EFFECT OF LEAD BIOACCUMULATION ON TESTICULAR MICROANATOMY

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Effect of lead bioaccumulation on testicular microanatomy

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Joelle Hassaniyeh

Abstract

Humans are exposed to a notable variety of toxicants including lead as a heavy metal resulting from occupations and through environmental accumulation as in the Lebanese coastal and freshwaters. It is well established that lead exposure in the workplace contributes to worker infertility and decreased reproductive efficiency. Several reports have reported the detrimental impact toxic effects on human male reproduction by subsiding libido, spermatogenesis, semen quality, hormonal production and regulation, and much more. The objectives of this study were to inspect the effects of lead chloride ($PbCl_2$) within the male reproductive system specifically on testicular microanatomy and spermatogenesis. Among the study outcomes was lead localization inside the testes and its spermatogenic series. In this respect, twenty healthy sexually mature male mice (Swiss white albino) aged between 72 and 80 with an average body weight ranging between 29.5 ± 2.03 g and 35.9 ± 1.99 g were treated with 0, 6, or 12 ppm of $PbCl_2$ in a highly controlled experiment and sacrificed at sequentially every 9 days indicating distinct stages of the spermatogenic cycle. The mice testes were collected for histological assessment to determine Johnson's Testicular Biopsy score and Spermatogenic cell counts, and to localize Lead (Pb) accumulation in the reproductive tissues via laser scanning microscope (LSM). Study findings report a significant decline in Johnson's score

from 10 during the first 9 days (Week1) of exposure to a score of 4.17 ±1.147 at day 36 (W4) of lead exposure (p-value: 0.000). Deterioration testicular histopathology from each subgroup was additionally reported. Similarly, spermatogenic cell counts were decreasing with week progression. Highest cell counts were delineated during the first 9 days of exposure (Week 1) with an average of 335 cells with 0 ppm Pb dose, reaching an average of 25 cells during day 36 (Week 4) with 12ppm dosage. Lead (Pb) fluorescence increased with the intensified dose and period of PbI_2 exposure. Pb accumulated in almost all spermatogenic populations. It was detected in Leydig and Sertoli cells from the first week of exposure. Having altered all seminiferous tubule cells, lead contamination may potentially employ a significant impact on spermatogenesis and sperm fertilization capacity, and reproduction success.

Keywords: lead bioaccumulation, spermatogenesis, spermiogenesis, testicular morphology

Table of Contents

List of Tables

List of Figures

List of Abbreviations

FSH: Follicle Stimulating Hormone

IACUC: Institutional Animal Care and Use Committee

IHC: Immunohistochemical

LCA: Leydig cells aggregations

LSM: Laser Scanning and Confocal Microscope

Pb: Lead concentration

$PbCl_2$: Lead chloride

PBS: Phosphate Buffer Saline

PBS: Phosphate Buffer Saline

ppm: Part Per Million

ROS: Reactive Oxygen Species

ROS: Reactive Oxygen Species

SAS: Statistical Analysis Software

Tif: Tagged Image File

Effect of Lead Bioaccumulation on Testicular Microanatomy

I. Introduction

Male reproduction involves complex and delicate processes and depends on normal development and organization during the fetal period as well as during growth and puberty (Jensen et al., 2000). Among the many external disruptors of male reproduction and spermatogenesis is exposure to substances related to occupation, such as pesticides, polychlorinated biphenyls (PCBs), dioxins and furans, ethanol, phenols, phthalates, as well as metals like cadmium, lead, and mercury (Melnick, 1999a; Eertmans et al., 2003).

Reproductive dysfunction has been described in men exposed to lead in the workplace (Lancranjan et al., 1975; Rom W. N., 1976). General health perturbations can lead to male infertility and/or sexual dysfunction. Erectile dysfunction and male infertility are considered proxies for general health (Lotti and Maggi, 2018).

Lead is a hazardous heavy metal and a ubiquitous environmental toxin. Metallic lead, lead salts, and organic lead-containing carbon are the three main types of lead found in the environment (Ahamed and Siddiqui, 2007). Traditional leaded gasoline, lead-based paints, and batteries are the most common sources of occupational lead exposure. The enhanced anthropogenic activities and vehicular

emissions are mainly responsible for the increase in the lead level in the human body through inhalation, ingestion, and dermal contact (Singh et al., 2018). Recognition of the adverse effects of lead has resulted in lead removal from major sources including gasoline, home paint, and consumer products (Lanphear, 2007). Lead exposure is considered to be detrimental and associated with behavioral abnormalities, hearing deficits, neuromuscular weakness, and impaired cognitive functions in humans and experimental animals (Flora et al., 2012).

Surprisingly, lead was identified in a significant proportion of the drinking groundwater consumed by animals and people in a recent study conducted in North Lebanon by (Tannous et al., 2013). Lead binds to erythrocytes and is broadly dispersed in soft tissues such as the liver, kidney, brain, heart, and testes once it is absorbed from the gastrointestinal system (Flora et al., 2008). Lead is a highly poisonous metal affecting almost every organ in the body (Wani et al., 2015). Indeed, no blood-lead levels appeared to be safe, and sub-clinical influences of lead toxicity were reported in recent years (Canfield et al., 2003).

Additionally, lead is known to cause several adverse outcomes in both men and women (Winder, 1993) with recognized innumerable modes of action that seem to influence profuse cellular processes and enzyme systems throughout the body (Wani et al., 2015). Lead toxicity entails damage primarily to major biomolecules (lipid, protein ,and nucleic acids) and liver (hepatotoxicity), nervous system (neurotoxicity), kidney (nephrotoxicity), and DNA (genotoxicity), present in animals and humans (Singh et al., 2018). Toxicity is additionally manifested in male reproductive function by the deposition of lead in the testes, epididymis, vas deferens, seminal vesicles, and seminal ejaculate (Elgawish and Abdelrazek, 2014).

The process of spermatogenesis is not initiated until puberty and is then maintained throughout the rest of life in normal and healthy men. Male fertility is dependent upon the successful perpetuation of spermatogenesis, the multi-step process of male germ cell expansion and development that occurs within the seminiferous tubules of the testes (Smith and Walker, 2014). As a result, the spermatogenic process itself is directly vulnerable during this period to adverse effects coming from a man's lifestyle and/or his exposure to toxic substances in the surrounding environment or as a result of occupation (Wani et al., 2015). Lead

directly targets testicular spermatogenesis and also the sperms in the epididymis inducing reproductive toxicity (Wadi and Ahmad, 1999).

The reproductive system of both males and females is distressed by lead. Exposure to lead has been associated with several reproductive dysfunctions in men. In males, when blood lead levels exceed 40 μg/dL, the sperm count was reduced and other changes in the volume of sperm occurred (Wani et al., 2015). Activities including the motility and the general morphology of sperm were also altered at this level (Navas-Acien et al., 2007). Low lead doses were noted to significantly reduce the number of sperms within the epididymis of mice, whereas high doses reduced both the sperm count and the percentage of motile sperms and led to an increased percentage of epididymal abnormal sperms (Wadi and Ahmad, 1999). In females, reproductivity complications are much more severe. Toxic levels of lead can lead to miscarriages, prematurity, low birth weight, and developmental issues during childhood (Park et al., 2008).

Researchers have become increasingly concerned about the health effects of heavy metal exposure over time, particularly its impact on fertility and reproduction, therefore experiments on mice, rats, and guinea pigs have been conducted (Chowdhury, 2009; Meeker et al., 2010).

On top of that, in both children and adults, the nervous system is the most commonly afflicted organ in lead intoxication (Wani et al., 2015). Lead has long been known to be toxic to male fertility. The effects of lead exposure on mammals are reported to be devastating (Singh et al., 2018). Preceding reports indicate that lead has toxic effects on male reproduction with a prevailing conclusion indicating that the exposure to low-to-moderate levels of environmental lead affects reproductive parameters (Massányi et al., 2020). As reported in a study by Martynowicz et al. (2005), chronic lead exposure can induce functional disruptions (decrease in testosterone synthesis) or morphological disorders (decrease in testicular weight and seminal vesicle, peritubular fibrosis, seminiferous tubular diameter decrease, and decrease in germ cell population related to an apoptotic process). Lead has been shown to affect the activity of antioxidant enzymes including glutathione peroxidase, catalase, and superoxide dismutase in mice and rats Flora et al. (2004); Apaydin et al. (2015), resulting in lower sperm counts and an increase in sperm abnormalities such double heads, hooked heads, and double tails, among others.

Despite all of the aforementioned research investigations, the precise mechanism of action of lead on the reproductive system remains unknown, as just a few studies have investigated this concept. With Pb reported inducing a significant impact on cellular function in reproductive organs, further investigating

testicular cellular activity would permit the explication of the diverse organismal and physiological manifestations occurring in the presence of this metal The mechanisms involved in lead-induced toxicity primarily include free radical-mediated generation of oxidative stress which promptly imbalance the prooxidants and the antioxidants in the body (Singh et al., 2018). Lead cytotoxicity is mostly dependent on the activation of the c-Jun NH2-terminal kinase (JNK), PI 3-kinase, Akt, and p38 mitogen-activated protein kinase (MAPK) signaling pathways. Lead promotes apoptosis via a signaling cascade and associated factors, and it greatly distresses cell differentiation and maturation. Moreover, lead has a significant influence on metabolic processes including heme synthesis, which causes anemia to develop in those exposed to lead (Singh et al., 2018).

Lead effects on calcium fluxes and calcium-regulated processes have been proposed as major mechanisms of lead neurotoxicity (Marchetti, 2003; Toscano and Guilarte, 2005). Lead's capacity to induce oxidative stress is another putative route of lead toxicity. Lead exposures can have harmful effects that include the production of reactive oxygen or nitrogen species (ROS/RNS) as well as a direct depletion of the antioxidant reserves (Patrick, 2006). Lead decreases glutathione levels by directly binding to thiol groups and inhibiting glutathione reductase (Gurer and Ercal, 2000). Lead also hampers δ-aminolevulinic acid dehydrogenase (ALAD) resulting in increased levels of δ-aminolevulinic acid (ALA) thus

prompting ROS production (Bechara, 1996). Furthermore, lead stimulates membrane lipid peroxidation by binding to phosphatidylcholine in the cellular membrane and inducing changes in membrane biophysical properties (Adonaylo and Oteiza, 1999b, a). Lead's carcinogenic ability may be mediated via the formation of ROS and other potentially genotoxic compounds (Patrick, 2006). Table 1 is a summary of the various studies which investigated the toxic effects of lead on male reproductive biology.

This experimental research is conducted to study the effect of lead on male mice's reproductive system and spermatogenesis. Thus, establishing a direct link between the contaminant, sperm quality, and the cells affected in the testis, mainly the Leydig, and Sertoli cells, as well as spermatogonial germ cells. Consequently, morphology assessments and fluorescence microscopy (LSM) methods were performed. We hypothesize that lead will significantly impair testicular weight, the number of various cell types as well as the function of the Sertoli, and Leydig cells in male mice subjected to lead.

To our knowledge, this study is among the few studies that assess the resultant effects of lead administration on mice testicular anatomy. The originality of this study lies mainly in assessing the anatomic effects of lead administration by inspecting the histologic changes. This study falls in the category of toxicology studies that would potentially benefit medical professionals, and industrial workers

frequently exposed to lead. While the current literature data makes it clear that minimal lead exposure is associated with detrimental harm, one can speculate that longer use/exposure to lead would lead to even more pernicious sequels.

In this research sexually mature male white mice (Swiss White albinos) were used as an animal model to study the impact of heavy metals on spermatogenesis, due to their high reproductive rate and short spermatogenic cycle between 36 and 42 days Johnson and Everitt (2013), and because they develop similar anatomical features as humans, their reproductive organs consist of the testes and a series of ducts and glands (L'hernault S, 2006).

II. Literature Review

1. Lead Impact on Human Health

Lead is known to be one of the most dangerous and cumulative environmental contaminants, affecting all biological systems through air, water, and soil exposure as well as food sources (Patra et al., 2011). Lead exposure induces clinical pathological changes through kidney and endocrine system toxicity (Poon et al., 1997). A high level of lead in animals yields reproductive failure (Ahmed et al., 2008).

Long-time exposure to lead has been linked to anemia, along with an increase in blood pressure, mainly in the elderly and middle-aged. Severe damage

to the brain and kidneys, both in adults and children, was detected after being exposed to high lead levels resulting in death (Wani et al., 2015). Lead toxicity is associated with a high rate of blood disorders and nervous system impairment (Wani et al., 2015). Chronic lead exposure was found to reduce fertility in males (Sokol and Berman, 1991).

Lead has negative impacts on both women's and men's reproductive systems. General effects found in men include abnormal spermatogenesis (decreased number and motility), reduced libido, abnormal prostatic function, chromosomal damage, changes in serum testosterone, and infertility. In women, lead exposure has been associated with spontaneous abortion Borja-Aburto et al. (1999), low birth weight Irgens et al. (1998), preterm delivery Cheng et al. (2017) , fetal growth restriction Rahman et al. (2016), premature rupture of membranes Huang et al. (2018), pregnancy hypertension Sowers et al. (2002), pre-eclampsia Bede-Ojimadu et al. (2018), and gestational diabetes (Tyrrell et al., 2017).

Maternal blood lead has also been associated with a decrease in length of gestation (Cantonwine et al., 2010). Fetuses and neonates are particularly vulnerable to the effects of lead given that they efficiently absorb ingested lead, host a limited lead excretion capacity, and their nervous system is rapidly developing ((WHO), 2010; Canada., 2013). Lead exposure in infants and children can cause irreversible neurodevelopmental consequences leading to impaired

cognition and learning, shorter attention spans, and disruptive behavior ((WHO), 2010; COUNCIL ON ENVIRONMENTAL HEALTH, 2016). Behavioral manifestations of early exposures entail hyperactivity and inattentiveness, followed by distal impacts including poor school performance, juvenile delinquency, and increased likelihood of restraint (COUNCIL ON ENVIRONMENTAL HEALTH, 2016).

Human exposure to lead occurs mainly via the digestive and respiratory tracts (Boskabady et al., 2018). Lead in drinking water differs from lead from other sources in that it disproportionately. affects developmentally vulnerable children and pregnant mothers. Children can absorb 40 % to 50 % of a water-soluble lead dose, whereas adults can only absorb 3% to 10% (US Department of Health and Human Services, 2007). In a dose-response relationship for children aged 1 to 5, every 1-ppb rise in water levels yields a 35% increase in blood lead (Ngueta et al., 2016).

2. Lead Impact on animal reproductive function

Animal studies appear to confirm lead's toxic effect on the reproductive system. Lead is a global environmental toxin that is primarily present in industrial regions further placing animals at risk of exposure. Lead poisoning in animals engenders from a variety of sources in the surrounding environment, and this can

be traced back to contamination of feed, and soil from industrial pollution and agricultural practices (Burki, 2012). Accumulated lead is toxic in most of its chemical forms, whether inhaled or ingested in water or feed. The extent to which orally administered lead is absorbed into the host is small. However, due to its slow rate of elimination, harmful levels of lead can accumulate in tissues after prolonged exposure to minimal quantities (Ercal et al., 2001).

A study by Foster et al. (1993) reported that animals exposed to lead exhibited lower levels of plasma luteinizing hormone (LH) following stimulation with gonadotropin-releasing hormone when compared to controls. They have as well, experienced a reduction in the inhibin/follicle-stimulating hormone (FSH) ratio. Saleh et al. (2009) stated that there is a significant drop in the mating index and fertility index in females that were mated with lead-exposed males

Lead has been proven to have a direct effect on the testes in rodents (Murthy et al., 1991). Meanwhile lead acetate administered orally to adult male rats at doses of 273 or 819 mg/L resulted in a significant decrease in the weight of the reproductive organs, reduction in epididymal sperm count, motile sperm, and viable sperm indicating a notable diminution in sperm production and deterioration in sperm quality and serum testosterone levels in treated rats indicating a decreased steroidogenesis (Anjum et al., 2011).

Continuous intrauterine lead exposure of male rodents also showed detrimental sequels on male sexual maturity and reduced neonatal sex steroid levels. Moreover, exposure during puberty induced mitigation in testosterone concentrations (Camoratto et al., 1990). Assennato et al. (1987) described a reduction in sperm concentration by a direct, non-hormonal effect, in sperm production or transport (Assennato et al., 1987; Murthy et al., 1991). Excess lead intake can derive decreased sperm production and testicular weight (Saxena et al., 1989) Studies in monkeys chronically exposed to lead showed alterations in Sertoli cell function (Gustafson et al., 1989; McGregor and Mason, 1990).

3. Effect of lead on the male reproductive system in humans

According to Elgawish and Abdelrazek (2014), lead acetate significantly decreased male reproductive organs functions, and promoted testicular tissue alterations in the histological patterns of the testis. Blood lead levels of >40 g/100 ml in men, have been linked to reduced libido, low semen volume and sperm counts, increased aberrant sperm morphology and decreased sperm motility, all of which can lead to reproductive dysfunction (Telisman, 2000; ASTDR, 2007).

In men, reproductive dysfunction includes oligozoospermia and dose-dependent asthenozoospermia (Lancranjan et al., 1975). A study by Telisman et al. (2000) revealed an inverse association between plasma lead levels and sperm

volume and concentration was reported following blood and semen analysis from battery factory workers. Lead levels were shown to have significant correlations with reproductive parameters, indicating a decrease in sperm density, motility, and viability counts, as well as an increase in aberrant sperm head morphology.

Other authors have also reported a reduction in spermatogenesis among battery workers as one of the findings in symptomatic lead poisoning (Assennato et al., 1987; McGregor and Mason, 1990). Another study by Rodamilans et al. (1988) evaluated lead exposure and damage over a timeframe. The study demonstrated a progressive decrease in LH in individuals exposed for less than one year, while those exposed for more than three years showed lowered testosterone and testosterone/steroid transport protein ratio, implying a correlation between testicular dysfunction and duration of exposure. In an alternative study in which men were exposed to lead in the workplace, increased lead blood levels were associated with decreased libido and an increase in semen abnormalities displayed a significantly surged incidence of asthenospermia, hypospermia, and teratospermia (Lancranjan et al., 1975).

4. Overview of the male reproductive system and spermatogenesis

The male reproductive system consists of the testes, genital excurrent ducts, accessory sex glands, and penis. Adding to the aforementioned organs are the

accessory sex glands comprising the seminal vesicles, the prostate, and bulbourethral glands. The testis hosts two primary functions which are spermatogenesis (the production of sperm, called *male gametes*), and steroidogenesis (synthesis of androgens, also called *sex hormones*). Androgens, primarily testosterone, are essential for spermatogenesis, play an essential role in the embryonic development of the male XY embryo into the phenotypic male fetus, and are responsible for sexual dimorphism (male physical and behavioral characteristics). A primary androgen produced is testosterone which stimulates the production of sperm as well as gives secondary sex characteristics with puberty onset (Ross, 2011).

4.1 Testis

The adult testes are paired ovoid organs that lie within the scrotum, located outside the body cavity. Each testis is suspended within the end of an elongated musculofascial pouch, which is continuous with layers of the anterior abdominal wall and projects into the scrotum. Testes are connected by the spermatic cords to the abdominal wall and tethered to the scrotum by scrotal ligaments. The scrotum helps maintain the temperature of the testicles. The testes develop on the posterior wall of the abdomen in close association with the urinary system retroperitoneally and later descend into the scrotum.

The testes have an unusually thick dense connective tissue capsule, the tunica albuginea that covers each. Additionally, each testis is divided nearly into 250 lobules by incomplete connective tissue septa that project from the capsule. Each lobule consists of several highly convoluted seminiferous tubules. It's important to note that each lobule of the testis consists of one to four seminiferous tubules, in which sperm is produced, and a connective tissue stroma, in which Leydig (interstitial) cells are contained.

Each tubule within the lobule forms a loop and, because of its considerable length, is highly convoluted, actually folding on itself within the lobule. Each seminiferous tubule is approximately 50 cm in length (range, 30 to 80 cm) and 150 to 250 μm in diameter. The seminiferous tubules are packed and compose 90% of the weight of the testes; it is the functional unit of the testis, where spermatogenesis takes place (Ross, 2011).

Inside the seminiferous tubules, the epididymis resembles a coiled tube on the posterior surface of each testis. The epididymis incorporates three parts caput, corpus, and cauda. The first is the caput transports the sperm from the testes to the proximal part of the epididymis. The second is the body or corpus epididymis which assists in sperm maturation. This middle section is a highly convoluted middle. The third part, or cauda epididymis, is the storing and nourishment center of the sperm till ejaculation to the vas deferens (Johnson and Everitt, 2013).

Blood supply constantly maintained into the testes ensuring proper hormonal, and nourishments for sperm development (Spiegel et al., 2013). At this point, testicular blood vessels could be a site for heavy metals transportation and storage (Michaut et al., 2000). Additionally, the prostate gland can as well be a site for heavy metal storage further reducing semen quality by impairing the process of spermatogenesis, steroidogenesis, Sertoli cell, and sperm functions, thereby leading to decreased male fertility (Michaut et al., 2000; Queiroz and Waissmann, 2006; Loukas et al., 2010; Selvaraju et al., 2021).

Seminiferous tubules contain epithelium consisting of Sertoli cells that envelop and support germ cells undergoing progressive differentiation and development into mature spermatozoa (Matsumoto and Bremner, 2016). The organization of the seminiferous epithelium and the physical support provided by Sertoli cells are crucial for the normal progress of spermatogenesis (Kotaja, 2013).

4.2Sertoli Cells

Sertoli cells are also known as supporting, or sustentacular, cells. Sertoli cells are columnar cells with extensive apical and lateral processes that surround the adjacent spermatogenic cells and occupy the spaces between them. Sertoli cells are known to provide structural organization to the tubules as they extend through

the full thickness of the seminiferous epithelium. Sertoli cells constitute about 40 % of the testicle and are known as “nurse cells” (De França et al., 1993) .

The first cells to differentiate, and to set the wider process of testis differentiation underway, are the Sertoli cells (Sharpe, 2010). Sertoli cells permit the development of adherent sperm cells through the stages of spermatogenesis. The appropriate development of the Sertoli cell population sets spermatogenic capacity through adulthood (Orth et al., 1988) . Thus, the regulation of Sertoli cell proliferation (establishing effective cell number) and maturation (establishing effective cell function) is vital for normal adult fertility (Tarulli et al., 2012). Further, Sertoli cells provide both secretory, and support, and assist in sperm maturation by consuming unwanted portions of the spermatozoa (Loukas et al., 2010). The sperm is afterward transported through a series of efferent ductules to the cauda epididymis for storage and further maturation (Kumar, 1987).

Sertoli cells are regularly outlined tall columnar cells that rest on the basal lamina. They exhibit oval or pear-shaped nuclei located in the broad basal portion of the cell and contain large nucleoli. Moreover, Sertoli cells have pale nuclei while prominent and dense nucleoli. A cross-section of the seminiferous tubule has about 20 evenly-spaced sustentacular cells.

4.3Spermatogenic cells

These cells frequently replicate and differentiate into mature sperm. Spermatogenic cells are derived from primordial germ cells originating in the yolk sac that colonize the gonadal ridges during early testis development. Spermatogenic cells are organized in poorly defined layers between adjoining Sertoli cells. The most immature spermatogenic cells, called spermatogonia, rest on the basal lamina. The most mature cells, called spermatids, are attached to the apical portion of the Sertoli cell, where they border the lumen of the tubule (Ross, 2011).

- Spermatogonia are immature spermatogenic cells lying on the basement membrane of the Seminiferous tubule.
- Primary spermatocyte: These are the largest germ cells occupying the middle region of the Seminiferous tubule. They have larger rounded nuclei with coarse chromatin clumps.
- Secondary spermatocytes: These are short-lived cells and intermediate in size between the primary spermatocytes and spermatids.
- Spermatids: They are much smaller and lie in groups along the margins of Sertoli cells. With the formation of spermatids, the first phase of spermatogenesis is completed. The second phase (Spermiogenesis) starts when the non-motile spermatids convert into motile spermatozoa. Early

spermatids rounded in shape with a spherical nucleus are in the middle layers of the spermatogenic epithelium. Late spermatids are in the layer adjacent to the lumen of the tubule, and have an elongated shape.

- Spermatozoa: Spermatozoa have dark elongated heads focused on the periphery of the tubule and tails hanging in the lumen of the tubule. Spermatozoa have a detectable flagellum.

4.4. Leydig Cells

Leydig cells (interstitial cells) are large, polygonal, eosinophilic cells that typically contain lipid droplets. Like other steroid-secreting cells, Leydig cells have an elaborate smooth endoplasmic reticulum (sER), a feature that accounts for their eosinophilia. The enzymes necessary for the synthesis of testosterone from cholesterol are associated with the sER. Mitochondria with tubulovesicular cristae, another characteristic of steroid-secreting cells, are also present in Leydig cells. Leydig cells differentiate and secrete testosterone during early fetal life. The seminiferous tubules are separated by the interstitial or, *Leydig Cells* (Figure 1).

5. Spermatogenesis: The essential steps

Spermatogenesis is the process by which spermatogonia develop into sperm. In humans, spermatogenesis shortly begins before puberty onset, under the influence of rising levels of pituitary gonadotropins, and continues throughout life (L'hernault S, 2006). Once the gonocytes have differentiated into fetal spermatogonia, an active process of mitotic replication begins very early in embryonic development Sosa (2016) , and arrests until puberty when spermatogenesis resumes in the seminiferous tubules during active sexual life (Saladin, 1998). The stimulation of anterior pituitary gonadotropic hormones (FSH, LH), begins at an average age of thirteen years old and continues throughout most of the remainder of life ensuring the proper progression of spermatogenesis (CarrellT. and Aston, 2013; Rasoulpour et al., 2016). Therefore, a marked decrease in old age leads to a decrease in fertility and sexual drive (Oliveira and Alves, 2015). Table 2 shows the time for completion of spermatogenesis and the approximate period of each seminiferous cycle in different mammalian species (Clermont, 1972; Wu, 2008; Johnson and Everitt, 2013).

Spermatogenesis entails a complex and unique series of events, and is divided into three distinct phases:

- **Spermatogonial phase:** It is the phase in which spermatogonia divide by mitosis to replace themselves to mass-produce a committed spermatogonia population that will further differentiate into primary spermatocytes (Spermatocyte I). The quiescent interphase pro- spermatogonial germ cells of the immature testis are reactivated at puberty to enter rounds of mitosis in the basal compartment of the tubule. Henceforth they are known as *spermatogonial stem cells*
- **Spermatocyte phase (meiosis):** Primary spermatocytes in this phase undergo two meiotic divisions to reduce both the chromosome number and amount of DNA thus producing haploid cells noted as spermatids.
- **Spermatid phase (spermiogenesis):** In the spermatid phase, at the end of spermatogenesis, spermatids undergo extensive cell remodeling as they differentiate into mature sperm. Spermatids are released from the supporting Sertoli cells into the lumen of the seminiferous tubule through a process known as spermiation. The extensive cell remodeling that occurs during the differentiation of the spermatid population into mature sperm

(spermiogenesis) consists of four phases. These phases occur while the spermatids are physically attached to the Sertoli cell plasma membrane by specialized junctions. The rate of spermiation in the testis determines the number of sperm cells in the ejaculate of semen. Numerous pharmacologic treatments, toxic agents including heavy metals, and gonadotropin suppression result in spermiation failure. In this case, spermatids are not released, instead, they are retained and phagocytosed by the Sertoli cell.

5.1 Events of spermiogenesis

Spermiogenesis is a complex process that transforms round spermatids after meiosis into a complex structure called the spermatozoa. During this process, morphological changes occur once the process of meiosis is completed. In humans, six different stages are identified similar to mice or rats stages , and have been described in the process of spermatid maturation and were termed as Sa-1 and Sa-2, Sb-1 and Sb-2, and Sc-1 and Sc-2 (Vries, 2012). During the Sa-1 stage, both the Golgi complex and mitochondria are well developed and differentiated. In addition, the acrosomal vesicle appears, the chromatoid body develops in one pole of the cell, and the proximal centriole and axial filament appear. During the Sb-1 and Sb-2 stages, acrosome formation is completed, the intermediate piece is

formed and the tail develops. This process is completed during the SC stages, where the nucleus becomes condensed (Cooper and Yeung, 2006; Gilbert, 2008).

5.2 Duration of spermatogenesis in humans

The entire period of spermatogenesis, from spermatogonia to spermatozoa, takes about 74 days in humans Har-Vardi (2014) and 36-42 days in mice and rats. The duration of the cycle of the seminiferous epithelium is constant, lasting about 16 days in humans. In humans, it would require about 4.6 cycles (each 16 days long), or approximately 74 days, for a spermatogonium produced by a stem cell to complete the process of spermatogenesis. It would then require approximately 12 days for the spermatozoon to pass through the epididymis. Approximately 300 million sperm cells are produced daily in the human testis. The length of the cycle and the time required for spermatogenesis are constant and specific in each species. Table 2shows the time for completion of spermatogenesis and the approximate period of each seminiferous cycle in different mammalian species (Clermont, 1972; Wu, 2008; Johnson and Everitt, 2013).

5.3 Blood Testicle-Barrier

Spermatozoa develop *within* the seminiferous tubules in close association with *Sertoli cells*, whereas androgens are synthesized *between* the tubules in the *Leydig cells.* Cellular barriers, which develop during puberty and hinder the free

exchange of water-soluble components, divide these two compartments not just physically but also physiologically. The physical basis for this barrier comprises multiple layers of adherents (inter-Sertoli cell anchoring), gap (inter-Sertoli cell-communicating), and tight (para-Sertoli cell occluding) junctional complexes completely encircling each Sertoli cell, and linking it firmly to the surrounding molecules.

The barrier constitutes the main element of the so-called *blood-testis barrier*. The term is a bit misleading as it is not a blood-organ barrier, but rather one that is formed by the tight junctions between Sertoli cells of the seminiferous tubules. For this reason, this barrier is also referred to as the "Sertoli cell barrier". The barrier isolates further developed germ cells from the blood. It is absent prepubertally but develops before the initiation of spermatogenesis. The presence of this barrier has two significant functional implications. Primarily, it inhibits intratubular spermatozoa from seeping into systemic and lymphatic circulations. This function is critical because the body's immune system is not tolerant of spermatozoa antigens, which might provoke an immunological response. Second, the composition of *intratubular fluid* significantly differs from that of *intertubular fluids*: blood, interstitial fluid, and lymph. Ions, proteins, and charged carbohydrates are observed to enter the interstitial fluid and lymph rapidly

confirming the absence of a significant barrier at the capillary level. In contrast, these molecules do not gain free diffusional access to the tubular lumen.

6. Toxicants and Blood-Testis Barrier Function

Environmental toxicants, such as heavy metals (e.g., cadmium), cause testicular injury Parizek and Zahor (1956); Parizek J (1960); Chiquoine (1964) and blood-testis barrier (BTB) disruption were first reported more than 5 decades ago (Setchell and Waites, 1970). A recent study concluded that exposure to environmental toxicants induced a trend of reduced sperm quality and increased testicular cancer in Finnish men Jørgensen et al. (2011), which is consistent with previous studies that associated reduced sperm count and reproductive dysfunction in men exposed to environmental toxicants (Phillips and Tanphaichitr, 2008; Benoff et al., 2009; Lucas et al., 2009; Bonde, 2010; Wong and Cheng, 2011). The testis is a primary male reproductive gonad beholding endocrine functions and responsible for spermatogenesis, which depends on the normal functions of Leydig cells and spermatogonia, respectively. In the testis, the normal process of spermatogenesis relies on the vigorous regeneration of spermatogonia (Huang et al., 2021). Due to the rapid division of spermatogonia during spermatogenesis, it is highly sensitive to toxins. Toxicant-induced testis damage has only recently been used as a model to study Blood-Testicle Barrier (BTB) regulation at the cellular

and molecular levels, both in vitro and in vivo (Janecki et al., 1992; Chung and Cheng, 2001; Fiorini et al., 2004; Gualtieri et al., 2011; Wong and Cheng, 2011; Wang et al., 2022). These findings further illustrate that the assembly of the BTB is tightly associated with Sertoli cells' maturation and that the BTB can be developed only with differentiated Sertoli cells that have ceased to divide (Cheng and Mruk, 2012). More important, Cd and other toxicants, such as heavy metals (e.g., lead, mercury) and estrogenic-based compounds (e.g., bisphenols) may account for the recent declining fertility in men in developed countries by reducing sperm count and testis function.

Apart from that, an experiment by Marchlewicz (1994), implemented on rats after long exposure to lead acetate has disclosed that the blood-epididymis barrier fails to provide a barrier against lead. Smooth myocytes, epithelial cells, and the epididymal duct lumen all showed Pb deposits. This correlated with a significant drop in the number of epididymal spermatozoa, as well as several ultrastructural defects. It has been demonstrated that lead is expelled from the male genital system with the sperm when it passes through the epididymis duct lumen through the cells and tissues that make up the organ's wall.

Finally, the present study helped inquest the repercussions of lead on the reproductive system specifically on spermatogenesis in male mice, further establishing a direct association between the contaminant and the cells impacted in

the testis including the spermatogenic population cells namely Leydig, and Sertoli cells. Laser scan microscopy (LSM) was used to co-localize the different spermatogenic cells and their contaminant accumulation. Lebanese residents are getting exposed to numerous chemicals and pollutants, particularly heavy metals, as a result of increased industrial and technological activities including transportation, and urban and industrial waste incineration (Hashim et al., 2011; Abi-Ghanem et al., 2013). Heavy metals including lead have been associated with increased prevalence of many human diseases and reproductive disorders Marouani et al. (2012) including infertility, oligospermia, and/or asthenospermia testicular abnormal growth, cryptorchidism, hypospadias, and altered sex ratio (Melnick, 1999b; Nelson, 2003).

Recent studies showed that there has been an increase in lead contamination in the Lebanese waters, rivers, and oceans Tannous et al. (2013); Nada et al. (2014) that exceeded the normal amount of 0.01 ppm recommended by the WHO (Tannous et al., 2013). In a recent report published in 2019 entitled “Lebanon Water Quality Report 2019 ” sample levels ranged from less than 1.0 to 6.4 ppb (LebanonUtilities, 2019).

Lead entails a toxic potential for human reproduction and reproductive outcomes (Kumar, 2018). Many studies have examined the extent to which lead alters male reproductive functions, however, the methodologies used in these

investigations were confined to hormone analysis tests, atomic absorbance spectrophotometry, ICP-AAS, and mass spectrometry MS.

This has further triggered our interest in developing a unique reliable detection tool for lead accumulation and their specific localization in the male reproductive tissues. Furthermore, to assess the morphological alterations caused by lead contamination to this system, fluorescence microscopy, and Laser Scan Microscopy (LSM), was done to locate the cells targeted by Pb inside of the testes.

Based on all previous data from the literature, our expected results would include an association between the lead dose, duration of exposure on the testes, and the amount of lead absorbed by the tissue. The reproductive capacity is expected to be reduced (testicle morphological abnormality, reduction in spermatogenic cell counts). Additionally, a diversified fluorescence activity should be observed in different cells in the testes of treated mice.

Disturbed spermatogenesis validates the diagnosis of impaired male reproductive processes. The designed study experiment utilized Swiss White Balb albino mice to question any consequential changes to lead exposure influencing the reproductive system.

This study allowed us to evaluate lead effects on the male reproductive system and spermatogenesis, hopefully establishing a direct link between the

contaminant and the cells affected in the testis, mainly the Leydig, and Sertoli cells as well as spermatogonial germ cells. Specifically, we

- Detected severe histological disruption in the male testicular tissues of the mice.
- Specified any cessations in spermatogenic stages.
- Identified lead propagation through testicular tissue and testicular cells

III. **Experimental design**

Previously, from Onessi et al. 2016, The impact of lead on spermatogenesis and reproductive tissue in male mice (Unpublished Data), this project entailed a single experiment, as summarized in Table 3 to assess the impact of lead at high doses on testicular cells in spermatogenesis. This experiment was performed with 20 Swiss White Balb Albinos sexually mature male mice with an average body weight ranging between 29.5 ± 2.03 g and 35.9 ± 1.99 g acquired from USJ-pharmacy. These mice were treated with 0, 6, and 12 ppm of PbCl2 for 36 days. Following dissection, the testes were collected and processed for fluorescent microscopy. Following the administration of high lead levels (6, and 12 (high dose) ppm PbCl2 for 36 days, testes were collected every 9 days corresponding to one seminiferous cycle. Every 9 days were referred to as a week. Week 1 was

designated at 9 days, Week 2 at 18 days, Week 3 at 27 days, and finally week 4 at day 36. The chosen lead concentrations were adopted from the previous literature search (Graça et al., 2004; Oliveira et al., 2009).

All procedures and animal operations were conducted according to Institutional Animal Care and Use Committee (IACUC) guidelines for laboratory animals following obtaining Institutional Review Board approval from Notre Dame University (NDU).

Previously from Onessi et al. 2016, The impact of lead on spermatogenesis and reproductive tissue in male mice (Unpublished Data), testicular tissues were processed for fluorescence staining. As a final step, testicular tissues were incubated in 2 ml of molten paraffin at 60 ºC for 24 hours before embedding into 5x3cm2 molds. Testis-mounted slides were prepared and spared for tissue re-slicing and staining (Graça et al., 2004).

IV. Materials and Methods

1. Slicing & Rehydration

Testicular tissues obtained were sent to the pathology laboratory fixed in Bouin's solution for at least 20-30 minutes and then submerged in formaldehyde and embedded in paraffin. A manual microtome Leika 600 was used to obtain, 5-8 µm thick slices, which were mounted onto appropriate glass microscope slides for

analysis. The sliced tissues were rinsed with xylene for 10- 30 minutes to remove the paraffin and clean the slide, and then rehydrated in ethanol at 30-60 second intervals in decreasing concentrations (100, 90, 80, and 70 percent). Furthermore, the slides were treated with a permeabilizing buffer for 5 minutes then stained with 25 µl of Hoechst 33258 (1:100) for 30 minutes, washed with 1X PBS then stained in the dark with Rhodamine B (1:1000) for 15 -30 seconds, then rewashed with 1X PBS, dried and sealed with a cover-slip and mounting media before observing them with a fluorescent microscope. Hoechst 33342 was used for specifically staining the nuclei of living or fixed cells and tissues. Rhodamine dyes as part of fluorescence microscopy for lead detection. On microscopic evaluation, a sample was considered satisfactory if at least 30 seminiferous tubules were visible for cell counting.

The laser for excitation and emission for Hoechst 33258, the nuclear dye, was set at 400 nm and 500 nm respectively with a red pseudo color showing the nucleus, while those for Rhodamine B, the lead-specific dye, were set at 500 nm and 570 nm respectively with the overlap of red color and green color to determine Pb contamination in testicular cells.

2. Laser scanning fluorescent microscopy analysis

The samples were studied in total darkness with a Zeiss LSM 700 confocal laser scanning microscope to reduce light bleaching. Zen 2011 software was

launched after turning on the laser keys, a power outlet, and a computer. The 10X objective lens was adjusted into position, and the light path alignment was checked. The stained tissue sections were inverted on the stage, focused with bright-field at minimal brightness, and then switched to confocal capture utilizing online ocular settings. The confocal fluorescence scanning was set under ocular settings offline by opening the smart system setup and selecting the proper configuration, dyes and colors, wavelengths, and lasers.

Snaps at three different magnifications, including 10X (dry) with an objective class set to Ph1, 40X, and 63X (with oil immersion) with an objective class set to DIC 1, were captured following appropriate optimization, which included modifying the laser gain to 600-700 for Hoechst and 500-600 for rhodamine B with a resolution varying between 8-16 bit, and the number of snaps was replicated four times with a snap speed reduced to 6 seconds while capturing the image. Images taken were saved as labeled image files (TIF), with the regions in the testes having pinched fluorescence activity representing the area damaged by lead. Testicular tissue samples collected during the experiment were utilized to confirm the presence of spermatogenesis disruption. Round seminiferous tubules or nearly round/oval were randomly chosen for analysis.

3. Johnsen's mean testicular biopsy score

Testicular histological damage and spermatogenesis were assessed using Johnsen's mean testicular biopsy score under light microscopy (Johnsen, 1970b); Johnsen (1970a). To assess histology, thirty tubules from each testis were evaluated and each tubule was given a score from 1 to 10 depending on the presence or absence of germ cell types including spermatozoa, spermatids, spermatocytes, spermatogonia, germ cells, and Sertoli cells in the testicular seminiferous tubules as detailed in table 3. Testicular tissue damage was measured as Johnsen's mean testicular biopsy score count, by week. To obtain the Johnsen score, slides were examined under an optical microscope (magnification, ×100). A score was assigned for each tubule counted. The number of tubules with a given score was multiplied by the score. The result was summed across different scores and then divided by the number of evaluated tubules, giving the final Johnsen score. Referring to Johnsen (1970a), cut-off values between few and many have not been exactly defined. "Few" was referred to as having less than 5 cells observed. To minimize variation, a single trained individual carried out all the analyses. A Johnson means score ≥8 is representative of a normal testicular histopathology pattern (Johnsen, 1970a; Lestari et al., 2019).

4. Cell Counting in the seminiferous tubules

This quantitative valuation aims to indicate the degree of detriment to spermatogenic cell types. The spermatogenic cells were manually counted, therefore cell counting conforming to a size cut-off criterion was hampered. The spermatogenic population was identified as per their morphologic appearance and location. Histologically, the seminiferous tubule is arranged as complex stratified epithelium which consists of stem cells (spermatogonia) at the base of the epithelium. The other cells are arranged in the order of development: spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. Primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa are in the adluminal compartment. The lumen also contains some spermatozoa, and cellular debris These spermatogenic cell types are represented in numerical values. They were quantified in 10 cross sections of seminiferous tubules during each week and based on the administered lead doses. The proportion of spermatogenic cell counts to the total cells was reported too.

5. Statistical Analysis

Statistical analysis was carried out through Statistical Package for the Social Sciences (SPSS) software. Data was exported from Microsoft excel (Excel V.16.29, 2019) to SPSS. Statistical tests are stated in the same order as the reported

results. Continuous variables specifically cell count is presented as mean ± standard deviation (SD). Cell counts were presented as percentages in bar charts and as proportions [N (%)] from the total cell count. Friedman Test was used to determine if there are statistically significant differences for spermatogenic cell distributions along week progression. P-value<0.05 was used to indicate the statistical significance.

V. Results

1. Localization of lead in the seminiferous tubules by LSM

The results are outlined as per week progression. With lead administration in week 1, lead absorption was boldly distinguished in spermatogonia cells and spermatocytes. The cellular organization was still maintained during the first week of administration. The lumen of the tubules was still intact at this stage. Spermatids, spermatozoa are present. Leydig cells and Sertoli cells were still visualized as the latter sustained close contact with different spermatogenic cells. The seminiferous tubules show a normal morphology even with 12ppm lead administration. Following the continuation of lead exposure over the second week, spermatogonia germ cells were noted to be more affected than other mature cells. However, additional spermatogenic cells were affected by lead as shown by the increase in rhodamine B fluorescence.

Further inspecting the microanatomy of the spermatogenic cells, Leydig cells in the interstitium of the seminiferous tubules became contaminated with lead as clearly visualized by the increased spread of rhodamine B fluorescent activity. This further explains drop in sperm numbers and the increase in morphological abnormalities of the seminiferous tubules beyond this stage. During week 3, it was noticed that the cellular organization of the tubules has been lost. The

spermatogonial cells and the spermatocytes have markedly degenerated and were viewed as spots under confocal microscopy. Fluorescence has extensively spread almost covering the whole tubular surface. The seminiferous tubules were shrunken in diameter by Week 4 (figure 2). Tubule content was exceedingly aggregated as visualized under confocal microscopy. Loosening of the seminiferous epithelium cell walls and the lack of spermatozoa in the lumen can be distinctly observed. The testes of control mice showed little fluorescence due to Hoechst staining of the nucleus, however, this fluorescence was not as apparent as compared to testes from mice drinking lead water. Furthermore, a particular observation of red dots at 63x in 6 ppm lead mice following 36 days (Week 4) of exposure, indicated parasitic organisms, possibly the result of reduced immunity in the testes as indicated in previous research (Elad et al., 1983; Schulz et al., 2015)(figure 3). Additional images of lead bioaccumulation in the seminiferous tubules are present in figure 4, 5 and 6.

2. Johnsen's mean testicular biopsy score results

Testicular biopsy is a crucial assessment in clinical reproductive medicine with diagnostic and prognostic importance. (Johnsen, 1970a)Our results showed complete spermatogenesis with many spermatozoa in Week 1. The seminiferous tubules were lined by a complex stratified and highly organized germinal epithelium leaving an open lumen. The tubules displayed a clear lumen with all cell types

represented including Sertoli cells, spermatogonia, spermatocytes, spermatids, and spermatozoa. The tubule basement membrane (BM) was seen with the interstitium containing Leydig cells. The arrangement of spermatogonia was very systematic and concentric and laid against the basement membrane. A normal morphology of seminiferous tubules was presented in week 1. The various spermatogenic phenotypes were present outlining normal spermatogenesis. The mean score was 10. No pronounced changes were reported between Week 1 and Week 2 regarding spermatogenesis disruption. Spermatozoa were abundantly observed during both weeks. Taking account of the absence of a cut-off value for the terminology "many" in Johnsons Score, Lowest number of spermatogenic cells reported in the control group was considered as the cut off value, therefore the threshold value was ≤80 cells.

As demonstrated in the below images (figure 3), complete spermatogenesis with many spermatozoa was observed in Week 1. The seminiferous epithelium normally lined the tubules leaving an exposed lumen. Different stages of the spermatogenic cycle were identified along the tubules at distinct depths of the epithelium. Spermatogenic cells were synchronously present including Spermatids and Spermatozoa. Sertoli cells were still recognized interspersed between the germ cells within the seminiferous epithelium. Among other images many spermatozoa were present but germinal epithelium was disorganized with marked sloughing or

demolition of lumen. The tubule membrane was disrupted as observed in some images. Sertoli cells and Leydig cells were still visible. The mean score for the cells in Week 2 was 10.

Week 3 denoted a clear disruption of spermatogenesis. Spermatogonial cells and spermatocytes were significantly downsized. The tubule lumen was clearly shrunken. Some cells were no longer attached to the basement membrane. Leydig cells were recognized as highly aggregated sets of cells. The borders of the tubules were no longer discrete. Spermatids were abundantly present. The mean score for week 3 was 7.8.

As for week 4, the images examined revealed a heterogeneous appearance of the seminiferous epithelium associated with testicular damage. The seminiferous tubules were abnormal with a varying alteration extent. With careful examination, cells within the tubules are seen to be fragmented. There was a drastic loss of germ cells, and vacuolation of spermatocyte cells is present. LCA is visible in the interstitial compartment. Seminiferous tubules are outlined without a lumen. No spermatozoa but many spermatids present. Week 4 outlined poor cellular morphology compared with the previous weeks. Epithelial barrier erosion. The basement membrane was broken and the germ cells sloughed into the interstitium. The mean score was 5.11. A significant difference was reported between the scores as demonstrated in table 5.

3. Effect of lead on testicular cell count

The present study shows that as lead dosage and the exposure period increase, the count of testicular cells per tubule (spermatogonia, spermatocytes, spermatid, sperm) decreases. As reported in figure 7, for the control group (0ppm lead), an average of 51 spermatogonia were present in week 1, 52 spermatogonia in week 2, and 46 cells in week 3. No data was acquired for week 4. With 6ppm lead administration, spermatogonia counts were the highest in week 2 with an average of 59 cells. This number dropped to reach an average of 16 cells by week 4. With 12ppm lead administration, spermatogonia counts were reported to be the highest in week 2 with an average of 61 cells before dropping to reach their lowest of an average of 9 spermatogonia in week 4. Similarly, all cell categories were decreasing with week and dose progression. Spermatocytes were the highest in the control group with an average of 88 spermatocytes per tubule. This value decreased to reach its lowest during week 4 (an average of 19 cells with 6ppm lead; average of 9 cells with 12ppm). Spermatid counts declined from an average of 89 cells per tubule to reach an average of 9 cells with 6ppm and 12ppm lead during the fourth week of lead dispensing. Similarly, spermatozoa count alleviated to attain almost negligible numerals specifically at week 4 with 12ppm lead administration (≈1 spermatozoa per tubule).

VI. Discussion

Heavy metals are publicly widespread in the environment and commonly occur in food, water, air and tissues even in the absence of occupational exposure. The present study was the first qualitative and quantitative description of testicular harm in white mice. In this respect, it allowed the investigation of lead contribution to inducing testicular morphological disorders. This study further revealed that lead induces significant degeneration in the male testicular structure and microanatomy. Significant alterations in the histological patterns in the testis were denoted. Lead toxicity has been extensively studied, but data on the mutagenic, and carcinogenic qualities of lead and lead compounds is still inconclusive and conflicting. The deterioration of male fertility associated with environmental toxins including lead is found in numerous epidemiological studies as presented in table 3. The IARC classified lead as a potential human carcinogen based on sufficient evidence for carcinogenicity in experimental animal models but insufficient evidence for carcinogenicity in humans (IARC, 2006).

It is known that histopathological changes in the testes are the histological basis of changes in spermatogenesis function, especially in the seminiferous tubule (Johnson, 2014). The testicular morphology was closely observed under light microscopy and confocal microscopy. The margins of the tubules in our samples range from being perfectly smooth to having a very irregular outline, and the nuclei

likewise lose their identity- some appear aberrant, others fragmented, while in some of the degenerate masses no nuclei are detected. Additionally, the testis of lead-treated rats showed a marked reduction in the seminiferous tubule diameter as demonstrated in figure 5. These findings support the results from other reports that lead can seriously alter the testes and reproductive tract in male rats treated with lead (Johansson and Pellicciari, 1988; El-Shafai et al., 2011). The chronic lead exposure has induced a supplemental morphological disruption demonstrated by a decrease in seminiferous tubular diameter specifically following the third week of exposure (Figure 3). This was supported by findings from Ahmed et al. (2012) while reporting the toxic effects of prolonged lead exposure in male rabbits.

The testicles resulting from this experiment are indeed atrophied. A maturation arrest has been described in the histological analysis. Further inspecting the microanatomy of the testicular cells in the studied seminiferous tubules at (63x spermatogonia germ cells were first distressed when compared to other more mature cells with 6 ppm lead exposure. Spermatogonial cells were the first to clearly fluoresce. Furthermore, at 12 ppm $PbCl_2$ Leydig cells, the interstitial seminiferous tubule cells become contaminated with lead as clearly visualized by the increase and spread of rhodamine B fluorescent activity, explaining the further decrease in sperm numbers and increase in abnormality after this phase (Figures 4, 5 and 6). The contents of the tubules were shattered and disarranged that they bear the slightest

resemblance to their normal state. Our result agreed with Aithamadouche et al. (2013) that reported sloughing of all layers of testicular seminiferous tubules of rats previously treated with lead acetate. Additionally , Al-Attar (2011) experiment on male rats documented the detachment of the germinal cell layer from the basal membrane, atrophy of Leydig cells plus interstitial edema and low density of seminal plasma in the rat testes. Madhavi et al. (2007) showed lead-induced cytogenetic damage in germ cells of mice.

Testicular damage is confirmed by histopathological lesions (Hamadouche et al., 2013). In their study, Hamadouche et al. (2013) demonstrated that lead can seriously alter the testicular tissue that started the changes with vacuolar degeneration until necrosis and atrophy of seminiferous tubules. The treated groups displayed vacuolation and degenerative changes in spermatogonia, arrest of spermatogenesis, and pyknotic changes in spermatocytes. This is, as well compatible with our study in which, the center of most seminiferous tubules showed a moderated number of spermatozoa, advanced degeneration of spermatogonia and interstitial cells, and abnormal distribution of spermatozoa especially after the third week of lead exposure.

A partial or complete loss of spermatogenic series from the lumen of seminiferous tubules was reported as well. The germ cell loss has progressively

induced shrinkage of the seminiferous tubules leading to the appearance of a frail phase in which only residual interstitial cells remain (Woldemeskel, 2017).

Our study demonstrated an exponential drop in the cell count of all populations of spermatogenic cells with the increase in lead period exposure and dose. It has reported an absence of sperm in the lumina of the histological sections following 4 weeks of lead exposure .This is further confirmed by Shubina and Dudenkova (2016) that have reported a decrease in the production of all populations of spermatogenic cells and a decline in the spermatogenesis index, thus indicating a decrease in the functional activity of the testes. Another study by Huang et al. (2021) reporting on the lead-induced spermatogonia and Leydig cell toxicity demonstrated that lead primarily exerted toxicity in spermatogonia and Leydig cells according to the increase of Pb concentration following treatment further indicating that Pb was accumulated in cells, induced cellular death and seminiferous tubule depletion.

Immunofluorescence microscopy additionally showed that the seminiferous tubules were degenerating faster with the increase of PbCl2 treatment as seen in figure 6 with three different magnifications. Such degenerative activity might lead to the loss of germ cells and an increase in dead and abnormal sperm (Makhlouf et al., 2008). Similarly, there was an increase in rhodamine B fluorescence activity with increases in both dose and days of exposure to PbCl2.

Knowing that Rhodamine B bind specially to lead in the tissues Beija et al. (2009) in another study done by Wu (2008) showed that Rhodamine B probes bind to Pb2+ exclusively thus it is a good chelators agent for PbCl2. With all these pronounced demolitions, it can be readily set that a destructive process has involved the seminiferous tubules and it's clear that lead can cross over the blood-testis barrier reach the seminiferous tubule epithelium and expose its components. As for the testes, the absorbed Pb was either transported to different cells inside of the seminiferous tubules or absorbed by the blood testicular veins to be transported by the blood and excreted from the mice's body (Graça et al., 2004; Wang and Jia, 2009; Vigeh et al., 2011).

VII. Conclusion and Future Directions

Lebanese marine water and freshwater regions are exposed to a multi-point source contaminant as reported by Korfali and Jurdi (2012) in their published manuscript. Heavy metal pollution is one of the most serious environmental issues globally Mohiuddin et al. (2011) , and it seems to affect Lebanon as such. Heavy metals like chromium, lead, cadmium, and arsenic tend to exhibit extreme toxicity even at trace levels (Mohiuddin et al., 2011). It was estimated that tanneries discharge nearly 40 tons of chromium yearly into the Mediterranean Sea in a report for the Lebanese Ministry of Environment ECODIT (2001) , and a fertilizer company discharges an approximation of 0.7 tons of cadmium, 2 tons of lead, and 2

tons of nickel into the sea yearly (ECODIT, 2001). The sustained exposure of the Lebanese coastal line to various types of contaminants specifically heavy metals resulting from, mostly, domestic and industrial activities immensely impact the seawater quality and subsequently human reproductive health.

Our study outcomes are clear indicative of the negative impact of lead acetate in the course of the process of spermatogenesis in the testes of male white mice. Spermatogenesis is a complex process involving a multitude of cells. Therefore, any minor disruption to these spermatogenic cells leads to dire consequences, including alteration of sperm quality and quantity, infertility, or conspicuous testicle alteration. This experimental research design was implemented to assess the impact of lead on male reproductive organs and specifically spermatogenesis in mice. White mice were fed, in different experiments, 0, 6, and 12 ppm of $PbCl_2$ and showed a positive interdependence between the lead consumed and its expansion into the testicle gradually infiltrating the seminiferous tubules as detected by Fluorescence microscopy. Fluorescence microscopy for the testes additionally demonstrated an increase in fluorescence when increasing lead concentration and when increasing the exposure period. Fluorescence levels reached not only spermatogonial germ cells, but reached spermatids, Leydig, Sertoli, and interstitial/intersemineferous tissues further depleting the cell count. In addition to the contamination of the various germ cells, lead exposure, predominantly 12 ppm, demolished the seminiferous tubule

morphology, with the absence of a tighter lumen.

Lead has been well-known to induce testicular injury with its prolonged exposure leading to atrophy of the testis and infertility. Most of the cross-sectional analyses in humans as well as in vivo and in vitro animal studies have generally confirmed that even moderate- to low-level exposure to lead affects certain reproductive parameters and leads to reproductive toxicity. Lead toxification is a censorious threat to human physiological systems having its chronic exposure remain asymptomatic for extensive periods before symptoms manifest. Epidemiological studies are needed to extrapolate the experimental data on humans. Yet, the detailed mechanisms of lead toxicity on the male reproductive system and spermatogenesis are yet to be fully addressed.

VIII. Study limitations

Despite the above strengths of this experimental study, we are aware of the several limitations of this study; this experimental design was implemented with two lead concentrations of $PbCl_2$ which were 6 ppm and 12 ppm and not more, hence our results may not be representative of the higher and lower concentrations. We were not able to maintain slide replicates due to tissue damage. The first experimental design was conducted back in 2016, therefore by the time the specimen and the ready-made slides were utilized, it was no doubt that a portion of

tissues was partially degraded, the tissue quality was indeed altered, and the quality of histology slides was negatively influenced. Several reports have confirmed that a long delay between cutting sections and immunohistochemical (IHC) staining can decrease the IHC reaction intensity (Mirlacher et al., 2004). The slicing, rehydration, and staining were not fully successful due to the tissue quality primarily and due to the deterioration in the fixation ability of the rhodamine stain. Therefore, in some slides, the efficient morphologic evaluation was hampered. Nine days following lead administration was the earliest stage studied, but it may be that the effects could have been noted a few days before.

Moreover, spermatogenic cell populations were manually counted and not counted through software like image J due to technical issues in maintaining the software. This may have acted as a confounding factor, whereby affecting the efficacy of the cell counting process. What we have obtained through this experimental study relates to qualitative descriptions of lead repercussions and reflects only the end stage of intoxication and not the detailed process and route.

Tables and Figures

Table 1: Highlights of lead exposure to human and animal male reproduction

Exposure to Lead	Observed Effects	References
Exposure to inorganic lead >40µ/dL of blood	Reduced sperm count, volume, density, changing sperm motility and morphology	Apostoli et al. (1998)
Reproductive endocrine function in healthy male industrial workers	Moderate exposure to Pb (BPb <400 µg/L) and Cd (BCd <10µg/L) reduces semen quality without conclusive evidence of impairment of male reproductive function	Telisman et al. (2000)
Reproductive effects were observed at low-level Pb environmental exposure (BPb median 49 µg/L range 11-149 µg/L)	A significant association of BPb, delta-aminolevulinic acid dehydrase, and/or erythrocyte protoporphyrin with reproductive parameters indicated a lead-related elevation in immature, pathologic, wide, round, and short sperm, serum testosterone and estradiol level, and a decline in seminal plasma zinc and serum prolactin	Telisman et al. (2007)
BLL ≥20 µg/dL and subjects with BLL< 20µg/dL	A non-significant reduction in the count, motility, and higher impaired sperm morphology with BLL ≥20 µg/dL compared with BLL< 20µg/dL subjects; chromatin condensation negatively correlated with BLL	Awadella et al. (2011)
Semen quality and endocrinal function in infertile painter	A significant negative correlation between BPb and sperm count, motility whereas no significant correlation between BPb and endocrinal parameters PbB≥20 µg/dL showed a significant decrease in sperm motility, increase in testosterone level	Hosni et al. (2013)
Seminal Pb and Cd at environmental level and semen quality	A negative association between seminal Pb and Cd and sperm concentration, and % abnormal morphology, exposure to, exposure to Pb (5.29-7.25 µg/dL) and Cd (4.07-5.92 µg/dL) might affect semen quality.	Pant et al (2015)
Accumulation of Lead	Affects the hypothalamic-pituitary axis, causing blunted TSH, GH and FSH/LH in response to TRH, and GnRH stimulation. Respectively, higher levels of prolactin in Pb toxification. In short-term Pb exposure, low testosterone levels do not induce high LH and FSH.	Doumachout et al. (2009)

Lead-exposed male workers and serum sex workers	Testosterone lower. Pb exposure alters male sexual hormone, which might harm endocrine function and Sertoli cells	Yu et al. (2010)
Role of Pb and Cd on sex hormones and molecules of the steroid biosynthesis pathway	After adjustment of age, race, BMI, smoking, diabetes, and alcohol. BPb was positively associated with testosterone and SHBG.	Kresovich et al. (2015)
The effect of lead on the process of spermatogenesis in sex glands of male albino rats	Lead acetate induced a decrease in the production of all populations of spermatogenic cells, decreased spermatogenesis index and an index of relaxation (tension spermatogenesis), and an increase in the index of ripening, index meiotic activity and germinative index, which indicates a decrease in the functional activity of the testes.	Shubina OS *et* al. (2016)
Lead-induced spermatogonia and Leydig cell toxicity and mitigative effect of selenium in chicken	Spermatogonia has a higher tolerance to Pb and Se than Leydig cells; Pb causes spermatogonia and Leydig cell injury and reduces testosterone secretion of Leydig cells; Pb induces heat shock response and autophagy under the burden of oxidative stress.	He Huang *et* al. (2021)

Table abbreviations: BLL: Blood Lead level; TSH: Thyroid Stimulating hormone; GH: Growth hormone; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; TRH: Thyrotropin-releasing hormone; GnRH: Gonadotropin-releasing hormone; BMI: Body mass index; SHBG: sex-hormone-binding globulin

Table 2: Kinetics of spermatogenesis among different mammalian species

Species	Spermatogenesis (days)	Seminiferous cycle (days)
Humans	74	16
Rats/Mice	36-42	9
Bulls	54	13.5
Boars	34	8.5

Reference source: Damm and Cooper, 2010; Johnson, 2013 #53

Table 3: Histological classification of seminiferous tubular cross-sections according to the Johnsen scoring system

Score	Description
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium is organized in a regular thickness leaving an open lumen.
9	Many spermatozoa are present but germinal epithelium disorganized with marked sloughing or obliteration of lumen.
8	Only a few spermatozoa (<5-10) are present in the section.
7	No spermatozoa but many spermatids present.
6	No spermatozoa and only a few (<5-10) are present.
5	No spermatozoa, no spermatids but several or many spermatocytes present.
4	Only a few spermatocytes (<5) and no spermatids or spermatozoa are present.
3	Spermatogonia are the only germ cells present.
2	No germ cells but Sertoli cells present.
1	No cells in the tubular section

Table 4: Average spermatogenic cells along 4 weeks of Lead exposure

	Lead Dose	Total Count	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
Day 9	0 ppm	335	51(15.2%)	88(26.3%)	96(28.7%)	100(29.9%)
	6 ppm	236	40(16.9%)	60(25.4%)	43(18.2%)	93(39.4%)
	12 ppm	183	36(19.7%)	68(37.1%)	30(16.4%)	49(26.8%)
Day 18	0 ppm	312	52(16.7%)	84(26.9%)	90(28.8%)	86(27.6%)
	6 ppm	154	34(22.1%)	59(38.3%)	28(18.2%)	33(21.4%)
	12 ppm	156	35(22.4%)	61(39.1%)	30(19.2%)	30(19.2%)
Day 27	0 ppm	294	46(15.6%)	78(26.5%)	89(30.3%)	81(27.6%)
	6 ppm	96	25(26%)	31(32.3%)	21(21.9%)	19(19.8%)
	12 ppm	55	16(29.1%)	20(36.4%)	6(10.9%)	13(23.6%)
Day 36	0 ppm	No Data	No Data	No Data	No Data	No Data
	6 ppm	53	16(30.2%)	19(35.8%)	6(11.3%)	12(22.6%)
	12 ppm	25	9(36%)	9(36%)	6(24%)	1(4%)

Table 5: Friedman 2-way ANOVA for distribution of Spermatogenic cells

Null Hypothesis	**Test Significance (p-value)**	**Decision**
The distributions of Spermatogonia0ppm, Spermatogonia6ppm and Spermatogonia12ppm are the same.	0.050*	Reject null hypothesis
The distributions of Spermatocyte0ppm, Spermatocyte6ppm and Spermatocyte12ppm are the same.	.097	Retain null hypothesis
The distributions of Spermatid0ppm, Spermatid6ppm and Spermatid12ppm are the same	.097	Retain null hypothesis.
The distributions of Spermatozoa0ppm, Spermatozoa6ppm and Spermatozoa12ppm are the same.	0.264	Retain null hypothesis.

***The Friedman 2-way ANOVA indicated that the distribution of spermatogonial cells were significantly different among the 3 administered lead doses.**

Table 6: Johnsen's mean scores by weekly exposure

Exposure Week	Mean scores ± S.D	P-value
Day 9 (W 1)	10 ±0.000	0.000*
Day 18 (W 2)	9.83 ±0.379	
Day 27 (W 3)	6.83 ± 1.464	
Day 36 (W 4)	4.17 ±1.147	

*Statistical significance was noted between the Johnson's mean scores during the 4 weeks of lead exposure; W: Week

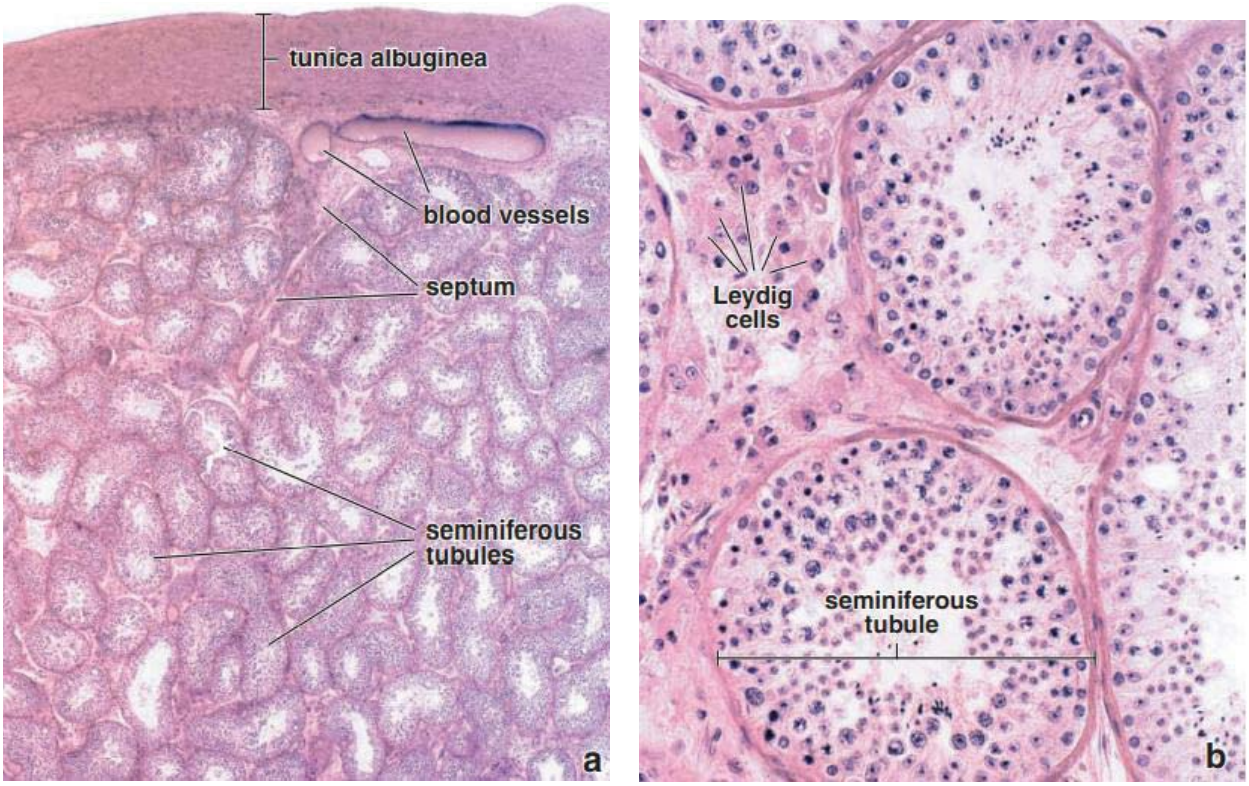

Figure 1: Photomicrographs of the human testis (Ross, 2011)

- Figure 1a. H&E–stained section of human testis showing seminiferous tubules and the tunica albuginea. Seminiferous tubules are highly convoluted

- Figure 1b. Seminiferous tubules depicted at a higher magnification. Leydig (interstitial) cells occur in small clusters in the space between adjoining tubules.

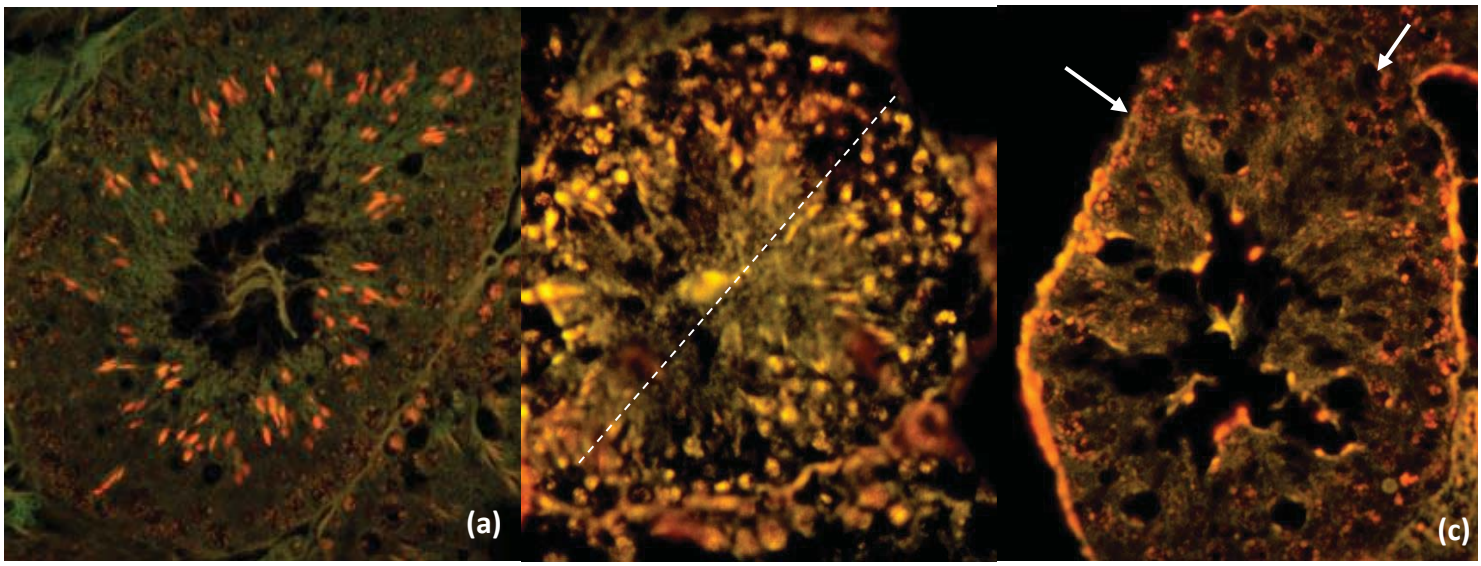

Figure 2: Effect of lead exposure on seminiferous tubule morphology (Day 36/ 63X).

- Panel (a): Seminiferous tubule with normal diameter
- Panel (b): Shrunken seminiferous tubule diameter
- Panel (c): Deformed seminiferous tubule

*Arrows in the above images refer to tubule diameter in panel(b) and to the deformed tubule outline in panel(c).

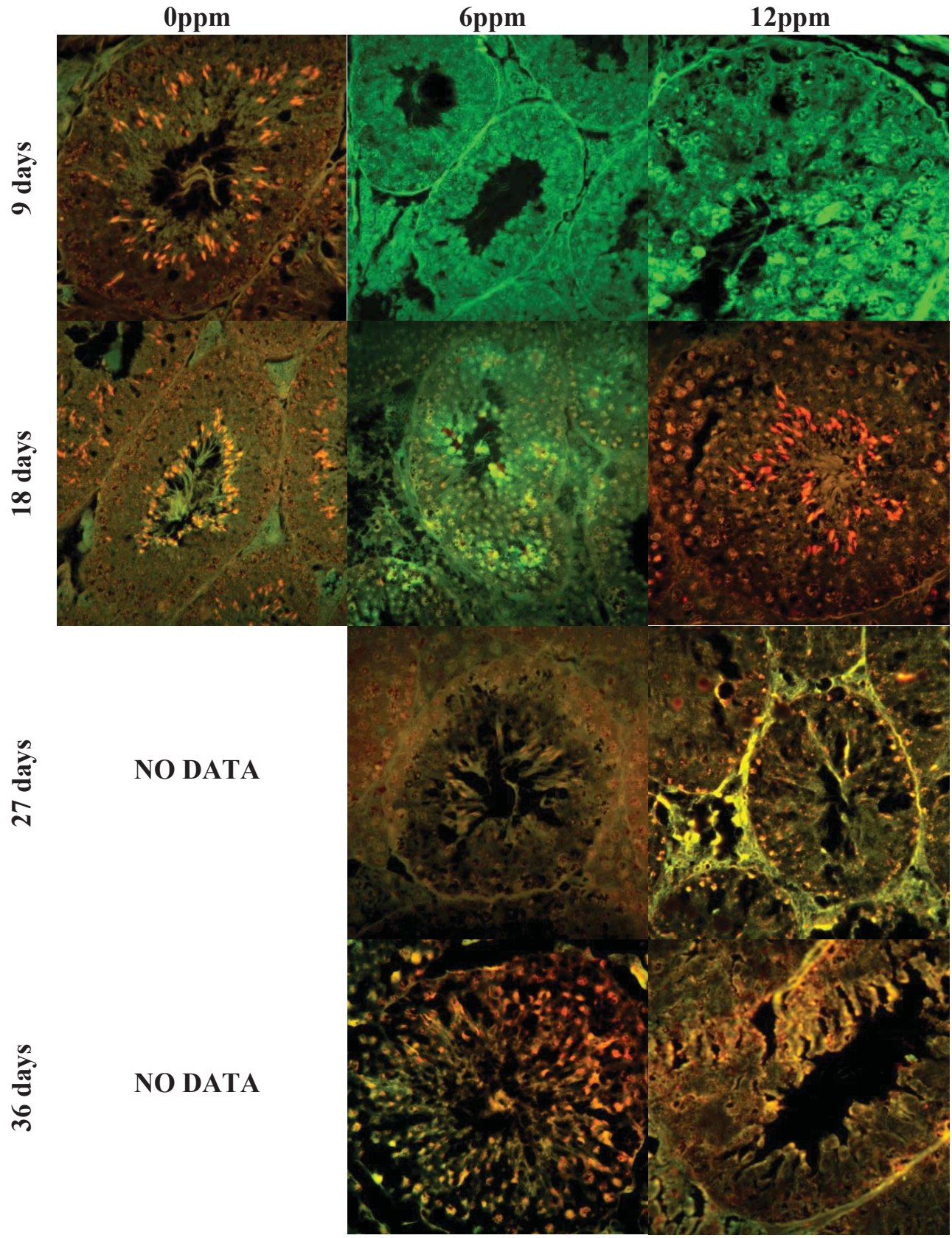
0ppm
6ppm
12ppm
9 days
18 days
27 days
36 days
NO DATA
NO DATA

Figure 3: Effect of lead bioaccumulation on the testes microanatomy (63X) magnifications via LSM where the tubule microanatomy was being observed along the 4 weeks of exposure. Fluorescence microscopy revealed that the seminiferous tubules were degenerating faster with the increase of PbCl2 treatment. Such degeneration led to tubule deformity and to the gradual loss of spermatogenic series

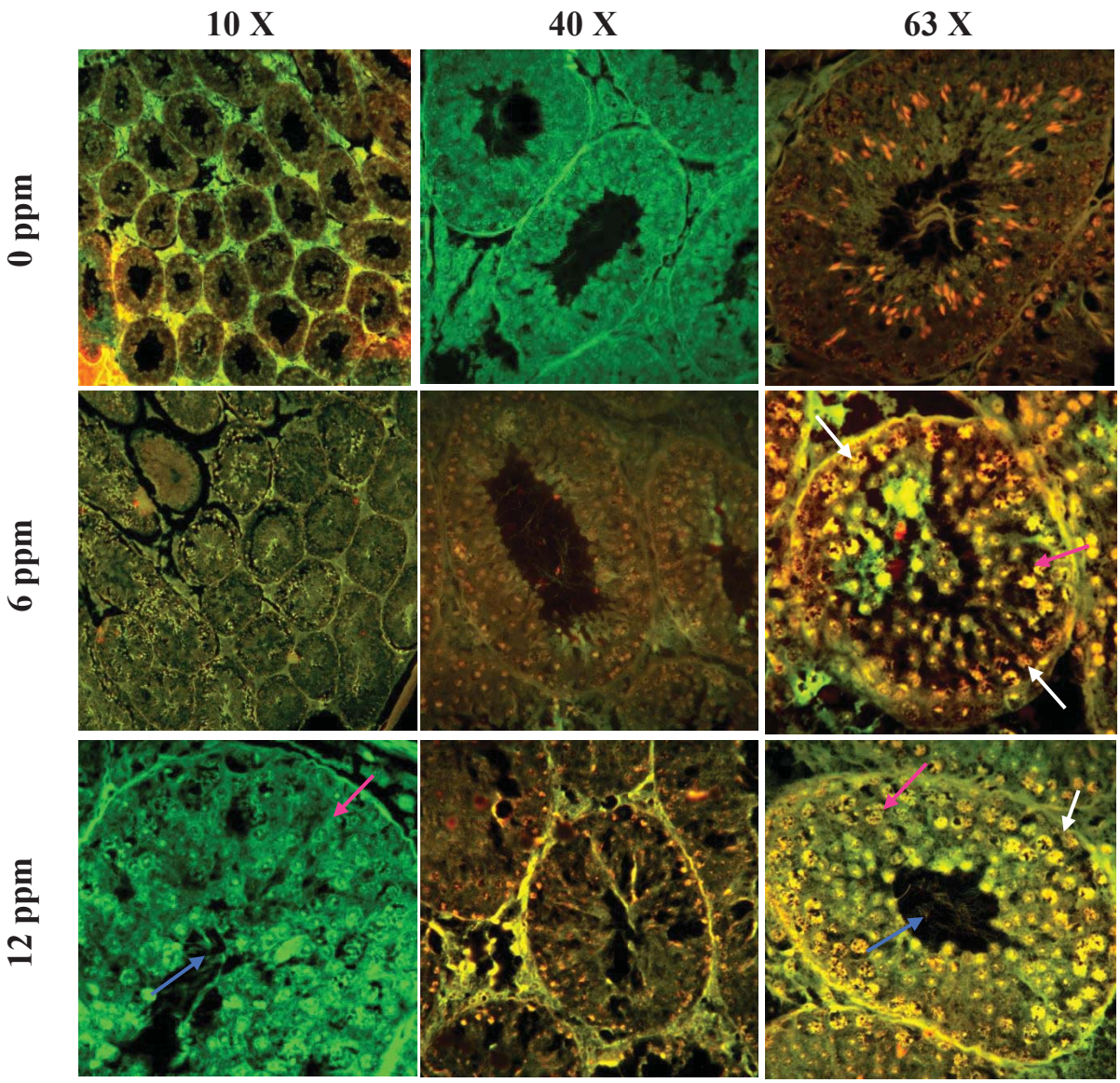

Figure 4: Effect of lead bioaccumulation on the testes microanatomy in white mice

Rhodamine B dye (Red to violet) was utilized for Pb detection. An increase in Rhodamine B is indicated by a flashy yellow colour. There was an increase with rhodamine B fluorescence activity with increasing the dose of $PbCl_2$ in pp. These micrographs were taken at 3 different magnifications via LSM (10X-40X-63X).

- White arrows indicate spermatogonia
- Pink arrows indicate spermatocytes
- Blue arrows indicate spermatozoa

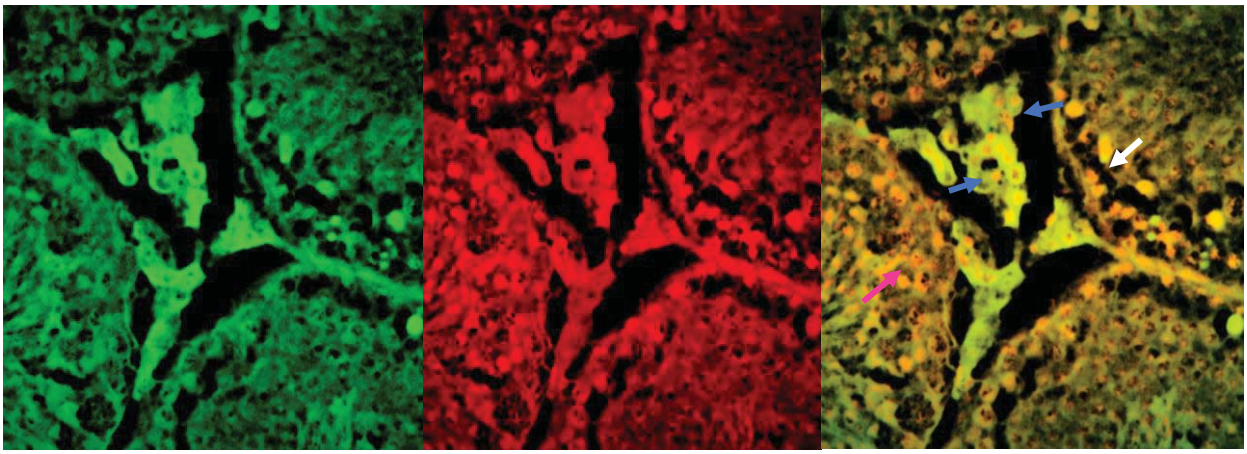

Figure 5: Lead bioaccumulation by spermatogenic cells and Leydig cells after 4 weeks of lead administration in drinking water

These micrographs were taken at 63X magnification via LSM with at 3 different channels (Phase contrast- Red- Blue). The micrographs show the four concurrent seminiferous tubules with Leydig cells present in the interstitial space outside the tubules. Following two weeks of lead exposure, lead uptake was visible in all tubules with a clear accumulates in Spermatogonia, Spermatocytes and Leydig Cells. White arrows indicate spermatogonia, pink arrows indicate spermatocytes, blue arrows indicate Leydig cells

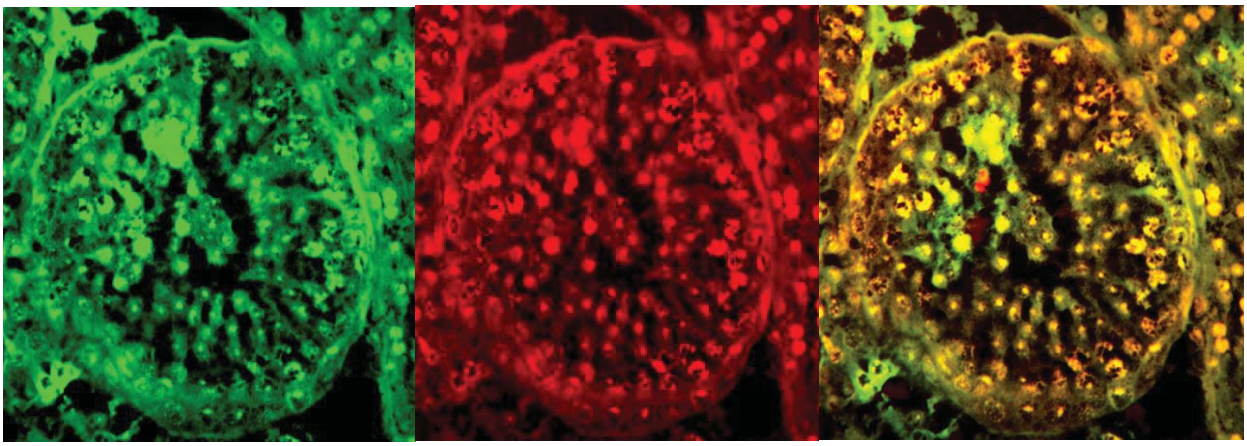

Figure 6: Lead uptake by all the spermatogenic population

These micrographs were taken at 63X magnification via LSM with at 3 different channels (Phase contrast- Red- Blue). The micrographs show a definite seminiferous tubule with visible spermatogenic cell series. This micrograph captured a sample from Week 2 of $PbCl_2$ with 6ppm dose. Lead accumulation is recognized in all spermatogenic populations.

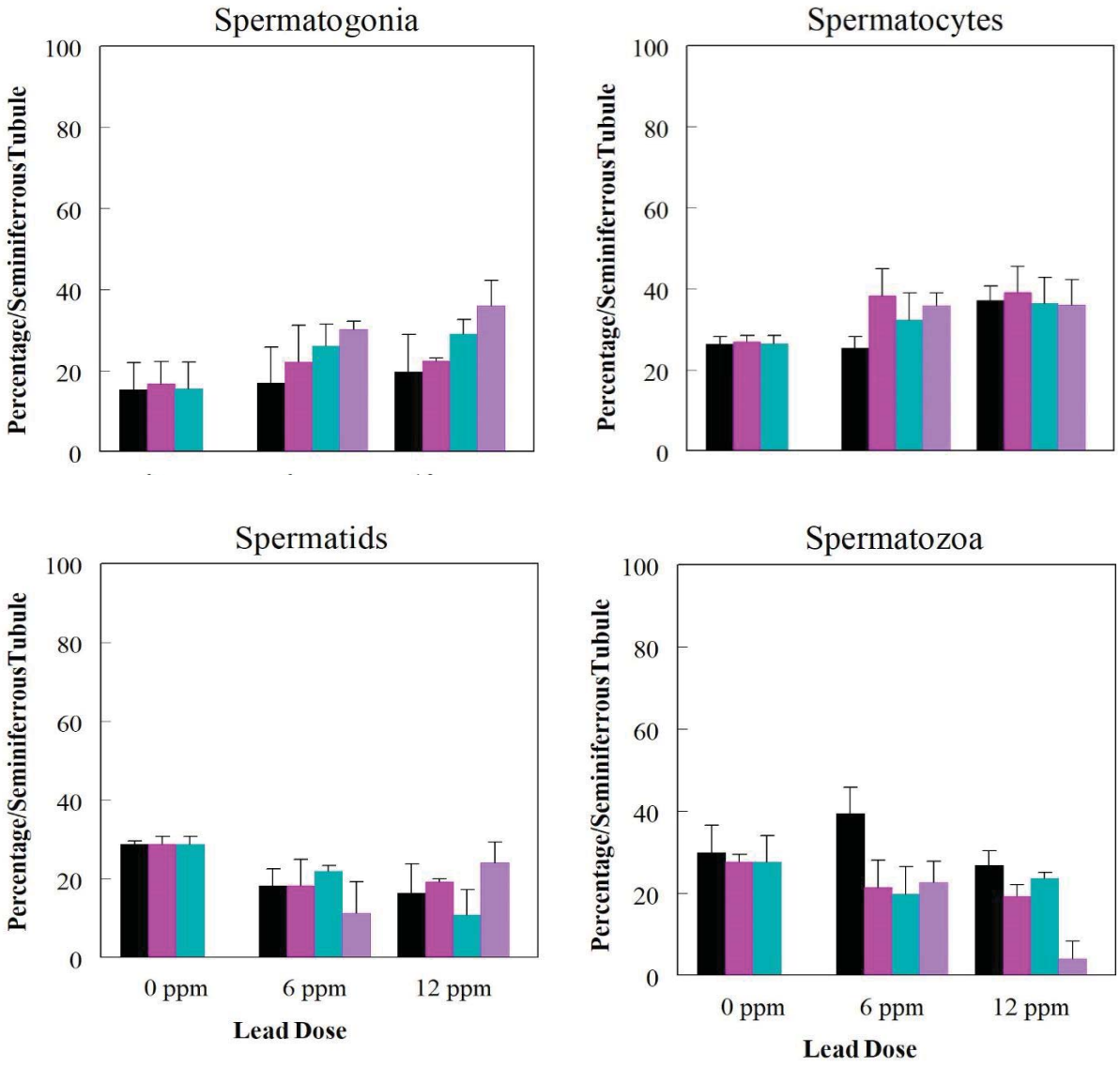

Figure 7: Effects of repeated lead exposure of spermatogenic cell percentages in mice

The percentages of the cells were calculated by dividing the counts of each cell type during a specific week and exposure dosage by the total cell count, all multiplied by 100. Cell counts were decreasing with lead exposure and dosage

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