hormone level (Min lee *et al*., 2018).

Arsenic

Arsenic is a toxic environmental pollutant and a toxic semi-metalloid neuroendocrine disruptor (Ommati *et al*., 2020). Arsenic has been placed among the top hazardous elements by ATSDR (1999). Arsenic is carcinogenic for a number of human body organs. It is in debate which form of arsenic is carcinogenic because arsenic present in the environment in mainly four forms: arsenate arsenite, methylarsonic acid and dimethylarsinic acid (Kitchin *et al*., 1999). Arsenite is highly toxic arsenic form. The main routes of entrance of arsenic are through respiration, skin absorption, drinking water and food (Laine *et al*., 2015; Ommati *et al*., 2019). In drinking water, concentration of arsenic must be below 10 µg/L (WHO, 1998), as rate of mortalities and abnormalities increase above 10 µg/L (IARC, 2004). Arsenic is a major water pollutant having genotoxic effects (Li *et al*., 2008). Arsenic is used repeatedly in aquatic environment as herbicides, insecticides and rodenticides and become hazardous because of its extensive use (Flora *et al*., 1995). Occupation exposure from arsenic occurs through metal-smelting and glass-making industries (Yih *et al*., 2002). Ovarian tumors (Waalkes *et al*., 2004), skin cancer (Saha, 1995), and severe diabetic disorders (Tseng *et al*., 2002) are outcomes of arsenic intoxication. Metabolic processing of arsenical compounds resulted in elevation of oxidative stress by producing free oxygen radicals (Tabacova *et al*., 1997), which damage DNA (Kato *et al*., 1994).

Female reproductive toxicity of arsenic

Arsenic causes morphological and functional changes in biological systems, mainly in reproductive system of males and females (Ommati *et al*., 2020). Normal reproductive functions disturb after arsenic exposure. Estradiol and progesterone plays important role in the reproductive function of females, which in-turn are controlled by gonadotropins LH and FSH, which are secreted from anterior pituitary gland. Ovary, which is the main source of estradiol, is affected badly by arsenic exposure (Chatterjee and Chatterji, 2010; Chattopadhyay and Ghosh, 2010). Rats exposed to arsenic showed decreased levels of E2 and gonadotropins (Chattopadhyay and Ghosh, 2010). Histological changes in ovaries resulted due to disturbed level of E_2. Arsenic induced lethal effects in experimental rodent model on reproductive system of female (Chattopadhyay *et al*., 1999). Arsenic treatment may alter ovary histological status. It may reduce number of healthy

follicles and increase follicular atresia. Arsenic intoxicated group of rats have disturbed estrous cyclicity. Diestrus phase may be prolonged after arsenic treatment (Chattopadhyay and Ghosh, 2010). Metabolism of arsenic compounds resulted in the production of free oxygen radicals that inturn produced increased oxidative stress, which damage DNA (Kitchin, 2001; Tabacova *et al*., 1997). ROS production caused ovarian toxicity (Akram *et al*., 2010; Chatterjee and Chatterji, 2010). Arsenic treatment suppressed peroxidase activity in ovarian tissues (Chattopadhyay and Ghosh, 2010). Arsenic also interrupts estrogen signaling through oxidative damage of proteins that play role in estrogen signaling pathway (Chatterjee and Chatterji, 2010).

Purpose of the study

While aforementioned studies have focused on the consequences of heavy metal contact on female reproductive health and estrous cyclicity, 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 woman.

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 females rats. The effects of lead, cadmium, and arsenic on female reproductive system of female rat is not yet entirely comprehended concerning its histology, reproductive cyclicity and antioxidant status; therefore, it is essential to assess likely alterations in these factors under simulated environmental and occupational exposure conditions.

Aims and objectives

The objective of the present research is to study the comparative effects of different doses of three heavy metals (lead, cadmium and arsenic) on female reproductive system of rats and to explore the effect of heavy metals exposure on the oxidative stress markers (ROS and TBARS) and antioxidant enzyme status (SOD, POD and CAT) of ovarian tissues of rats.

Materials and methods

Current study was performed in the Laboratory of Reproductive Physiology, Department of Zoology, Quaid-i-Azam University, Islamabad, Pakistan. Handling of animals and all 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 female adult Sprague Dawley rats (Rattus norvegicus) of pubertal age (weight 160-250g) were acquired from the animal house of Biological Discipline Faculty, Quaid-i-Azam University, Islamabad, to be used for the present research. Animals were separated randomly into seven groups containing each (n=5) 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. Prior to initiation of the experiment, the rats were allowed to properly adjust to the environment for atleast seven days.

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 ordered 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 female rats corresponded to the lowest and highest doses of the heavy metals previously used for subchronic exposure 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 1.5mg/kg body weight of $Pb(CH_3COO)_2$ was selected for low level sub chronic exposure to lead acetate (Uchewa and Ezugworie., 2019).

For high level sub chronic exposure, a dose of 8mg/kg body weight $Pb(CH_3COO)_2$ was selected as described earlier by Junaid *et al*. (1997).

Cadmium chloride:

A dose of 0.09mg/kg body weight $CdCl_2$ was used for low level sub chronic exposure to cadmium chloride, as suggested by Nasiadek *et al*. (2019).

For high level sub chronic exposure to cadmium chloride, a daily dose of 5mg/kg body weight $CdCl_2$ was chosen (Li *et al*., 2018).

Sodium arsenite:

A daily oral dose of 1.6mg/kg $NaAsO_2$ was selected for low level arsenic exposure (Brown and Kitchin, 1996).

For high level sub chronic exposure to arsenic, a daily dose of 24.6mg/kg body weight $NaAsO_2$ was selected as used by (Brown and Kitchin, 1996).

Preparation of heavy metal stock solutions

Solutions of heavy metal salts were prepared in distilled water according to the body weight of experimental animals.

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 purified water. Analytical ranking chemicals were used during the course of the experiment.

Experimental design

The current experiment aimed to evaluate the reproductive toxicity of three metals (Pb, Cd, As) on female rats. All animals used in this experiment were divided into seven groups with five female 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. 1.5mg/kg $Pb(CH_3COO)_2$.

Group III: Rats received high oral dose of lead acetate i.e. 8mg/kg $Pb(CH_3COO)_2$.

Group IV: Animals were orally provided with low dosage of cadmium chloride i.e. 0.09mg/kg $CdCl_2$ dissolved in distilled water.

Group V: Rats of this group were orally given high cadmium chloride dosage 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. 1.6mg/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. 24.6mg/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 were sacrificed on 29th day of the experiment by decapitation.

Determination of body weight

Rats were weighed on day 1^{st}, 14^{th} and 28^{th} 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 physical euthanasia at day 29 of the experiment and blood (upto 3-5ml) was drained into heparinised tubes via cardiac puncture. Blood was centrifuged at 3000rpm for 15 minutes. Plasma was separated and kept at -20°C until analyzed. Immediately after blood collection, reproductive organs, including, ovaries and uterus, 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.

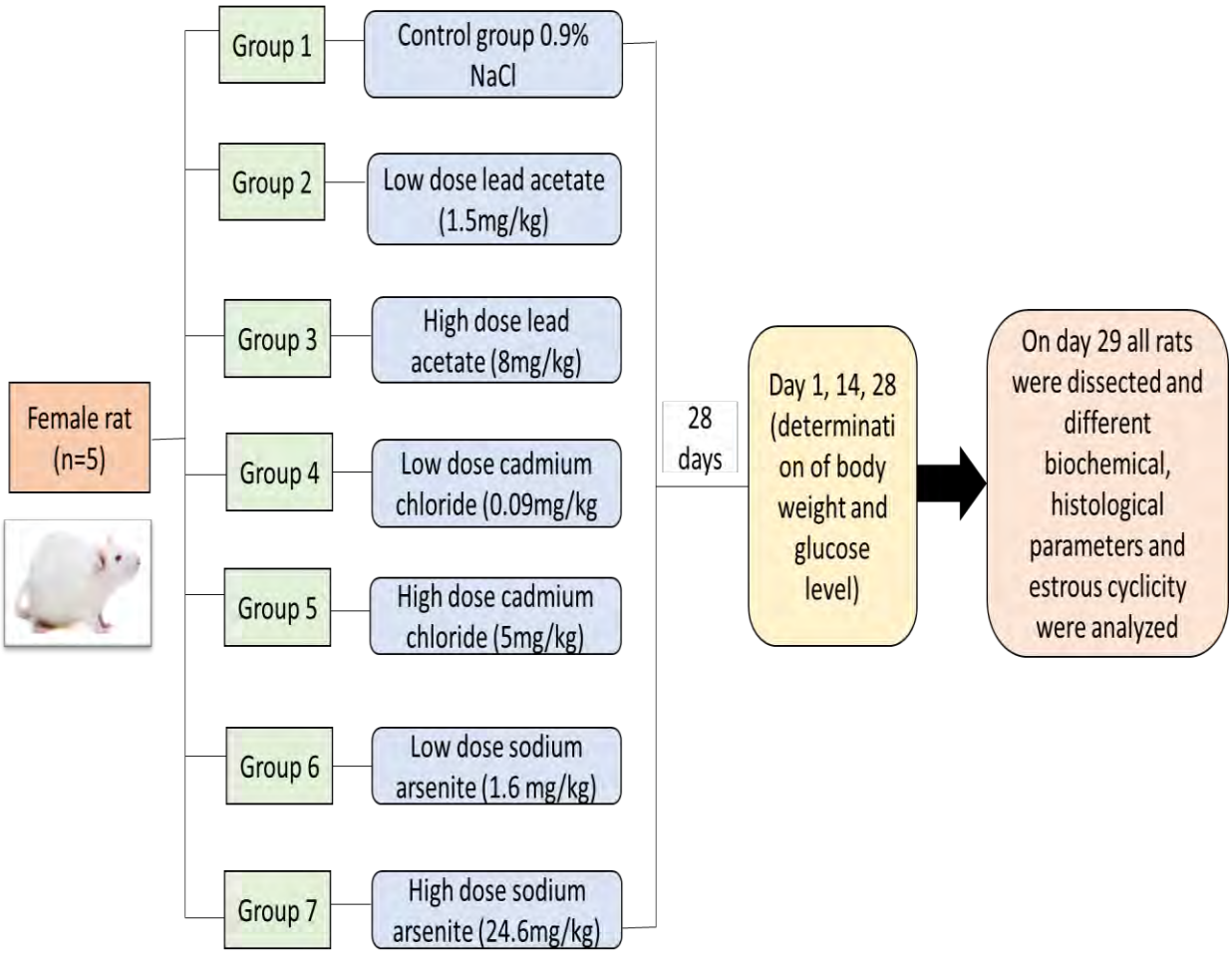

Figure 1. Schematic representation of oral sub chronic administration of different doses of lead acetate, cadmium chloride and sodium arsenite in adult female rats.

Tissue Histology

Histology of ovarian tissues were carried out to measure the reproductive toxicity of contrasting doses of lead acetate, cadmium chloride, and sodium arsenite in the rats. Once ovaries were collected after dissection of rats, the processes done subsequently are as follows;

Fixation

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

Dehydration

After fixation, tissues were removed of water in different 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 ^{o}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 ^{o}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 atleast 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. The slides were placed 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
3. Hydration
4. Sections were rehydrated in descending grades of alcohol
5. 100% alcohol I --- 3 min
6. 100% alcohol II --- 3 min
7. 90% alcohol --- 3 min
8. 70% alcohol --- 3 min
9. Washed with water --- 3 min
10. Slides were stained in different grades as follows:
11. Hematoxylin --- 8 min
12. Washing --- 2 min
13. Acidified alcohol --- 1 min
14. Washing --- 2 min
15. Bluing solution (1mL NH_4OH + 300mL water) --- 2 min
16. Washed with water --- 2 min

17. Dehydration
18. 90% alcohol --- 10 dips
19. Eosin ---2 min
20. Washed with water --- 2 min
21. 90% alcohol --- 5 min
22. Absolute alcohol I --- 5 min
23. Absolute alcohol II --- 5 min
24. Absolute alcohol III --- 5 min
25. Xylene I --- 5 min
26. Xylene II --- 5 min
27. 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 Olympus light microscope (Japan). 2-3µm sections were observed at 10X magnification. Folliculogenesis were recorded through histo-morphometric analysis. Olympus light microscope (Japan) paired with Canon digital camera (Japan) was used for microphotography of the sections.

Follicular classification:

Sections from each ovary were observed and follicles were counted in each section.

Follicles were evaluated in accordance with previous work by Luo *et al*., 2008 as:

A primordial follicle; an oocyte surrounded by a single layer of flattened granulosa cells.

Early primary follicles; which possessed predominantly cuboidal granulosa cells with some squamous granulosa cells.

A primary follicle; an oocyte surrounded by a single layer of cuboidal granulosa cells.

A prenatal follicle; when containing two to four layers of cuboidal granulosa cells without antral space (secondary follicle).

An antral follicle; a clearly defined antral space with three sheets of granulosa cells.

A Graafian follicle; granulosa layers with antral space and an oocyte surrounded by cumulus cells and zona pellucida (Luo *et al*., 2008).

An atretic follicle; consisting of at least 20 apoptotic granulosa cells, a deteriorating oocyte or a fragmented nucleus of oocyte (Luo *et al*., 2008).

Biochemical analysis

Analysis of antioxidant enzymes and cellular stress markers was done using ovarian tissue of control and treated animals for the determination of oxidative stress. Frozen ovarian 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 procedure defined by Aebi (1984) was used for estimation 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 ovarian tissue homogenate.
2. 50mM PBS (2.5 ml) at pH 5.0.
3. 5.9mM H2O2 (0.4ml).
4. Thoroughly mix them and record their absorbance at 240nm at 0 second and 1minute 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 procedure initially defined by Chance and Maehly (1955) was used to determine the Peroxidase (POD) activity.

Procedure:

1. 0.1ml of ovarian tissue homogenate.
2. 0.1ml of freshly prepared (20mM) guaiacol.
3. Take 0.3ml of (40mM) hydrogen peroxide.
4. Take 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. 4.1g of Sodium acetate (CH3COONa) 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 CH3COONa buffer (100 ml).
3. 50 mg of ferrous sulphate (FeSO4) added to 10 ml of CH3COONa 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' ovaries.

Procedure:

1. 0.02 ml (100mM), Ferric chloride (FeCl3).
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 mixture was kept in pulsating water bath for incubation at 37^{o}C for 60 min.
6. 10% trichloroacetic acid (1 ml) used to halt the reaction.
7. Put in 0.67% thiobarbituric acid (1 ml) and then kept in waterbath at 95^{o}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.

Protein Estimation Assay

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

Principle of test:

Peptide bonds present in the protein form a chelate with the Cu2+ 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 synthesized and one ml of each was taken and put it into the reagent (1.0 ml).
3. To prepare standards of different concentrations, one ml of standard was shaked well in one 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^{o}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 ovarian tissue.

10. A linear graph was plotted with the standard absorbance against the sample absorbance to measure the protein level of samples.

Estrous cyclicity:

For examination of alterations that appear during the reproductive cycle, the short length of estrous cycle in rats, renders them an ideal animal model. At the onset of puberty, the estrous cyclicity of all female rats was evaluated through different types of cells present in vaginal smear, collected every 7^{th} day over a period of 28 days. As an indicator of ovarian activity, vaginal smear histology has consistently been used (Long and Evans, 1922).

Vaginal smears:

Vaginal smear of the females were inspected on every 7^{th} day for 28 days period.

Material:

1. Pubertal female rat
2. Fine tip micropipettes (tip inner diameter 1 mm)
3. Saline 0.9% (0.15M) or Phosphate buffer saline (PBS)
4. Light microscope with 10x objective
5. Cleaned glass slides

Procedure:

1. For this purpose a pipette was used
2. 10-20 µL of saline was filled in pipette and the tip of pipette was placed on the vaginal opening of female rat.
3. Vagina was flushed with saline for three to five times and at the sixth time it was collected in tip of pipette.
4. For observation of vaginal cytology, use 10µL of normal saline.
5. The vaginal fluid was placed on glass slide (Caligioni, 2009).

Staining:

1. The vaginal smear was spreaded on the slide to make a smear.
2. When the smear get dried up, it was stained with few drops of haematoxylin stain.
3. Shake the slide for few seconds, then extra stain was removed from the slide by tilting the slide.
4. Further put few drops of eosin stain on the smear, and again shake it well to spread the stain on whole smear.

5. Tilt the slide again to remove extra stain. Then slide was washed gently with water four to five times to remove excessive stain.
6. All the procedure were done carefully to avoid washing of the vaginal cells.
7. This process is repeated foe each slide.

Morphometric analysis:

Determination of estrous cycle stage was carried out by light microscopy of stained vaginal smears. The analysis of the estrous cycle stage is based on the percentage amongst the 3 cell forms, viewed at 10x objective lens. The comparative ratio of cell types observed in smears was used as a determination of estrus cycle. The length of reproductive cycle in rat and mice, is about four to five days and it can be distributed into 4 stages;

Proestrus:

This stage is characterized by the presence of round nucleated epithelial cells, which may appear as clusters or discretely. Rarely, some cornified cells may seem in the sample. During day, estradiol level rises and during the night, gonadotropin surge occurs resulting in ovulation (Walmer *et al*., 1992).

Estrus:

This stage is distinctively characterized by the presence of cornified squamous epithelial cell, in clusters with no visible nucleus .The cytoplasm is granular, with irregular in shape (Walmer *et al*., 1992).

Metestrus:

This stage is characterized by the presence of equal proportion of 3 different cell types. Estradiol level in plasma is low (Walmer *et al*., 1992).

Diestrus:

Diestrus stage is characterized by high leukocyte number.

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 determined for all values.

Results

Effect on body weight

Mean ± SEM body weights of female rats exposed to different concentrations of lead acetate, cadmium chloride and sodium arsenite are presented in Table 1. At day 14th of treatment, low dosage group of lead acetate (1.5mg/kg) and high dosage group of lead acetate (8 mg/kg) depicted significant decrease ($P<0.05$) and ($P<0.001$) respectively on body weight with reference to the control group. However, at day 14th of treatment, low dosage groups of cadmium chloride (0.09mg/kg), and sodium arsenite (1.6mg/kg) and high dosage groups of cadmium chloride (5 mg/kg), and sodium arsenite (24.6mg/kg) showed no effect on body weight in comparison to control and it increased normally in these treated groups similarly as in control.

At day 28th of treatment, low dose of lead acetate (1.5mg/kg) and high dose of lead acetate (8mg/kg) indicated significant decrease ($P<0.001$) in the rat's body weights as compared to control. Whereas low dose of cadmium chloride (0.09mg/kg) and sodium arsenite (1.6mg/kg) and high dose of cadmium chloride (5mg/kg) and sodium arsenite (24.6mg/kg) presented no effect on body weight as compared to control and body weight increased normally.

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 dose of lead acetate (1.5mg/kg) showed significant increase ($P<0.05$) in glucose level of female rats in comparison with control. However, low dose of cadmium chloride (0.09mg/kg), showed no significantly difference in glucose level of the rats. But, significant rise ($P<0.01$) was detected in glucose level of animals exposed to high doses of lead acetate(8mg/kg), cadmium chloride(5mg/kg), and sodium arsenite (24.6mg/kg) when compared to the control. Furthermore, significant elevation ($P<0.01$) was obseved in blood glucose level of animals exposed to low dose of sodium arsenite (1.6mg/kg) when compared to control.

At 28th day of treatment low dose lead acetate (1.5mg/kg) and high dose lead acetate (8mg/kg) exposure showed significant elevation ($P<0.01$) in glucose level of female rats. However, low dose group of cadmium chloride (0.09mg/kg) displayed significant elevation ($P<0.05$) in glucose level in comparison with control. At day 28th, exposure to high dose (5mg/kg) of cadmium chloride displayed noteworthy rise ($P<0.001$) in glucose level of exposed animals. Similarly, at day 28th high dose of sodium arsenite (24.6mg/kg) and low dose of sodium arsenite (1.6mg/kg) indicated significant elevation ($P<0.001$) in the glucose level of rats in comparison with control.

Table 1. Mean ± SEM body weight (g) of rats at different days of treatment with different doses of heavy metals {lead, cadmium and arsenic (n=5 / group)}.

	Body Weight (g)		
Treatment	**Day 1**	**Day 14**	**Day 28**
Control	207.0 ± 3.74	232.4 ± 4.18	260.2 ± 5.03
$Pb(CH_3COO)_2$			
1.5mg/kg	209.4 ± 4.04	216.0 ± 4.31*	206.6 ± 4.55***
8mg/kg	207.4 ± 2.79	209.0 ± 3.05***	194.8 ± 1.16***
$CdCl_2$			
0.09mg/kg	211.0 ± 3.02	233.0 ± 2.70	260.8 ± 2.01
5mg/kg	212.6 ± 3.52	232.8 ± 2.54	261.0 ± 3.62
$NaAsO_2$			
1.6mg/kg	214.0 ± 2.21	233.4 ± 2.54	260.6 ± 3.17
24.6mg/kg	213.8 ± 4.32	233.2 ± 3.92	260.4 ± 2.66

Values are stated as Mean ± SEM. *, **, *** showing significant variance at $P<0.05$, $P<0.01$ and $P<0.001$ vs control.

Table 2. Mean ± SEM blood glucose level (mg/dl) of rats at different days of treatment with different doses of heavy metals {lead, cadmium and arsenic (n=5 / group)}.

	Blood Glucose Level (mg/dl)		
Treatment	**Day 1**	**Day 14**	**Day 28**
Control	108.2 ± 2.44	111.4 ± 2.8	109.0 ± 2.72
$Pb(CH_3COO)_2$			
1.5mg/kg	109.6 ± 3.75	128.4 ± 3.85*	128.4 ± 5.1**
8mg/kg	110.8 ± 3.31	130.6 ± 3.75**	131.0 ± 3.42**
$CdCl_2$			
0.09mg/kg	111.0 ± 4.24	117.8 ± 4.97	126.0 ± 4.89*
5mg/kg	111.6 ± 3.83	131.0 ± 2.77**	136.0 ± 3.84***
$NaAsO_2$			
1.6mg/kg	109.2 ± 4.4	130.2 ± 3.57**	135.0 ± 3.3***
24.6mg/kg	110.2 ± 2.94	132.4 ± 4.38**	142.0 ± 1.96***

Values are stated as Mean ± SEM. *, **, *** showing significant variance at $P<0.05$, $P<0.01$ and $P<0.001$ vs control.

Antioxidant enzyme (CAT, POD, & SOD) activity, and oxidative stress markers (ROS & TBARS) in ovarian tissues of rats treated with different doses of heavy metals (lead, cadmium and arsenic)

Mean ± SEM of the antioxidant enzymes activity and level of oxidative stress markers in ovarian tissue of female rats exposed with (low and high) lead acetate dosage, cadmium chloride, and sodium arsenite are presented in Table 3. Significant diminution ($P<0.05$) was observed in the activity of POD and SOD in ovaries of rats treated with low dosage of lead acetate (1.5mg/kg). After treatment with high dose lead acetate (8mg/kg), significant decline ($P<0.01$) in POD and SOD activity was observed in ovarian tissue of animals. However, a significant increase ($P<0.01$) in CAT activity was detected in ovaries of animals exposed to low dose (1.5mg/kg) of lead acetate. The activity of CAT significantly rise ($P<0.001$) in ovarian homogenate of animals exposed to high dose (8mg/kg) of lead acetate.

After exposure to low dose of cadmium chloride (0.09 mg/kg), noteworthy decrease ($P<0.05$) in superoxide dismutase and catalaze activity and significant decline ($P<0.001$) in POD activity was detected in ovarian tissue of animals. The activity of CAT, POD and SOD significantly declined ($P<0.001$) in ovarian homogenate of animals exposed to high doses (5mg/kg) of cadmium chloride and (24.6mg/kg) of sodium arsenite. A significant lessening ($P<0.01$) was detected in catalase and SOD levels and significant decrease ($P<0.001$) in POD activity in the ovarian tissue of animals exposed to low concentration of sodium arsenite.

No significant increase was noted in TBARS in the ovaries of animals treated with low dose of lead acetate (1.5mg/kg).A significant rise ($P<0.05$) was detected in TBARS in the ovarian tissue of animals treated with high concentration of lead acetate(8mg/kg).After exposure to low dose of cadmium chloride (0.09mg/kg) and high dose of cadmium chloride (5mg/kg), a significant rise ($P<0.001$) was noted in TBARS levels in the ovaries of treated rats. The levels of TBARS significantly elevated ($P<0.01$) in ovarian homogenate of animals exposed to low dose of sodium arsenite (1.6mg/kg) and high sodium arsenite dosage (24.6mg/kg).

A significant increase ($P<0.05$) was detected in ROS in the ovarian tissue of animals treated with low amounts of lead acetate (1.5mg/kg), and sodium arsenite (1.6 mg/kg). Activity of ROS in ovarian tissue of animals treated with high concentration of lead acetate (8mg/kg), low concentration of cadmium chloride (0.09mg/kg) and high concentration of sodium arsenite

(24.6mg/kg), indicated significant increase ($P<0.01$). A significant rise ($P<0.001$) in ROS in the ovarian tissue of animals was observed when exposed to high concentrations of cadmium chloride (5mg/kg).

Table 3. Mean ± SEM Antioxidant enzyme (CAT, POD, & SOD) activity, and oxidative stress markers (ROS & TBARS) in ovarian tissues of rats treated with different doses of heavy metals {lead, cadmium and arsenic (n=5 / group)}.

Treatment	CAT (U/mg protein)	POD (U/mg protein)	SOD (U/mg protein)	ROS (U/g tissue)	TBARS (min/mg tissue)
Control	23.84 ± 0.86	27.51 ± 0.71	20.68 ± 1.68	0.73 ± 0.01	0.52 ± 0.06
$Pb(CH_3COO)_2$					
1.5mg/kg	29.82 ± 1.07**	20.32 ± 2.23*	15.29 ± 1.38*	0.78 ± 0.01*	0.72 ± 0.05
8mg/kg	34.62 ± 1.33***	18.15 ± 1.64**	13.90 ± 0.73**	0.78 ± 0.00**	0.73 ± 0.02*
$CdCl_2$					
0.09mg/kg	19.04 ± 0.94*	11.78 ± 1.54***	15.53 ± 1.19*	0.79 ± 0.01**	0.85 ± 0.05***
5mg/kg	13.21 ± 0.97***	13.05 ± 1.26***	11.10 ± 0.74***	0.8 ± 0.02***	0.87 ± 0.04***
$NaAsO_2$					
1.6mg/kg	18.21 ± 1.83**	13.12 ± 2.12***	15.13 ± 1.00**	0.78± 0.01*	0.78 ± 0.08**
24.6mg/kg	12.02 ± 0.71***	13.64 ± 1.69***	13.53 ± 0.74***	0.79± 0.01**	0.79 ± 0.06**

Values are stated as Mean ± SEM. *, **, *** showing significant variance at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Ovaries and uterine weight (g) of rats treated with different doses of heavy metals (lead, cadmium and arsenic)

Ovaries and uterus weight of rats treated with different doses of lead acetate, cadmium chloride, and sodium arsenite in Table 4. Ovarian weight of animals treated with lead acetate(1.5mg/kg), high dose of lead acetate(8mg/kg), and high dose sodium arsenite(24.6mg/kg) displayed significant reduction($P<0.001$) when matched to control. However, weight of ovaries of animals exposed with low dose of cadmium chloride (0.01mg/kg) was condensed significantly ($P<0.05$) when compared to control. The ovarian weight in animals treated with high dose of cadmium chloride (5mg/kg), and low dose of sodium arsenite(1.6mg/kg) presented significant decline($P<0.01$) in comparison to control.

Noteworthy decline($P<0.01$) in uterus weight observed in animals exposed to low lead acetate dosage (1.5mg/kg), lead acetate high dosage (8mg/kg), low cadmium chloride dose (0.09mg/kg), and low dose sodium arsenite(1.6mg/kg). Significant decline ($P<0.001$) in uterine weight was noted in animals exposed to high dose of cadmium chloride (5mg/kg) and high dose of sodium arsenite (24.6mg/kg).

Table 4. Mean ± SEM Ovaries and uterine weight (g) of rats treated with different doses of heavy metals {lead, cadmium and arsenic (n=5/ group)}.

	Reproductive organs weight	
Treatment	**Paired Ovaries (g)**	**Uterus (g)**
Control	0.27 ± 0.01	0.86 ± 0.03
$Pb(CH_3COO)_2$		
1.5mg/kg	0.19 ± 0.02***	0.72 ± 0.02**
8mg/kg	0.19 ± 0.01***	0.71 ± 0.01**
$CdCl_2$		
0.09mg/kg	0.22 ± 0.02*	0.73 ± 0.03**
5mg/kg	0.21 ± 0.01**	0.69 ± 0.02***
$NaAsO_2$		
1.6mg/kg	0.21 ± 0.01**	0.73 ± 0.03**
24.6mg/kg	0.19 ± 0.00***	0.68 ± 0.03***

Values are stated as Mean ± SEM. *,**,*** showing significant variance at $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs control

Modifications in the estrous cyclicity of rats at different days of treatment with different doses of heavy metals (lead, cadmium and arsenic)

Estrous cycle regularity was determined through vaginal smears, obtained every 7th day morning during a period of 28 days, for heavy metals (lead, cadmium, arsenic) treated groups as well as for control females. Comparison of control and heavy metals treated animals' vaginal smears was made. Animals treated with low dose (1.5mg/kg) lead acetate and high dose (8mg/kg) lead acetate remained at diestrus stage for maximum days, displaying abnormality in the reproductive cycle in comparison to control group. Animals exposed to low dose of cadmium chloride (0.09mg/kg) showed increase in estrus phase length compared with control. The length of proestrus shortened and the length of diestrus phase increased in animals treated with high dose of cadmium chloride (5mg/kg) compared with control. Prolonged diestrus phase was observed in animals' vaginal smears treated with sodium arsenite contrasting doses (1.6mg/kg) and (24.6mg/kg), presenting irregularity of the sexual cycle when matched with control group presented in Table 5. Estrous cycle of control group showed all stages, with different types of cells as shown in Figure 2 and exhibit normal estrous cycle of 4-5 days duration.

Table 5. Modifications in the estrous cyclicity of rats at different days of treatment with different doses of heavy metals {lead, cadmium and arsenic (n=5 / group)}.

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	+	+++	++++	+	+++
Pb ($CH_3COO)_2$					
1.5mg/kg	+++	++++	++++	++++	++++
8mg/kg	+++	++++	++++	++++	++++
$CdCl_2$					
0.09mg/kg	+++	++++	+	++	++
5mg/kg	++++	++++	++++	++++	++
$NaAsO_2$					
1.6mg/kg	++	++++	++++	++++	++++
24.6mg/kg	++++	++++	++	++++	++++

+ = Proestrus, ++ = Estrus, +++ = Metestrus, ++++ = Diestrus

Table 6. Evaluation of Estrous cyclicity of rats through different types of cell present in vaginal smear.

Stages of Estrous cycle	Round Nucleated epithelial cells	Anucleated cornified squamous epithelial cells	Leucocytes	mixed: having same proportion among three cell types
Proestrus	+++	+	−	−
Estrus	−	+++	−	−
Estrus	++	++	++	+++
Diestrus	+	+	+++	+

+ = present in small number, ++ = present in moderate amount, +++ = present abundantly, − = absent

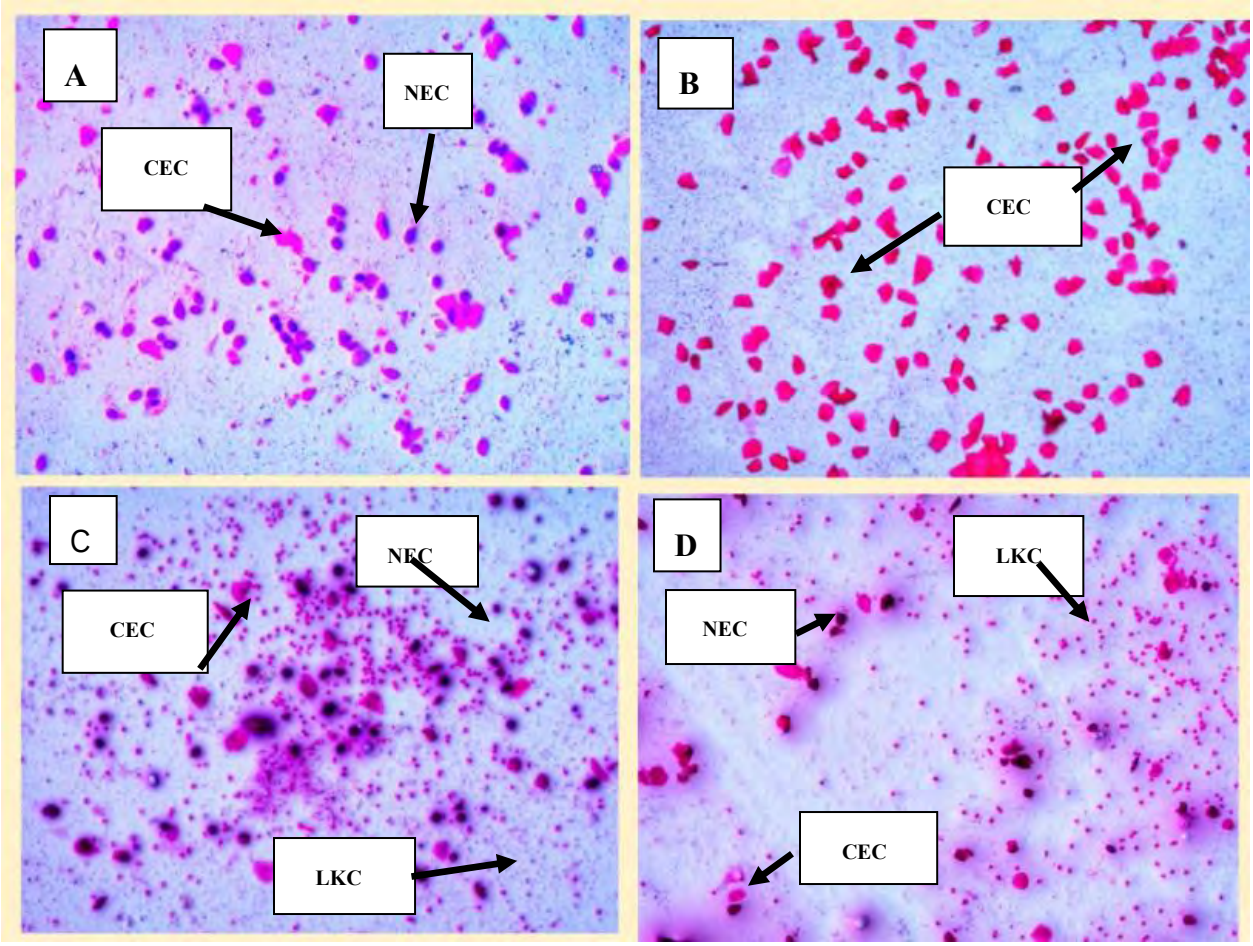

Figure 2.Vaginal smears stained during different phases of the estrous cycle. (A) Proestrus, with numerous round nucleated epithelial cells (NEC). (B) Estrus, clumps of cornified epithelial cell (CEC). (C) Metestrus, with round nucleated epithelial cells, cornified epithelial cells and a high number of leukocytes. (D) Diestrus, with numerous leukocytes (LKC). (10X magnification).

Histomorphometric analysis of ovaries

Effect of different doses lead, cadmium, and arsenic on morphology of rat ovaries was examined through histology study. Figure 3 indicated control group with normal structure of ovary showed normal stages of folliculogenesis. Figure 4 shows reduction in number of ovarian follicles, elevation in number of atretic follicles, visible empty spaces, and follicles necrosis in group exposed to low dose (1.5mg/kg) lead acetate. Figure 5 shows follicular cell dispersion, optical empty spaces, increased atretic follicles quantity, and corpus luteum cyst in group treated with high dose (8mg/kg) of lead acetate. Figure 6 shows greater number of atretic and antral follicles in group treated with low dose (0.01mg/kg) cadmium chloride. Figure 7 showed deterioration of corpus lutea, broken oocytes and less number of oocytes in group exposed to high dose (5mg/kg) cadmium chloride. Figure 8 and Figure 9 indicated follicular atresia in groups treated with contrasting doses (1.6mg/kg) and (24.6mg/kg) of sodium arsenite in comparison to the control group.

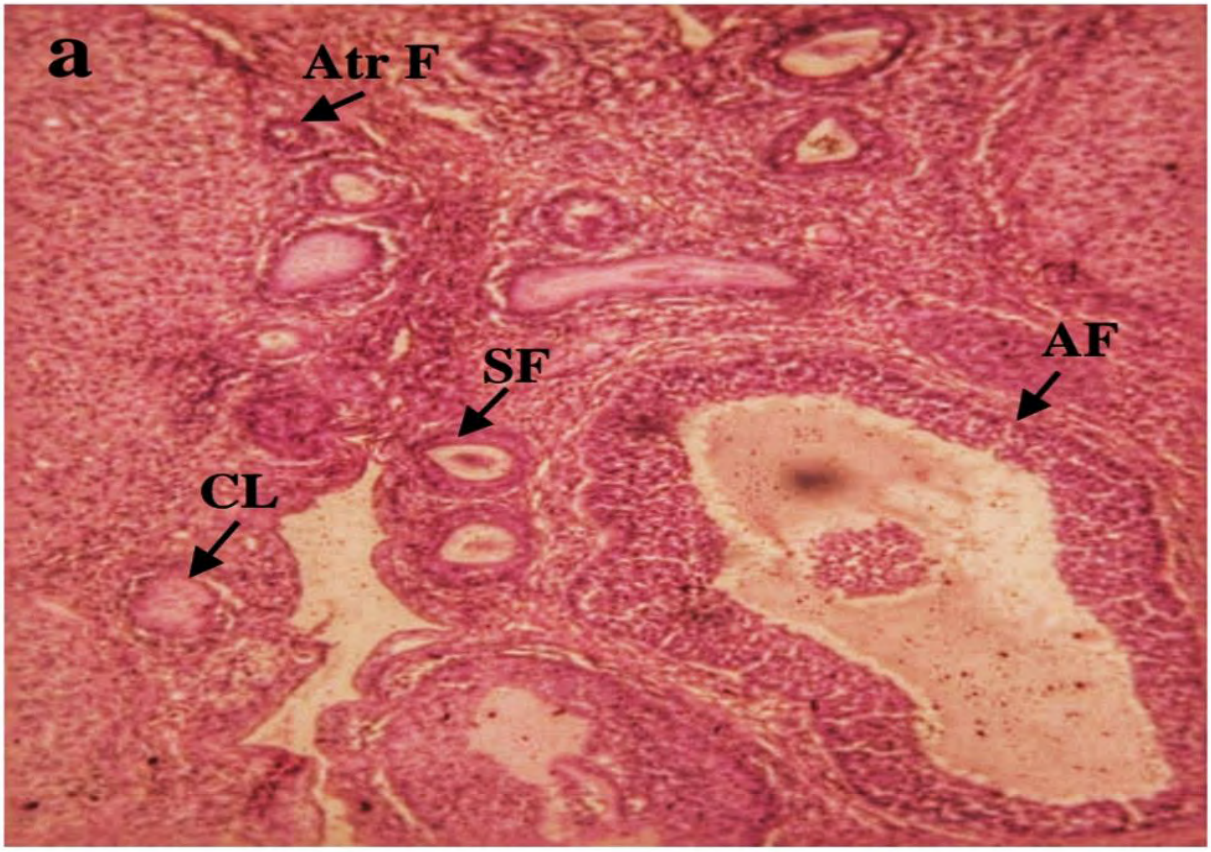

Figure 3.Microphotograph from cross-sectional area of ovarian tissue of rat taken as control group. (a) Control group displaying normal stages of folliculogenesis. Atr F, Atretic follicles; SF, Secondary follicles; AF, Antral follicles; CL, Corpus luteum. (10X magnification)

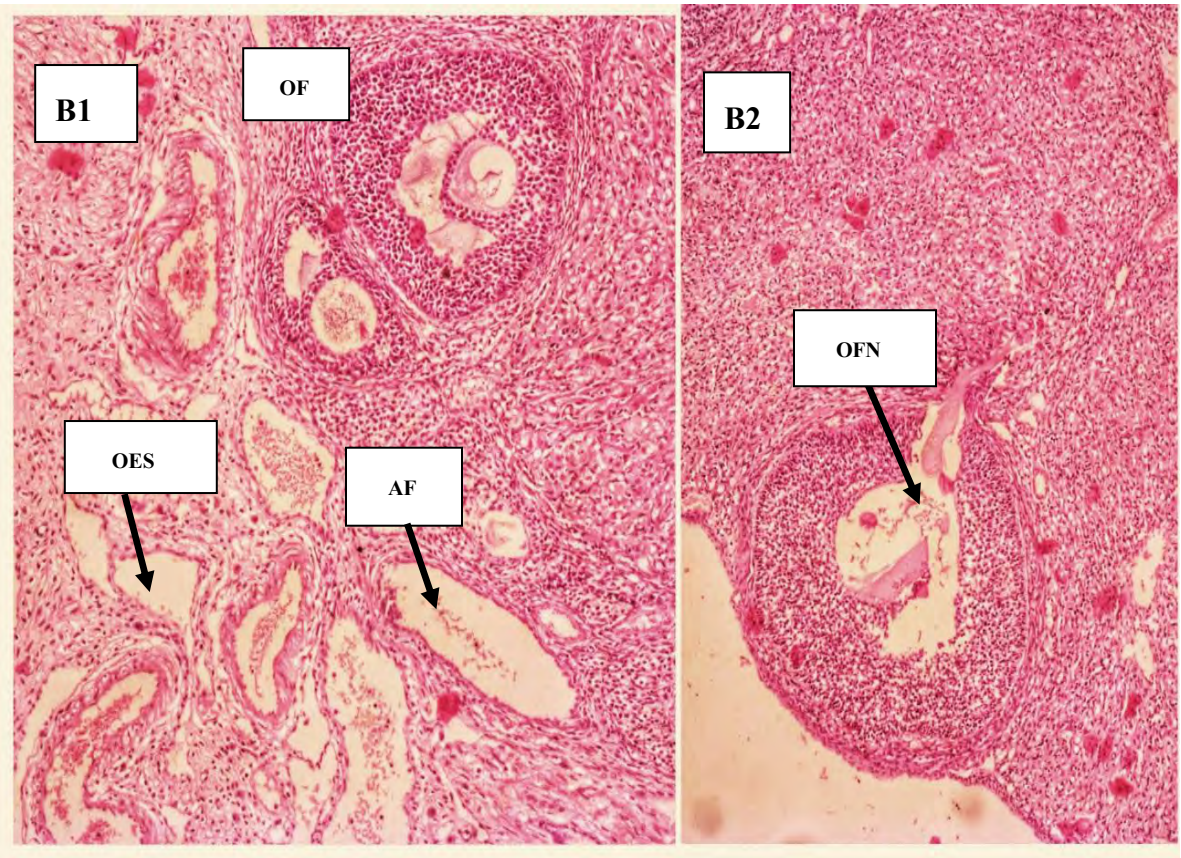

Figure 4.Microphotograph from cross-sectional area of ovarian tissue of rat exposed to 1.5mg/kg lead acetate in comparison to control. (B1) Condensed number of ovarian follicles, elevated atretic follicles quantity, and optical empty spaces; (B2) Ovarian follicles necrosis were observed in the group administered with low dose of lead acetate.

OES, Optical empty spaces; OF, Ovarian follicle; AF, Atretic follicles; OFN, Ovarian follicles necrosis. (10X magnification)

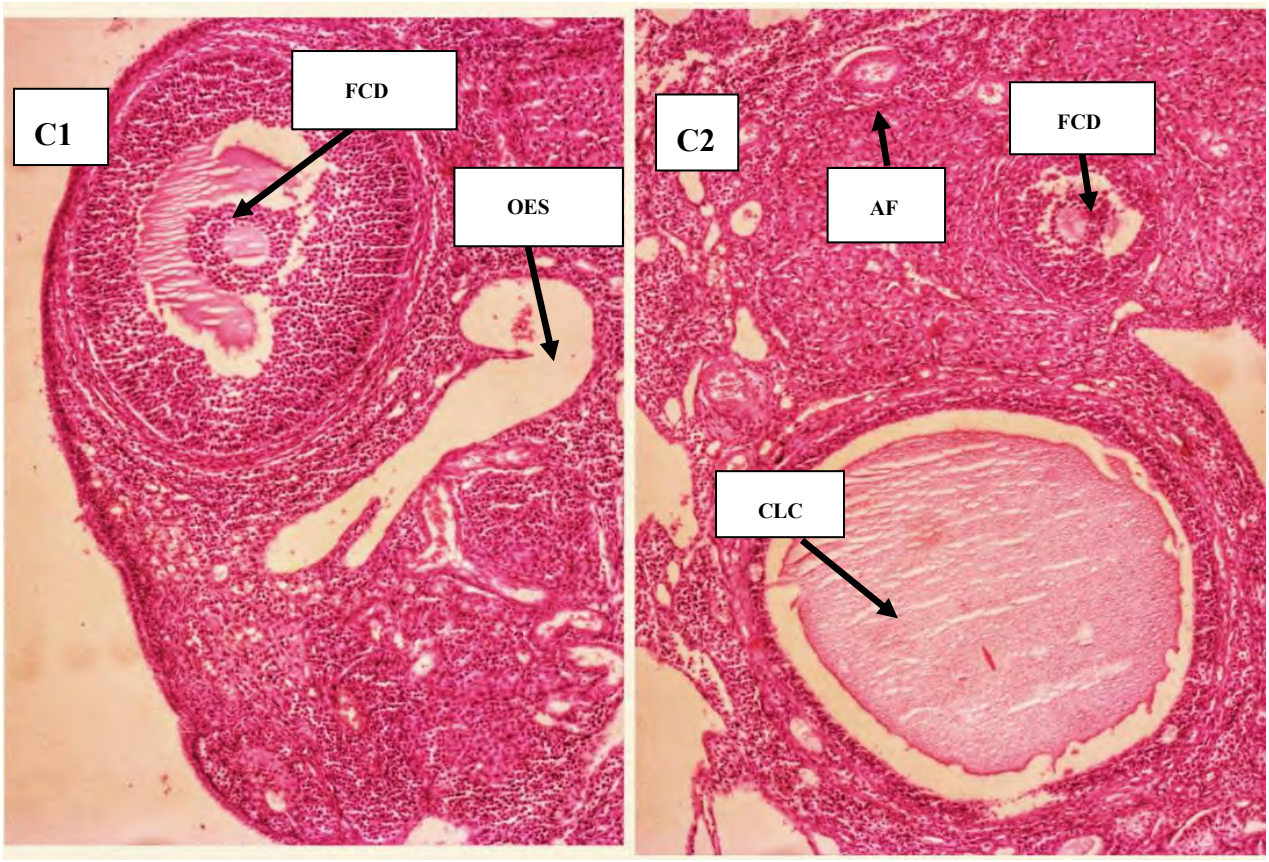

Figure 5.Microphotograph from cross-sectional area of ovarian tissue of rat exposed to (8mg/kg) lead acetate in comparison to control. (C1) Follicular cell dispersion (FCD) and enlarged optical empty spaces(OES) : (C2) Corpus luteum cyst(CLC), Atretic follicles(AF) , and Follicular cell dispersion were observed in the group exposed to high lead acetate dosage. (10X magnification)

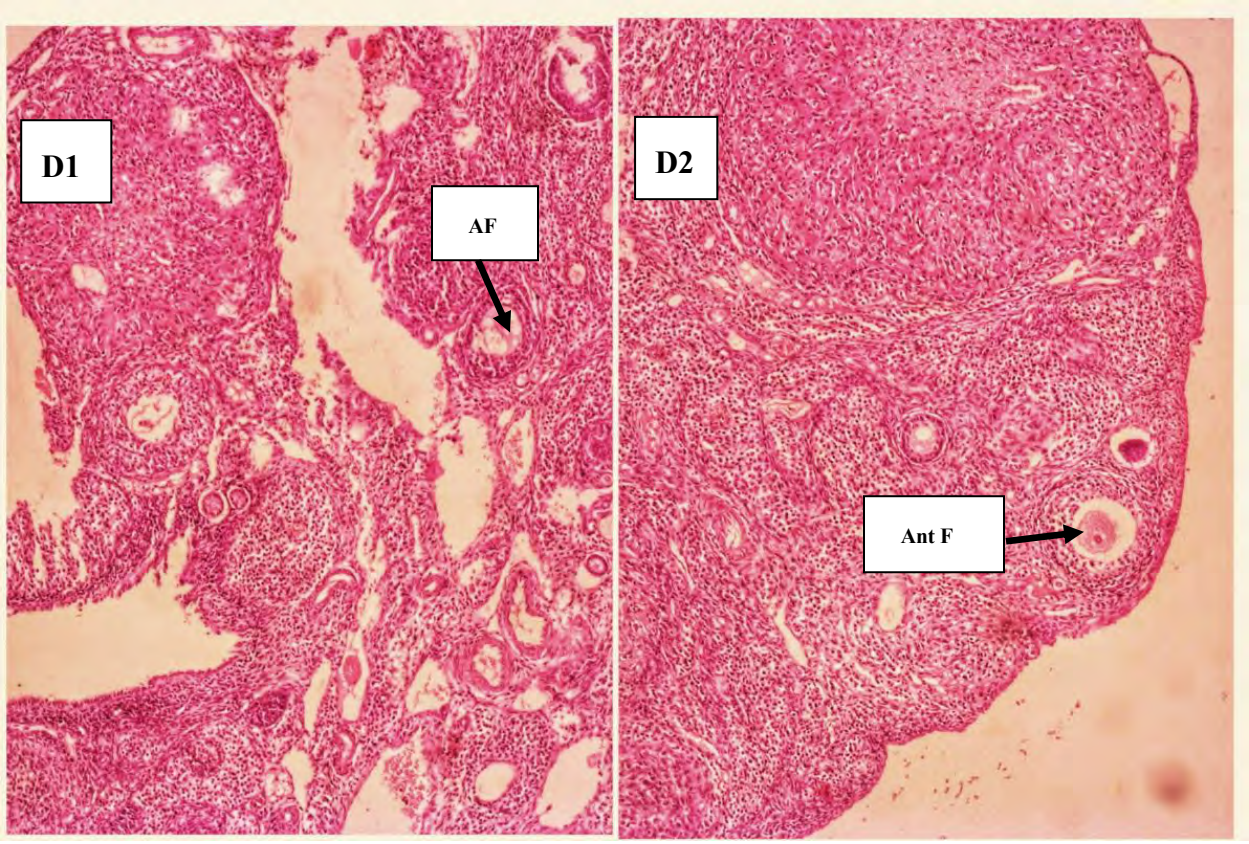

Figure 6.Microphotograph from cross-sectional area of ovarian tissue of rat exposed to (0.09mg/kg) cadmium chloride in comparison to control. (D1) Higher atretic follicles(AF) amount: (D2) Higher antral follicles (Ant F) quantity were detected in group exposed to low dose cadmium chloride. (10X magnification)

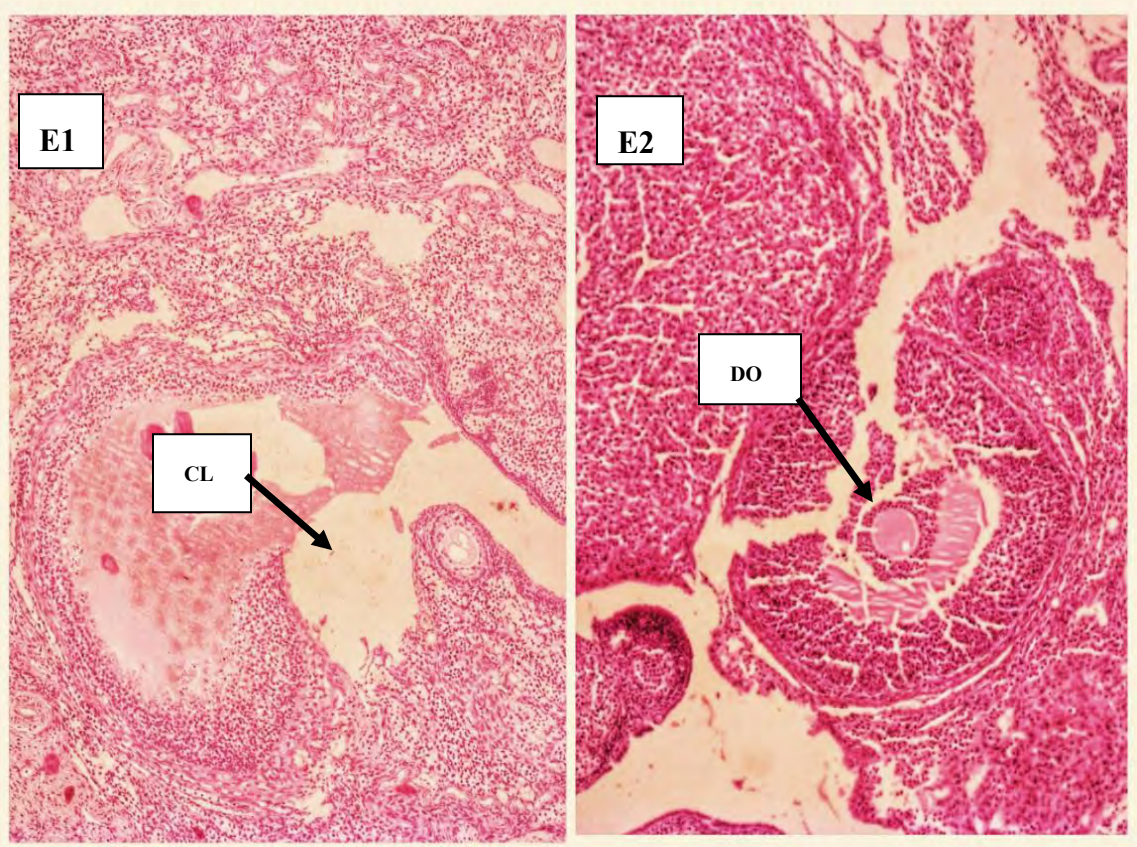

Figure 7.Microphotograph from cross-sectional area of ovarian tissue of rat exposed to (5mg/kg) cadmium chloride in comparison to control. (E1) Degeneration of corpus luteum (CL): (E2) Damaged oocytes (DO) and less numerous oocytes were observed in group treated with high dose of cadmium chloride. (10X magnification)

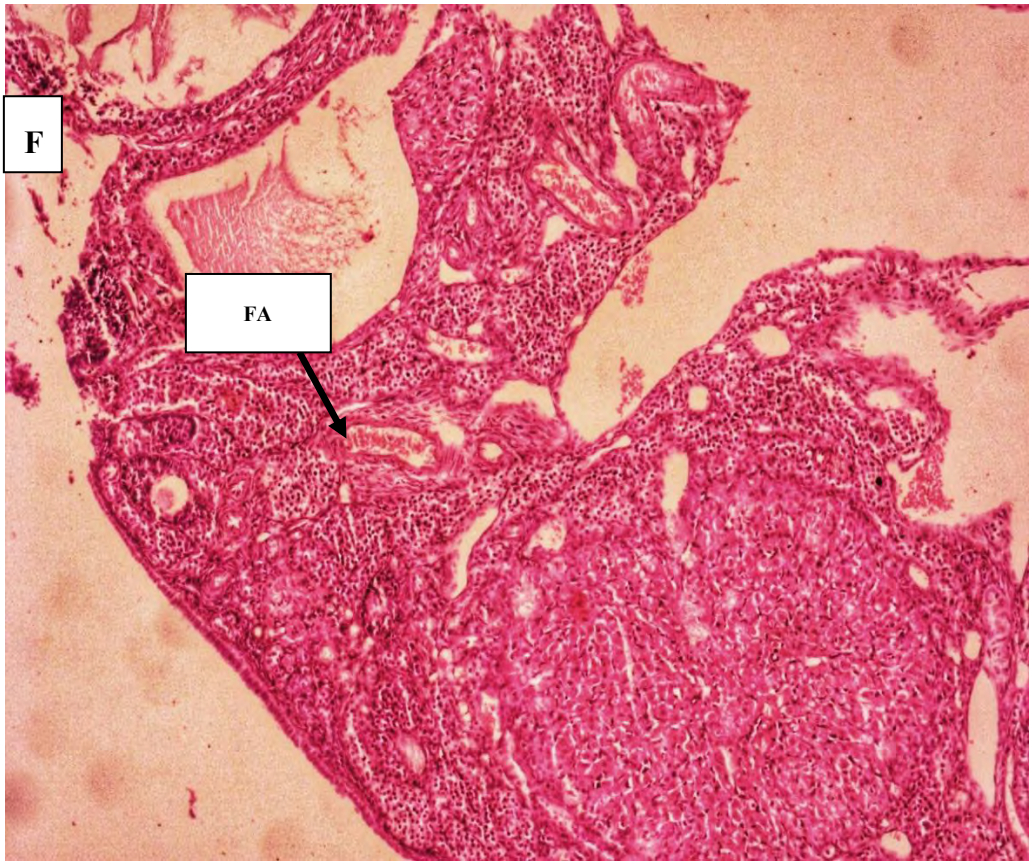

Figure 8.Microphotograph from cross-sectional area of ovarian tissue of rat exposed to (1.6mg/kg) sodium arsenite in comparison to control. (F) Follicular atresia (FA) were observed in group treated with low dose of sodium arsenite. (10X magnification)

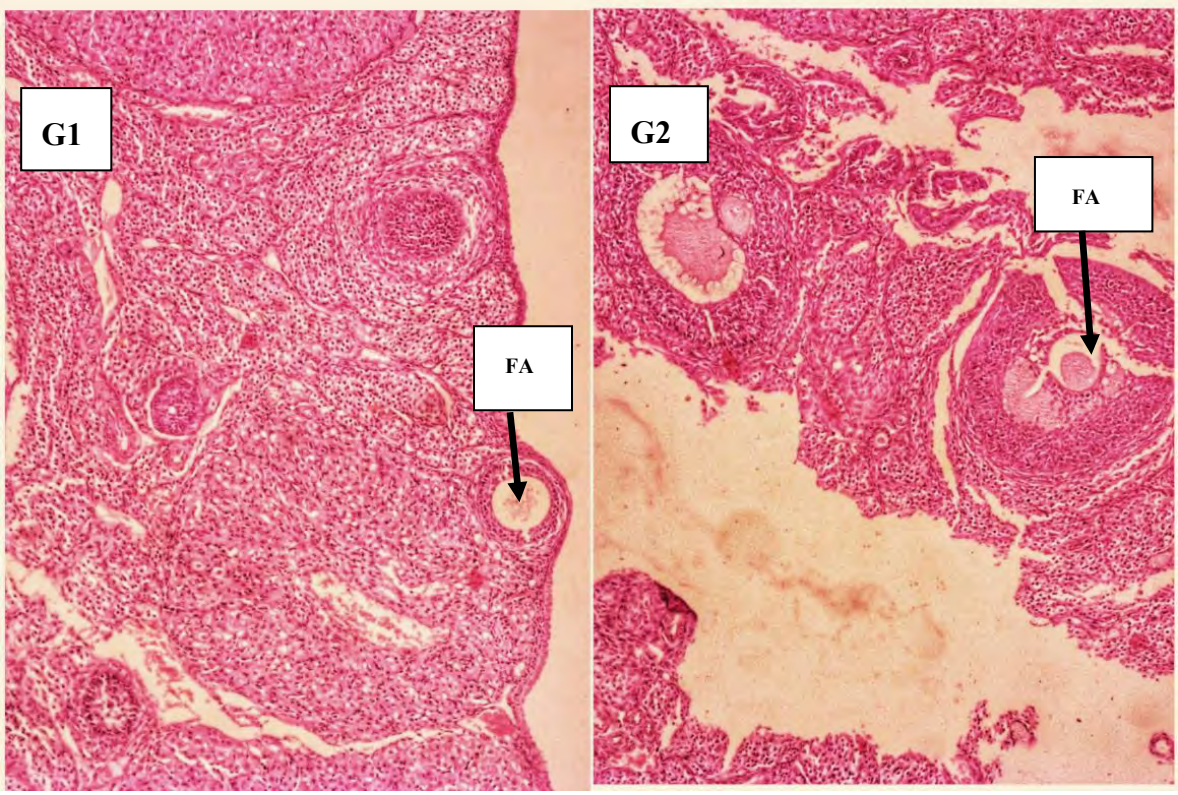

Figure 9.Microphotograph of cross-sectional area of ovarian tissue of rat exposed to (24.6mg/kg) sodium arsenite in comparison to control. (G1) and (G2) Follicular atresia (FA) were observed in group treated with high dose of sodium arsenite. (10X magnification)

Table 7. Evaluation of number and size of ovarian follicles and corpora lutea per section of rats treated with normal saline and different doses of heavy metals {lead, cadmium and arsenic (n=5 /group)}.

Treatment	Changes within ovary structure			
Control	1. Normal stages of folliculogenesis		2. Normal ovary structure	
$Pb(CH_3COO)_2$				
1.5mg/kg	1-Reduced number of ovarian follicle	2-Increased number of atretic follicles	3-Optical empty spaces	4-Ovarian follicles necrosis
8mg/kg	1-Follicular cell dispersion	2-Increased number of atretic follicles	3-Enlarged optical empty spaces	4-Corpus luteum cyst
$CdCl_2$				
0.09mg/kg	1-Higher antral follicles amount		2-Larger quantity of atretic follicles	
5mg/kg	1-Deterioration of corpus lutea	2-Damaged oocytes	3-Less numerous oocytes	
$NaAsO_2$				
1.6mg/kg	Follicular atresia			
24.6mg/kg	Follicular atresia			

Discussion

A continuous debate regarding the potential increase in human female infertility has been emerging during the last few decades. Environmental contaminants, mainly heavy metals, which have endocrine disrupting properties are the possible cause for the rise in female factor infertility (Rzymski *et al*., 2015). Lead, cadmium and arsenic are the most common heavy metals, affecting general and reproductive health (Tarin *et al*., 2015; Hanson *et al*., 2017). These are present in nature as earth's crust component and come in environment mainly by human activities. Humans exposed to heavy metals chiefly through polluted air, food or water. 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 evaluate the consequence of metal contact (lead, cadmium, arsenic) on the body weight revealed decrease in body weight of experimental rats treated with contrasting doses of lead acetate in comparison with control. These findings are in accordance with the Suradkar *et al*. (2010) {1, 100, and 1000 ppm $Pb(CH_3COO)_2$ for 28 days}, coinciding with the outcomes of Shakoor *et al*. (2000) and Chen *et al*. (2004), all reporting a diminution in body weight. However, Uchewa and Ezugworie (2019) {1.5mg/kg $Pb(CH_3COO)_2$ for 21 days} proposed that weight of lead exposed group elevated in second week and noteworthy reduced in third week. Lead may required time to cause damage in various organs of body, thus as a result body weight elevated and after few days, body weight decreases rapidly. Current study revealed that body weight increases normally in experimental rats exposed to cadmium chloride contrasting doses as similar as in control. These outcomes resemble to those observed by Nasiadek *et al*. (2018) {0.09, 1.8, and 4.5mg/kg $CdCl_2$ for 30 days} reported that there were no noteworthy change in the final weight of body between females receiving cadmium doses and of control group. However, Borzelleca *et al*. (1989) {25, 51, 107 and 225mg/kg $CdCl_2$ for 10 days by gavage; or 13 to 323mg/liter $CdCl_2$ in drinking water for 10 consecutive days}, reported that increase in body weight was depressed depending upon dose amount in male and female receiving dose through gavage, but there were no dose related effect in females receiving dose through drinking water. Analysis of current study showed normal rise in weight of rats exposed with low and high dose of sodium arsenite similar in comparison to control group. These findings are in accordance to the previous studies by Chattopadhyay and Ghosh (2010) {0.4ppm sodium arsenite for 28 days}, reported that body weight increased normally in arsenic-treated animals similarly as in control;

and Mondal *et al*. (2013) {3ppm/rat/day As_2O_3 for 30 days} reported that arsenic have no effect on body weight and body weight increased normally in treated group corresponding to the control.

Our current results revealed effective increase in glucose level of female rats after exposure to lead acetate, cadmium chloride and sodium arsenite contrasting doses when matched with control. These results are in accordance with the earlier studies by Stevenson *et al*. (1976), and Leff *et al*. (2018) that proposed a dose dependent rise in glucose level of blood because lead treated animals also displayed a dose-dependent rise in PEPCK and G6Pase enzyme levels in the liver. Chapatwala *et al*. (1982) {0.25, 0.75, and 1.25mg/kg cadmium chloride for 28 days} reported significant elevation in blood glucose level because in kidney and in liver, activities of glucose-6-phosphatase (G-6-Pase), fructose-1,6-diphosphatase (FD-Pase), and phosphoenolpyruvate carboxykinase (PEPCK) were changed in time-related and dose dependent manner. Navas-Acien *et al*. (2006), and Ghafghazi *et al*. (1980) reported that in arsenic treated experimental rats glucose level raised in dose dependent manner.

The cellular antioxidant enzymes can be categorized into catalase, peroxidase and superoxide dismutase that form a vital part of the cell´s homeostasis mechanism to combat oxidative stress within the body (Stahl *et al*., 1998). Significant decrease in ovarian SOD activity, revealed in the current study, is in agreement with the findings of Nampoothiri *et al*., 2007, {0.05mg/kg lead acetate or cadmium acetate for 15 days} who presented inhibition of SOD activity and elevation of CAT activity in ovarian granulosa cell signifying that lead and cadmium replaced zinc and bind in its place because SOD needs zinc as a cofactor for their normal functioning (Kofod *et al*., 1991; Hussain *et al*., 1987). Ariza *et al*. (1998) showed analogous kind of inhibition but Uchewa and Ezugworie, (2019) {1.5mg/kg lead acetate for 28 days} reported increase in SOD activity. Pillai *et al*. (2010) {0.05mg/kg lead acetate or cadmium acetate for 56 days} also reported decrease in SOD and CAT activity. Pillai *et al*. (2010) {0.05mg/kg lead acetate or cadmium acetate for 56 days} also reported noteworthy rise in MDA amount in lead and cadmium exposed female rats in comparison to normal group (control). Roopha and Padmalatha, (2012) {50 and 200 ppm $CdCl_2$}, and Nna *et al*. (2017) {$CdCl_2$ 5mg/kg for 14 days} presented decreased in SOD and CAT activity. Nna *et al*. (2017) {$CdCl_2$ 5mg/kg for 14 days} stated that MDA and H_2O_2 elevated significantly in Cd exposed group when matched to control. Roopha *et al*. (2011){50 and 200 ppm cadmium

chloride for 65 days}, reported that ovaries lipid peroxidation rise in treated animals might be due to concomitant rise in production of hydrogen peroxide in treated animals.

Mondal *et al*. (2013) {arsenic trioxide 3 ppm/rat/day for 30 consecutive days}, Biswas *et al*. (2019) {As_2O_3 at 3mg/kg for 28 days}, and Yu *et al*. (2019) {sodium arsenite 8mg/kg for 16 days} stated that ovarian activities of SOD decreased in the treated groups. Biswas *et al*. (2019) {As_2O_3 at 3mg/kg for 28 days} proposed that ovarian activity of CAT reduced and MDA levels enhanced in the arsenic treated group. Yu *et al*. (2019) {sodium arsenite 8mg/kg for 16 days} informed that ROS and MDA levels in ovarian tissue of treated animals increased, SOD activity reduced and elevated ROS level results in disturbance of equilibrium between oxidants and anti-oxidants within ovary, reduction in number of developed oocytes, ovulated oocytes, and fertilization rate. Also sodium arsenite repressed cell division and prompted apoptosis by icreasing oxidative stress.

The animals treated with heavy metals; lead, cadmium and arsenic in the present study revealed reduction in antioxidant enzyme peroxidase activity. These findings are in accordance with the study of Pillai *et al*. (2010) {0.05mg/kg lead acetate or cadmium chloride for 56 days} who presented remarkable decrease in enzymatic activity of POD in ovarian tissue of treated animals propose that lead and cadmium chiefly target three enzymes (POD, SOD, CAT). Most of these enzymes require transition metals for their normal funtion. Lead and cadmium can replace transition metals from their binding sites, causing a diminution in enzyme action. In conclusion, information from the current study suggests that oxidative stress generated by heavy metals caused transformation of ovarian steroidogenic process. Chattopadhyay and Ghosh, (2010) {0.4 ppm sodium arsenite for 4 weeks} showed suppressed POD activity in ovarian tissues of arsenic treated rats in contrast to control group.

The animals after exposure with low and high dosage of lead acetate, cadmium chloride and sodium arsenite in the present study revealed decline in antioxidant enzymes and rise in oxidative stress markers (ROS and TBARS). These findings resembled with Mondal *et al*. (2013) {arsenic trioxide 3 ppm/rat/day for thirty sequential days} that proposed noteworthy rise in malondialdehyde content (MDA) of the ovary. Arsenic caused high production of ROS, which damage proteins associated with estrogen signaling pathway and then interrupts estrogen signaling

(Chatterjee and Chatterji, 2010). Arsenic harmfulness reduced ovarian SOD activity and overproduced MDA.

A prominent decline in weight of ovaries and uterine tissue was noted in rats exposed with contrasting doses of lead acetate in the current study, which elucidates the findings of Uchewa and Ezugworie (2019) {Lead acetate 1.5mg/kg for 21 days}, who reported a similar significant reduction in ovarian and uterine weight after exposing to lead acetate. McLellan *et al*., 1978 reported that half-life of cadmium is long and cause toxic effects and ovary is the main target tissue affected by cadmium. Our results also revealed effective decrease in ovarian and uterine weight of rats treated with cadmium chloride. These outcomes are in accordance to the previous study by Nna *et al*. (2017) {$CdCl_2$ 5mg/kg for 2 weeks}, reporting a decline in weight of ovarian and uterine tissue. Exposure to sodium arsenite in the current study decreased the ovarian and uterine weight of treated rats in comparison with the control group. These findings resembled with the earlier studies by Mondal *et al*. (2013) and Chattopadhyay and Ghosh (2010), all reporting a decrease in ovarian and uterine weight.

Female rats are polyestrous. The reproductive cycle in rat is about 4-5 days and the length of each phase of cycle was: proestrus 12-14 hours, estrus 25-27 hours, metestrus 6-8 hours, and diestrus 55-57 hours (Long and Evans, 1922; Antunes *et al*., 2016; Sanabria *et al*., 2019). Sexual cyclicity is regulated through activation of HPG axis by a cascade of neuroendocrine events.

Lead acetate treated rats in the present analysis revealed irregularity in the estrous cycle with prolong diestrus phase, as described earlier by Shubina and Dudenkova (2014) in which lead acetate exposure altered the length of sexual cyclicity, mainly the diestrus phase. In the current analysis, rats exposed to cadmium chloride (0.09mg/kg for 28 days) showed disturbed estrous cycle with long estrus phase. And in experimental rats treated with high dose of cadmium chloride (5mg/kg for 28 days), diestrus phase prolonged and proestrus shortened. These outcomes are in harmony to the previous study by Nasiadek *et al*. (2018) stated that before treatment, normal extent of sexual cycle in rats stretched from 3- 4 days and low dose of cadmium (0.09 and 1.8mg/kg) have no effect on length of estrous cycle but high dose of cadmium (4.5mg/kg) has impact on its length and causes prolonged diestrus phase when matched with the control. Nasiadek *et al*. (2019)

proposed that low dose cadmium treated rats showed elongation of the estrus stage and high dosage Cd (4.5 mg/kg) treated rats showed no alteration in the period of cycle extent.

In current study, animals treated with arsenic showed irregularity in estrous cyclicity with prolonged diestrus phase. Davila-Esqueda *et al*. (2012) reported that rats after treatment with arsenic exhibited extended diestrus stage, with low level of E_2. Arsenic exposure may disturbed the morphology of ovaries by damaging their genetic material DNA. Halt in follicular development was also observed in arsenic exposed rats. Chattopadhyay and Ghosh (2010) propose that experimental rats treated with sodium arsenite showed prolonged diestrus phase.

Oral administration of low dose of lead acetate causes decline in ovarian follicles quantity. There exists a strong link between amount of lead in blood and ovarian follicular atresia, as previously suggested by Ahmed (2008). In the current analysis, examination of the ovary of rat exposed to low dosage lead acetate, under microscope, revealed damage in folliculogenesis with elevated atretic follicles number. The outcomes of the current histomorphometric analysis are in accordance with the working of Patrick (2006); Igile *et al*. (2015); and Durgesh and Lata, 2014. The histomorphometry of ovaries in the present study also demonstrated that the ovaries undergo structural changes including optical empty spaces and ovarian follicle necrosis. These findings correspond to the working of Dumitrescu *et al*. (2015) who stated that low dose lead acetate exposure led to alterations in the ovarian structure comprising mortification of follicles, empty spaces within ovary, and presence of atretic follicles. When ovaries of experimental rats exposed to high dosage lead acetate are observed under microscope, showed follicular cells dispersion and enlarged optical empty spaces, alike to the previous studies done by Waseem *et al*. (2014), and Qureshi *et al*. (2010). In the current analysis, treatment with low dose of cadmium chloride had shown increase in number of antral and atretic follicles. These findings are in accordance to the previous study (Massanyi *et al*., 2020; Massanyi *et al*., 1995). They also reported that cadmium exposed rats showed increased in ovarian weight and in number of antral and atretic follicles in comparison to control. Cadmium exposure may decrease level of LH and FSH. High dose cadmium exposed rats in the current study showed deteriorated corpus luteum, and smashed oocytes as compared to controls, these outcomes are in accordance to the study of Nasiadek *et al*. (2018). In

the current analysis, rats treated with sodium arsenite showed follicular atresia (Chattopadhyay and Ghosh, 2010).

Conclusion

The outcomes of current comparative analysis showed that contrasting doses of lead acetate reduced body weight depending upon dose but low and high dose of cadmium chloride and sodium arsenite have no effect on body weight of treated rats. Present study also depicts exposure of female rats with contrasting doses of lead, cadmium, and arsenic led to increased glucose level. All doses of these three heavy metals (low and high) caused reduction in reproductive organ weights (ovary and uterus). Heavy metals (lead, cadmium, and arsenic) disrupt oxidant/antioxidant balance through oxidative stress, damaged reproductive organs of females, leading to infertility. The outcomes of present comparative study also disclosed that treatment of female rats to low and high dosage of lead, cadmium, and arsenic caused irregularity in normal estrous cyclicity by changing duration of different phases depending upon nature of metal. Heavy metals exposure also altered the normal structure of ovary. All these alteration caused by heavy metals exposure enhanced infertility in female rats. It may be due to endocrine disrupting properties of heavy metals. However, further studies are required to understand the cellular and molecular mechanisms mediating the reproductive toxicity of these three metals (Pb, Cd, As). Moreover, the understanding of how these heavy metals penetrate into the reproductive organs also needs to be elucidated and will be useful in understanding the current problems of female infertility.

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