MARLI DO CARMO CUPERTINO

MINERAL AND INFLAMMATORY DISTURBANCES ON REPRODUCTIVE SYSTEM TRIGGERED BY CADMIUM EXPOSURE

Thesis submitted to the Federal University of Viçosa, in partial fulfillment of the requirements in the Graduate Program in Structural and Cell Biology, for the degree of *Doctor Scientiae*.

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(Adviser)

To God,

For showing me that humility is the main force that I need to walk day after day in the pursuit of my dreams;

To My Parents, Brother, Sisters and Nephews,

The base that strengthens me and heals me every day. My safe haven;

To my husband, Rodrigo,

My guardian angel. My greatest example of human being;

I dedicate.

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CONTENTS

RESUMO

CUPERTINO, Marli do Carmo, D.Sc., Universidade Federal de Viçosa, março de 2016. **Distúrbios inflamatórios e minerais, no sistema reprodutor, causados por exposição ao cádmio.** Orientador: Sérgio Luis Pinto da Matta.

Estudos sobre os mecanismos de toxicidade causados por poluentes ambientais representam um campo de investigação amplo. O efeito tóxico do Cd (cádmio) no sistema reprodutor não é um assunto novo, mas ainda possui muitas lacunas a serem preenchidas, principalmente sobre os mecanismos pelos quais o Cd desencadeia lesões. O objetivo deste estudo foi avaliar o padrão inflamatório e mineral apresentado por ratos expostos a quatro baixas doses de cádmio e analisar após a fase inflamatória inicial e antes da fibrose crônica. Ratos machos adultos foram expostos a doses únicas intraperitoneais de cloreto de cádmio, juntamente com um grupo controle salina:1- 0.9% NaCl; 2- $CdCl_2$ 1.1 mg/kg (0.67 mg Cd/kg); 3- $CdCl_2$ 1.2 mg/kg (0.74 mg Cd/kg); 4-$CdCl_2$ 1.4 mg/kg (0.86 mg Cd/kg); e 5- $CdCl_2$ 1.8 mg/kg (1.1 mg Cd/kg). Sete dias após a exposição foram coletados sangue e órgãos reprodutores sendo feitas análises: biométricas, morfométricas, estereológicas, estresse oxidativo, peroxidação lipídica e proteica, dano em DNA, ultraestrutura por microscopia eletrônica de varredura e transmissão, dosagem de minerais teciduais, testosterona sérica, citocinas inflamatórias e análises histopatológicas que incluíram marcações para apoptose e calcificação. Observou-se que em todos grupos expostos ao Cd um padrão inflamatório marcado por neutrofilia no intertúbulo e ambiente basal, sem alterações nos marcadores de peroxidação lipídica, sugerindo ausência de danos na barreira de células de Sertoli. Marcadores de estresse oxidativo e citocinas apresentaram alterações indicando ser um dos mecanismos pelos quais as lesões são induzidas. Houve redução da produção de testosterona e reduções volumétricas nos parâmetros de Leydig. Foram observadas intensa apoptose e calcificação de células germinativas e das fibras colágenas. Houve incremento do cálcio tecidual com o aumento da dose de Cd, porém outros minerais sofreram queda. Histopatologias e alterações morfométricas, estereológicas e biométricas apresentaram

aumento dose-dependente. Concluímos que as lesões causadas pelo Cd são dose-dependentes e que as vias minerais e inflamatórias desempenharam papel-chave para o desenvolvimento das lesões.

ABSTRACT

CUPERTINO, Marli do Carmo, D.Sc, Universidade Federal de Viçosa, March, 2016. **Mineral and inflammatory disturbances on reproductive system triggered by cadmium exposure** Advisor: Sérgio Luis Pinto da Matta.

The study of toxicity mechanism caused by the environmental pollutants is a broad research field. The toxic effect of Cadmium (Cd) in reproductive system is not a novel issue, but many gaps still remain to be filled, especially on the mechanisms by which the Cd triggers injuries. The objective of this study was to evaluate the inflammatory and mineral pattern presented by rats exposed to four low doses of Cd and to analyze after initial inflammatory phase and before chronic fibrosis. Thus, adult male rats were intraperitoneally exposed to single doses of Cd chloride, along with a saline control group: 1- 0.9% NaCl; 2- $CdCl_2$ 1.1 mg/kg (0.67 mg Cd/kg); 3- $CdCl_2$ 1.2 mg/kg (0.74 mg Cd/kg); 4-$CdCl_2$ 1.4 mg/kg (0.86 mg Cd/kg); e 5- $CdCl_2$ 1.8 mg/kg (1.1 mg Cd/kg). Seven days after exposure, blood and reproductive organs were collected and the following analyses were performed: biometric, morphometric and stereological, oxidative stress, lipid and protein peroxidation, DNA damage, ultrastructure by transmission and scanning electron microscopy, dosage of tissue minerals, serum testosterone, inflammatory cytokines and histopathological analyses that included markings for apoptosis and calcification. It was observed that the inflammatory pattern is marked by neutrophils in intertubule and basal environment without changing markers of lipid peroxidation, suggesting the absence of damage in the Sertoli cell barrier. Markers of oxidative stress and cytokines presented changes indicating this is one of the mechanisms by which lesions are induced. There was reduction in testosterone production and changes in the Leydig parameters. Intense apoptosis and calcification of germ cells and collagen fibers were observed. There was increase of calcium in the tissue along with the increase of Cd dose, but the other minerals decreased. Histopathology and morphometric, stereological and biometric changes increased depending

on the dose. We concluded that the injuries caused by Cd are dose-dependent and mineral and inflammatory pathways play a key role in the development of lesions.

1. INTRODUCTION

1.1. Human fertility

In comparison to most animals, human fertility is remarkably low. The prevalence of couple infertility is extremely high in many countries and affects one in seven couples, with the most commonly cause identified as 'male factor' (Sharpe, 2010). Indeed, in a series of prospective and well-standardized studies undertaken across Europe over the past 10 years, it has been found that the prevalence of an abnormally low sperm count (less than 20 million sperm/ mL; the limit for normal based on World Health Organization (WHO) standards) in young men (18-25 years old) is as high as 15-20% (Jorgensen et al., 2006). Another remarkable feature about semen quality in normal young men is that only a low percentage (5-15%) of the sperm that are actually produced can be classified as normal (WHO), which is remarkably lower than domestic or laboratory animals in which more than 90 per cent of sperm can usually be classified as normal. This suggests that there are fundamental differences between spermatogenesis in humans and other species that result in production of overall lower quality sperm. This may cause spermatogenesis in humans inherently more vulnerable to disruption by external factors (Sharpe, 2010). These adverse factors trigger alterations main under Sertoli or Leydig cells number or integrity, which are the mainly cells in spermatogenic process. Sertoli cells are responsible for maintaining the Sertoli cell barrier and keep spermatogenic cells protected against the immune system. Leydig cells are usually the most abundant components of the intertubular compartment, and they are responsible for the synthesis of testosterone, the hormone involved with the maintenance of spermatogenic process, secondary sexual characteristics and libido, as well as the functioning of accessory glands (Payne et al., 1996). The two factors that affect the spermatogenesis process are resulting from lifestyle of the man (obesity, sedentariness) and/or his exposure to toxic agents from the general environment, as Cadmium (Cd) exposure (Sharpe, 2010).

1.2. Cd and spermatogenesis

Cd is a nonessential metal and a prevalent environmental pollutant in industrial countries. The source of Cd intake is mostly food and drinking water. Cd can accumulate in different organs, depending on the dose and exposure pathway. Cd is a natural element in earth's crust, found as a mineral combined with other elements such as oxygen (Cd oxide), chlorine (Cd chloride), or sulfur (Cd sulfate, Cd sulfide). All soils and rocks, including coal and mineral fertilizers, contain some Cd and it is readily mobilized by human activities such as mining. Most Cd used is extracted during the production of other metals like zinc, lead, and copper (Singh, 2005). Natural disasters (volcanoes) or mining disasters are a large source to human exposition. In Brazil in 2015, a mining disaster exposed exposing millions of people to contaminated unknown substances (Lambertz and Dergam, 2015), among them heavy metals like Cd. The issue of Cd pollution is accompanied by human industrial civilization. Every year, thousands of tons of Cd-containing pollutants are disposed into our environment, which increases health risk to our food and drinking water. It is now well accepted that Cd can accumulate in many organs, including liver, kidney, pancreas, and testis, and adversely affect the function of these organs (Yang and Shu, 2015).

Cd does not oxidize easily and since the metal cannot undergo metabolic degradation to less toxic species and is only poorly excreted, long-term storage is a viable option for dealing with this toxic element. Besides, Cd undergoes bioaccumulation and bio magnification processes becoming a toxic dose in biological systems (Menon et al., 2016). Cd toxicity may be more common among natural populations of vertebrates than has been appreciated and may often go undetected or unrecognized. Ingestion of even trace quantities of Cd can influence not only the physiology and health of individual organisms, but also the demographics and the distribution of species (Larison et al., 2000).

It is well known that testes are very sensitive to Cd toxicity causing biochemical and functional changes. Growth and various cellular events, including proliferation and survival are affected by Cd. The toxic effects of Cd often result from interference with metabolic processes mediated by many metals. Among Cd mechanisms that trigger testicular lesions, induction of oxidative stress, modulation of inflammatory response and in essential mineral dynamics, can be highlighted. Since several experiments using antioxidant, anti-inflammatory and mineral therapies have been tested and none of them were able to block the toxic effects of Cd on the testis (Marettová et al., 2015).

The mechanism that Cd-induced lesions are controversial in many organs (Luevano and Damodaran, 2014; Marettová et al., 2015). The testicular negative effects of Cd are widely described since the middle of last century (Parizek, 1957). In humans and other mammals, the range of effects depends on the dose, route, ways, and duration of exposure. Many studies in man and in various species of mammals showed that Cd induces several changes in testicular histopathology that was revised and summarized in the figure bellow:

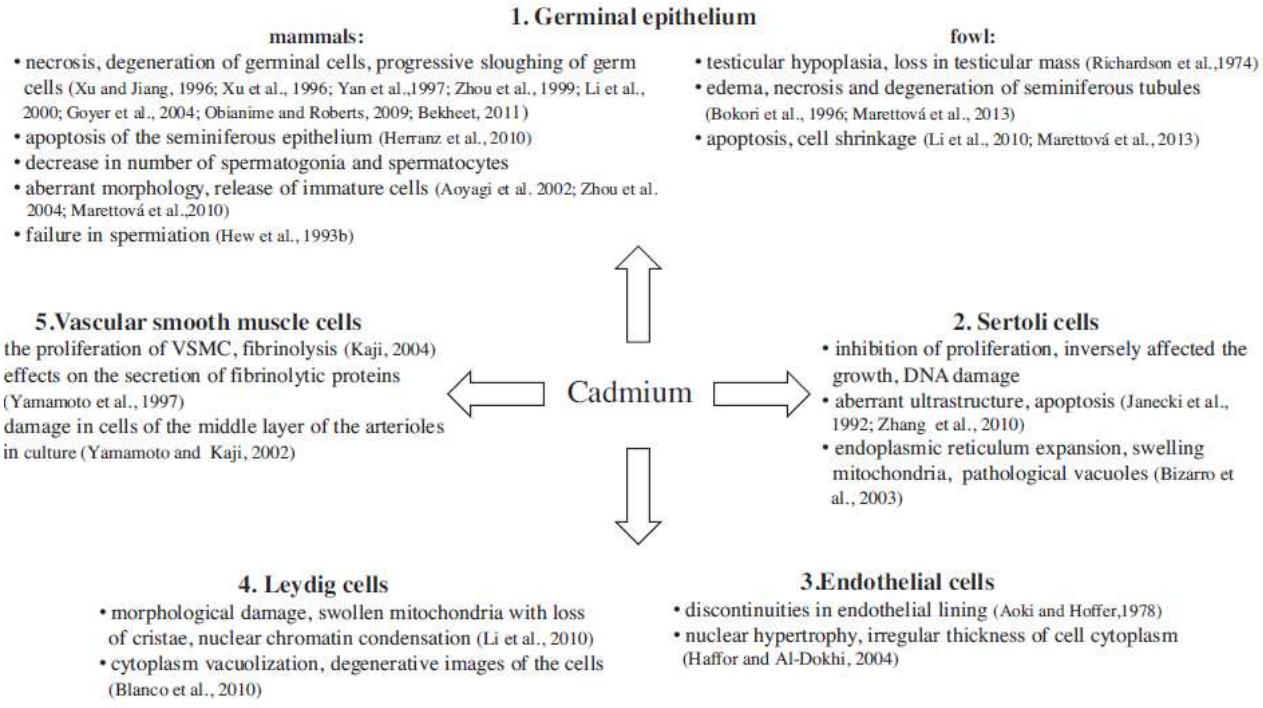

Figure 1. Review about the main changes on testicular structures described in the literature from 1974 to 2015 by Marettová et al. (2015).

Many efforts are necessary to explain the events that occur during Cd-induced testis injury and the reconstruction mechanisms of testicular tissue in animals and humans (Marettová et al., 2015).

1.2.1. Cd and mineral dynamics

Essential metals can affect the metabolism of nonessential metals. Transition metals are essential for the proper maintenance of body functions through a number of biological processes. Calcium and iron deficiency are worldwide health problems, and deficiency of these minerals increases Cd absorption (Min et al., 2008; Shawki and Mackenzie, 2010; Menon et al., 2016).

Cd exerts negative effects through physical and chemical properties of the Cd^{2+} ion, given its similarities to bivalent metals, like calcium, iron and zinc. Therefore, Cd is likely to substitute calcium or zinc in crucial physiological processes that are mediated by these ions, resulting in the activation and/or inhibition of several signaling pathways (Siu et al., 2009). Zinc has a relationship with many enzymes in the body and can prevent cell damage through activation of the antioxidant defense system (Powell, 2000; Ozturk et al., 2003). Decreased utilization of zinc by spermatogenic cells due to competitive action of Cd may cause disturbances in sperm development process (Amara et al., 2008).

Several important transition metals including iron (Fe), cobalt (Co), manganese (Mn), and copper (Cu) are required for optimal physiological function in different organs. Some functions of these metals are catalysts for biochemical reactions, regulators of gene expression, second messengers in signaling pathways and cofactors for many vital enzymes. For example, several enzymes involved in antioxidant functions use metals as cofactors; superoxide dismutase requires Mn in the mitochondria and Zn/Cu in the cytosol (Crapo et al., 1992). Also, both Zn and Fe are cofactors for serine/threonine phosphatases and kinases. Imbalanced metal homeostasis either by deficiency or by overload of metals is associated with organ dysfunction

that leads to various disorders. For example, anemia due to nutritional iron deficiency or gene mutations result in impaired production of iron-requiring proteins (e.g. hemoglobin), inhibition of cell growth and ultimately cell death. In contrast, iron overload causes tissue damage due to the elevated oxidative stress. Because of the consequences of metal imbalance, the homeostasis of metals in healthy organisms is tightly regulated at the levels of uptake, metabolism and excretion (Menon et al., 2016).

Non-essential metals can exert toxic effects even at low levels. Inorganic salts of several metals, such as Cd, exert toxic effects by the disruption of metal homeostasis, which is expressed by DNA damage, alterations of gene expression and damage by oxidative stress (Yuan et al., 2015). Alteration of cell metabolism results in dysregulation of cell proliferation, apoptosis and cell cycle progression, which ultimately leads to cancer, probably by distinct mechanisms occurring in metal deficiency.

The absorption of metals from dietary sources occurs by a number of metal transporters to provide specific needs for individual nutrient metals. In particular, the Divalent Metal Transporter 1 (DMT1) that is a major divalent metal transporter and plays an important role in the uptake of several non-essential divalent metals, including Cd (Cd) and lead (Pb) which are significant toxicants in our environment. Since the expression levels of DMT1 are regulated by several factors, such as body metal status, gene polymorphism and inflammation, changes in these factors could also alter the transport and toxicity of metals. For example, iron deficiency up-regulates intestinal DMT1 levels and increases the absorption of Mn and Cd, which in turn alters body metal status (Shawki and Mackenzie, 2010; Menon et al., 2016).

1.2.2. Cd and testosterone production

Another possible mechanism of Cd-mediated toxicity has been associated with exposure to Cd in primary cultures of rat Leydig cells. It was found that Cd exposure to result in decreased

Leydig cell viability, thereby leading to decreased testosterone secretion (Yang et al., 2003). Reduced testosterone levels in the testis stimulated the release of gonadotropin releasing hormone from the hypothalamus. An increased Cd accumulation in the hypothalamus, pituitary and testis and decreased plasma levels of follicle stimulating hormone in rats suggests a possible effect of Cd on the hypothalamic-pituitary-testicular axis (Lafuente et al., 2000). Recent studies on mammalian testis demonstrated that effects of Cd on the endocrine and reproductive systems are correlated. Therefore, Cd toxicity induced in the testis is probably the result of interactions of a complex network of causes (Siu et al., 2009).

Zn-Cd interactions in testosterone production is an important pathway to Cd-cytotoxicity, since there is described correlation with Zn reducing testosterone production (Al-Ani et al., 2015; Liu et al., 2015; Omu et al., 2015). However, the mechanisms behind alterations in Zn testicular levels triggered by Cd intoxication remain largely unexplored (Said et al., 2010). According to Waalks (2000), Cd competes with zinc for binding to some proteins and blocks cellular zinc accumulation. Omu et al., (2015) reported the existence of significant negative correlations between rats exposed to zinc deficient diet and levels of Cd serum compared to control and zinc supplementation groups. Said et al. (2010) exposed rats to high Cd doses and observed a significant decrease in plasma and testicular concentrations of Zn, which was accompanied by decreased serum testosterone level. According to these authors, Zn depletion seems to occur due to its sequestration by Cd-induced hepatic metallothioneins from the plasma, thereby increasing its concentration in the liver and probably restricting Zn supply of other tissues, like testicular tissue (Bonda et al., 2004). Previous studies have showed that Cd impairs the testosterone production in isolated Leydig cells without affecting their viability (Laskey and Phelps, 1991), demonstrating that steroidogenic disruption in Leydig cells is likely to be an initial target of Cd toxicity as an endocrine modulator. Animals exposed to zinc deficient diet showed a reduction of Leydig cell volume, serum concentrations of Zn and testosterone, as well

as increased levels of Cd serum, and apoptosis of the germ cells when compared to control and zinc supplementation groups (Omu et al. 2015). Cd is a well-known metal for its inhibitory effect of testosterone synthesis on Leydig cells (Laskey and Phelps, 1991). Zn is critical for the function of over 300 enzymes in the body (Bettger and Odell, 1993). On a study with rats pre-treated with Zn and exposed to Cd, testosterone reduction was abolished (Omu et al. 2015), deducing that Zn is essential to testosterone production. Inadequate zinc levels prevent the pituitary gland from releasing luteinizing and follicle stimulating hormones, which stimulate Leydig cells to produce testosterone (Omu et al., 2015). Zinc also inhibits the aromatase enzyme that converts testosterone into excess estrogen and the presence of testosterone is essential for normal function and survival of germ cells in seminiferous tubules (Al-Ani et al., 2015).

1.2.3. Cd, oxidative stress and inflammation

Cd is one of the inflammation-related xenobiotics (Marettová et al., 2015). Prolonged exposure to low concentration of Cd resulted in up regulation of proinflammatory cytokines and its exposure in the tissues is often accompanied by infiltration of inflammatory cells (Kundu et al., 2009). Effects of antioxidants, in most cases, improve testis conditions, but do not avoid the harmful effects (Marettová et al., 2015; Siu et al., 2009; Ige et al., 2012), and anti-inflammatory drug treatment could not totally inhibit the proliferation process, whereas inflammation was prevented (Kundu et al., 2009).

Cd induces oxidative stress damage by decreasing the biological activities of some antioxidants, such as superoxide dismutase and glutathione peroxidase (Kara et al., 2007) with significant reduction in testicular function and androgen secretion. Oxidative stress induced by Cd was associated with production of reactive oxygen species mainly comprising superoxide radical anion, hydrogen peroxide and hydroxyl radical. The degree of Cd-induced oxidative stress depends on the dose, duration and frequency of Cd exposure. It is well established that

testicular oxidative stress is commonly induced under different, normal and/or pathophysiological conditions leading to male infertility (Siu et al., 2009). Oxidative stress either leads to oxidative damage directly or activates signal transduction pathways to initiate defense responses, causing damage in DNA, proteins and lipids (Nair et al., 2013).

2. OBJECTIVES

- To investigate the inflammatory pattern triggered by Cd in the testis, after initial inflammation and before chronic fibrosis, in order to provide data to establishe the role that cytokines and oxidative stress play in this process according to the Cd dose exposure **(Chapter 1)**.
- To provide an overview of the mechanisms correlating low doses of Cd able to cause changes in Zn concentrations and negative effects in testis, with emphasis on testosterone production, and morphological alterations in Leydig cells **(Chapter 2)**.
- To investigate the interactions between metals calcium (Ca), magnesium (Mg), iron (Fe), Selenium (Se), copper (Cu), and Cd **(Chapter 3)**.

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CHAPTER 1: Short-term inflammatory pattern in testicular tissue from rats exposed to cadmium: role of cytokines, and blood testis barrier

(Manuscript in preparation for publication and edited according to journal guidelines)

Short-term inflammatory pattern in testicular tissue from rats exposed to cadmium: role of cytokines, and blood testis barrier

Abstract

The inflammatory pattern depend on the ethiology, route, organs and other issues. The inflammatory pattern that cadmium (Cd) triggers in the testis, after initial inflammation and before chronic fibrosis, is unknown. In order to elucidate the morphological remodeling according to the dose exposure and establishes the role of cytokines and oxidative stress play in Cd exposure, male rats were divided in five group (Cd0=control saline, and exposed to Cd - Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg). Blood and testes were collected seven days after exposure. The following analyses were performed: stereological and pathological tissue measurements, evaluation of oxidative stress markers (SOD, CAT, and glutathione s-transferase), final products of lipids and protein oxidation (malondialdehyde, isoprostane and protein carbonyl), tissue cytokines and nitrite quantification. Markers of oxidative stress were elevated. Adiponectin, which is an anti-apoptotic inflammatory marker, was reduced and the seminiferous epithelium presented high rate of apoptosis in germ cells. There was also increasing of carbonyl proteins in the highest dose. Only in the intertubular tissue and in the base of seminiferous tubules, intense inflammatory cell infiltration was observed. Hemorrhagic areas were observed in intertubular regions. Lipid peroxidation markers remained unchanged. We suggest that depending on the dose and through activation of cytokines and oxidative pathways, Cd triggers apoptosis in germ cells without cellular infiltration in seminiferous epithelium and damage in the blood testis barrier, which is confirmed by the absence of inflammatory cells in ad luminal environment of seminiferous epithelium and alterations in lipid peroxidation markers.

Keywords: Cytokines, lipid peroxidation, antioxidant enzymes, inflammatory infiltration, apoptosis.

Introduction

Many infertile men are victims of spermatogenesis disorder (Zhu et al., 2016). Spermatogenesis is a process that occurs in the testes and is characterized by sequential transitions of multiple processes: self-renewal of spermatogonial stem cells, differentiation of stem cells into differentiating spermatogonia, and meiotic events leading to the production of functional sperm (Kawasaki et al., 2015). Sperm are haploid cells that contain half of the full, diploid number of chromosomes, and need to be protected against the male immune system and nourished by Sertoli cells in the seminiferous tubules (Monteiro et al., 2014; Yao et al., 2016).

The mechanisms of testis preventing haploid cells destruction by the immune system include the existence of an immunological incomplete Sertoli cell or blood testis barrier (BTB), a structure that limits the access of germ cell antigens to the immune cells and antibodies, the presence of regulatory immune cells in the testicular interstitial areas, and the secretion of multiple cytokines factors by testis cells inducing an immunosuppressive microenvironment (Tung et al., 2002; Hedger and Meinhardt, 2003). Some cytokines are able to modulate the opening and closing of the inter-Sertoli tight junctions that constitute the BTB (Lui and Cheng, 2007) and receptors present in Sertoli cells increase the production of inflammatory cytokines, suggesting that these cells may play important roles protecting the seminiferous epithelium against invading pathogens and autoantigens (Wu et al., 2008).

Two main factors that affect the sperm production process result from the lifestyle of the man (obesity, sedentariness) and/or his exposure to toxic agents from the general environment, such as cadmiumd (Cd) exposure (Sharpe, 2010). Cd is a well-recognized environmental pollutant with numerous adverse health effects, and daily exposure to this heavy metal is inevitable (Niknafs et al., 2015). In potatoes from European soils the Cd levels can be ten times higher (0.14 mg/kg) than permissible limit, and in Italy soil can be 0.96 mg/kg. In Japan data

from 2000 to 2009 suggested Cd enter into human body 3.0 µg/kg body weight per week via mostly rice intake (Jha and Bohra, 2015).

Human exposure to Cd and decrease in fertility are a reality, but and the precise mechanisms underlying its toxicity to the testes remain controversial (Siu et al., 2009; Luevano and Damodaran, 2014; Marettová et al., 2015). According to Marettová et al., (2015) Sertoli cells are considered the most vulnerable cells to Cd exposure. However, this result was contradicted by Niknafs et al., (2015) affirming that supportive cells such as Sertoli cells in seminiferous tubules did not exhibit high sensitivity to Cd. Findings on Cd-induced testicular injury, highlighting the disruption of the blood-testis-barrier or the impact of Cd actions as an endocrine disruptor in males need to be clarified. Cd is one of the inflammation-related xenobiotics. Prolonged exposure to low concentration of Cd resulted in up regulation of proinflammatory cytokines and its exposure in the tissues is often accompanied by infiltration of inflammatory cells (Kundu et al., 2009). Several studies using antioxidants, in most cases, improve the conditions of testis, but do not avoid the harmful effects (Marettová et al., 2015; Siu et al., 2009; Thompson and Bannigan, 2008; Wang et al., 2012; Ige et al., 2012), and anti-inflammatory drug treatment could not totally inhibit the proliferation process (Kundu et al., 2009).

Since human exposure to Cd and decrease in fertility are a reality, and the inflammatory pattern that Cd triggers in the testis, after initial inflammation and before chronic fibrosis, is unknown, the aim of this study was to elucidate the inflammatory process according to the doses exposure and establishes the role of cytokines and oxidative stress play in Cd low doses exposure.

Material and Methods

Animals and ethics statement

Thirty male Wistar rats, 70 days old, were provided by the Central Animal Facility of the Federal University of Viçosa (UFV) and maintained under controlled photoperiod (12:12 h light/ dark), temperature (21 ± 2ºC) and relative humidity (60-70%). The animals received food and water *ad libitum*. The study was conducted according to international standards for the care and use of laboratory animals. The research protocol was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (CEUA/UFV) (protocol 030/2010).

Experimental design

Cadmium chloride ($CdCl_2$/ Sigma, St Louis, MO, USA) was dissolved in distilled water and administered by intraperitoneal route (i.p.) in a single dose. Male Wistar rats (n=30) were randomized into five groups of six animals: (1) Saline (control), 0.9% NaCl; (2) Cd1, $CdCl_2$ 1.1 mg/kg (0.67 mg Cd/kg); (3) Cd2, $CdCl_2$ 1.2 mg/kg (0.74 mg Cd/kg); (4) Cd3, $CdCl_2$ 1.4 mg/kg (0.86 mg Cd/kg); and (5) Cd4, $CdCl_2$ 1.8 mg/kg (1.1 mg Cd/kg). The sample size was rationalized according to ethical aspects and was determined considering the probability P=1/2 to increase or decrease the variables of interest. Thus, considering the significance level α=0.05, the minimal significant number of animals used in the statistical analysis was P = (1/2) events; so, if n=6, P = (1/2)6 or P = 0.03; thus, P<0.05 (Novaes et al., 2013). In a previous study, Souza Predes et al. (2010), concluded that 1.2 mg/kg CdCl2 is the minimal dose to causes significant testicular negative effects. So, in this experiment were used a dose low than minimum, the minimum and two doses more than minimum necessary to causes significant negative effects. The doses were calculated to ensure that there would be animals with injury, thus making the analysis of possible pathways and mechanisms by which these alterations are formed possible.

The use of Cd at low doses is more appropriate for studies, since environmental contamination usually occurs at lower doses (Souza Predes et al., 2010). Ogawa et al. (2013), considered 3 mg $CdCl_2$ kg i.p. as low dose and suggest that low dose allows tracking minor and initial effects that Cd triggers in the tissue, becoming possible to search the role this metal plays in lesion processes (Halder et al., 2015). Seven days after exposition was the time chosen to collect samples, since studies about acute (immediately or until 5 days after exposition) (Fouad et al., 2013; Li et al., 2015; Horiguchi and Oguma, 2016) and chronic inflammation (Schuppe et al., 2008; Ogawa et al., 2013) are described in literature. So the inflammation pattern triggered by Cd in the testis, after initial inflammation and before chronic fibrosis is not described in low doses.

Sample collection

Seven days after exposure, the animals were euthanized by deep anesthesia (ketamine 45 mg/kg and xylazine 5 mg/kg, i.p.) followed by cardiac puncture. The testes were quickly removed and weighed on an analytical balance. The right testis was divided; one part was frozen in liquid nitrogen and stored in a freezer at -80°C while the right testis was used to measure the water content per unit of tissue weight (mL/g). The left testis was immersed in Karnovsky solution (Karnovsky, 1965) and then divided into smaller fragments that were used for morphological and morphometric analyses.

Morphometric and Histopathological analyses

The left testes that were immersed in Karnovsky solution for 24 h were transferred to 70% ethanol. Testicular fragments were dehydrated in increasing ethanol series for inclusion either in glycol methacrylate (Historesin®, Leica) for light microscopy analyses, or paraffin wax for the detection of apoptotic cells. Semi-serial sections (3 µm thick for Historesin and 5 µm thick for paraffin wax) were obtained using a rotary microtome (Leica RM2255), observing

a minimum interval of 40 µm between different cuts. The Historesin preparations were stained with toluidine blue-sodium borate (1%). Morphometry was performed using digital images captured at different magnifications with the light microscope AX-70 Olympus. All images were analyzed by the software Image-Pro Plus (Media, Cybernetics).

The volumetric ratio between the seminiferous tubules and the intertubular tissue were estimated by counting 2660 points projected over 10 digital images (per animal, 200× magnification) obtained from histological slides. The volumetric proportions of all intertubular tissue components (blood and lymphatic vessel, macrophage, connective tissue, and Leydig cell) were calculated by counting one thousand coincident points over the intertubular components. The counting was performed using the photomicroscope Olympus AX-70 and Image-Pro Plus software (Media Cybernetics, Bethesda).

Qualitative histopathological analyses were made screening two glass slides per animal with semi serial sections from testes stained with toluidine blue, searching for tissue alterations.

Apoptosis

Apoptosis was detected by the TUNEL assay (Terminal deoxynucleotidy l transferase dUTP nick end labeling), which was performed according to the protocol of the detection kit (Calbiochem, Merck KGaA, Darmstadt, Germany). Histological sections were deparaffinized, rehydrated and incubated with proteinase K for 20 min at room temperature, then washed in distilled water and incubated with hydrogen peroxide plus methanol for 5 min, in order to stop endogenous peroxidase activity. The preparations were then incubated with Tdt equilibrium buffer in distilled water at room temperature and kept inside a humid chamber for 20 minutes. The incubation proceeded with Tdt mix enzyme and DTT for 60 min at 37uC. Immunoreactivity in cells was detected by incubating the slides with a mixture of 3, 3 -diaminobenzidine tetrahydrochlorid (DAB) and hydrogen peroxide for 13 min in a humid chamber protected from

light. The sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted. Preparations of the same material were used as negative and positive controls. TDT enzyme was omitted for the negative control, and apoptotic nuclei were stained with hematoxylin. The positive control slides were incubated with 1.00 U/mL DNase I (Invitrogen) in DNase buffer for 10 min before blocking endogenous peroxidase activity. Thus, the same steps were followed as described for the TUNEL technique. Preparations of different groups were stained at the same time in order to avoid discrepancies when comparing results. The identification of apoptotic cells was made based on nuclear characteristics and DAB staining.

Lipid and protein oxidation

Lipid oxidation was measured by enzyme-linked immunosorbent assay (ELISA)-based oxidation of tissue phospholipids. Fragments of the testicular tissue (100 mg) were homogenized with a protease inhibitor (Protease Inhibitor Cocktails; Sigma Chemical Co. St. Louis, MO, USA) in portable tissue homogenizer (YO-04727-09; LabGEN) and centrifuged at 3000 ×*g* for 10 min. The supernatant was collected for the 8-Isoprostane assay. The concentration of isoprostane was measured by using a 96-wells commercial kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). For analysis of tissue malondialdehyde (MDA), an end product of lipid peroxidation, an aliquot of frozen testicular tissue (100 mg) was homogenized in phosphate buffer (pH 7.0), the supernatant centrifuged (3000g/10min) and the homogenate reacted with thiobarbituric acid solution (trichloacetic acid 15%, thiobarbituric acid 0.375% and 0.25 N HCl) for 15 minutes. The formation of thiobarbituric acid reactive substances was monitored at 535 nm as described previously (Buege and Aust, 1978). All samples were measured simultaneously in duplicate.

Protein carbonyl content was measured biochemically in the testicular tissue pellets by adding 0.5 mL of 10 mM 2, 4-dinitrophenylhydrazine (DNPH). The reaction involved derivatization of the carbonyl group with DNPH, leading to the formation of a stable 2, 4-dinitrophenyl (DNP) hydrazone product. The optical density (OD) was measured spectrophotometrically at 370 nm (Levine et al., 1990). The total protein levels in testicular tissues were determined by using the Bradford method (Bradford, 1976).

Nitric oxide analysis

Nitric oxide (NO) production was indirectly quantified through nitrite content in the supernatants of the testis homogenate by the standard Griess reaction. Briefly, 50 µL of testicular homogenate supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediaminedihydrochloride, and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance was measured at 550 nm in a microplate scanning spectrophotometer (Power Wave X). The nitrite concentration was calculated with reference to the sodium nitrite ($NaNO_2$) standard curve (Ricart-Jané, 2002).

Edema index

Testicular fragments weighing about 100 mg were obtained and dried. The edema index was obtained by calculating the water concentration in the tissue from the ratio of fresh weight and dry weight of the organ (wet weight - dry weight) and the dry mass was acquired after drying the organ in an oven (70ºC), until a constant dry weight was reached (Novaes et al., 2012). The value of water content obtained was subtracted from the water concentration in control animals and defined as the edema index.

Antioxidant enzyme assays

Activity assay of catalase (CAT), glutathione s-transferase (GST), and superoxide dismutase (SOD) were performed with the supernatant obtained from an aliquot of frozen testicular tissue (100 mg). Those aliquot parts were homogenized in ice-cold phosphate buffer (pH 7.0) and centrifuged at 3000 g (5°C) for 10 min. Catalase activity was evaluated according to the method described by Aebi, (1984) by measuring the kinetics of hydrogen peroxide (H_2O_2) decomposition. GST activity was followed spectrophotometrically at 340 nm as described by Habig et al., (1974) and calculated from the rate of NADPH oxidation. SOD activity was estimated by pyrogallol method based on the ability of this enzyme to catalyze the reaction of the superoxide (O^{-2}) and hydrogen peroxide (Sarban et al., 2005).

Enzyme-linked immunosorbent assay (ELISA) for cytokines

Fragments of testicular tissue were homogenized with a protease inhibitor (Protease Inhibitor Cocktails; Sigma-Aldrich) in portable tissue homogenizer **(**YO-04727-09; LabGEN) and centrifuged at 3000 ×*g* for 10 min; the supernatant was collected for the cytokine assay. The concentrations of cytokines were measured by sandwich ELISA. The cytokines adiponectin, interleukin 10 (IL-10), tumor necrosis factor (TNF-α), and high molecular weight (HMW) adiponectin were measured by using commercial kits according to the manufacturer's instructions (Uscn Life Science Inc., Wuhan, China).

Statistical analysis

Results were expressed as means and standard deviations (mean ± SD). The data were submitted to unifactorial one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) post-hoc test for multiple comparisons. Statistical significance was established at $p < 0.05$. All tests and graphics were performed using Graph Pad Prism 5.01® statistical software (Graph Pad Software, Inc., San Diego, CA, USA).

Results

Morphometric parameters

The volumetric density between seminiferous tubules and interstitial tissue did not change in all animals exposed to Cd in relation to control. The volumetric density of interstitial components (blood and lymphatic vessels, macrophages, connective tissue and Leydig cells) were not different in all animals exposed to Cd in relation to control. (Table 1).

Table 1. Morphometric parameters from components of testicular tissue.

Parameters (%)	Sal	Cd1	Cd2	Cd3	Cd4
Tubule	92.94±0.17	88.9±7.54	79.65±11.5	88.5±7.6	85.82±8.36
Intertubule	7.06±0.17	11.1±7.54	20.35±11.50	11.5±7.6	14.2±8.4
Blood vessels	4.63±2.94	5.56±4.86	4.52±2.10	3.35±2.15	2.85±0.66
Lymphatic vessels	46.38±2.61	39.16±20.00	28.46±19.61	54.85±6.96	33.36±22.37
Connective tissue	14.06±5.22	16.24±9.04	47.33±30.98	30.67±32.29	40.33±35.92
Macrophages	3.10±1.36	3.27±1.47	1.95±1.86	3.08±1.99	1.71±1.65
Leydig cells	31.84±6.4	35.78±17.16	17.74±10.2	19.02±8.82	18.44±14.57

Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Data are expressed as mean and standard deviation (mean ± SD).

Inflammatory infiltrate

The figure 1 shows testicular tissues stained with toluidine blue from rats control (Fig. 1A and 1B) and exposed to Cd (Fig. 1C, D, E, F, G, and H). Animals exposed from Cd2 dose showed pathologies in seminiferous tubules and intertubular tissue. Among them, there was abundant presence of inflammatory cell infiltration in the intertubular tissue (Arrows: fig. 1C, 1E and 1F), and in the base of seminiferous tubules (Arrows: fig 1G and 1H), but it was absent within seminiferous tubules. The regions of seminiferous epithelium in contact with inflammatory cells were absent of nucleus (Fig. 1G and H). Vacuolization and lack of germ cells were observed in the seminiferous tubules (Arrowheads: fig. 1C and 1D) as well as characteristics of germ cell loss (fig.1D). Some hemorrhagic areas were observed at

specific points of intertubular region, but not diffusely across the intertubular tissue (Stars: fig. E and 1F).

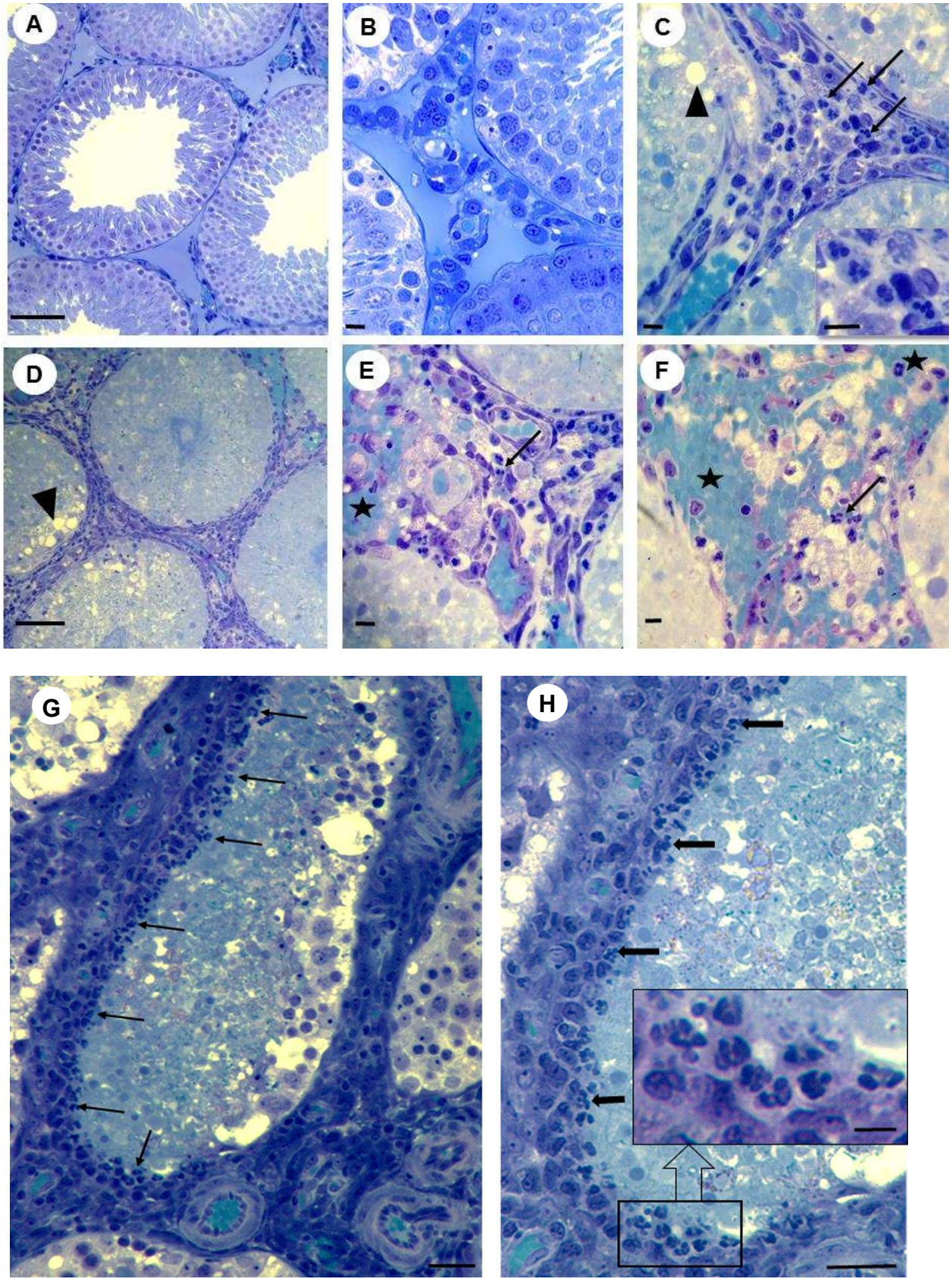

Figure 1. Testicular tissues from control and rats exposed to Cd and stained with toluidine blue. Arrows indicate inflammatory cells infiltration areas; Arrowheads indicate vacuolization in seminiferous tubules; Stars indicate hemorrhagic areas. A and B: Control animals. A: Bar = 100 µm; B: Normal intertubular area. Bar = 10 µm; C (Cd2 group); D and E (Cd3 group); F, G and H (Cd4 group) = Absence of cells into seminiferous epithelium and inflammatory cells in intertubular tissue and in the base of seminiferous tubules. C: Bar = 10 µm Detail: inflammatory cell infiltration areas. Bar = 10 µm. D: Bar = 100 µm; E and F: Bar = 10 µm; G and H: Bar: 50 µm; H detail: Inflammatory cells. Bar: 5 µm.

Apoptosis

Animals exposed to Cd from Cd2, Cd3 and Cd4 groups showed high amounts of markings for apoptotic cells. Figure 2a and 2b represent, respectively, the negative and positive controls for TUNEL technique. Figure 2c shows apoptotic cells in the saline control group (arrows indicate apoptotic cells). Figures 2d, 2e and 2f are representative of apoptosis patterns found in animals exposed to the doses of Cd2, Cd3 and Cd4 groups, respectively.

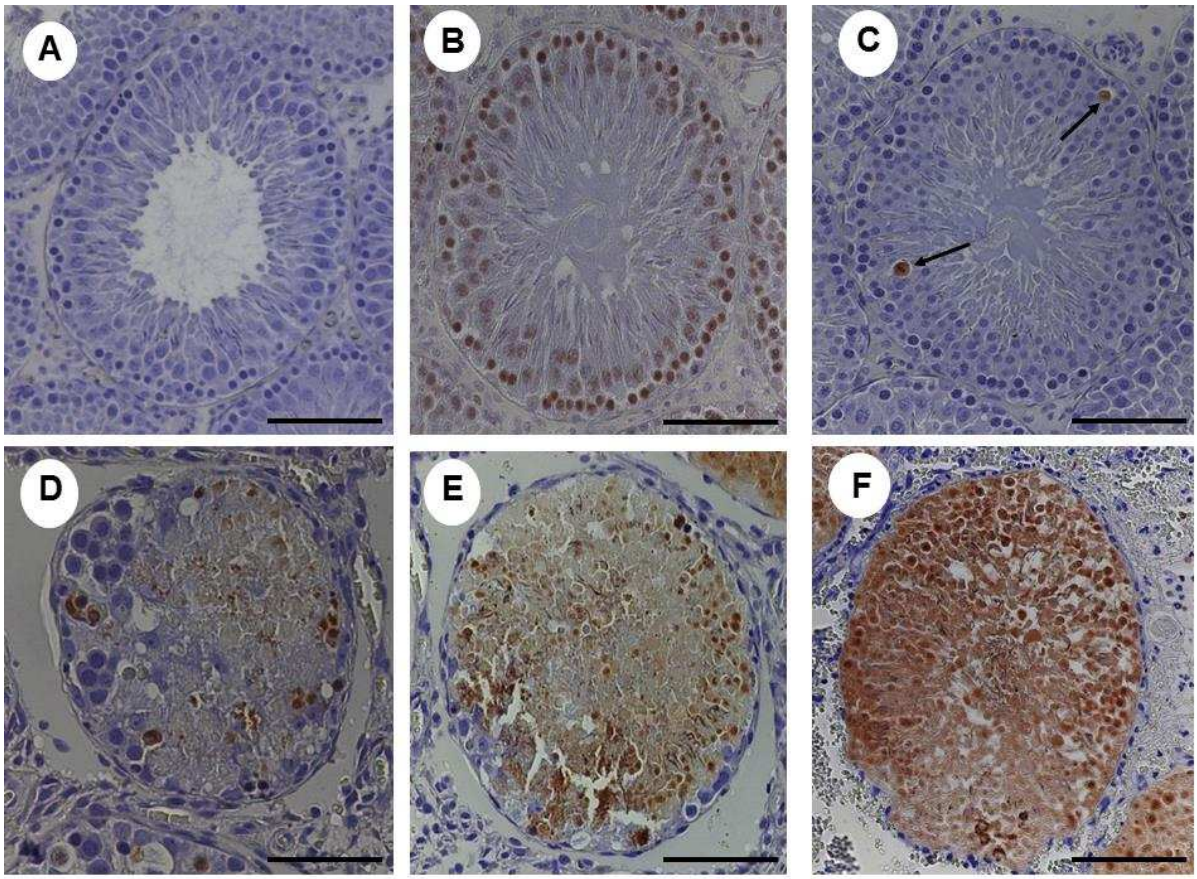

Figure 2. Testes of control and rats exposed to Cd subjected to TUNEL technique. A - Animals from negative control for TUNEL technique; B - Animals from positive control for TUNEL technique; C - Animals from saline control group. Arrows show apoptotic cells; D: Cd 2 group; E: Cd 3 group, F – Cd 4 group. Brown nuclei show positivity for apoptosis. Magnification of 400 X. Bar: 100µm.

Oxidative stress and edema index

Figure 3 shows superoxide dismutase (SOD), catalase (CAT), and glutathione s-transferase (GST) enzymatic activities and total carbonyl protein. SOD activity increased in Cd2, Cd3 and Cd4 groups in comparison with control and Cd1 groups (fig. 3A). CAT activity increased in all animals exposed (fig. 3B). GST activity did not alter in exposed animals compared to control (fig. 3C). The total carbonyl protein quantity increased in the Cd4 group when compared to control and other doses (fig. 3D). The values of 8-isoprostane,

malondialdehyde, nitrite, and edema index presented no difference between control and animals exposed (complementary material).

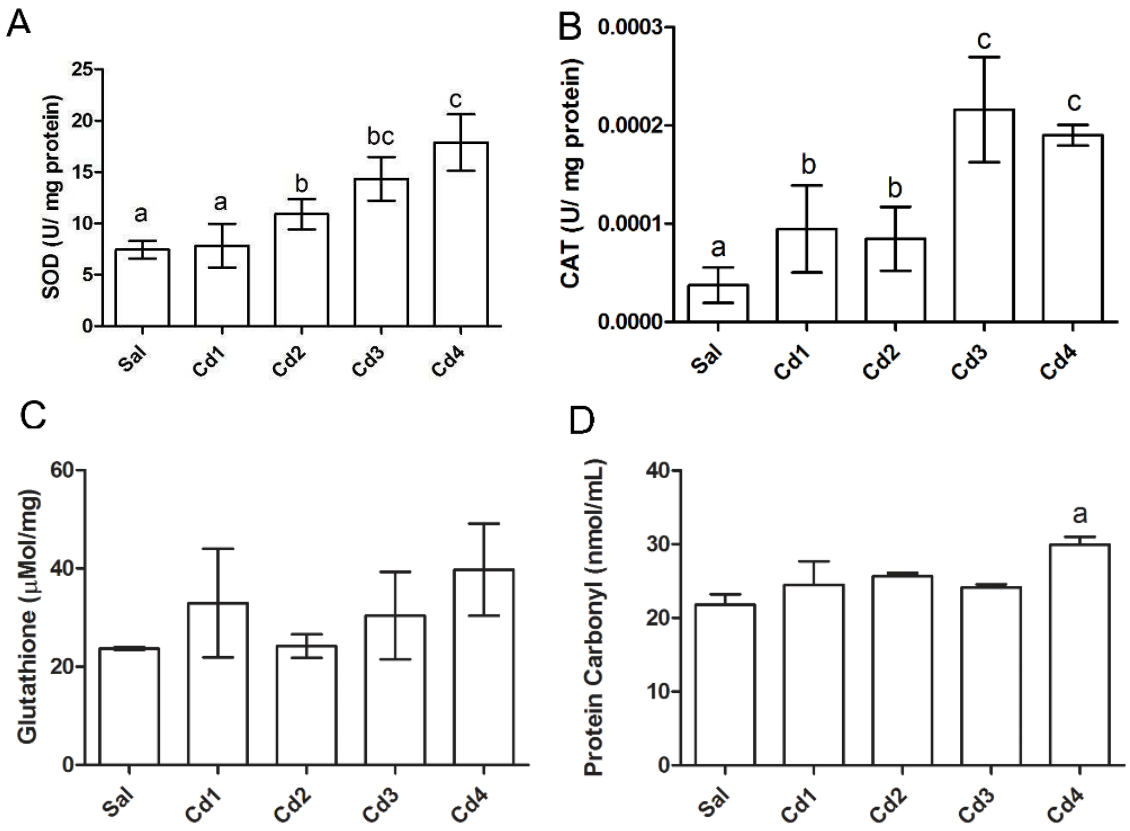

Figure 3. Superoxide dismutase (SOD), catalase (CAT), and glutathione s-transferase (GST) enzymatic activities and total level of protein carbonyl in testicular tissue. A – Superoxide dismutase activity (SOD); B – Catalase activity (CAT); C – Glutathione s-transferase (GST); D – Carbonyl Protein. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Different letters indicate statistical difference between groups ($p<0.05$).

Cytokines

Tumor necrosis factor alpha (TNF-α) values decreased in animals from the Cd4 group. High molecular weight adiponectin (HMW) and interleukin 10 (IL 10) increased in animals from the Cd4, Cd3 and Cd2 groups when compared to the animals from control and Cd1 groups. Adiponectin values decreased in all animals exposed to Cd (Figure 4).

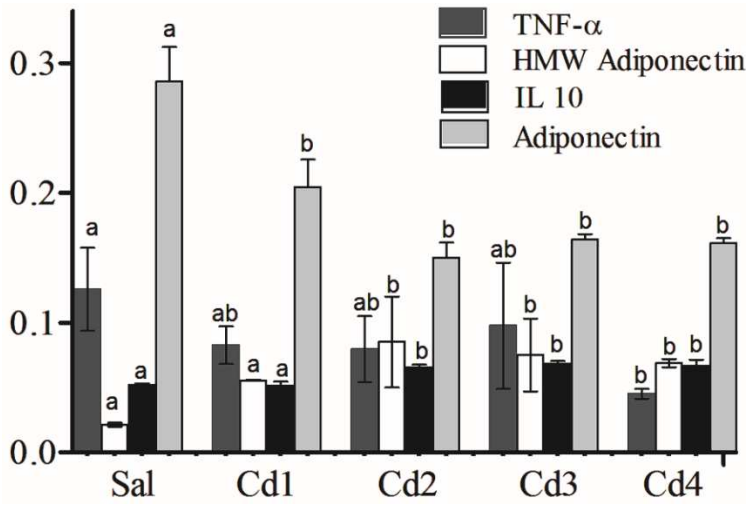

Figure 4. Inflammatory cytokines measurements in testicular tissue. TNF-α = Tumor necrosis factor α; HMW Adiponectin = High Molecular Weight Adiponectin; IL10 = Interleukin 10; Adiponectin = Adiponectin. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Different letters indicate statistical difference between groups ($p<0.05$).

Discussion

In this study the dose Cd1 did not induce apoptosis in germ cells or intertubular inflammatory cell infiltration in animals. However, Cd1 dose decreased adiponectin and increased catalase levels indicate that those components can be the first mechanism activated by Cd in the inflammatory process. Fouad et al., (2013) exposed rats to $CdCl_2$ (2 mg/kg ip), analyzed 3 days after Cd exposition and observed significant catalase activity. Ogawa et al., (2013) in chronic analysis (45 days after the single dose exposition to 3 mg $CdCl_2$ kg i.p)

observed that Cd had slightly accumulated in the testes, not affecting the spermatogenic state at the light microscopic level.

From Cd2 dose there was intense inflammatory cell infiltration, mainly of neutrophils, in intertubular tissue. There was an amplification of the inflammatory response when increase Cd dose exposition. In liver studies it was observed that a single injection of Cd into mice induced neutrophilia that occurred slowly, but continued to increase until 24 h, in contrast to bacteria, which rapidly induced neutrophilia with a peak at 12 h followed by a decrease (Horiguchi and Oguma, 2016). According to Guazzone et al., (2009) the main histopathological characteristic of testicular inflammation is the presence of inflammatory cells in the interstitial areas, mainly lymphocytes, macrophages, and mast cells. Mast cells were not observed and the macrophage percentage did not change in this study. The testicular inflammatory patter caused by bacteria is different with acute massive cell infiltration, mainly of neutrophils in both interstitial and tubular compartments, and in chronic inflammatory reactions peritubular and interstitial lymphomononuclear cell infiltrates present (Schuppe et al., 2008). Our results indicated that seven days of Cd-induced inflammation in the testis is characterized by prolonged neutrophilia with a different pattern when compared to inflammation caused by bacteria: it does not alter the number of macrophages and mast cells and it does not occur diffusely in the seminiferous tubules, only in basal compartment.

This study found no increase in end-products of lipid peroxidation, MDA and 8-isoprostane, indicating that the harmful effects of Cd, are possibly not achieved by changing the membrane structure and probably do not alter the blood-testis barrier. The cell membrane phospholipids are particularly susceptible to the peroxidation process, which involves changes in the structure and permeability of the membrane, resulting in loss of ion exchange selectivity, releasing the contents of organelles such as lysosomes and creating cytotoxic products by hydrolytic enzymes, among them, malondialdehyde (MDA) and 8-isoprostane (Montuschi et al.,

2004; Owen 2005). Since blood testis barrier (BTB), separate two distinct environments into seminiferous epithelium, basal and apical (ad luminal) (Cheng and Mruk 2012), inflammatory cells only in the basal environment can indicate that inter-Sertoli tight junctions that constitute the BTB were not opened by Cd. Furthermore, damage in cell membranes constituting this barrier did not seem to have occurred since lipid peroxidation markers remained unchanged in all animals exposed to Cd.

Edema was not observed, in this study, in all animals exposed. According to Malaguarnera et al., (2013), injury induced by Cd consists of three overlapping inflammatory phases: edematous, proliferative and fibrogenic. According to the pattern found in seven days, the main phase is the proliferative, suggesting that edema phase occurred before. In this current study there were hemorrhagic areas only in the Cd4 group. In animal experiments, high-dose exposure to Cd induced severe testicular interstitial hemorrhage with edema (Takiguchi and Yoshihara, 2006).

In this study, an increase in carbonyl protein was reported only in the Cd4 group. The proteins are subjected to a variety of modifications produced by reactive species in pathological conditions. These oxidative modifications can inactivate the enzymatic functions and cause structural degeneration of the proteins, or activate transcription factors and proteolytic systems and oxidants can potentiate the effects of proteases (Tuder et al., 2006; Toyokuni and Akatsuka, 2007; Barnes et al., 2003). The rise in the damage intensity according to the increase in dose exposition can be related to other mechanisms that are dose-dependent activated, causing protein degradation. Reactive nitrogen species (RNS) are formed from the synthesis of nitric oxide (NO) (Mak 2008; James et al., 2004), and together with reactive oxygen species (ROS) are the mainly cause of damage in proteins. Superoxide dismutase (SOD) and catalase (CAT) activities increase after Cd exposure. Hydrogen peroxide, which is the product of the reaction of SOD, is the substrate for CAT activity. Thus, these enzymes act in combination to protect the body from

injury caused by free radicals (Mukherjee, 2003). Glutathione s-transferase (GST) represents a major group of detoxification enzymes, and did not change in animals exposed to Cd. Patra et al., (2011) affirm that Cd can causes necrosis and atrophy in testicular tissue by suppression of GST activity. Adult male mice intraperitoneally injected with Cd at a dose of 2 mg/kg body weight per day for seven consecutive days showed decrease in glutathione (GSH) and superoxide dismutase (SOD) activities (Li et al., 2015). Studies before concluded than low concentrations of Cd induced antioxidants, while higher levels of metal reduced the antioxidant defense mechanisms like the activity of SOD and CAT (Wolf and Baynes, 2006; Ognjanović et al., 2007). In this study, increase in antioxidant enzymes can indicate oxidative stress triggered by Cd. However, it does not seem to be the only pathway to Cd causes testis cytotoxicity, since antioxidant compounds improve testicular parameters changed by Cd, but do not eliminate the negative effects completely (Marettová et al., 2015; Siu et al., 2009; Thompson and Bannigan, 2008; Wang et al., 2012; Ige et al., 2012).

Seminiferous epithelium showed a high rate of apoptosis and loss of germ cells in Cd-exposed animals compared to control animals. Those damages were not observed in the low dose and were amplified according to the increase in exposure dose. Vacuolization is the first step to degeneration and apoptosis (Creasy, 2001). Increasing in the apoptosis rate of the cells results in tissue destruction and lesion development (Tuder et al., 2006; Sönmez and Tascioglu, 2015). Apoptosis predominates in tissues under oxidative stress and the experimental block of apoptosis sharply reduces the expression of markers for oxidative stress; i.e., the apoptotic process can be a potent generator of reactive species. The administration of compounds with antioxidant activity can prevent the development of apoptosis, suggesting an interaction by positive feedback between oxidative stress and apoptosis (Tuder et al., 2003). Increased apoptosis, and increased expression markers of oxidative stress may have had a positive feedback effect on this study. These facts together may have caused the massive apoptosis in

tubular tissue. Apoptosis and cell loss occurred mainly in seminiferous tubule regions with inflammatory cell infiltration, indicating that some substance produced by these cells is an apoptosis inducer. Increase in apoptosis can be triggered by cytokines produced by inflammatory cells infiltrate from spreading of immune cells within the testicular interstitium. These cells alter the normal immunosuppressive microenvironment mainly through the secretion of pro-inflammatory cytokines (Guazzone et al., 2009). Inthis study, hemorrhagic points were observed in interstitial compartment with positive correlation with increasing apoptosis in seminiferous epithelium.

Studies documented that Cd is a potent immunotoxicant (Colombo et al., 2004; Pathak and Khandelwal 2008) affecting both cellular and humoral immune response (Lafuente et al., 2004). Various inflammatory mediators are involved in this response to Cd exposure; among them, we can mention those with anti-inflammatory activity, such as adiponectin, highlighting the high molecular weight (HMW) adiponectin and interleukin 10 (IL 10), as well as pro-inflammatory cytokines, such as TNF-α. Studies confirm that adiponectin plays an important role in the metabolism of glucose and lipids (Pereira et al., 2012). Adiponectin is a plasma protein, mainly secreted by adipocytes, with anti-diabetic, anti-inflammatory, anti-apoptotic, anti-atherogenic and immunomodulatory properties (Bobbert 2011). There are three major isoforms of adiponectin (Hickman et al., 2012), but it is believed that the HMW adiponectin isoform is the most active in peripheral tissues (Goto et al., 2013). There is evidence that adiponectin is capable of reducing oxidative stress (Wang et al., 2013), inhibiting the expression of TNF-α and increasing IL-10 expression which is also a TNF-α inhibitor (Lo et al., 2012). IL-10 is a potent anti-inflammatory cytokine released by immune cells, which triggers the regulation of a variety of anti-inflammatory processes (Kwilasz et al., 2014). In this study, adiponectin, which is a powerful anti-apoptotic, decreased in all animals treated and the apoptosis increase in all seminiferous tubules. IL10 and HMW adiponectin increased in the three highest Cd doses, and

both have the potential to decrease the expression of TNF-α, which is a potent pro-inflammatory factor. In fact, TNF-α levels decreased in animals exposed to the highest dose and the apoptosis rate increased. TNF- α may act as a proapoptotic factor during inflammatory conditions of the testis and also in human testicular pathologies such as Sertoli cell-only syndrome and germ cell arrest (Theas et al., 2008).

Inflammatory reaction was observed in the intertubular region, despite the inhibition of pro-inflammatory markers (TNF-α) and the increase in anti-inflammatory markers (IL10/HMW adiponectin); this may have occurred because after seven days without exposure, there was extensive apoptosis in cells in order to produce TNF-α. In the seminiferous tubules, TNF-α is synthesized by pachytene spermatocytes and by round and elongated spermatids (De et al., 1993; Siu et al., 2003). Several interstitial cells such as mast cells (Rodriguez et al., 2006), lymphocytes, and macrophages synthesize TNF-α (Bryniarski et al., 2005). Testicular macrophages release more TNF-α when stimulated by bacteria and modify the normal immunosuppressive microenvironment of the testis (Suescun et al., 2003; Xiong and Hales, 1993).

Recent data highlighted TNF-α regulatory role on Sertoli tight junction dynamics (Lui and Cheng, 2007; Yan et al., 2008). The intratesticular administration of acute doses of these cytokines in rats has been shown to open the BTB, suggesting that TNF-α facilitates the opening of tight junction by lowering occludin levels. The administration of an endotoxin from bacteria induces TNF-α by activating immune cells, as well as the increase of tissue and plasma levels of NO (nitric oxide) (Hales et al., 1999), and *in vitro* TNF- α stimulates rat Sertoli cells to release NO (Bauche et al., 1998). The rats received a single intraperitoneal (i.p.) injection of Cd chloride (2 mg/kg) and when analyzed 3 days after, revealed that Cd increased testicular nitric oxide levels (Fouad et al., 2013). On the basis of the widespread distribution of NOS in the male reproductive tract, we suggest the potential role for NO in the mediation of many reproductive

functions. NOS localizations include nerves and vascular endothelium. The presence of NOS in neuronal plexuses within the adventitia and endothelium of arteries supplying reproductive organs indicates that NO regulates the vasoactivity and therefore, blood delivery to these organs. In combination with other cytokines, TNF-α stimulates rat Sertoli cells to release NO *in vitro* (Burnett et al., 1995). Since Cd did not change the number of macrophages and the other cells that produce TNF-α undergone apoptosis, the inflammation pattern triggered by Cd is different compared to bacteria, with no damage in BTB and no alterations in NO levels.

In summary, the possible mechanism Cd triggers the testicle lesions is inducing oxidative stress and an initial increase in TNF-α (Li et al., 2015) which induces apoptosis of germ cells (Theas., et al., 2008). TNF-α triggers an initial (before seven days) increase of nitric oxide (Hales et al., 1999), leukocyte infiltrate and dilation of blood vessels (Burnett et al., 1995) with edema and hemorrhagic areas (Takiguchi and Yoshihara, 2006).

In this study we concluded than, a second moment (seven days), the decrease of adiponectin, triggers the increasing apoptosis of germ cells producing TNF-α, leading to decrease its levels. The increase of IL-10 and high molecular weight adiponectin also influences the decrease of TNF-α. The decreasing of TNF-α triggers decreasing in nitric oxide and reduces injuries in the BTB. Hemorrhagic areas and neutrophilia are the result of an initial increase of oxidative stress, and TNF-α that no longer exists. The mechanism by which Cd, in seven days, and at these doses causes the inflammatory process is different from the caused by bacteria because Cd maintains prolonged neutrophilia without changing the number of macrophages and mast cells in intertubule. Besides, inflammatory cells were not present in the ad luminal environment of seminiferous tubules.

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Competing interests

The authors declare that there is no conflicts of interests.

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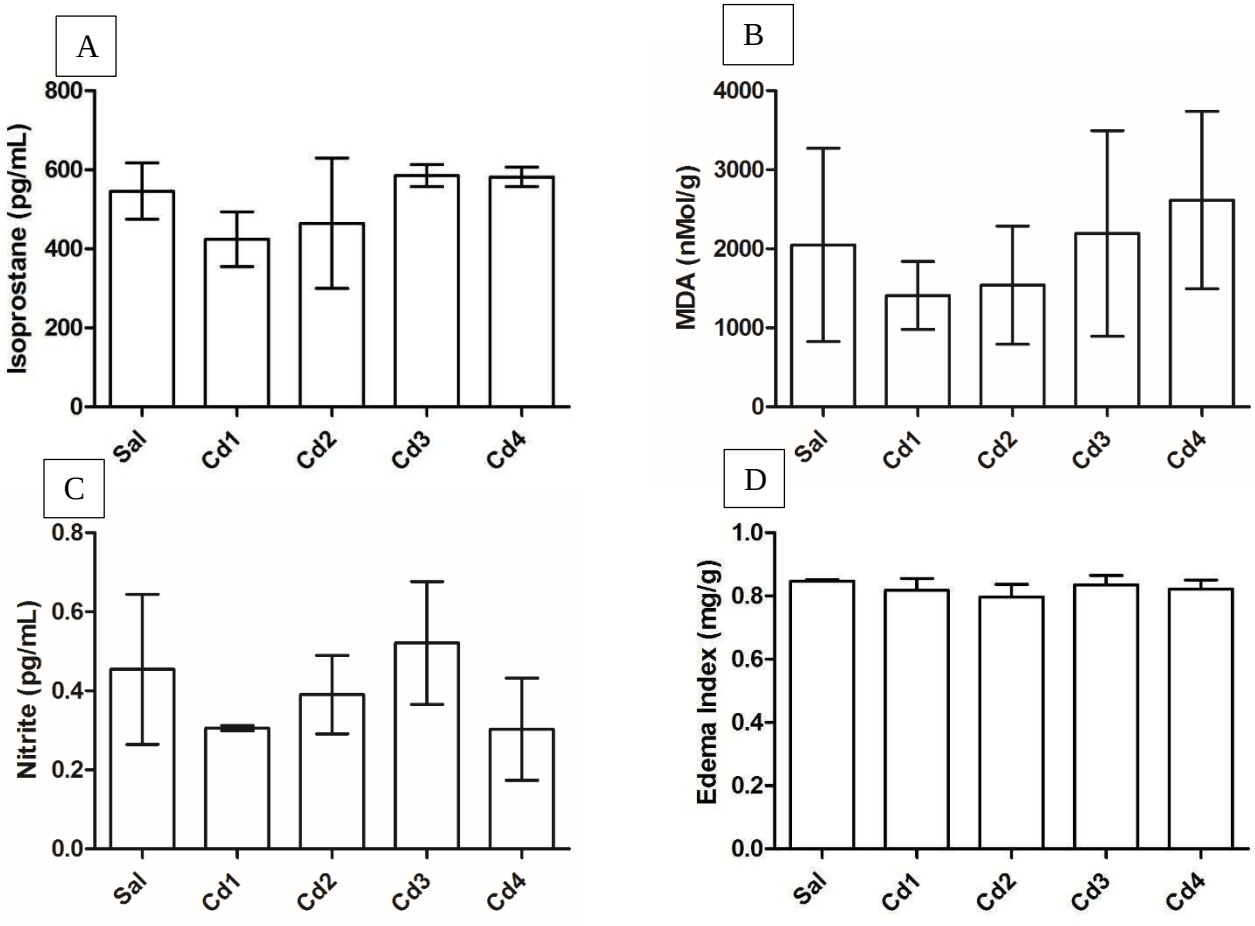
Supplementary material

The values of end products of lipid peroxidation (8-isoprostane and malondialdheyde), nitrite and edema index did not show difference between control and animals exposed (supplementary figure 1).



Supplementary Figure 1. Quantification of end products of lipid peroxidation (8-isoprostane, malondialdheyde), Edema index and Nitrite in testicular tissue of control and animals exposed to Cd. A - 8-Isoprostane; B – MDA: Malondialdheyde; C – Nitrite; D – Edema. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Different letters indicate statistical difference between groups ($p>0.05$).

CHAPTER 2: Cadmium exposure triggers reduction in testicular zinc levels and causes damage in spermatogenesis by reducing testosterone production: dose-response effect

(Manuscript in preparation for publication and edited according to journal guidelines)

Cadmium exposure triggers reduction in testicular zinc levels and causes damage in spermatogenesis by reducing testosterone production: dose-response effect

Abstract

The treatments in cases of cadmium (Cd) exposure are complex. Anti-inflammatories, antioxidants and Zinc (Zn) overdoses therapies are used. Cd competes with Zn to absorption sites and reduce Zn concentration in several tissues. In testis Zn is correlated with testosterone production. However there is a lack of data about dose-response relation of low doses of Cd exposure, Zn concentration and testosterone production in testis tissue. To investigate this relation, male rats were divided into five groups, Cd0= saline control and the others were exposed to a single dose at different concentrations of Cd (Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg). Blood and organs from reproductive system were collected seven days after exposure. The analyses performed were: biometrics measurements from reproductive organs; quantitative and qualitative measurements from testis tissue; Cd, Zn and testosterone concentration levels and genomic DNA integrity. Cd testicular levels increased in all animals exposed. The animals from Cd3 and Cd4 group showed decrease in testicular Zn concentrations and in plasma testosterone, with degenerative and apoptotic cells in seminiferous epithelium and reduction in volume of leydig cytoplasm and nucleus, and DNA damage. The weight of the epididymis did not change, however seminal vesicle and testicular parenchyma reduced in all groups exposed. Prostate reduced in animals exposed to Cd4 dose. We concluded that, in exposition to values equal to and greater than 0.86 mg Cd/kg, Cd is able to alter Zn and testosterone concentration and increase the damage in germ cells and Leydig cells that result in reducing sperm production. Seminal vesicle and testicular parenchyma are organs more sensible to Cd exposure and the dose of 0.67 mg Cd/kg is able to trigger alterations in these organs without significant alterations in Zn or testosterone concentration. Prostate was more resistant to alterations and the dose 1.1 mgCd/kg was the threshold to alterations in weight.

Keywords: Heavy metal, reproduction, hormone, Cd-Zn interactions, organs testosterone-dependent, Leydig cell, infertility.

Introduction

The prevalence of infertility is extremely high and increases in many countries, with the most commonly identified cause being 'male factor' infertility (Jorgensen et al. 2006; Sharpe 2010). A study made in Europe over ten years, showed that semen quality in young men is decreasing and the levels of sperm quality is already very low (Jorgensen et al. 2006). In adulthood, testes have endocrine and exocrine functions: the first is testosterone production, in interstitial tissue, by Leydig cells, while the second is sperm production, in seminiferous epithelium, by germ cells, which are supported by Sertoli cells (Fijak et al. 2015). Leydig cells are usually the most abundant components of the interstitial tissue, and are responsible for the synthesis of testosterone, the hormone involved in the maintenance of the spermatogenic process, secondary sexual characteristics, and libido, as well as the functioning of accessory glands (Payne 1996), and adverse factors may trigger alterations in Leydig cell number or integrity, causing alterations in the spermatogenesis process. The two factors that affect the sperm production process result from the lifestyle of the man (obesity, sedentariness) and/or his exposure to toxic agents from the general environment, such as Cd (Cd) exposure (Sharpe 2010).

Daily exposure to Cd is inevitable (Niknafs 2015; Jha and Bohra, 2015). There are several sources of human exposure to Cd, including its utilization in primary metal industries, production of certain batteries, some electroplating processes, food, water, alcoholic beverages and consumption of tobacco products (Chater 2008; Luevano and Damodaran 2014; Jha and Bohra, 2015). The toxic effects of Cd are often associated with oxidative stress and zinc treatments frequently reduce or abolish the adverse effects of Cd (Goering 1994). This might be considered as molecular mimicry, since these two elements are closely located in the periodic table and favor similar bio ligands. Excess of zinc can antagonize many adverse effects of Cd,

including tumor formation (Waalkes 2003), indicating a mechanistic role for Cd-zinc interaction in Cd toxicity.

Although the testicular damage induced by Cd was recognized decades ago (Parizek 1957) and continues to be described (Marettová et al. 2015; Niknafs et al. 2015), the precise mechanisms underlying its toxicity in testes remain unclear (Siu et al. 2009; Luevano and Damodaran 2014; Marettová et al. 2015). Effects of antioxidants, in most cases, improve testicular conditions, but do not avoid the harmful effects (Thompson et al. 2008; Siu et al. 2009; Ige et al. 2012; Wang et al. 2012; Marettová et al. 2015).

There is some Zn correlation in reducing testosterone production (Prasad et al. 1996; Yu et al. 2014; Liu et al. 2015; Omu et al. 2015) and Zn treatment against testicular negative effects caused by Cd (Al-Ani 2015). However, the dose response alterations in Zn testicular levels triggered by Cd exposure remain largely unexplored (Saïd et al 2010) and there are no established doses of Cd able to cause changes in testicular concentrations of Zn, testicular testosterone and organs testosterone dependent effects. In this study we aimed to obtain data in order to explain dose-response relation of low doses of Cd exposure, Zn concentration and testosterone production in testis tissue triggering damage in spermatogenesis process.

Materials and methods

Animals, ethics statement and experimental design

Thirty male Wistar rats, 70 days old, were provided by the Central Animal Facility of the Federal University of Viçosa (UFV) and maintained under controlled photoperiod (12:12 h light/ dark), temperature (21 ± 2ºC) and relative humidity (60-70%). The animals received food and water *ad libitum*. The study was conducted according to international standards for the care

and use of laboratory animals. The research protocol was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (CEUA/UFV) (protocol 030/2010).

Cd chloride ($CdCl_2$/ Sigma, St Louis, MO, USA) was dissolved in distilled water and administered by intraperitoneal route (i.p.) in a single dose. The 30 rats were randomized into five groups of six animals: (1) Saline (control), 0.9% NaCl; (2) Cd1, $CdCl_2$ 1.1 mg/kg (0.67 mg Cd/kg); (3) Cd2, $CdCl_2$ 1.2 mg/kg (0.74 mg Cd/kg); (4) Cd3, $CdCl_2$ 1.4 mg/kg (0.86 mg Cd/kg); and (5) Cd4, $CdCl_2$ 1.8 mg/kg (1.1 mg Cd/kg). The sample size was rationalized according to ethical aspects and was determined considering the probability P=1/2 to increase or decrease the variables of interest. Thus, considering the significance level α=0.05, the minimal significant number of animals used in the statistical analysis was P = (1/2) events; so, if n=6, P = (1/2)6 or P = 0.03; thus, P<0.05 (Novaes et al., 2013). In a previous study, Souza Predes et al. (2010), concluded that 1.2 mg/kg $CdCl_2$ is the minimal dose to causes significant testicular negative effects. So, in this experiment were used a dose low than minimum, the minimum and two doses more than minimum necessary to causes significant negative effects. The use of Cd at low doses is more appropriate for studies, since environmental contamination usually occurs at lower doses (Souza Predes et al., 2010).

Sample collection

The animals were weighed before and after the experiment. Seven days after exposure, the animals were euthanized by deep anesthesia (ketamine 45 mg/kg and xylazine 5 mg/kg, i.p.) followed by cardiac puncture, when the blood was collected, centrifuged for 15 minutes at 3000 g to collect serum.

The organs from reproductive system were quickly removed, dissected, and weighed on an analytical balance. The gonadosomatic index (GSI), which quantifies the investment in testis related to body mass, was estimated by dividing the testis weight by the body weight, multiplied

by 100 (testis weight/ body weight ×100). The right testis was used to measure Cd and Zn concentrations and DNA damage in the tissue. The left testis was immersed in Karnovsky solution (Karnovsky 1965) and used for analysis in light microscopy. Tunica albuginea were removed and weighed.

Cd and Zinc tissue concentration

The Cd and Zn content in testicular tissue was investigated by Energy Dispersive X-ray Spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with an attached x-ray detector system (Tracor TN5502, Middleton, WI, USA). Small pieces of testis from each animal were dehydrated in ethanol, submitted to critical point drying (CPD 030, Baltec, Witten, North Rhine-Westphalia, Germany) and coated with a thin film of evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The EDS microanalysis was performed at ×1000 magnification, with an accelerating voltage of 20kV and a working distance of 19 mm.

Morphometric and stereological analyses

The left testes that were immersed in Karnovsky solution for 24 h were transferred to 70% ethanol. Both testes and the tunica albuginea of one of them were removed and weighed and the tunica albuginea weight was subtracted from the gonad weight to calculate the volume of testicular parenchyma. Testicular fragments were dehydrated in increasing ethanol series for inclusion in glycol methacrylate (Historesin®, Leica) for light microscopy analyses. Semi-serial sections (3 µm thick) were obtained using a rotary microtome (Leica RM2255), observing a minimum interval of 40 µm between different cuts. The preparations were stained with toluidine blue/sodium borate 1%. Morphometry and stereology were performed using digital

images captured at different magnifications with the light microscope AX-70 Olympus. All images were analyzed by the software Image-Pro Plus (Media, Cybernetics).

The volumetric density between seminiferous tubules and the interstitial tissue were estimated by counting 2660 points projected over 10 digital images (per animal, 200× magnification) obtained from histological slides. Individual volume of Leydig cells (LC) was obtained from the nuclear volume and the proportion between the nucleus and cytoplasm. The mean diameter of the LC nucleus was obtained by counting 30 cells per animal, choosing those with the most spherical nuclei and evident nucleoli, using the software Image-Pro Plus. The nuclear volume was obtained by using the mean nuclear diameter and the formula $4/3\ \pi R^3$, where R= nuclear diameter/2. The cytoplasmic volume was estimated by multiplying the percentage of cytoplasm by the nuclear volume, divided by the nuclear percentage. The single cell volume was estimated by adding the nuclear and cytoplasmic volumes. These values were expressed in μm^3. The number of LC per testis was estimated from the LC individual volumes and the total volume occupied by LC in the testicular parenchyma. This value was divided by the gonadal weight, in order to estimate the number of LC per gram of testis. The leydigosomatic index (LSI), which quantifies the investment in LC related to body mass, was estimated by multiplying the LC volume in the testicular parenchyma by the body weight, divided by 100.

Testosterone Concentration Measurement

Plasma testosterone concentration was obtained by chemiluminescence, using Access® Testosterone (Beckman USA) kit and the measurements were performed on Access II equipment (Beckman, USA). The results were expressed in ng/dl.

Histopathology

Qualitative histopathological analysis were performed screening Histological preparations from each animal with semi serial sections from testes stained with toluidine blue, searching for tissue alterations.

DNA Extraction and genomic integrity

Fragments from testicular tissue weighing about 100 mg were transferred for a 2 mL Eppendorf and 800 µL of extraction solution (100 mM Tris-HCl, pH8.0, 50mM EDTA and 500 mM NaCl), 30µL 20% SDS and 20 µL 20 µg/µL of the Proteinase K (Sigma) were added. The extract was thoroughly mixed and incubated at 65°C for 24 h followed by inactivation in a dry bath at 95°C for 10 minutes. After this procedure, 1.5µL 20 mg/mL of RNAse was added and the sample was mixed and incubated at 37°C for 30 minutes. After that, proteins and cellular debris were precipitated by adding 300 µL 6 M NaCl, kept at 4°C for 15 min. Centrifugation was performed at 25,000 x g for 20 min. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the proteinase K digests, thoroughly mixed, and centrifuged to separate the aqueous and organic layers. The upper aqueous layer containing the nucleic acid was collected and transferred for a new Eppendorf and the extraction was repeated. At the end of the extraction, the nucleic acid was precipitated with two volumes of cold absolute ethanol (-20°C) and one fraction of 10 M ammonium acetate solution (pH 5.2) at -20°C for one day, washed twice with 70% ethanol, spun down at 3000 x g for 15 minutes, dried, and then suspended carefully in 50 µL of milliQ water. The quantity and purity of genomic DNA was determined in a NanoDrop micro volume sample retention system (Thermo Scientific NanoDrop Products); about 1000 ng/µL and molecular marker (1000bp DNA ladder, New England BioLabs Inc., Ipswich, USA) were subjected to 1.5% agarose gel (containing 0.5µg/ml gel red) electrophoresis with the Mupid electrophoretic apparatus (Advance, Tokyo, Japan).

DNA ladder formation was visualized under a UV trans-illuminator (VilberLourmat, Cedex, France). Photographs were taken with a Polaroid DS-300 camera (Coombs 1999).

Statistical analysis

Results were expressed as means and standard deviations (mean ± SD). The data were submitted to unifactorial one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) post-hoc test for multiple comparisons. Statistical significance was established at $p < 0.05$. All tests and graphics were performed using Graph Pad Prism 5.01® statistical software (Graph Pad Software, Inc., San Diego, CA, USA).

Results

Cd and Zinc testicular levels

The Cd concentration per gram of dry matter of testicular tissue increased in all animals exposed to Cd and Zn concentration decreased in animals exposed to Cd3 and Cd4 doses. The box represents the interquartile range with the median indicated (horizontal line), and whiskers represent the superior and inferior quartiles (Figure 1).

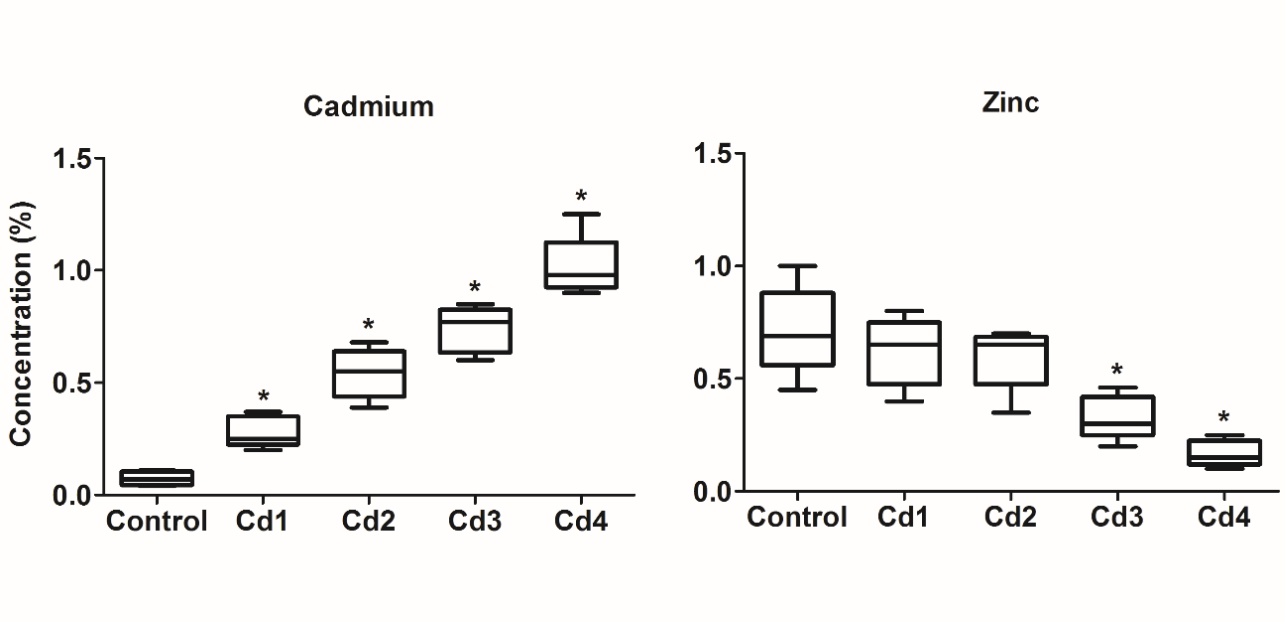


Figure 1. Cd and Zn testicular levels. Control= Saline 0.9%; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86 mg Cd/kg; Cd4 = 1.1 mgCd/kg. (*) indicates statistical difference between groups ($p<0.05$).

Biometrics parameters

Final body weight, testicular and albuginea weight, gonadosomatic index (GSI) did not present variation (Supplementary data). The weight of the epididymis did not change, however seminal vesicle and testicular parenchyma reduced in all groups exposed. Prostate reduced in animals exposed to Cd4 dose.

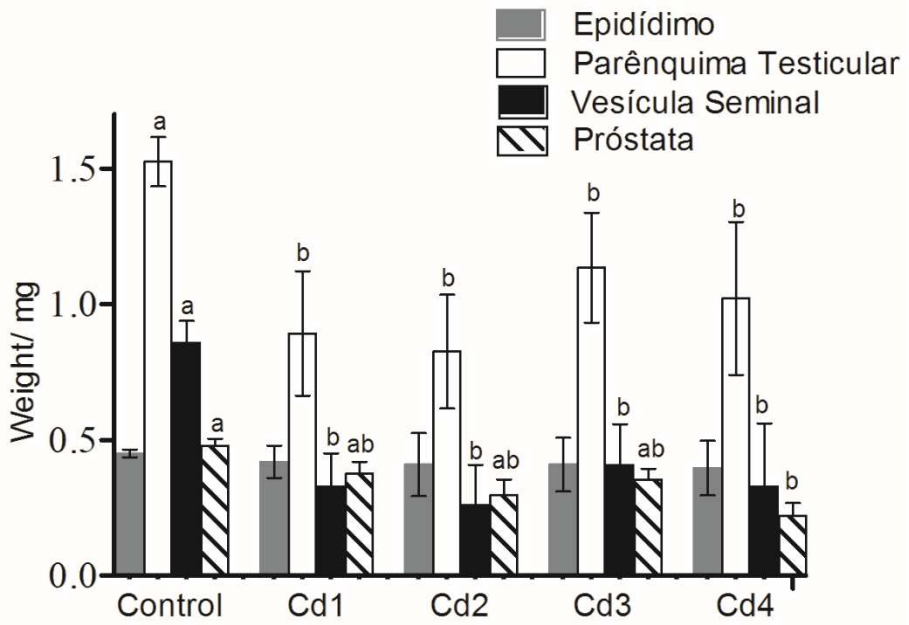


Figure 2. Biometrics parameters from control and animals exposed to Cd. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Different letters indicate statistical difference between groups ($p<0.05$).

Morphologic and stereological parameters

Leydig cells volume decreased in animals exposed to the three highest doses and Leydig cells volume of nucleus and cytoplasm decreased at the two highest doses (Figure 3).

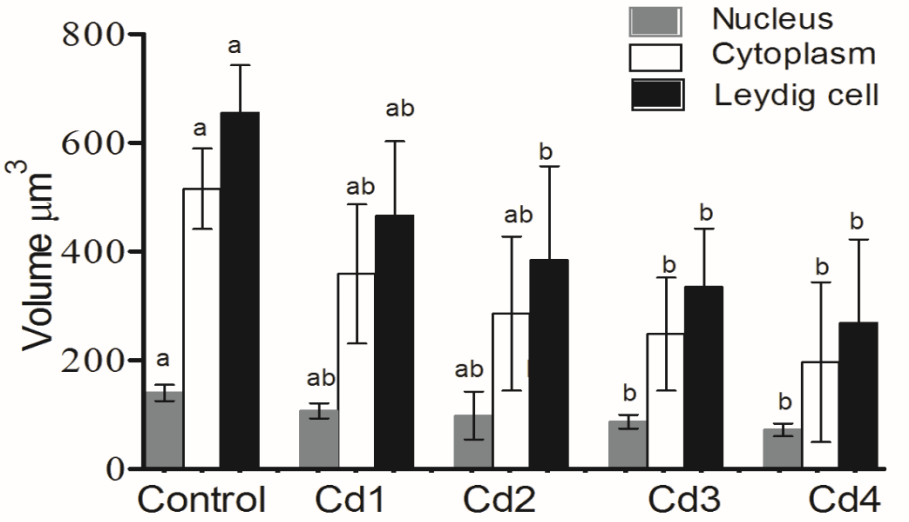


Figure 3. Leydig cells volume and Leydig cells volume of nucleus and cytoplasm from testis of control and rats exposed to four different doses of Cd. Control= Salina 0.9%; Cd1 = 0.67 mg

Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg Different letters indicate significant difference ($p<0.05$).

The volumetric density between the seminiferous tubules and the interstitial tissue did not change. The number of Leydig cells per testis and per gram of testis, the Leydigsomatic index (LSI), and the nucleus-cytoplasmic ratio showed no alterations, but the Leydig nuclear diameter decreased in the animals exposed to the highest dose (Table 1).

Table 1. Leydig cell morphometric data from testis of control and exposed rats to four different Cd concentrations (Mean±SD).

Parameters	**Control**	**Cd1**	**Cd2**	**Cd3**	**Cd4**
Tubule (%)	92.94±0.17	88.9±7.54	79.65±11.5	88.5±7.6	85.82±8.36
Intertubule (%)	7.06±0.17	11.1±7.54	20.35±11.50	11.5±7.6	14.2±8.4
Nucleus/Cytoplasm Ratio	21.43±1.15	23.98±5.31	28.52±10.82	28.41±11.3	31.72±12.4
Nuclear diameter (µm)	6.44±0.23^{a}	5.88±0.25^{ab}	5.62±0.85^{ab}	5.49±0.27^{ab}	5.16±0.27^{b}
Number of cells per testis ($\times 10^8$)	7.55±2.2	10.8±5.89	6.09±3.57	8.35±4.38	12.24±4.7
Number of cells per testis gram ($\times 10^8$)	6.09±2.54	11.9±6.73	7.03±3.23	7.33±3.5	13.4±5.9
Leydigsomatic index	0.16±0.03	0.18±0.09	0.09±0.06	0.12±0.06	0.09±0.08

Control= Salina 0.9%; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Different letters in the same line indicate significant difference ($p<0.05$) by Student-Newman-Keuls test. SD: Mean ± Standard deviation.

Testosterone serum levels

The serum values of testosterone concentration decreased in animals exposed to Cd3 and Cd4 doses of Cd (figure 4).

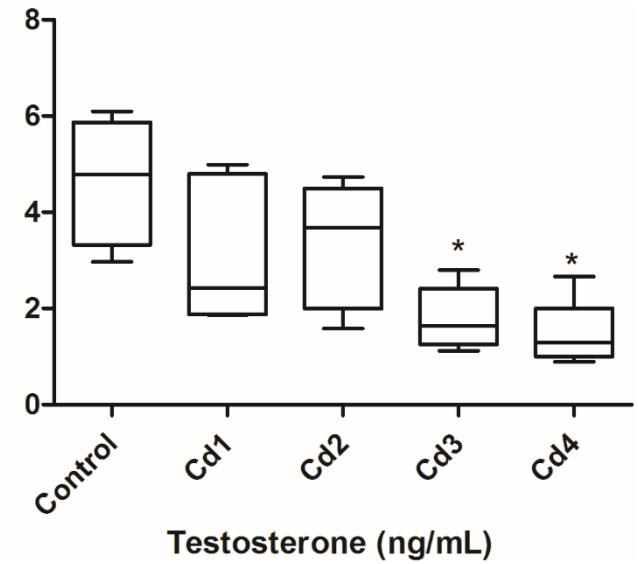


Figure 4. Serum testosterone levels from testis of control and rats exposed to four different Cd doses. Control= Salina 0.9%; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Symbols (asterisk) indicate statistical difference between groups ($p<0.05$).

Histopathology

In seminiferous tubules, cytoplasmatic vacuolization (figure 5b) and dysplastic cells showing a polyploidy nucleus (figure 5b – detail) were observed in animals from Cd3 group, and animals from Cd4 showed tubules with characteristics of germ cell loss (figure 5c).

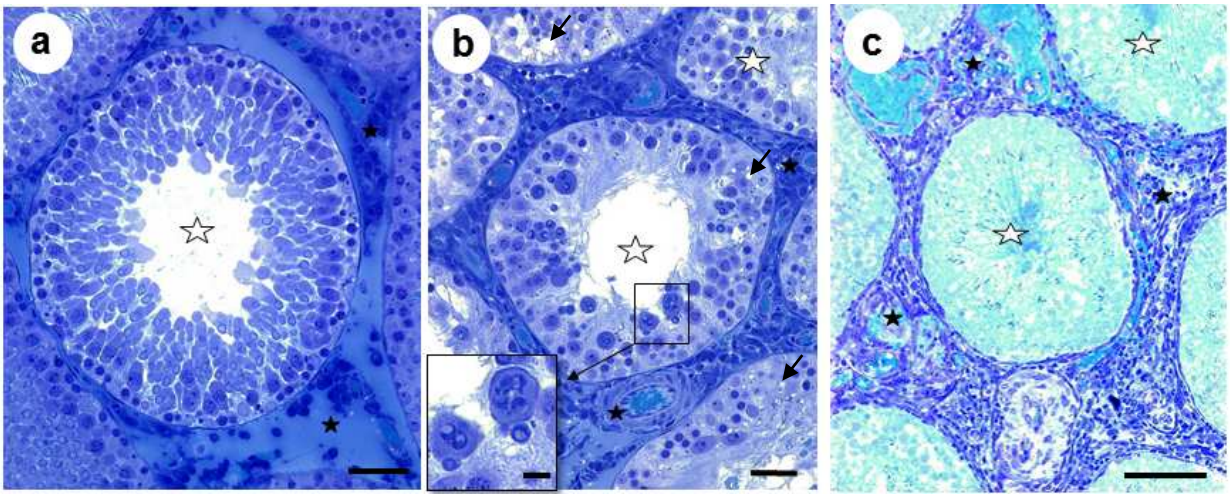


Figure 5. Testes tissues from control and rats exposed to Cd, stained with toluidine blue. White stars indicate seminiferous tubules areas; Black stars indicate interstitial tissue. A: Control animals. Bar: 50 μm; B: Cd 3 group. Arrows indicate cytoplasmic vacuolization. Bar: 50 μm Detail: Cells showing polyploid nucleus Bar: 10 μm; C: Cd4 group Bar: 50 μm.

DNA damage

By analyzing the integrity of the genomic DNA of testes, banding was observed in animals exposed to Cd, which was not seen in control and Cd1 animal groups, indicating the occurrence of DNA fragmentation in animals exposed to the highest doses of Cd. In some animals from Cd2 group is possible to see a light band (Figure 6).

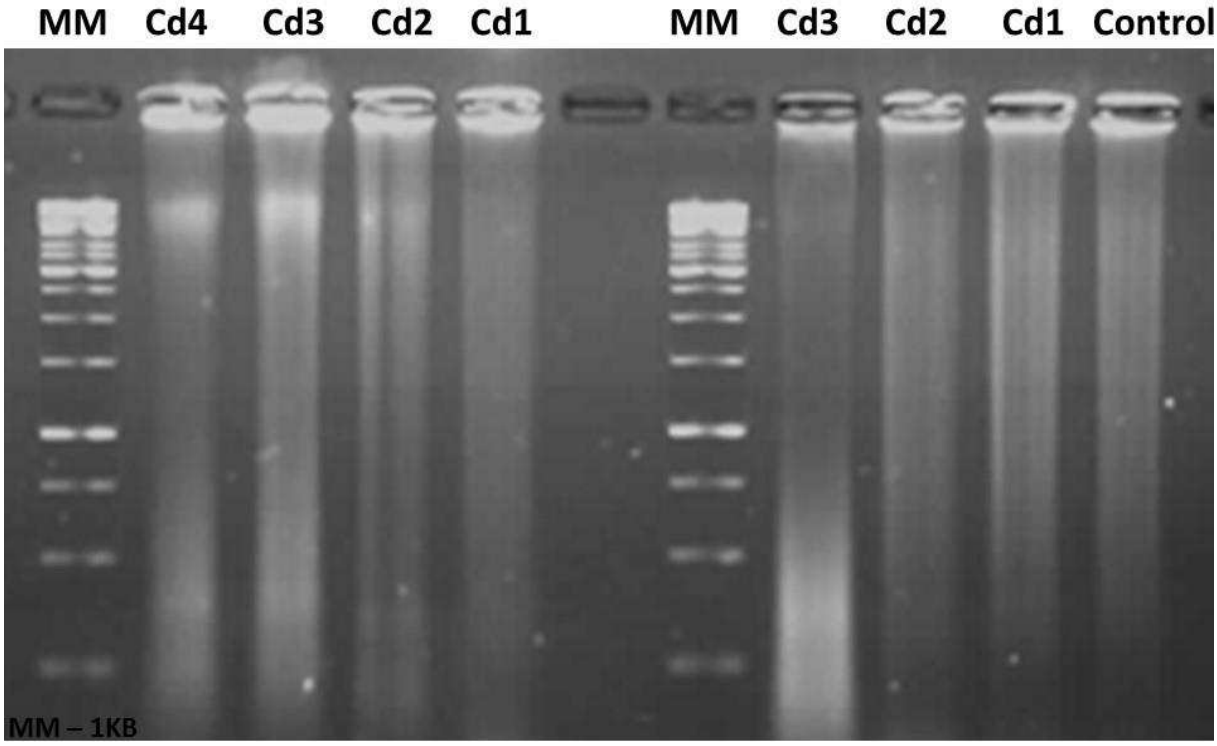


Figure 6. Agarose gel with testicular DNA samples of control and rats exposed to Cd. DNA fragmentation patterns from Cd treated present a band co-migrating with some markers. MM = molecular marker. Control= Salina 0.9%; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg

Discussion

Testicular levels of Cd increased depending on the Cd dose which animals were exposed. Negative relation was observed between Zn and Cd testicular levels in Cd3 and Cd4 groups. According to Waalks (2000), Cd competes with zinc for binding to some proteins and blocks cellular zinc accumulation. Said et al. (2010) exposed rats for thirty five days to high Cd doses and observed a significant decrease in plasma and testicular concentrations of Zn, which was accompanied by decreased serum testosterone level. According to these authors, Zn depletion seems to occur due sequestration by Cd-induced hepatic metallothioneins from the plasma, thereby increasing its concentration in the liver and probably restricting Zn supply to other tissues, like testis tissue (Bonda et al. 2004). Thus, on the basis of our results and the aforementioned reports, it is reasonable to assume that there is a threshold dose of Cd concentration that is able to compete with the binding sites of Zn and decrease its tissue concentration in testis. In our study this threshold dose of Cd was 0.86 mg/kg.

The volumetric density of seminiferous tubules and interstitial tissue, the total number of Leydig cells per testis and per testis gram, the nucleus-cytoplasm ratio and the Leydigsomatic index showed no alterations in all animals exposed to Cd. This may indicate that the cells decreased metabolic activity, but apoptosis did not occurred in Leydig cells. Previous studies have showed that Cd impairs testosterone production in isolated Leydig cells without affecting their viability (Laskey and Phelps, 1991), demonstrating that steroidogenic disruption in Leydig cells is likely to be an initial target of Cd toxicity as an endocrine modulator. In spite of Leydig cells volume decrease in animals from Cd2, Cd3 and Cd4 groups, reduction in the volume of cytoplasm and nuclei were only observed in Cd3 and Cd4, followed by a significant reduction in testosterone serum levels at the same doses that decreased Zn levels in testis. Animals exposed to zinc-deficient diet presented a reduction of Leydig cell volume, serum concentrations of Zn, and testosterone as well as increased levels of serum Cd, and apoptosis of the germ cells when

compared to control and zinc supplementation groups (Omu et al. 2015). Cd is a metal well known for its inhibitory effect on Leydig cell testosterone synthesis (Laskey and Phelps, 1991). According to Cavaliere and Wang (2015) Zn is an important metal involved in testosterone production. In animals with supplementation of Zn it was observed increase in testosterone levels. Dietary zinc deficiency alters 5 alpha-reduction and aromatization of testosterone in rat (Om and Chung, 1996). Previous studies showed decrease in the level of testosterone hormone after Cd administration. On a study with rats pre-treated with Zn and exposed to Cd, testosterone reduction was abolished (Omu et al. 2015), deducing that Zn is essential to testosterone production. Zinc also inhibits the aromatase enzyme that converts testosterone into excess estrogen (Al-Ani et al. 2015). The exact mechanism of Cd effects on rat testicular tissue is not obtained from this study. However, Cd triggered decrease in Zn tissue levels, Leydig cells volume and testosterone serum levels by dose dependence way. The dose 0.74 mg Cd/kg/BW was able to reduce Leydig volume, but not testosterone production, indicating that this may be the threshold dose or close to a threshold that triggers hormonal alterations. The reduction in Leydig cell nucleus and cytoplasm in the 0.86 mg Cd/kg/BW, together testosterone reduction in plasma indicates that there is a positive correlation in the number of effects and the Cd dose exposed. For example, it remains to be determined if Cd modulates Zn and if these effects are dose-dependent (Lafuente et al., 2004; Siu et al., 2009). Collecting these data is possible to confirm that Leydig cell steroidogenesis capability is dose-dependent and impaired due to Cd exposition, and is correlated with Zn levels in testicular tissue.

In this study the final body weight, testicular and albuginea weight, gonadosomatic index (GSI) and the weight of the epididymis showed no variation in all Cd doses exposed. Previous studies using lower doses (1 mg/kg $CdCl_2$, i.p.) showed that testis index, epididymis index and seminal vesicle index did not change (Zhou et al., 2016). Lamas et al. (2015) exposed animals to 1.2 mg/kg BW of $CdCl_2$ and observed no change in final body weight, testes weight and GSI.

In this study, seminal vesicle and testicular parenchyma reduced in all groups exposed. Prostate reduced in animals exposed to Cd4 dose. These tissues are named androgen-dependent reproductive organs, and reduction in their weight is related with reduction of testosterone level (Trentacoste et al., 2001). The epididymis weight no alterations can be related with germ cell death than acumulate in this organ, together with other necrosed materials from seminiferous tubule. El-Demerdash et al, 2004 have reported reductions of accessory sex organs weight after Cd administration. Pires et al. (2013) attributed the reduction in testis parenchyma weight to organ atrophy, with the presence of necrosis, inflammatory infiltrates and vascular congestion.

Cd-exposed animals showed decrease in nuclear volume, and DNA damage. The possible protective effect of Zn against apoptosis has been attributed to its inhibition of a Ca^{2+} and Mg^{2+} dependent endonuclease, thereby preventing DNA fragmentation, a terminal step and hallmark of apoptosis (Perry et al. 1997). Seminiferous epithelium showed a high rate of dysplasia, cells with polyploidy and a loss of germ cells in Cd3 and Cd4 groups compared to control animals, Cd1 and Cd2 groups. Recently, Yamaguchi et al. (2009) suggested that zinc is an essential trace element for the maintenance of germ cells and the progression of spermatogenesis. Seven days after exposure Zn may have resumed their concentrations in the animals from Cd2 group, and this may explain the lesions of lower intensities in this group, since Zn-Cd interactions are very dynamic and the body tries to eliminate the negative effects and recover homeostasis after exposure. Increasing in apoptosis rate of the cells, not counterbalanced by an increase in proliferation of these cells, result in tissue destruction and lesion development (Tuder et al. 2006; Sönmez and Tascioglu 2015), as observed in animals exposed to the highest dose of Cd. Both in vitro and in vivo models, Zn supplementation prevents apoptosis induced by a variety of agents (Perry et al. 1997; Omu et al. 2008). Leydig cells synthesize and secrete testosterone to blood stream and testicular tissue (Moridian et al. 2015), and the presence of testosterone is essential for normal function and survival of the germ cells in seminiferous tubules (Sinha Hikim and

Swerdloff 1999). When the testicular environment cannot support spermatogenesis, specific mechanisms leading to germ cell death are activated (Hardy et al. 2005). Reduction in the testicular level of Zn on tissue and testosterone serum levels can be caused by massive apoptosis in tubular tissue, with alterations increasing in number and intensity depending on the Cd dose exposed. However Zn is not totally benefic to the reproductive system. It can, for example, increase the progression of prostatic tumors (Waalks and Misra 1996).

In summary, this study has established that Cd caused a dose-dependent reduction in Zn levels on testicular tissue, triggering a reduction in the volume of Leydig cells, from dose of 0.86; mg Cd/kg/ BW. The reduction in the volume of Leydig cells led to a decrease in testosterone concentrations and this could induce germ cell death. Increase in germ cell loss is the main cause to the decrease of overall spermatic production. This investigation contributes with importants datas about dose-response relation of low doses of Cd exposure, Zn concentration and testosterone production in testis tissue and relation between the intoxication concentrations of elements and alterations in reproductive system and spermatogenesis process.

Conclusions

(1) Cd levels in testicular tissue increase depending on the Cd dose which animals were exposed.

(2) Negative relation exists between Zn in testicular tissue and serum testosterone levels with Cd dose exposition from dose 0.86 mg Cd/kg/BW i.p.

(3) Cd decreases Leydig cells volume in a dose dependent way. Cd threshold dose to trigger alterations in Leydig cells parameters (total cell volume) was 0.74 mg Cd/kg/BW i.p.

(4) DNA damage was observed in testis exposed to the doses from 0.74 mg Cd/kg/BW i.p.

(5) There is a direct relation between the Cd dose exposure and the number of alterations from dose 0.74 mg Cd/kg/BW i.p.

(6) The dose 0.67 mg Cd/kg was not capable to trigger alterations in the histological parameters in testis tissue, but is able to increase Cd concentration levels in testis tissue and decrease testosterone serum levels.

(7) Organs testosterone dependend showed decrease in the weight according to dose exposed. Seminal vesicle and testicular parenchyma showed alteration in the weight from dose 0.67 mg Cd/kg, and prostate from 1.1 mgCd/kg.

Supplementary table 1. Biometry of reproductive organs from rats control and exposed to Cd.

	Sal	**Cd1**	**Cd2**	**Cd3**	**Cd4**
Body weight (g)	304.1±11.73	271.17±34.74	267.3±41.97	246.17±18.9	267±24.27
Testes weight (g)	2.56±0.32	2.59±0.7	2.46±0.69	2.9±0.6	2.62±0.73
Albuginea weight (g)	0.073±0.013	0.048±0.005	0.068±0.027	0.10±0.047	0.11±0.07
Gonadosomatic Index	0.84±0.073	0.97±0.29	0.94±0.27	1.18±0.26	0.97±0.2

Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. g: gram. Data are expressed by mean ± standard deviation (Supplementary data).

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CHAPTER 3: Dystrophic calcification in collagen fibers triggered by cadmium exposure: Mineral Mapping of Testicular Tissue by Energy Dispersive X-ray Spectroscopy

(Manuscript in preparation for publication and edited according to journal guidelines)

Dystrophic calcification in collagen fibers triggered by cadmium exposure: Mineral Mapping of Testicular Tissue by Energy Dispersive X-ray Spectroscopy

Abstract

Few studies have focused on the interaction between cadmium (Cd) and other minerals, mainly calcium (Ca) causing dystrophic calcification. The investigations with respect to the toxic interactions of essential and no essential metals on the reproductive system and their underlying mechanisms of toxicity could provide several new data and therapeutic targets to treat metals associated to reproductive disorders. In this study, thirty adult male rats were divided into five groups, Cd0= saline control, and the others were exposed to a single dose in different concentrations of Cd (Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg). The testis was collected seven days after exposure. The organs were weighted and a part of the left testis was fixed and prepared according to routine histology techniques, stained with Toluidine blue for morphometric and stereological analyses and Von Kossa method for calcified areas. Another part of the left testis was used for transmission and scanning electron microscopy and mineral mapping by EDS method. The right testis was used to measure total protein. In the results morphometric and stereological testicular response to Cd was milder than described in previous studies. Calcium and Cd have a positive synergistic concentration in rat testis. Magnesium, Iron, Copper, and Selenium have a negative synergistic concentration with Cd in rat testis. Von Kossa positive staining was observed in collagen fiber in the testis albuginea, and in interstitium area, and in germ cells into the seminiferous epithelium. Total protein decrease in groups exposed to the higher doses. In conclusion, Cd exposure to induced alteration in mineral dynamics in testis tissue, triggers dystrophic calcification in collagen fibers and germ cells and this impair the spermatogenesis process.

Keywords: Mineral interactions, reproduction, heavy metal, spermatogenesis

Introduction

The toxic effect of cadmium (Cd) in testicular function is not a novel issue (Parizek 1957). It is speculated that the Cd mechanism that triggers reproductive disturbances is given, at least in part, by an imbalance of blood testis barrier - BTB (Setchell, Waites, 1970), caused by loss of junctions between cells as a result of replacement by Cd^{2+} in binding sites where the Ca^{2+} (Calcium) action is necessary (Prozialeck, Lamar, 1999), increase in lipid peroxidation, and decreased expression and activity of antioxidant enzymes such as superoxide dismutase and catalase (Wong Cheng, 2011). An interesting mechanism of Cd indirect role in the production of free radicals is that Cd can replace iron and copper in several cytoplasmic proteins and membrane. The displacement of iron and copper by Cd can explain its toxicity, since copper binds with the essential site to catalyze the decomposition of hydrogen peroxide by the Fenton reaction (Jomova, Valko, 2011). Some trace elements are important in processes involving free radicals. Cupper (Cu), Zinc (Zn) and Manganese (Mn), form part of superoxide dismutase (SOD). Iron (Fe), Cu and Mn also act as catalysts in the production of free radicals by the Fenton reaction and classical Haber Weiss reaction. Selenium is important because of the presence of the enzyme glutathione peroxidase which reduces H_2O_2 to H_2O (Coyle, Puttfarken 1993). The protein metallothionein (MT) plays a key role in Cd toxicity because Cd has a high affinity for it. MT not only regulates and binds with zinc and copper, but can act also as scavenger for free radicals (Panayi et al., 2002). The Cd toxic action is due to its affinity for protein radicals and its competitive action with other essential functional elements such as Cu, Fe and Ca. A major interaction of Cd is the strong union of Cd to -SH groups of proteins that inhibit intracellular enzymes having these groups (Klaassen et al, 1996; 2001).

Cadmium affects the metabolism of calcium, and phosphorus triggering alterations with calcification, decalcification and bone remodeling (Klaassen et al, 2001). The effects of Cd can

be difficult to assess, since metal binding proteins, stress proteins and antioxidants can individually modulate the toxicity of the element. However, there are many other metals which Cd can interact and by direct or indirect pathways triggers deleterious effects in cells and tissues and these interactions are poorly described in the spermatogenic process. The investigations regarding the toxic interactions of essential and no essential metals on reproductive system are essential to fill data gaps in the literature on this topic and help in future studies of therapies and mechanisms of Cd triggers reproductive disorders. In this study we aimed to get the interactions between cadmium and other metals linked to reproduction in order to obtain data that can explain testicular damage caused by different low doses of cadmium.

Materials and methods

Chemicals and experimental design

Cd chloride ($CdCl_2$/ Sigma, St Louis, MO, USA) was dissolved in distilled water and administered by intraperitoneal route (i.p.) in a single dose. The 30 rats were randomized into five groups of six animals: (1) Saline (control), 0.9% NaCl; (2) Cd1, $CdCl_2$ 1.1 mg/kg (0.67 mg Cd/kg); (3) Cd2, $CdCl_2$ 1.2 mg/kg (0.74 mg Cd/kg); (4) Cd3, $CdCl_2$ 1.4 mg/kg (0.86 mg Cd/kg); and (5) Cd4, $CdCl_2$ 1.8 mg/kg (1.1 mg Cd/kg).

In a previous study, Souza Predes et al. (2010), concluded that 1.2 mg/kg CdCl2 is the minimal dose to causes significant testicular negative effects. So, in this experiment were used a dose low than minimum, the minimum and two doses more than minimum necessary to causes significant negative effects. The doses were calculated to ensure that there would be animals with injury, thus making the analysis of possible pathways and mechanisms by which these alterations are formed possible. The use of Cd at low doses is more appropriate for studies, since environmental contamination usually occurs at lower doses (Souza Predes et al., 2010).

Animals and ethics statement

Thirty male Wistar rats, 70 days old, were provided by the Central Animal Facility of the Federal University of Viçosa (UFV) and maintained under controlled photoperiod (12:12 h light/ dark), temperature (21 ± 2ºC) and relative humidity (60-70%). The animals received food and water *ad libitum*. The study was conducted according to international standards for the care and use of laboratory animals. The research protocol was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (CEUA/UFV) (protocol 030/2010).

Sample collection and biometric analyses

The animals were weighed before and after the experiment. Seven days after exposure, the animals were euthanized by deep anesthesia (ketamine 45 mg/kg and xylazine 5 mg/kg, i.p.) followed by cardiac puncture. The testis were quickly removed, dissected, and weighed on an analytical balance. The right testis was frozen in liquid nitrogen and stored in a freezer at -80°C for total protein quantification. The left testis was fixed in Karnovsky solution (Karnovsky, 1965) and then divided into smaller fragments that were used for analysis in light microscopy, transmission and scanning electron microscopy and mineral microanalysis.

Morphometry and stereology

The left testes that were fixed in Karnovsky solution for 24 h were transferred to 70% ethanol. The tunica albuginea weight was subtracted from the gonad weight to calculate the volume of testicular parenchyma. Testicular fragments were dehydrated in increasing ethanol series for inclusion either in glycol methacrylate (Historesin®, Leica) for light morphometric analysis, or in paraffin wax for the detection of calcification areas. Semi-serial sections 3 µm thick were obtained from resin samples and sections 5 µm thick were obtained from paraffin wax samples using a rotary microtome (Leica RM2255), observing a minimum interval of 40

µm between different cuts. The resin preparations were stained with toluidine blue/sodium borate 1% and paraffin wax preparations were stained by Von Kossa method. Morphometry and stereology were performed using digital images captured at different magnifications with the light microscope AX-70 Olympus. All images were analyzed by the software Image-Pro Plus (Media, Cybernetics).

The volumetric ratio between the seminiferous tubule compartment and the intertubular tissue were estimated by counting 2660 points projected over 10 digital images (per animal, 200× magnification) obtained from histological slides. The volume occupied by seminiferous tubules and the intertubular tissue volume, expressed in milliliters, were calculated from the percentage of the testis occupied by them and from the volume of the testicular parenchyma. The volumetric proportions of all seminiferous tubules components (tunica propria, seminiferous epithelium and lumen) were calculated. The volume of the seminiferous epithelium was obtained by multiplying its percentage within the seminiferous tubules by the tubular volume, dividing the result by 100. The tubulesomatic index (TSI) was calculated in order to quantify the investment in the seminiferous tubules regarding the total body mass. It was obtained by dividing the tubular volume by the body weight and multiplying the result by 100. The diameter of the seminiferous tubules and the height of the seminiferous epithelium were obtained from the measurement of 20 tubular cross-sections, regardless the stage of the cycle. The epithelium height was measured from tunica propria to tubular lumen. Seminiferous tubule length (TL, in meters) per testis and per gram of testis was estimated using the formula: $TL=STV/ \pi R^2$ (STV=seminiferous tubule volume; R^2 =area of the seminiferous tubules cross section; R=tubular radio). The TL was divided by the testes weight in order to calculate the length of the seminiferous tubules per gram of testis.

Transmission electron microscopy

For ultrastructural analysis, testicular fragments that were fixed in Karnovsky solution for 1 hour were transferred to a 2.5% glutaraldehyde solution for 24 h. After being rinsed with phosphate buffer, specimens were post-fixed in 1% osmium tetroxide in the same buffer for 2 h. Dehydration was performed in ethanol and acetone, followed by adding embedding resin (Epon 812). Ultrathin sections were contrasted with 3% uranyl acetate and 3% lead citrate and observed under a transmission electron microscope (Zeiss EM 109) at the Center for Microscopy and Microanalysis of Federal University of Viçosa (Basile et al. 2012).

Scanning electron microscopy

For ultrastructural analysis, fragments of the left testis that were fixed in Karnovsky solution for 1 hour were transferred to a 2.5% glutaraldehyde solution for 24 h, dehydrated in ethanol, submitted to critical point drying (CPD030; Bal-Tec, Witten, NorthRhine-Westphalia, Germany), mounted on stubs, metalized with gold, and examined in a scanning electron microscope (Leo 1430VP; Carl Zeiss, Jena, Thuringia, Germany) (Dos Santos et al., 2013).

Mineral microanalysis

The mineral content in testicular tissue was investigated by Energy Dispersive X-ray Spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with an attached x-ray detector system (Tracor TN5502, Middleton, WI, USA). Small pieces of testis from each animal were dehydrated in ethanol, submitted to critical point drying (CPD 030, Bal-tec, Witten, North Rhine-Westphalia, Germany) and coated with a thin film of evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The EDS microanalysis was performed at ×1000 magnification, with an accelerating voltage of 20kV and a working distance of 19 mm. The proportion of the elements calcium (Ca),

magnesium (Mg), iron (Fe), Selenium (Se), copper (Cu), and cadmium (Cd) were measured by EDS (Novaes et al., 2013).

Histopathology and calcification

Qualitative histopathological analyses were made screening two glass slides per animal with semi serial sections from testes stained with Toluidine blue, searching for tissue alterations. The von Kossa staining method was used to reveal calcium concretions.

Total Protein

Fragments of the testicular tissue were homogenized with a protease inhibitor (Protease Inhibitor Cocktails; Sigma Chemical Co. St. Louis, MO, USA) in portable tissue homogenizer, and centrifuged at 3000 $\times g$ for 10 min. The total protein levels in testicular tissues were determined by using the Bradford method (Bradford 1976).

Statistical analysis

Results were expressed as measures of central tendency, means and standard deviations (mean ± SD). The data were submitted to unifactorial one-way analysis of variance followed by the Student-Newman-Keuls (SNK) post hoc test for multiple comparisons. Statistical significance was established at $p<0.05$. All tests were performed using GraphPad Prism 5.01® statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Morphometry and stereology

The percentage of seminiferous epithelium, tunica, and lumen, the intertubular volume, seminiferous tubule diameter, total seminiferous tubule length and per testis gram showed no significant changes (Supplementary data). The volume of seminiferous epithelium and seminiferous tubules decreased in all animals exposed to Cd (Fig. 1).

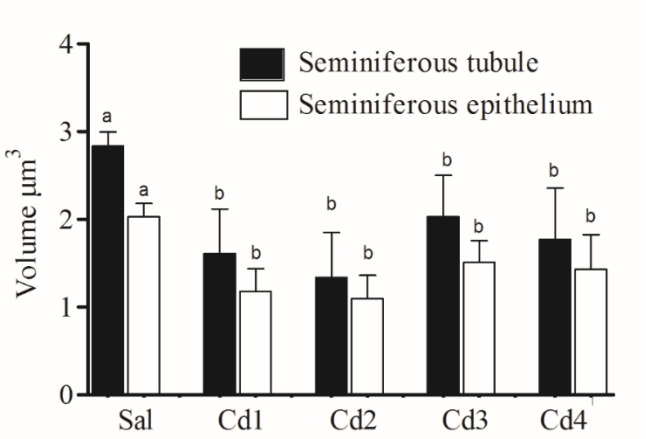

Figure 1. Biometrics parameters from control and animals exposed to Cd. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg Different letters indicate statistical difference between groups ($p<0.05$).

Mineral microanalysis

Animals exposed to Cd1 dose only increased Cd concentration in testicular tissue. Animals exposed to Cd 2 dose showed increased Cd concentration, while Cu and Mg decreased. Animals exposed to Cd 3 and Cd4 doses showed increased concentrationof Cd and Ca, while Se, Cu, and Mg decreased. Only in Cd4 dose was observed decreased concentration of Fe in testicular tissue (Fig. 2).

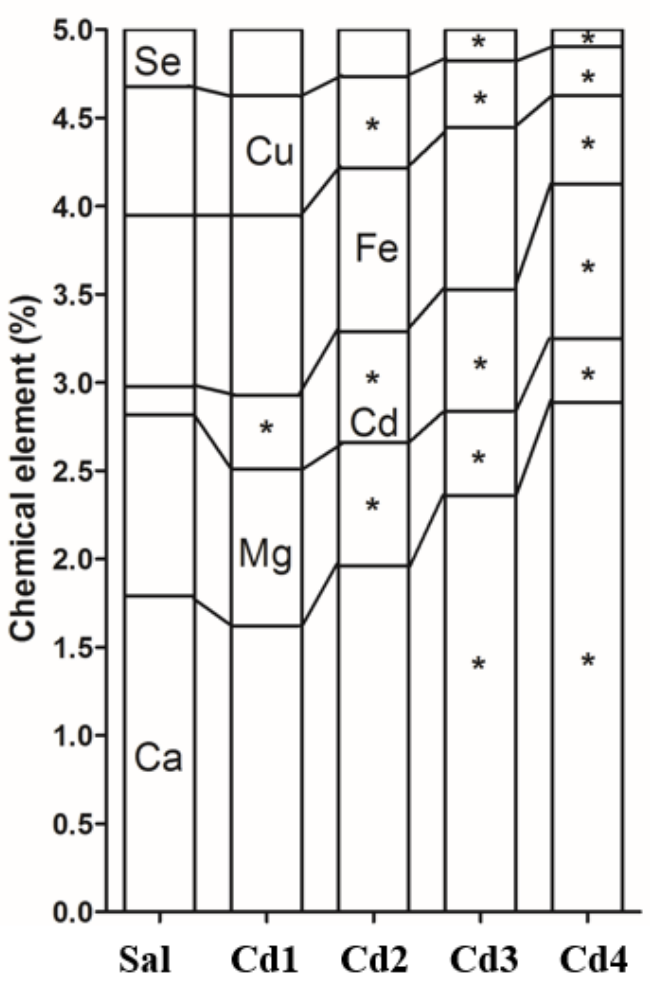

Figure 2. Mineral content in testicular tissue from rats control and exposed to Cd. The numbers represent the proportion of each mineral in the testis by group. Ca: calcium; Mg: magnesium; Fe: iron; Se: Selenium; Cu: copper; and Cd: cadmium (*) Asterisk means statistical difference among groups ($p<0.05$). Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg

Testicular ultrastructure

Seminiferous tubules electron microscopy evaluation as shown (Figure 3) revealed the ultrastructure of germ cells and extracellular matrix. In control animals, germ cells and normal spaces between cells could be observed, with nucleus showing a normal chromatin pattern. Furthermore, the germ cells of the seminiferous tubules of Cd1 presented vacuolization between cells. Germ cells from Cd2 groups show few and small cytoplasmic vacuolization. In animals

from Cd3 group, several and big cytoplasmic vacuolization were observed (Figure 3-Cd3). In animals from Cd4 group germ cells apoptosis with increasing chromatin condensation were observed (Figure 3-Cd4) compared to the othersgroups (Figure 3). The ultrastructural alterations intensified with the increase of Cd dose exposed.

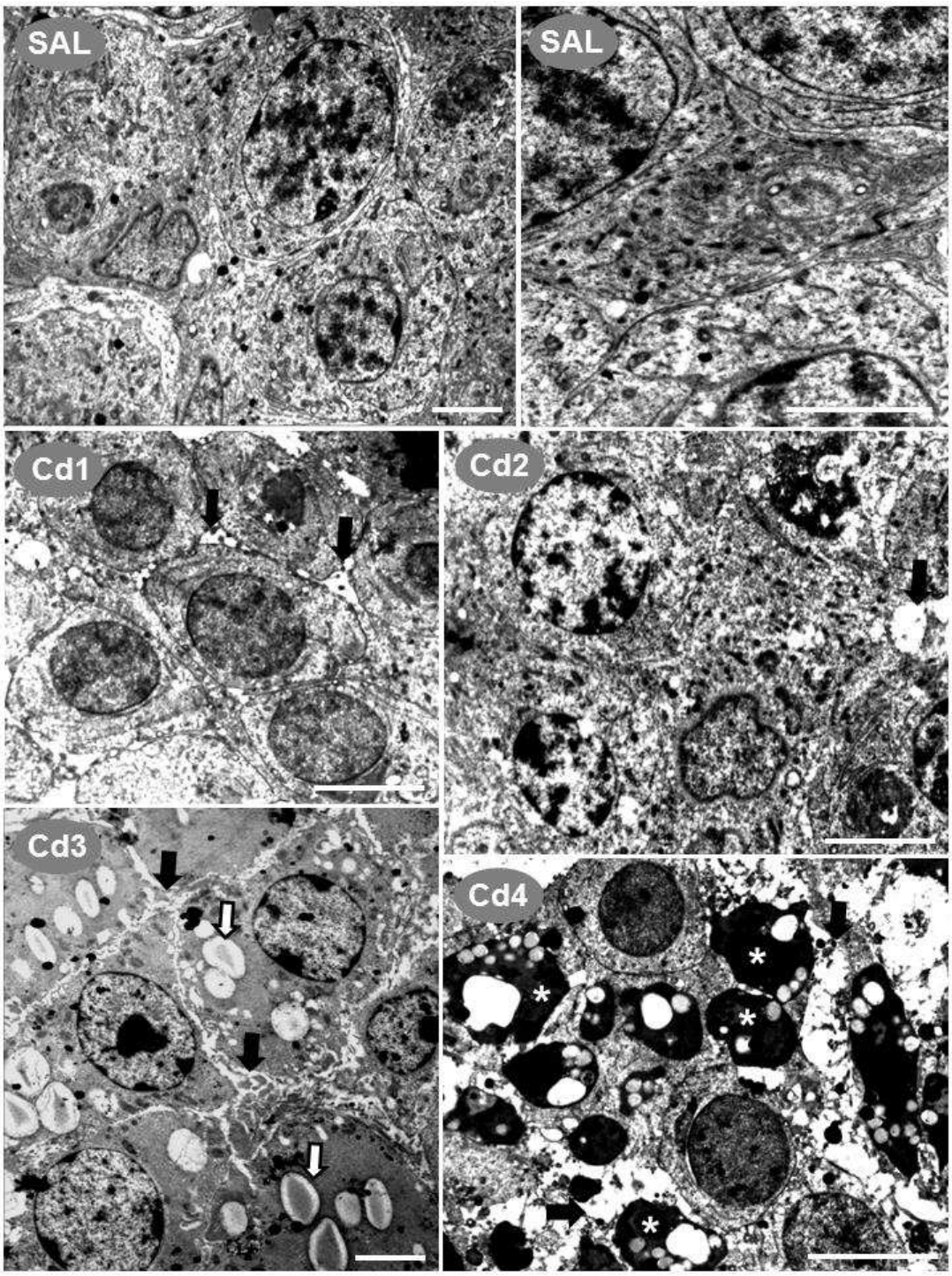

Figure 3. Seminiferous epithelium photomicrographs captured by transmission electron microscope. Black arrow: space between cells; White arrow: cytoplasmic vacuolization; asterisk: apoptotic bodies; Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mg Cd/kg. Bar: 5μm

Scanning electron microscopy

Electron microscopy evaluation of testicular tissue as shown (Figure 4) revealed the ultrastructure of seminiferous tubules. In animals from saline group is possible to observe seminiferous tubules with large lumen when compared to rats not exposed to Cd. The number of blocked seminiferous tubules increased with positive correlation to Cd exposed dose. In animals from Cd3 and Cd4 groups, almost all tubular lumens were blocked.

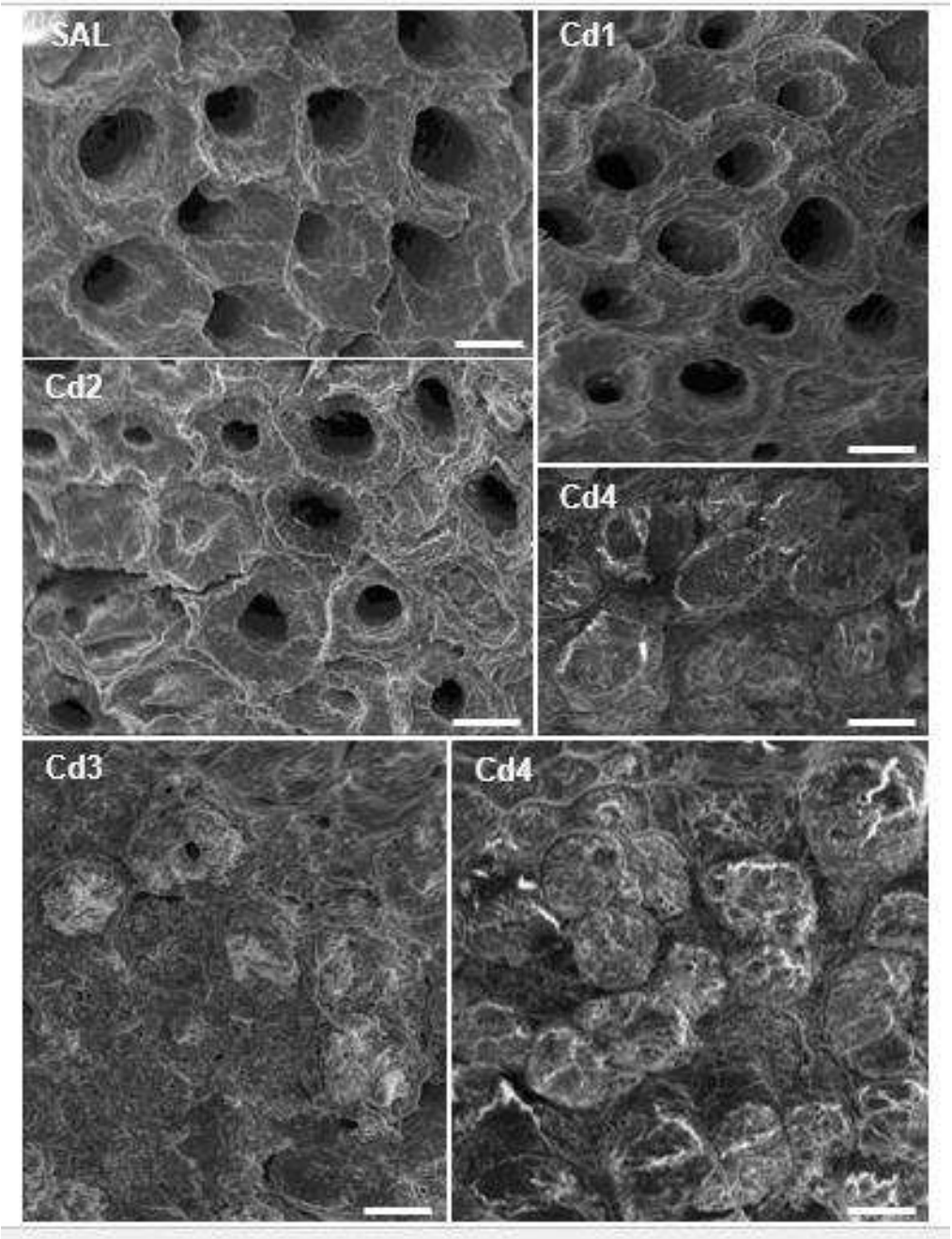

Figure 4. Testis tissue photomicrographs captured by scanning electron microscope. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mg Cd/kg. Bar: 15 0μm

Dystrophic calcification

Figure 5 shows abnormal positive staining for Von Kossa, which is used to indentify calcification in tissue sections. Abnormal calcium deposits may be found in tunica albuginea which is constituted by dense connective tissue rich in collagens fibers (asterisk: Fig. 5 A). Figure 5B shows an arteriole, which adventitious tunic, the outermost layer consisted of connective tissue, shows Von Kossa positive staining (black arrows). Figure 5C, D, E and F show collagen fibers in intertubular region with Von Kossa positive staining (black arrows) and Von Kossa positive staining in germ cells cytoplasm into the seminiferous epithelium (white arrows and Fig 5C detail). Fragmented collagen fibers in intertubular region with calcium positive staining (black arrows) in animals from Cd4 group is showed in Figure 5F.

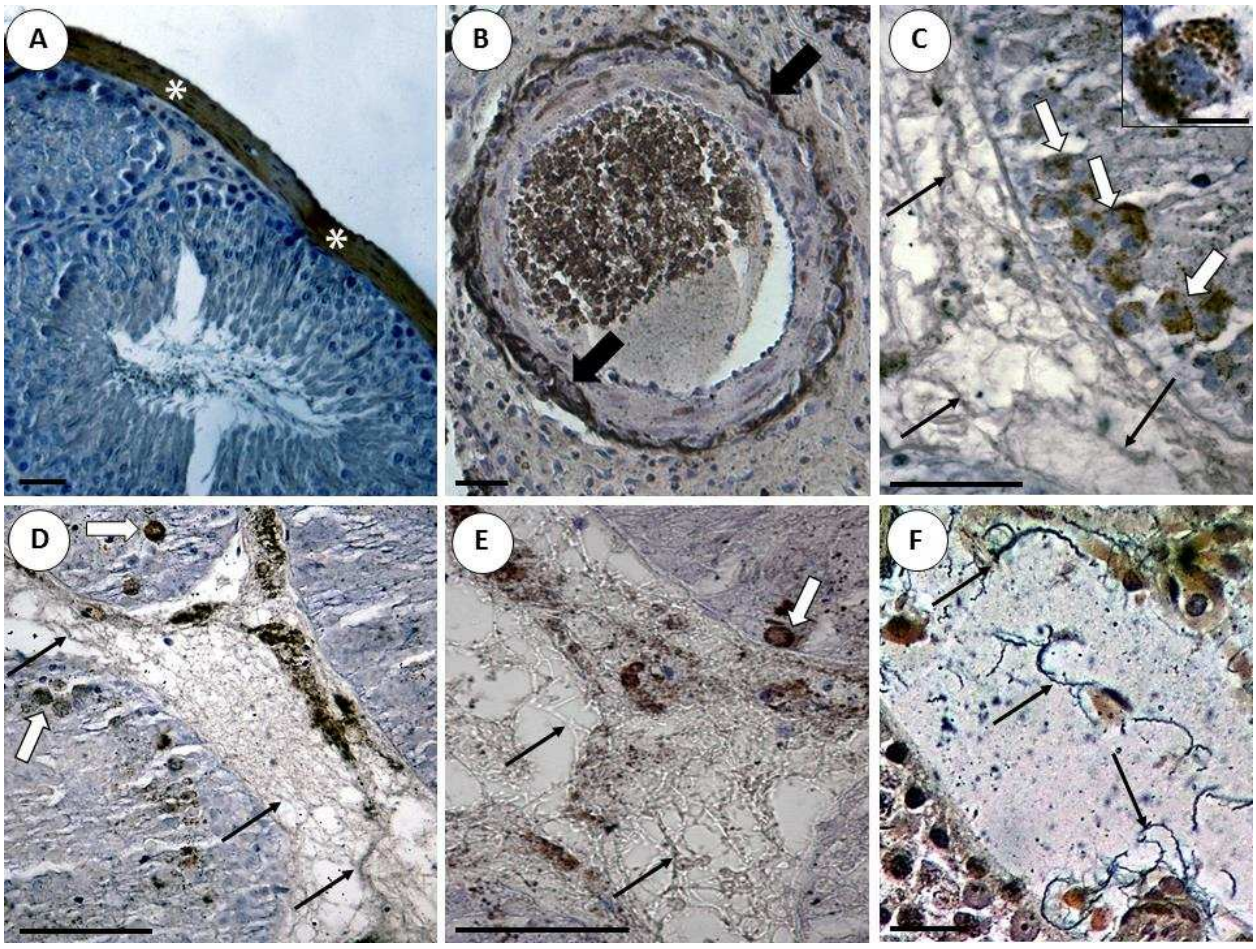

Figure 5. Rat testes exposed to Cd and subjected to Von Kossa staining technique. A - Abnormal calcium deposits in tunica albuginea (asterisk) Bar: 30µm; B - Adventitious tunica

from arteriole showing calcium positive staining (black arrows) Bar: 50µm. C, D and E - Collagen fibers in intertubular region with calcium positive staining (black arrows) and calcium deposit in germ cells cytoplasm into the seminiferous epithelium (white arrows and Fig 5C detail Bar: 10µm), from animals groups Cd1, Cd2 and Cd3 respectively Bar: 50µm; F - Fragmented collagen fibers in intertubular region with calcium positive staining (black arrows) in animals from Cd4 group Bar: 20µm

Total Protein

Total protein decreased in animal testis from Cd3 and Cd4 groups, compared to control and Cd1 groups (Fig. 6)

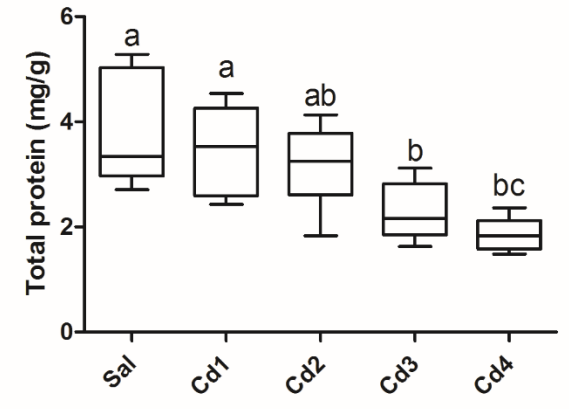

Figure 6. Total protein in testicular tissue from control and rats exposed to different Cd doses. The box represents the interquartile range with the median indicated (horizontal line), and whiskers represent the superior and inferior quartiles. Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mg Cd/kg. Different letters indicate statistical difference between groups ($p < 0.05$).

Discussion

The present results showed that the toxic effects of Cd in the male reproductive system were dose-dependent. The difference between doses clearly triggered very different morphologic and morphometrical results. The animals from Cd1 group showed increase in Cd tissue levels, decrease in seminiferous epithelium and seminiferous tubule volumes. The animals from Cd4 group showed alterations in all metals analyzed and a large variety of morphological damage. According to Souza Predes et al. (2010) the defenses of the testis against Cd contamination are defficient up to a very precise level, possibly depending on the level of cysteine-rich metal-binding proteins, which stands out metallothionein (MT), which helps in Cd detoxification. It is widely accepted that the Cd bound to MT is nontoxic, thus MT protects tissue against Cd toxicity in this way. The balance between Cd-MT and free Cd in the tissue has been shown to be crucially important for toxicity, but is dependent on MT quantities. Toxic effects probably result when the amount of MT becomes insufficient to bind with the Cd present, resulting in damage (Xu et al., 2005).

The percentage of seminiferous epithelium, tunica propria, intertubular volume, and tubulesomatic index did not change in this study, similar to Lamas et al., 2015 that exposed animals to 1.2 mg/kg BW of $CdCl_2$. Lamas et al., 2015 exposed animals to 1.2 mg/kg BW of $CdCl_2$ and observed reduction in seminiferous tubules volume without reducing the intertubular volume, similar to this study. Souza Predes et al., (2010) found decreased volume density of seminiferous tubules, accompanied by an increase in interstitium volume density. In this study there was no increase in intertubular volume. The tubular lumen percentage did not change in this study, but were filled with degenerated germ cells and multinucleated spermatid aggregates. Similar results were found by Souza Predes et al., (2010).

At ultrastructural analyses the animals Cd exposed showed nuclear abnormalities in germ cells in animals exposed to Cd4 dose. The possible mechanism Cd induces apoptosis has been

attributed to its inhibition of a Ca2+ and Mg2+ dependent endonuclease, thereby preventing DNA fragmentation, a terminal step and hallmark of apoptosis (Perry et al. 1997; Oteiza 1999). Inter and intracellular vacuolization and apoptotic bodies were observed in animals exposed to Cd3 and Cd4 dose, and intact cellular membranes with small vacuolization were observed in ultrastructural analysis in animals exposed to Cd1 and Cd2 doses. Vacuolization is the first step to degeneration and apoptosis (Creasy 2001).

We unknown studies that focus on the interaction between Cd and other minerals, mainly Ca causing dystrophic calcification in testis tissue. Essential metals can affect the metabolism of nonessential metals (Mim et al., 2008). In this study there were increased levels of Cd and Ca in testicular tissue and decreased levels of Se, Cu, Mg and Fe in testicular tissue. Divalent metal transporter 1 (DMT1) has a broad substrate specificity and favors most divalent metals, including Fe^{2+}, Zn^{2+} and Mg^{2+}, but not Ca^{2+} (Gunshin et al., 1997). It has been suggested that DMT1 is a nonspecific metal transporter that can transport not only essential metals, but probably toxic metals as well (Park et al., 2002; Ryu et al., 2004). A recent report demonstrated that functional iron depletion probably upregulates (DMT1), and increases Cd uptake with subsequent transfer of Cd to the body tissues (Park et al., 2002). Expression of the DMT1 is correlated with Cd body burden in rats, suggesting an important role in Cd absorption (Ryu et al., 2004).

The previous studies fed male mice over 4 weeks on a purified diet containing low doses of Ca and observed that Cd accumulation increased in liver and kidneys (Mim et al., 2008). Rats exposed to Cd and fed on low-Ca diet accumulated approximately 60% more Cd, suggesting that Cd retention is enhanced by a low-Ca diet and that the increased calcium binding protein activity caused by Ca restriction is responsible for the observed increase in Cd uptake. These reports suggest that Ca-related proteins, such as Ca transporters and channels, are important pathways for Cd absorption. A recent report shows that Ca transporter is involved

in both absorption and tissue distribution of Cd in mice (Bronner et al., 1986). In this study the diet had normal quantities of Ca and maybe induced Cd transport by DMT1 instead of by Ca transporter, decreasing other metals absorption, and for this reason only Ca absorption increased.

Cd can substitute Ca and bind molecules, such as calmodulin, in Cd-induced testis damage. Cd damage induced in the testis is probably the result of the similarity between Cd^{2+} and Ca^{2+}, with the improper and unintentional induction of cellular events mediated by Ca. Cd exerts its effects by physical and chemical properties of the Cd^{2+} ion, due to its similarities with calcium (Menon et al., 2016).

Reducing in Magnesium, Iron, Copper, and Selenium dose dependence can be related with morphological alterations. According to Rao et al., 2016 supplementation of organic form of Se improved performance and antioxidant responses in commercial broiler chickens. Iron is the most abundant transition metal in the body. This metal is essential for several body functions, including ATP production, DNA synthesis and oxygen transport. Magnesium (Mg), an abundant cation in the human body, plays an important role in multiple physiological functions. It is important for enzyme activation, muscular contraction and relaxation, neuronal activity and neurotransmitter release, and cardiovascular activities (Swaminathan, 2003). The important interaction between phosphate and magnesium ions makes magnesium essential to the basic nucleic acid chemistry of all cells of all known living organisms. It has a stabilizing function for the structure of DNA and RNA chains. More than 300 enzymes require magnesium ions for their catalytic action, including all enzymes using or synthesizing ATP and those that use other nucleotides to synthesize DNA and RNA. The ATP molecule is normally found in a chelate with a magnesium ion (Romani, 2013). Copper proteins play diverse roles in biological electron transport and oxygen transportation. In mitochondria it is found in cytochrome c oxidase, which is the last protein in oxidative phosphorylation. Copper is also found in many

superoxide dismutases, proteins that catalyze the decomposition of superoxides, by converting it to oxygen and hydrogen peroxide (Kumar et al., 2016).

Thus, it is not surprising that the body has developed complex physiological processes to limit the absorption, distribution and excretion of these metals. The regulation of body metal status is achieved by altering the expression of DMT1 and other metal transporters. Metal absorption is increased in that specific metal deficiency and decreased upon overload. Since both uptake and clearance of metals are affected by the concentrations of other metals in the body, altered body metal status could influence the susceptibility to metal toxicity and potentially to mental disorders, and spermatogenesis injuries triggered by Cd can occur by metal imbalance (Menon et al., 2016).

Von Kossa positive staining was observed in collagen fibers in the testicular albuginea, in interstitium area, and in germ cells into the seminiferous epithelium in all exposed animals. Ectopic soft tissue calcification can be separated into five main categories: metastatic, tumoral, calciphylaxis, idiopathic and dystrophic. Metastatic calcification occurs in the setting of malignancy or calcium physiology derangements such as hyperparathyroidism. Tumoral calcification is a familial disorder, and calciphylaxis is characterized by small vessel vasculitis and skin necrosis in case of renal failure. In contrast, idiopathic and dystrophic calcifications occur in case of normal calcium physiology. Dystrophic calcification is found in damaged or necrotic tissues as a result of trauma, infection, or inflammation (Boulman et al., 2005). The exact mechanism of dystrophic calcification is unknown, but it has been shown to be associated with necrosis as well as apoptosis (Kim, 1995), and can be a protective mechanism against Cd injuries. Calcification in collagen fibers could trigger lysis in these fibers, reducing the total protein in testicular tissue.

Conclusions:

1) Morphometric and stereological alterations in testicular tissue exposed to Cd was dose-dependent
2) Ca and Cd have a positive synergistic concentration in rat testis from 0.86 mg Cd/kg/BW i.p.
3) Magnesium, Iron, Copper, and Selenium have a negative synergistic concentration with Cd in rat testis. Each mineral showed a different threshold for this ratio. At dose 1.1 mg Cd/kg/BW i.p all minerals showed a negative synergistic concentration with Cd.
4) Cd induced calcification in collagen fibers in the testicular albuginea, in interstitium area, and in germ cells into the seminiferous epithelium from 0.67 mg Cd/kg/BW i.p.
5) Cd causes lysis in collagen fiber, reducing total protein from 0.86 mg Cd/kg/BW i.p.

*Supplementary table 1***.** *Morphometric and stereologic analyses of testicular components from rats control and exposed to Cd.*

	Sal	**Cd1**	**Cd2**	**Cd3**	**Cd4**
Seminiferous ephitelium (%)	66.47±3.11	66.68±3.83	66.67±4.54	66.72±3.13	69.91±4.19
Tunica (%)	3.27±0.26	2.95±1.12	3.74±0.59	2.89±0.35	3.13±0.59
Lumen (%)	23.21±3.47	19.27±12.34	9.24±10.70	18.87±8.41	12.78±12.23
Intertubular Volume (µm^3)	0.22±0.02	0.17±0.08	0.31±0.16	0.24±0.09	0.27±0.14
Tubulesomatic Index	0.93±0.02	0.6±0.22	0.51±0.19	0.83±0.21	0.66±0.23
Seminiferous tubule diameter (µm)	308.78±31.5	264.36±28.31	270.6±28.44	275.64±28.43	286.35±17.83
Seminiferous tubule Length (m)	38.87±9.12	28.75±5.69	23.05±6.59	33.75±5.91	28.29±12.63
Seminiferous tubule length/ testis gram (m)	25.32±4.69	33.08±6.24	28.28±5.84	30.08±4.01	26.94±4.67

Sal = control; Cd1 = 0.67 mg Cd/kg; Cd2 = 0.74 mg Cd/kg; Cd3 = 0.86; mg Cd/kg; Cd4 = 1.1 mgCd/kg. Lines with different superscripts indicate statistical difference between values. Data are expressed by mean ± standard deviation (Supplementary data).

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5. FINAL CONSIDERATIONS

1) Morphometric and stereological testicular response to Cd was milder than described in previous studies
2) Testicular tissue alterations triggered by Cd were dose-dependent.
3) Animals showed an individuality of response when exposed under the same conditions and the same doses of Cd. This fact creates a high standard deviation in the data and may be the main factor explaining findings in the literature of different results for similar experiments.
4) Cd, by activation of cytokines and oxidative pathways, triggered apoptosis in germ cells without cellular infiltration in seminiferous epithelium and damage in the blood testis barrier, which is confirmed by the absence of inflammatory cells in adluminal environment of seminiferous epithelium and alterations in lipid peroxidation markers.
5) The mechanism by which Cd, in seven days, caused the inflammatory process is different from the caused by bacteria, because Cd maintains prolonged neutrophilia without changing the number of macrophages and mast cells in intertubule. Besides, inflammatory cells were not present in the adluminal environment of seminiferous tubules.
6) Cd caused a dose-dependent reduction in Zn levels on testicular tissue, triggering a reduction in volume of Leydig cells, from a specific Cd dose exposition, resulting in apoptosis of germ cells and DNA damage from this dose.
7) Decreasing in testosterone concentrations occurred at lower doses levels than alterations in Leydig cells stereological parameters, and Zn tissue levels. These testosterone reductions were not able to trigger apoptosis in germ cells and DNA damage.

8) Cd altered mineral dynamics depending on the mineral involved: Calcium and Cd have a positive synergistic concentration, and Magnesium, Iron, Copper, and Selenium have a negative synergistic concentration with Cd in rat testis
9) Cd induced calcification in collagen fibers in the testicular albuginea, and in interstitium area, besides germ cells into the seminiferous epithelium, decreasing total protein.