



Toxicology of arsenate, arsenite, cadmium, lead, chromium, and nickel in testes of adult Swiss mice after chronic exposure by intraperitoneal route

Francielle de Fátima Viana Santana ^{a,b,*1}, Janaina Da Silva ^{a,c}, Amanda Alves Lozi ^a, Diane Costa Araujo ^a, Luiz Carlos Maia Ladeira ^a, Leandro Licursi De Oliveira ^a, Sérgio Luis Pinto Da Matta ^{a,b}

^a Department of General Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

^b Department of Animal Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

^c Institut national de la santé et de la recherche médicale (Inserm), Institut de recherche en santé, environnement et travail (Irset), Université de Rennes 1, UMR 1085 Rennes, France

ARTICLE INFO

Keywords:

Heavy metals

Toxicology

Spermatogenesis

Testicles

Male infertility

ABSTRACT

Background: Some residues such as the heavy metals cadmium (Cd), lead (Pb), chromium (Cr VI), nickel (Ni), and arsenic (As), this last one in its oxidized forms + 5 (arsenate) and + 3 (arsenite), can cause injuries to human health, so they are currently considered environmental health emergencies. In the testis, heavy metals can cause morphological and functional damage due to constant exposure acting chronically in individuals. Thus, we aimed to determine the toxicological mechanism of As⁺⁵, As⁺³, Cd, Cr VI, and Ni that leads to testicular damage and establish for the first time an order of toxicity among these studied heavy metals.

Methods: Forty-two Swiss mice at reproductive age (140 days) were used, randomly distributed into seven experimental groups ($n = 6$). Exposure to heavy metals was weekly performed, by intraperitoneal route. Group 1 received 0.7 mL 0.9% saline (control), and the other groups received 1.5 mg/ kg of As⁺⁵, As⁺³, Cd, Pb, Cr VI, or Ni, for six weeks.

Results: These studied heavy metals did not accumulate in the testis tissue. However, exposure to Ni induced moderate pathologies in the seminiferous tubules, plus changes in the tunica propria, blood vessels, lymphatic space, and carbonyl protein levels. Cd exposure caused moderate tubular histopathologies and changes in the blood vessels and lymphatic space. Cr VI induced slight tubular histopathologies, changes in the lymphatic space, blood vessels, and SOD activity. Pb and As⁺³ exposure triggered moderate tubular pathologies and changes in the SOD activity and carbonyl protein levels, respectively. Finally, As⁺⁵ induced only slight tubular pathologies.

Conclusion: The testicular histopathologies caused by the studied heavy metals are mainly triggered by changes in testicular oxidative balance. Based on our findings of histomorphological alterations, the toxicity order among the heavy metals is Ni>Cd>Cr(VI)>Pb=As⁺³>As⁺⁵. However, considering oxidative stress results, we propose the following testicular toxicity order for these heavy metals: Ni>As⁺³ > Cd>Cr(VI)>Pb>As⁺⁵. Ni exposure shows the most harmful among the heavy metals to the testis.

1. Introduction

Heavy metals are identified to have the potential to become toxic in biological systems [1]. Among the heavy metals, the toxicology of arsenate (As⁺⁵), arsenite (As⁺³), cadmium (Cd), lead (Pb), chromium (Cr VI), and nickel (Ni) has been the focus of studies due to their use in various sectors as well as some disasters that make these metals

available to the environment. Furthermore, all these heavy metals are included in the Substance Priority List (SPL) from the Toxic Substances and Disease Registry Agency - ATSDR [2], which is a list of prioritization of substances based on a combination of their frequency and their significant potential threat to human health due to their known or suspected toxicity and potential for human exposure. Exposure to these heavy metals is inevitable, since they are abundant in nature and largely

* Correspondence to: Av. Peter Henry Rolfs, s/n - Campus Universitário, Viçosa, Minas Gerais 36570-900, Brazil.

E-mail address: francielle.santana@ufv.br (F.F.V. Santana).

¹ <https://orcid.org/0000-0003-3492-6517>

used in industry, with long-term persistence in the environment [3]. Human contamination generally occurs accidentally through contaminated food and water or inhalation of contaminated air [4]. In addition, road transport is one of the main sources of pollution with heavy metals (such as Cd, Pb, Cr, and Ni) in roadside soils and groundwater, which significantly contributes to the increase of these toxic elements in the food chain and ecosystems due to vehicle emissions [5].

Biological activities can be affected under high-exposure environmental conditions [6]. Systemically, cases of mutation, carcinogenesis, and impact on the metabolism of essential metals and nutrients, as well as on the enzymatic processes have been reported due to exposure to these heavy metals [7–14]. They can also affect the structure and function of cell membranes, and induce oxidative stress impacting cell growth, development, and differentiation which is crucial to reproductive health. Epidemiological studies indicate a temporal decline in male fertility, characterized by poor semen quality and increased reproductive disorders, associated with the increase of heavy metals in the environment due to their extraction and processing, industrial waste, domestic effluents, agricultural inputs, disposal of commercial products, and burning of fossil fuels [15,16].

In the testis, As and Cr VI can induce apoptosis of germ cells and significantly reduce the total number of sperm [17,18]. Furthermore, studies have evidenced that both heavy metals inhibit testosterone production by Leydig cells [19,20]. Likewise, exposure to Cd causes irreversible damage, such as apoptosis of Leydig cells, which impairs the testicular steroidogenic function [21]. Additionally, Cd exposure causes germ cell depletion and induces testicular necrosis [22]. It was also confirmed that exposure to Pb significantly increases the number of apoptotic cells in the testicular parenchyma [23], which can be explained by the excess of reactive oxygen species (ROS) in the testis after exposure to this metal [24]. Moreover, sperm quality after exposure to Ni is prejudiced, as well as the testosterone level resulting from testicular damage [25]. The main testicular impact caused by Ni contamination was increased cell death in the intertubule and decreased seminiferous epithelium [26]. Thus, the determination of the mechanisms by which each heavy metal acts and establishing the order of toxicity of these metals have become research priorities [8].

This way, heavy metals constitute an important toxicological aspect of male fertility. Therefore, this study aimed to introduce a new approach in the heavy metal area evaluating comparatively, through histomorphometric, stereological, and oxidative status analyses, the toxicological mechanisms of As⁺⁵, As⁺³, Cd, Cr VI, and Ni, after chronic exposure by intraperitoneal route, in the testis of adult mice. In addition, we aimed to determine the gonadal accumulation of these metals and establish an order of toxicity between them, within the scope of male fertility to direct clinical research.

2. Materials and methods

2.1. Animals

Adult male Swiss mice ($n = 42$, 140 days old) were provided by the Central Animal Facility of the Center for Biological and Health Sciences, Federal University of Viçosa (UFV). The animals were kept in individual polypropylene cages at a controlled temperature ($21 \pm 1^\circ\text{C}$) and photoperiod (12–12 h light/dark). The animals received water and a standard rodent diet ad libitum. The research protocol followed the National Council for the Control of Animal Experiments (CONCEA) standards and received the approval of the Ethics Committee on the Use of Animals (CEUA-UFV, protocol 07/2018).

2.2. Experimental design

The animals were weighed and randomly distributed into 7 experimental groups ($n = 6$ animals/group). Exposure to heavy metals was performed in weekly doses by intraperitoneal route (*ip*) for 42 days,

totalizing six doses. Group 1 received 0.7 mL of 0.9% saline solution (control), group 2 received 1.5 mg/kg of As⁺⁵ (6.24 mg/kg of HAs-Na₂O₄·0.7 H₂O), group 3 received 1.5 mg/kg of As⁺³ (2.60 mg/kg of AsNaO₂), group 4 received 1.5 mg/kg of Cd (2.45 mg/kg of CdCl₂), group 5 received 1.5 mg/kg of Pb (2.45 mg/kg of (CH₃COO)₂Pb 0.3 H₂O), group 6 received 1.5 mg/kg of Cr VI (2.88 mg/kg of CrO₃), and the group 7 received 1.5 mg/kg of Ni (6.07 mg/kg of Cl₂Ni 0.6 H₂O) for six weeks.

The dose chosen was based on an experimental study performed by our team, in which the lowest single dose of 0.67 mg/kg Cd *ip* was able to cause testicular damage [27]. A double dosage, equivalent to approximately 1.5 mg/kg, was used to make sure that testicular damage would be induced at least by one of the heavy metals in order to perform a comparative toxicological evaluation between these heavy metals at the same dose. Although the *Ip* route is not the main route of exposure in nature [21], it is generally used in animal experiments to avoid degradation and modification of the administered substance through the gastrointestinal tract. Furthermore, the vast blood supply of the peritoneum facilitates the absorption of substances and causes the total transfer of the compound from the peritoneal cavity to systemic blood circulation [28]. The period of 42 days was established because it corresponds to the complete duration of spermatogenesis in mice [29] and allows the observation of chronic changes and the process of accumulation of heavy metals.

At the end of the experiment, the animals were weighed, anesthetized with 30 mg/kg thiopental *ip*, and euthanized by cardiac puncture. Then, after the scrotal incision, the testes were dissected and weighed. Finally, the right testis was frozen at –80°C for analysis of the oxidative status and the left testis was destined for histological processing and determination of heavy metal accumulation, fixed in Karnovsky's solution [30].

The albuginea was removed from the fixed testis and weighed to determine the weight of the testicular parenchyma. Based on body weight, the gonadosomatic index (GSI) and parenchymosomatic index (PSI) were calculated using the testicular and parenchymal weights, respectively, according to Mouro et al. [21].

The animals were randomly selected for outcome assessment to avoid the risk of bias and increase the methodological quality in all the analyses described below. Furthermore, the outcome assessors were blinded to which intervention each animal received.

2.3. Histological processing

Testis fragments intended for study under light microscopy were dehydrated in increasing concentrations of ethanol, included in 2-hydroxyethyl methacrylate (Historesin®, Leica), sectioned at a thickness of 3 µm and stained with toluidine blue/ sodium borate 1%. Preparations were mounted with Entellan® (Merck, Frankfurt, Germany). Tissue images were obtained on an Olympus AX-70 microscope and analyzed using the ImageJ software.

2.4. Testicular histopathology

For the testis histopathological evaluation, 200 tubules per animal were recorded and classified into four categories, adapted from the Johnsen index [31]: 1) Normal - tubules with germ cells arranged at their normal localization site and few vacuoles; 2) Slight pathologies - vacuoles at the base and/or apex of the seminiferous epithelium; 3) Moderate pathologies - tubules with a detachment of the seminiferous epithelium and even tubules with only basal cells; 4) Severe pathologies - tubules with only Sertoli cells and even tubules without Sertoli cells and germ cells).

2.5. Tubular morphometry and stereology

The proportion of the testicular elements was estimated by counting

5.320 points per animal, using a reticulum with 266 intersections (points), at 200X magnification, onto 20 randomly distributed fields in the histological preparations.

Points were recorded on the seminiferous tubule, specifically on the seminiferous epithelium, tunica propria, lumen, and the intertubular compartment. The proportion of these components and the volume of the seminiferous tubule and intertubule, as well as the elements that compose them, were calculated according to Mouro et al. [21]. Briefly, the proportion of each component was determined by the ratio between the total number of counted points and the number of counted points at the component. The volume of each component was also calculated by multiplying the proportion of each component by the testicular parenchyma volume. The testis volume was considered equal to the testis weight, as it is known that the testicular density is close to 1 in mammals [32]. Based on the body weight and seminiferous tubule volume, we calculated the tubulesomatic index (TSI) [33]. Based on the body weight and seminiferous epithelium volume, we calculated the epithelium-somatic index (ESI) [34].

The mean tubular diameter per animal was obtained based on the random measurement of 20 cross-sections of seminiferous tubules with the most circular outline possible, without considering the stage of the seminiferous epithelium cycle, according to Mouro et al. [21]. The height of the seminiferous epithelium was measured from the tunica propria to the tubular lumen in the 20 cross-sections used to obtain the tubular diameter. The height of the seminiferous epithelium was considered, in each tubule, by the mean of two diametrically opposite measurements. The diameter of the tubular lumen was calculated by subtracting the height of the epithelium from the diameter of the seminiferous tubule. The seminiferous tubule and lumen areas were calculated according to the following equation: area = $\pi \cdot R^2$, in which R is the tubular radius or the lumen radius, respectively. The epithelium area was obtained after subtracting the luminal area from the tubular area. The tubule epithelium ratio was calculated by dividing the tubule area by the epithelium area. Finally, the total length of the seminiferous tubules was estimated based on the knowledge of the volume occupied by the seminiferous tubules in the two testes and the mean of the seminiferous tubule area [35].

2.6. Intertubular morphometry and stereology

The proportion of intertubular elements in the testis was estimated by counting 1.000 points per animal, using a reticulum with 540 intersections (points), at 400X magnification, in fields randomly distributed in the histological preparations of each testis of the animals. Points were recorded on the components of the intertubular compartment, such as Leydig cells (nucleus and cytoplasm), blood vessels, lymphatic space, connective tissue, and macrophages. As described by Mouro et al. [21], the proportion of each component was determined by the ratio between the total number of counted points and the number of counted points at the component. The volume of each component was also calculated by multiplying the proportion of each intertubule component by the testicular parenchyma volume.

The mean nuclear diameter of Leydig cells was measured at 400x magnification, onto 30 nuclei for each animal. The measured nuclei were the most spherical, with perinuclear chromatin and evident nucleoli. The nuclear volume of a single Leydig cell was obtained using the equation for calculating the volume of a sphere ($4/3 \pi R^3$), in which R is the nuclear diameter. The cytoplasmic volume of a single Leydig cell was estimated by multiplying the cytoplasm percentage by the nuclear volume and dividing it by the nuclear percentage. Finally, the single Leydig cell volume was calculated by adding the nuclear and cytoplasmic volumes.

The volume of Leydig cells in the testes was calculated by multiplying the proportion of Leydig cells in the testicular parenchyma by the weight of the parenchyma of the two testes. The volume of Leydig cells, per gram of testis, was calculated by the ratio between the volume that

the Leydig cells occupy in the testes, and the weight of the testes [34]. The Leydigosomatic index (LSI) was calculated [35] based on the volume of Leydig cells and body weight.

The number of Leydig cells per testis was also estimated based on the individual volume of the Leydig cell and the total volume occupied by these cells in the testis. Finally, this value was divided by the gonadal weight to determine the number of Leydig cells per gram of testis, thus allowing comparisons among different species [35].

2.7. Oxidative status analyses

The frozen testes were homogenized in potassium phosphate buffer (pH 7.4) in the proportion of 100 mg of organ per 1000 µL of a buffer. The suspension was centrifuged (12000 rpm at 4°C for 10 min), and the supernatant was used to determine the activity of antioxidant enzymes, the concentration of nitric oxide, malondialdehyde, and the carbonyl protein content.

The enzymatic activity of superoxide dismutase (SOD) was determined based on the ability of this enzyme to catalyze the reaction of superoxide O₂⁻ in H₂O₂ and, thus, decrease the auto-oxidation ratio of pyrogallol [36]. The enzymatic activity of catalase (CAT) was measured according to Goth [37] and Korolyuk et al. [38], and modified by Hadwan and Abed [39]. The method is based on the reaction of hydrogen peroxide with ammonium molybdate to produce a yellowish color. The enzymatic activity of glutathione S-transferase (GST) was measured according to Habig et al. [40] by the formation of the glutathione-2,4-dinitrobenzene (CDNB) conjugate.

Oxidative and nitrosative stress markers such as malondialdehyde (MDA), nitric oxide (NO), and carbonyl protein (CP) were measured. The MDA concentration was accessed by adding the TBARS solution (15% trichloroacetic acid, 0375% thiobarbituric acid, and 0,25 M hydrochloric acid) to the homogenate and kept for 40 min in a water bath, at 90°C. The substances that react to thiobarbituric acid are mostly products of lipid peroxidation, and MDA is an important marker to verify the rate of lipoperoxidation [41]. To quantify NO, the Griess Reactive that reacts with nitrite was incubated with the sample, and the concentration of nitric oxide was determined using a standard curve with known concentrations of sodium nitrite [42,43]. The CP content was measured using the method described by Levine et al. [44], which uses the reaction of the carbonyl groups with 2,4-dinitrophenyl-hydrazine (DNPH), which promotes better specificity and sensitivity to the analysis. The values of enzyme activities SOD and CAT and the oxidative marker MDA were normalized by the total protein levels in testicular tissue, which were determined by using the Bradford [45] method.

2.8. Determination of heavy metals accumulation

The accumulation of metals As, Cd, Cr VI, and Ni was determined in testicular fragments fixed and dried at 60 °C, for 96 h. The samples were coated with carbon (Q150 T quorum, East Grinstead, West Sussex, England, UK), at the UFV Microscopy and Microanalysis Nucleus, and analyzed by energy-dispersive X-ray spectroscopy (EDS) and in a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Germany), with an X-ray detector system (Tracor TN5502, Middleton, WI, USA) [46], at the Scanning Electron Microscopy Laboratory of the UFV Department of Physics.

2.9. Statistical analysis

Statistical analysis was performed comparing exposure to the heavy metals with the control group. The studied data were submitted to normality assessment using the Shapiro-Wilk test, and, in sequence, those with normal distribution were compared using analysis of variance (ANOVA) followed by the Student Newman-Keuls (SNK) post hoc method. Nonparametric data were compared using the Kruskal-Wallis test. The GraphPad Prism software v8.0.1 (GraphPad Software, USA)

was used considering a significance level of $p \leq 0.05$. The results were expressed as mean \pm standard deviation (mean \pm SD).

3. Results

3.1. Biometric parameters

No significant differences were observed in the body, testes, alboginea, or parenchyma weights after chronic administration of As^{+5} , As^{+3} , Cd, Cr VI, and Ni. Furthermore, the GSI and PSI were not affected by the exposure, as shown in Table 1.

3.2. Testicular histopathology

The percentages of normal seminiferous tubules and seminiferous tubules with slight, moderate, and severe pathologies in the testis after administration of heavy metals As^{+5} , As^{+3} , Cd, Cr VI, and Ni are presented in Fig. 1. There was a significant increase in the frequency of abnormal seminiferous tubules and seminiferous tubules with slight pathologies after chronic administration of all studied heavy metals. The moderate pathologies also increased after the exposure to As^{+3} , Cd, Pb, and, Ni. There were no significant changes regarding severe pathologies in the testes of animals exposed to metals compared to the control group. Representative images of the testicular sections from the control and heavy metals-exposed animals, showing the pathologies observed in the epithelium of seminiferous tubules, are presented in Fig. 2.

3.3. Tubular morphometry and stereology

Regarding the proportion and volume of the elements that compose the seminiferous tubules, no significant changes were found in the tubule, seminiferous epithelium, or lumen, considering both of these parameters. However, the volume of tunica propria increased around 51% in the group contaminated with Ni (Fig. 3). The TSI, ESI, seminiferous epithelium height, tubular diameter, lumen diameter, length of seminiferous tubules, seminiferous tubule, lumen, epithelium areas or tubule-epithelium ratio were not significantly affected either (Sup. Table 1).

3.4. Intertubular morphometry and stereology

The proportion of intertubule and intertubular components (nucleus and cytoplasm of Leydig cell, blood vessels, lymphatic space, macrophages, and connective tissue) are presented in Table 2. The intertubule proportion was not significantly affected by chronic exposure to heavy metals. Regarding the intertubular components, the blood vessel proportion decreased around 51%, 44%, and, 52% in the groups contaminated with Cd, Cr VI, and Ni, respectively. In addition, the lymphatic space proportion increased by around 81% in the animals exposed to Cd, around 23% in the animals exposed to Cr VI, and 82% in animals exposed to Ni. The proportions of the other intertubular components presented no significant variations after the chronic administration of heavy metals used in this study.

Table 1

Biometric parameters of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni).

Parameters	Sal	As^{+5}	As^{+3}	Cd	Pb	Cr (VI)	Ni
Body Weight (g)	45.23 ± 7.78^a	42.18 ± 4.35^a	43.69 ± 6.46^a	43.46 ± 4.52^a	46.51 ± 2.36^a	43.51 ± 6.17^a	46.81 ± 4.75^a
Testicular Weight (g)	0.20 ± 0.03^a	0.18 ± 0.04^a	0.17 ± 0.06^a	0.19 ± 0.01^a	0.22 ± 0.03^a	0.18 ± 0.03^a	0.23 ± 0.03^a
Albuginea Weight (g)	0.05 ± 0.02^a	0.03 ± 0.01^a	0.03 ± 0.01^a	0.04 ± 0.00^a	0.06 ± 0.02^a	0.05 ± 0.03^a	0.03 ± 0.01^a
Parenchyma Weight (g)	0.15 ± 0.02^a	0.15 ± 0.03^a	0.14 ± 0.05^a	0.15 ± 0.01^a	0.16 ± 0.05^a	0.13 ± 0.05^a	0.20 ± 0.03^a
Gonadosomatic index (%)	0.44 ± 0.06^a	0.43 ± 0.05^a	0.39 ± 0.10^a	0.45 ± 0.08^a	0.47 ± 0.07^a	0.41 ± 0.09^a	0.50 ± 0.07^a
Parenchymosomatic index (%)	0.34 ± 0.06^a	0.35 ± 0.04^a	0.31 ± 0.06^a	0.36 ± 0.08^a	0.33 ± 0.10^a	0.33 ± 0.10^a	0.48 ± 0.08^a

Sal - Saline (control). Data expressed as mean \pm SD. Same letters in the same row, for each evaluated parameter, indicate no significant difference between the heavy metal groups and the control by Student Newman Keuls test (parametric data) or Kruskal-Wallis (non-parametric data) ($p \leq 0.05$)

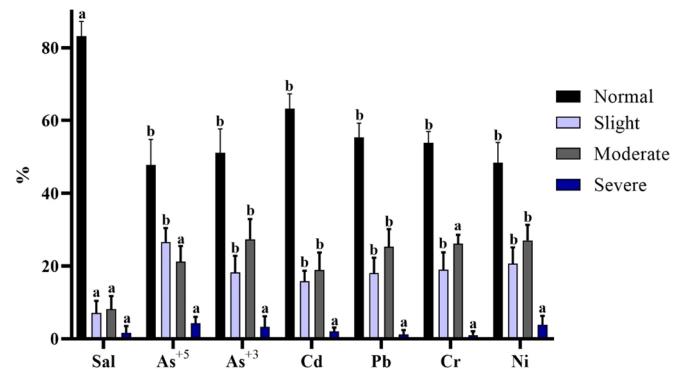


Fig. 1. Percentage of normal seminiferous tubules and seminiferous tubules with pathologies in the Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni). The seminiferous tubules with pathologies were grouped according to the presence of slight pathologies (vacuoles at the base and/or apex); moderate pathologies (detachment of seminiferous epithelium and tubules only with basal cells); and severe pathologies (tubules with only Sertoli cells and tubules without Sertoli cells or germ cells). Data expressed as mean \pm SD. Different letters indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis test (non-parametric data) ($p \leq 0.05$). Sal - Saline (control).

The volumes of the intertubule and intertubular components are presented in Table 3. The intertubule volume was not significantly affected by chronic exposure to heavy metals. However, the lymphatic space volume in the testis has increased by around 135% after chronic exposure to Ni and around 86.6% after exposure to Cd. The other intertubular component volumes were not significantly altered after exposure to the heavy metals compared to the control group.

The morphometric and stereological parameters of Leydig cells and the LSI did not present significant differences after exposure to the heavy metals compared to the control group (Tab. Sup. 2).

3.5. Oxidative status analyses

Regarding the enzyme activities, the superoxide dismutase (SOD) activity increased by around 51% in the group contaminated with Pb and 62% with Cr VI (Fig. 4). However, catalase (CAT) and glutathione-S-Transferase (GST) activities did not change significantly after chronic exposure to the heavy metals used in this study. Furthermore, no oxidative stress markers, such as malondialdehyde (MDA), or the nitrosative stress marker, nitric oxide (NO), were significantly affected after exposure to the heavy metals, compared to the control group. In contrast, the carbonyl protein content (CP) increased by 42% after contamination with As^{+3} , and 114%, after contamination with Ni (Fig. 5).

3.6. Determination of heavy metals accumulation

The concentrations of the heavy metals As^{+5} , As^{+3} , Cd, Cr VI, and Ni

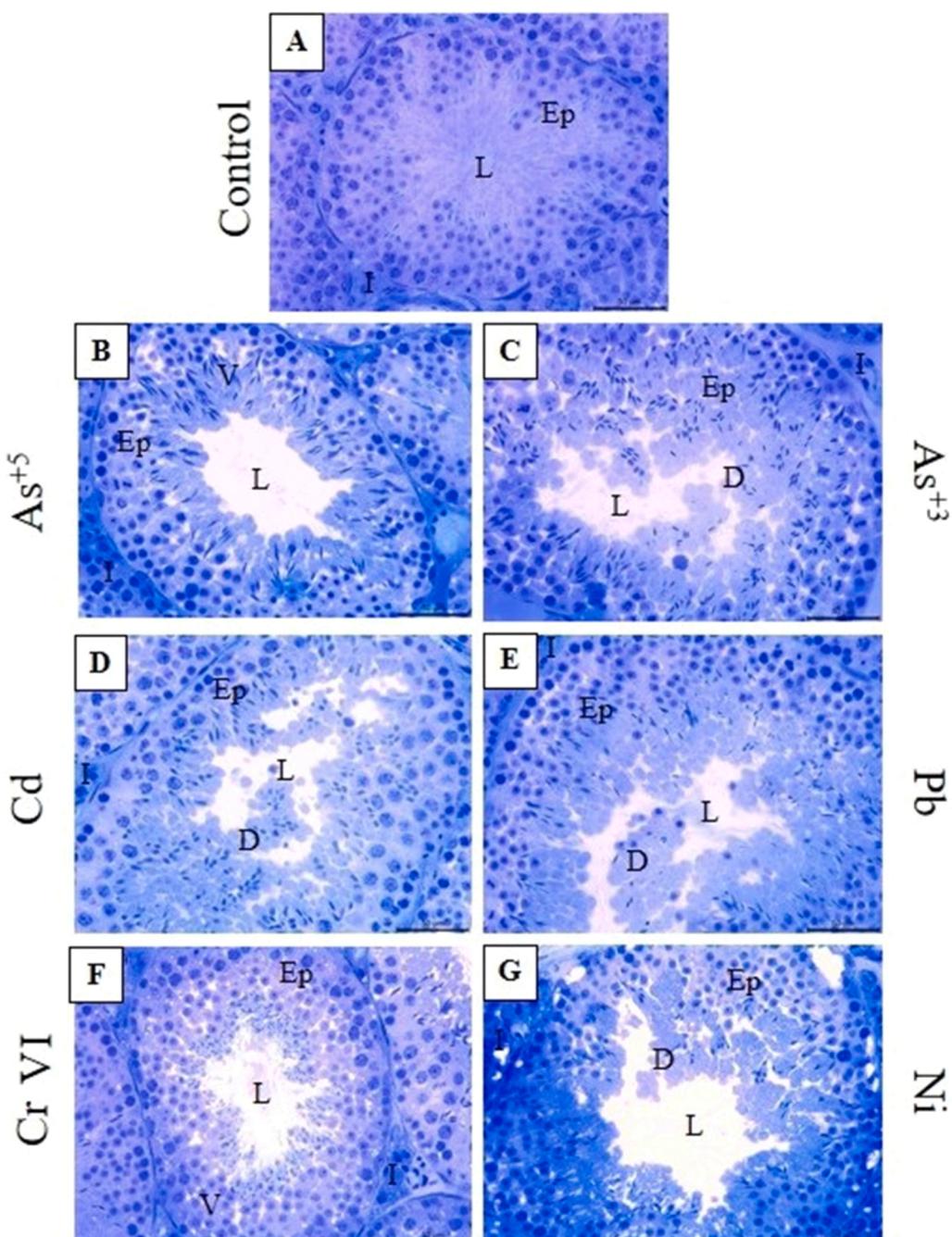


Fig. 2. Representative images of the histological sections from the testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI), and nickel (Ni). Section of 3 μm with toluidine blue staining. Scale bars = 50 μm . L – Lumen, Ep - Epithelium, I – Intertubule, V- Vacuoles, D- Detachment of seminiferous epithelium.

in the testis tissue are presented in Fig. 6. The weekly administration of the studied heavy metals by *ip* route for 42 days did not cause a significant accumulation of these heavy metals in the testes of the animals when compared to the control group.

4. Discussion

We present for the first time comparative data about testicular changes caused by chronic *Ip* exposure to the heavy metals As^{+5} , As^{+3} , Cd, Cr VI, Pb, and Ni, as well as the established order of toxicity among them. The results show that the exposition to the studied heavy metals causes slight histopathological damage in the seminiferous tubules and especially the heavy metals As^{+3} , Cd, Pb, and Ni induce moderate

histopathological alterations in the seminiferous tubules. Furthermore, Cd, Cr, and Ni alter the testicular tissue morphology by increasing the lymphatic space and decreasing the blood vessels, identified by morphometric analyses. Regarding the oxidative status, our findings reveal that Cr increases the SOD activity and As^{+3} and Ni increase the CP levels in the testicular tissue.

Although bioaccumulation was not verified by energy-dispersive X-ray spectroscopy (EDS) analysis, it was detected damage caused by the heavy metals. It is important to observe that the cumulative nature of heavy metals differs individually for several factors. Abiotic and biotic parameters can affect the rate at which organisms accumulate heavy metals in their tissues and organs [47]. An explanation for the results found in this study may be the low dose adopted to compare the metals

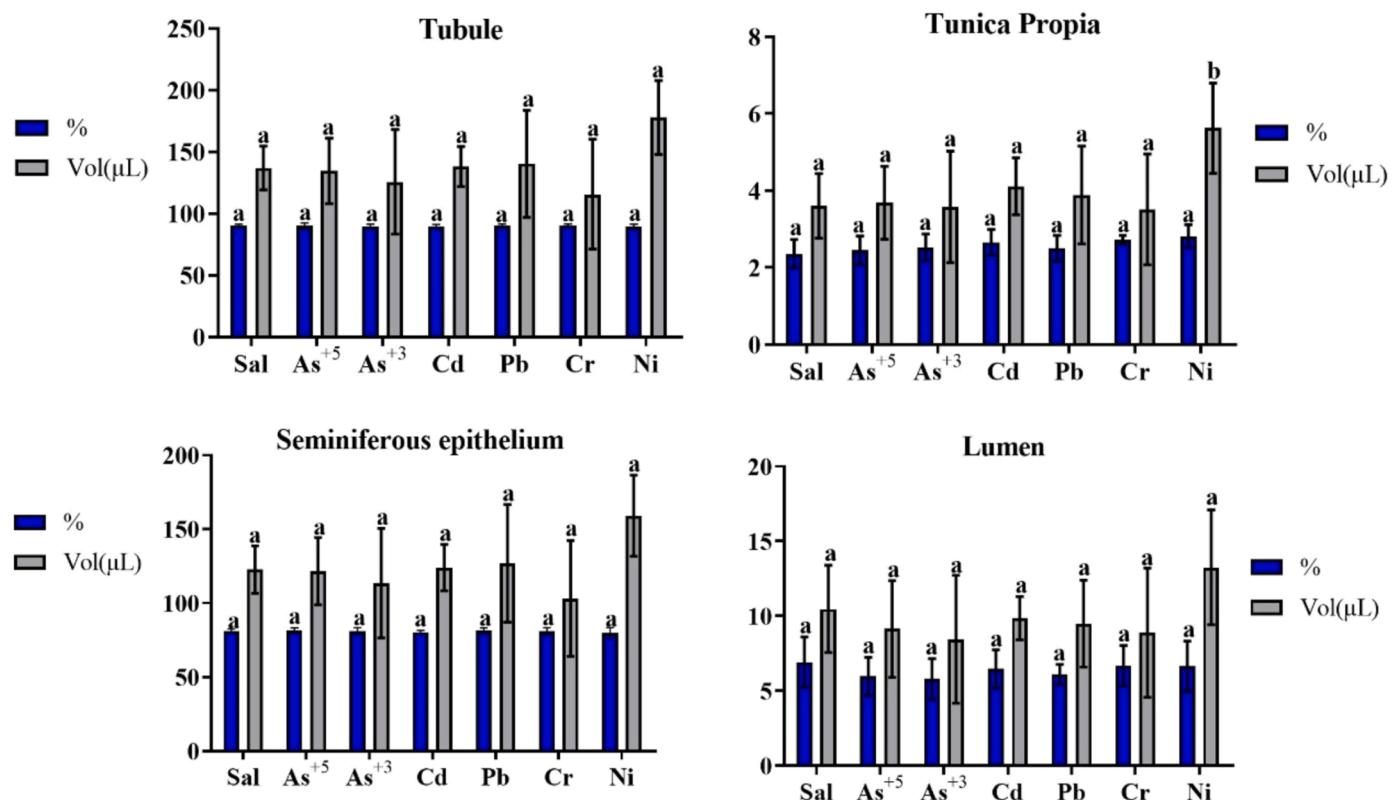


Fig. 3. Proportion (%) and volume (μL) of tubule, seminiferous epithelium, lumen, and tunica propria in the testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI), and nickel (Ni). Data expressed as mean \pm SD. Different letters indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis test (non-parametric data) ($p \leq 0.05$).

Table 2

Proportion of Intertubule and intertubular components of the testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni).

Parameters	Sal	As^{+5}	As^{+3}	Cd	Pb	Cr (VI)	Ni
Intertubule (%)	9.60 ± 0.87^a	10.18 ± 2.54^a	10.51 ± 1.86^a	10.43 ± 1.51^a	10.05 ± 1.66^a	9.53 ± 1.14^a	10.58 ± 1.97^a
Blood vessels (%)	0.96 ± 0.29^a	0.80 ± 0.21^a	0.64 ± 0.29^a	0.47 ± 0.09^b	0.73 ± 0.15^a	0.54 ± 0.13^b	0.46 ± 0.09^b
Lymphatic space (%)	1.38 ± 0.14^a	1.78 ± 0.62^a	1.67 ± 0.39^a	2.50 ± 0.65^b	1.94 ± 0.54^a	1.70 ± 0.15^b	2.51 ± 0.91^b
Leydig nucleus (%)	1.46 ± 0.24^a	1.59 ± 0.49^a	1.87 ± 0.66^a	1.51 ± 0.29^a	1.42 ± 0.32^a	1.55 ± 0.24^a	1.57 ± 0.48^a
Leydig Cytoplasm (%)	5.49 ± 0.74^a	5.68 ± 1.62^a	6.02 ± 1.39^a	5.55 ± 0.93^a	5.62 ± 0.97^a	5.45 ± 0.81^a	5.65 ± 0.86^a
Leydig cells (%)	6.95 ± 0.81^a	7.27 ± 1.94^a	7.88 ± 1.90^a	7.07 ± 1.12^a	7.04 ± 1.21^a	7.01 ± 0.95^a	7.22 ± 1.16^a
Connective tissue (%)	0.31 ± 0.08^a	0.31 ± 0.14^a	0.31 ± 0.06^a	0.38 ± 0.11^a	0.33 ± 0.09^a	0.39 ± 0.19^a	0.38 ± 0.13^a
Macrophages (%)	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a

Sal - Saline (control). Data expressed as mean \pm SD. Different letters in the same row indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis (non-parametric data) ($p \leq 0.05$).

Table 3

Volume of intertubule and intertubular components of the testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni).

Parameters	Sal	As^{+5}	As^{+3}	Cd	Pb	Cr (VI)	Ni
Intertubule (μL)	14.56 ± 2.42^a	14.72 ± 2.05^a	14.63 ± 4.57^a	16.13 ± 3.29^a	15.22 ± 3.81^a	12.59 ± 5.42^a	20.84 ± 4.20^a
Blood vessels (μL)	1.46 ± 0.50^a	1.20 ± 0.41^a	0.87 ± 0.43^a	0.73 ± 0.19^a	1.17 ± 0.51^a	0.72 ± 0.38^a	0.90 ± 0.08^a
Lymphatic space (μL)	2.10 ± 0.47^a	2.56 ± 0.71^a	2.43 ± 1.12^a	3.92 ± 1.28^b	2.96 ± 0.91^a	2.20 ± 0.90^a	4.94 ± 1.78^b
Leydig nucleus (μL)	2.23 ± 0.59^a	2.31 ± 0.59^a	2.57 ± 0.95^a	2.33 ± 0.50^a	2.10 ± 0.43^a	2.05 ± 0.99^a	2.70 ± 0.33^a
Leydig Cytoplasm (μL)	8.27 ± 1.25^a	8.20 ± 1.38^a	8.31 ± 2.71^a	8.55 ± 1.67^a	8.48 ± 2.03^a	7.08 ± 2.91^a	11.17 ± 2.12^a
Leydig cells (μL)	10.51 ± 1.71^a	10.50 ± 1.57^a	10.89 ± 3.46^a	10.88 ± 2.05^a	10.59 ± 2.39^a	9.13 ± 3.86^a	14.25 ± 2.74^a
Connective tissue (μL)	0.49 ± 0.17^a	0.45 ± 0.19^a	0.44 ± 0.17^a	0.59 ± 0.16^a	0.50 ± 0.18^a	0.54 ± 0.42^a	0.74 ± 0.23^a
Macrophages (μL)	0.01 ± 0.01^a						

Sal - Saline (control). Data expressed as mean \pm SD. Different letters in the same row indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis (non-parametric data) ($p \leq 0.05$).

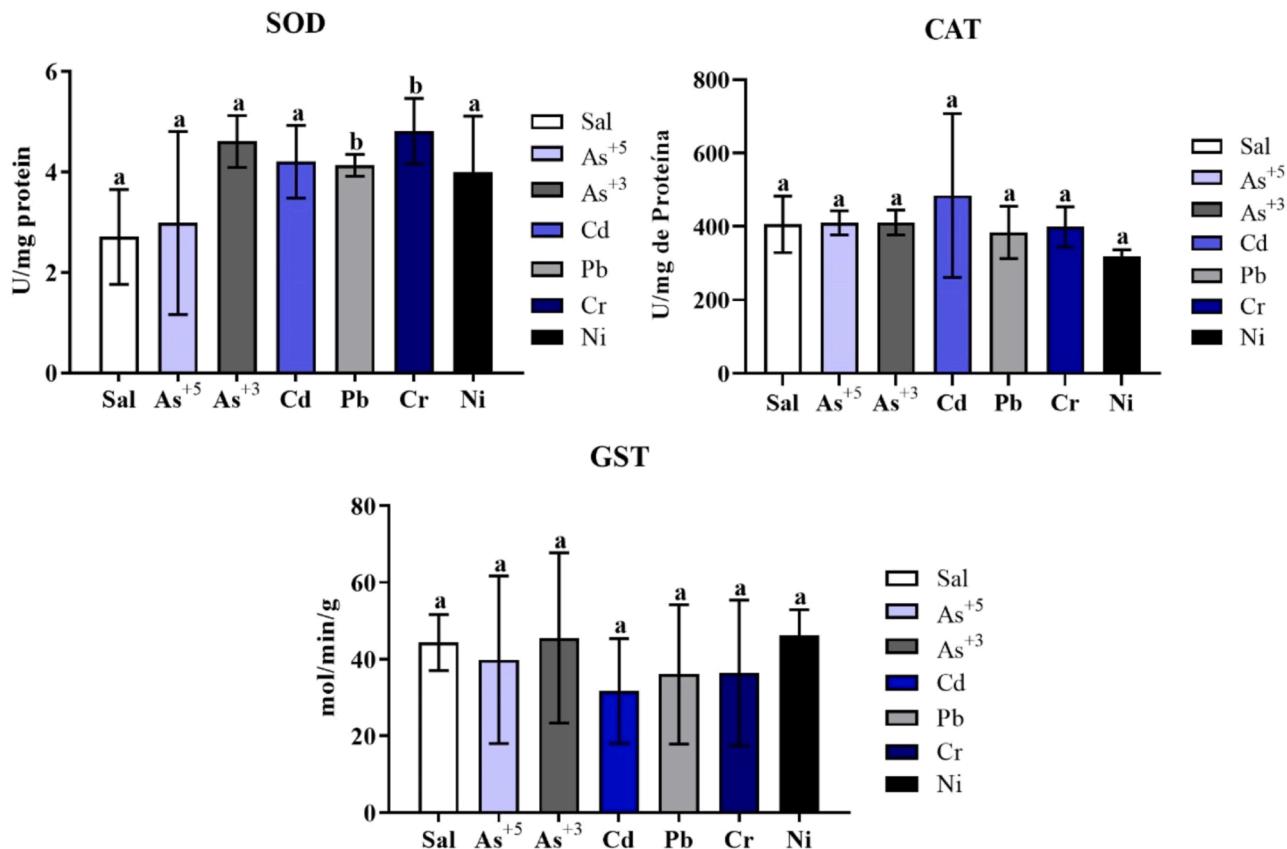


Fig. 4. Activity of antioxidant enzyme activity SOD, CAT, and GST in the testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As^{+5}), arsenite (As^{+3}), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni). Data expressed as mean \pm SD. Different letters indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis test (non-parametric data) ($p \leq 0.05$). Sal – Saline (control).

since studies that used higher doses indicate the bioaccumulation of these elements. Obone et al. [48] verified Ni accumulation in the testis in an experiment with 223.5 mg Ni/L. Oliveira et al. [49] found a considerable accumulation of Cd in the testis after exposure to 3 mg/kg, and Adhikari et al. [21] reported that rats exposed to Pb (5, 10 and, 20 mg/kg for two weeks) presented Pb accumulation in the testis at the highest doses. Massany et al. [25] emphasize that low doses of Cd alter testicular parameters without drastic changes, while moderate doses strongly reduce spermatogenesis. In addition, high doses of Cd cause aberrant morphology in the seminiferous tubules, a markedly low level of spermatogenesis, and other abnormalities in the testicular stroma [27].

The proportion and volume of the seminiferous tubule, seminiferous epithelium, and lumen presented no significant difference with the administered heavy metals, but the volume of tunica propria increased in the animals exposed to Ni. Lozi [50] found similar results using the same heavy metals, while Mouro et al. [21] recorded increased tunica propria after Cd administration at 1.2 mg/kg. Carvalho [51] suggests that, since tunica is part of the blood-testicular barrier, its growth may represent a protective mechanism against the activities of heavy metals. Carvalho [51] reported that the seminiferous tubules underwent an adaptation in the tunica propria and thickened it, to keep the tubular structure intact, when 1.0 mg/L of sodium arsenite was administrated for 42 and 84 days. The same could have happened in our study since it was also a chronic exposure. Nascimento [52] proposes that tunica propria may increase after exposure to heavy metals due to exacerbated production of collagen fibers, as a result of oxidative stress, which may corroborate the results of the present study, since exposure to Ni also induced an increase in CP levels, a marker of oxidative stress.

Our findings show that the proportion of blood vessels decreased in

animals exposed to Cd, Cr VI, and Ni, while the proportion of lymphatic space increased in the same groups and the lymphatic space volume increased in animals treated with Cd and Ni. These results suggest that the reduced percentage of blood vessels was compensated by the increased percentage of lymphatic space, which maintained the structure of the intertubule. According to Da Silva et al. [53], since the lymphatic system is organized in lymphatic sinusoids around the seminiferous tubules, it functions as an exit door for toxic substances present in the tissue. Thus, a proliferation of the lymphatic space may have occurred after exposure to Cd and Ni to maintain testicular homeostasis after intoxication. Another explanation for this change might be associated with the accumulation of reactive oxygen species (ROS) due to exposure to heavy metals. This mechanism can result in inflammation effects and, consequently, increased lymphatic space to deliver immune cells to the damaged tissue for its regeneration [53]. Chen et al. (2022) [54] demonstrated a decrease in the antioxidant enzymes in chicken testes after exposure to mercuric chloride (HgCl_2). The same study also showed the relationship between oxidative stress and the p38 MAPK signaling pathway inducing an inflammatory response that is the cause of many morphological alterations in the testis. Although these alterations in the interstitial tissue, the Leydig cells, responsible for the testosterone production, presented preserved morphology, determined by morphometry.

Oxidative stress is one of the main mechanisms that heavy metals can trigger testicular histopathological alterations and cause degeneration of the seminiferous epithelium and cell detachment into the lumen interrupting the spermatogenic process [55,56]. In our study, the initial vacuolization of the seminiferous epithelium was visualized in all groups exposed to studied heavy metals, and epithelial detachment processes were identified after exposure to As^{+3} , Cd, Pb, and Ni. In line, As^{+3} , Cr,

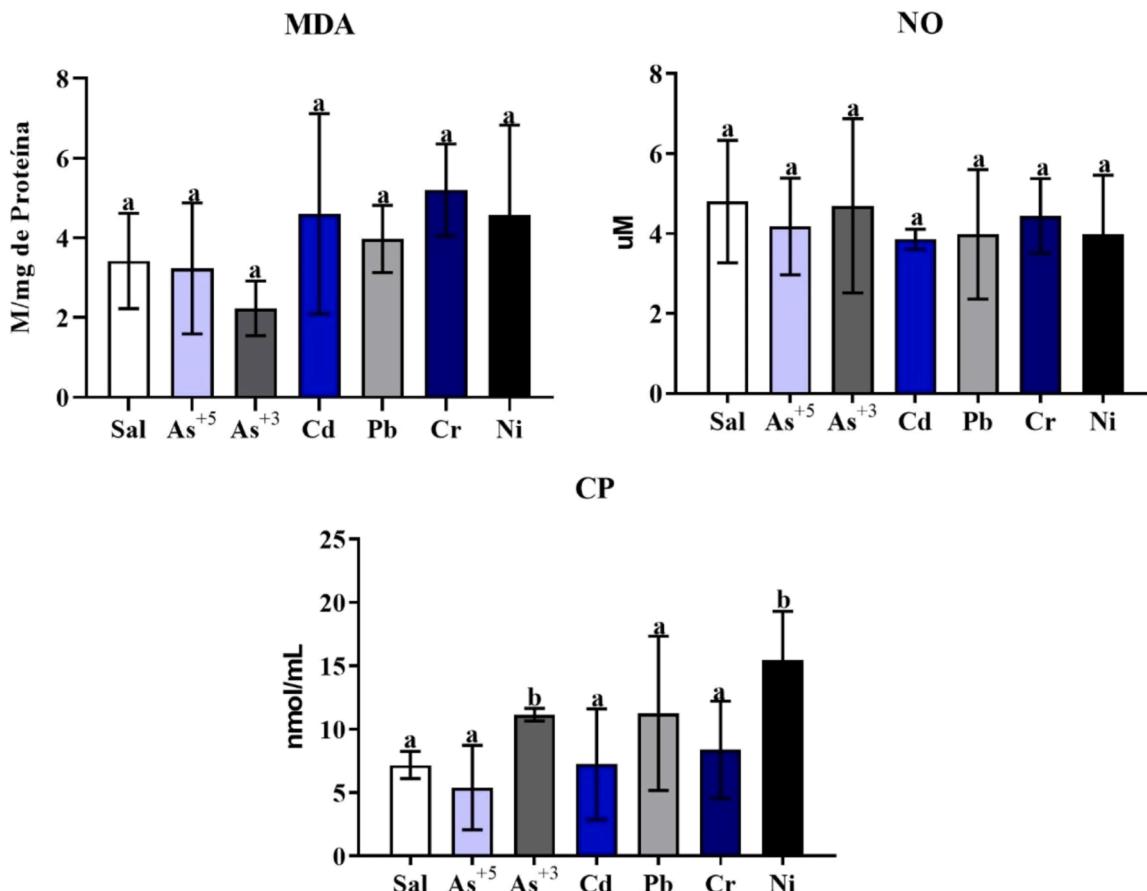


Fig. 5. Oxidative stress markers malondialdehyde (MDA), nitric oxide (NO), and, carbonyl protein (CP) in testis of Swiss mice after chronic intraperitoneal exposure to arsenate (As⁺⁵), arsenite (As⁺³), cadmium (Cd), lead (Pb), chromium (Cr VI) and nickel (Ni). Data expressed as mean \pm SD. Different letters indicate significantly different values compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis test (non-parametric data) ($p \leq 0.05$). Sal – Saline (control).

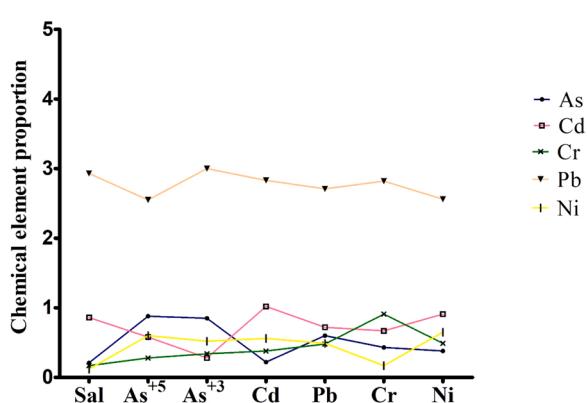


Fig. 6. Proportion of the heavy metals Arsenic (As), Cadmium (Cd), Lead (Pb), Chromium (Cr), and Nickel (Ni) in the testis of Swiss mice, after chronic intraperitoneal exposure. No statistical difference compared to the control group by Student Newman Keuls test (parametric data) or Kruskal-Wallis test (non-parametric data) ($p \leq 0.05$). Sal - Saline (control).

Pb, and Ni altered the testicular oxidative balance by increasing the SOD enzyme activity (Pb and Ni) and CP levels (As⁺³ and Cr VI). SOD is the first cell defense mechanism against the adverse effects of free radicals [57]. This antioxidant enzyme catalyzes the dismutation of superoxide anion radicals (O_2^-) into hydrogen peroxide (H_2O_2), which is degraded by the enzyme CAT into water and oxygen [58]. Mouro et al. (2019) [21] found pathologies in the seminiferous tubules associated with

changes in the activity of antioxidant enzymes after Cd exposure. Chen et al. (2022) showed the relationship between heavy metal-induced oxidative stress and testicular injuries in chickens after $HgCl_2$ exposure, including disorganization and detachment of germ cells. In addition, their study revealed that selenium mitigated $HgCl_2$ -induced testicular injuries by alleviating $HgCl_2$ -induced oxidative stress. Furthermore, CP levels represent another indicator of oxidative stress in the testis caused by heavy metal exposure since proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators [59]. Thus, our results indicate a process of intoxication caused by the administered heavy metals, in which the increase of pathological tubules may be due to an imbalance in the oxidative status.

5. Conclusions

Our findings reveal that although the heavy metals As⁺⁵, As⁺³, Cd, Cr VI, and Ni did not accumulate in the testis after exposure during one cycle of spermatogenesis, they can induce testicular histopathologies by mainly affecting the testicular oxidative balance. Based on our findings of histomorphological alterations, the toxicity order among the heavy metals is Ni>Cd>Cr(VI)>Pb=As⁺³>As⁺⁵. However, including the oxidative stress results, in which Ni and As⁺³ increased the CP levels and Pb and Cr increased the SOD activity, we propose the following testicular toxicity order for these heavy metals: Ni>As⁺³>Cd>Cr(VI)>Pb>As⁺⁵. This way, Ni is pointed out as the most harmful heavy metal for the testis, for the ability to induce pathologies and alter the oxidative status and As⁺⁵ is the least dangerous heavy metal since it

induced only slight tubular pathologies. The data from this study certainly contribute to improving the understanding of the toxicity of heavy metals in reproductive health and might guide future therapies for the appropriate repair of the damage caused by exposure to these metals.

Funding

This research was funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

CRediT authorship contribution statement

Conceptualization, Investigation, Methodology, and Writing – original draft: FFVS, JS and SLPM. Methodology, Investigation, and formal analysis: FFVS, DCA, AAL and LCML. Supervision and Project administration: LLO and SLPM. Writing – review & editing: FFVS, JS, SLPM, DCA and AAL.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Not applicable.

Acknowledgments

The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship provided to Santana, F. F. V. Also, to thank Elizabeth Lopes de Oliveira for receiving and caring for the animals in Animal Facility during its acclimatization period and one more week later and thank the students: João Vitor de Souza Ferreira, Pablo Fernandes Braga, Alex Filipe Ramos de Sousa and Priscila Gonçalves Silva for the help including in the animals' euthanasia process.

Code availability

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jtemb.2023.127271](https://doi.org/10.1016/j.jtemb.2023.127271).

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