

Highlights

- Effects of Cd and Zn on testicular development were assessed.
- Cd accumulation and Zn depletion in testis during lactation were noted.
- Cd-induced abnormal seminiferous tubules and a plasmatic testosterone decrease.
- Zn supply induced a significant protection against Cd toxicity.
- Cd toxicity observed in pups is mediated by disruption of maternal Zn metabolism.

**Protective role of zinc against the toxicity induced by exposure to cadmium during
gestation and lactation on testis development**

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Abstract

To assess the effects of exposure to Cd and Zn on rat testicular development, offspring, from mothers receiving either tap water, Cd, Zn or Cd+Zn during gestation and lactation periods, were observed on gestational day (GD) 20 and on postnatal days (PND) 12, 21 and 35. During gestation, Cd induced maternal hypozincemia and less transfer of Zn to the fetus. During lactation, progressive Cd accumulation and Zn depletion in testis at PND12 and PND21 were noted. An increase of abnormal seminiferous tubules and a decrease in testis weight and plasmatic testosterone concentration **were** also observed at PND21 and PND35 respectively. **Interestingly**, Zn supply induced a significant protection against Cd toxicity. These results suggest that the toxic effects of Cd observed during development are mediated by the disruption of Zn metabolism, which is established in mothers during pregnancy causing Zn deficiency in fetuses and continues to become more pronounced during lactation.

Keywords: Cadmium, Development, Gestation, Lactation, Testis, Zinc.

Introduction

Cadmium (Cd) is a toxic metal and an endocrine disruptor in humans and animals [1-3]. Various organs (e.g. kidney, liver) are affected by Cd and several studies have illustrated that the testis is exceedingly sensitive to Cd toxicity. The lower limit for testicular effects in the rat has been reported by Gunn et al. [1] to be approximately 4 μ mol (0.44 mg Cd/kg body weight). Acute and chronic Cd exposure to adult rats, either orally or subcutaneously (SC), is known to cause severe testicular degeneration, seminiferous tubule damage and necrosis [2, 3]. In addition, Cd induces testicular toxicity, not only when the exposure takes place during adulthood, but also in adult animals exposed to the metal in utero and through lactation, which causes deleterious effects on germ cell population, sperm morphology and motility and an increase in the rate of cell death in the testis [4-7].

Although the damage induced by Cd was recognized decades ago, the precise mechanisms underlying its toxicity remain unclear. The interaction between Cd and essential trace elements, such as zinc (Zn) could be one of the reasons for Cd-induced reproductive toxicity. Zn is ubiquitously distributed in hundreds of cellular proteins and it is required for a variety of biological activities such as growth, development, and reproduction. Both the relatively high Zn concentrations in male reproductive organs and the gonadotoxic effects of Zn deficiency suggest that male fertility may be affected by Zn status. An earlier study showed that zinc deficiency impairs testicular development and steroidogenesis [8].

Cd-Zn interactions in adult testes are well established [9-11]. These interactions could be due to the similarity between these ions and their 'competition' for the physiological binding sites of Zn. Decreased utilization of Zn by spermatogenic cells due to competitive action of Cd may cause disturbance in sperm developing process [10]. It has been reported also that testicular and plasmatic depletion of Zn, under Cd influence, may be a causal factor in Cd-

induced adverse effects on male rat reproductive processes [9, 11]. **On the other hand, it has been shown that Zn can protect against Cd-induced testicular damage in adult rat [11-13].**

Although, Cd and Zn interactions during early life have been reported for several years [14, 15], there is no data concerning the consequence of these interactions on the male reproductive function. In a recent study, we have demonstrated that Cd exposure during lactation decreased significantly the neonatal Zn absorption and lead to Zn redistribution in suckling rat organism by changes in Zn transporter expression profile in the mammary gland of mother rat and in the intestine of their pups [15]. Keeping in view these findings, the current study was conducted to test the hypothesis that the impairment of testicular development under Cd influence could be attributed to Cd-induced disturbance of Zn metabolism in female rats and their offspring. Moreover, we tested the hypothesis that Zn-supplementation could protect against Cd-induced gonadotoxic effects.

Materials and Methods

1. Animals and experimental design

Parental generation of male and female *Wistar* rats, purchased from a local supplier (CYPHAT, Tunisia), was subjected to 2-week acclimatization period. The animals were housed in individual stainless steel cages at $23 \pm 1^\circ\text{C}$ and exposed to 12-hour light-dark cycle. They had access to a standard rodent laboratory diet and drinking water *ad libitum*. The animals were housed according to the EEC 609/ 86 Directives regulating the welfare of experimental animals.

After acclimatization, male and female rats were mated to obtain the first-generation offspring. During mating, rats were separated after positive identification of a vaginal sperm plug, after which a designation of gestational day zero (GD0) was made. At GD 0, pregnant rats were housed individually in plastic cages and randomly divided into four groups. A

control group of animals received tap water and three experimental groups received either Cd (50 mg/L Cd as CdCl₂), Zn (60 mg/L Zn as ZnCl₂) or Cd+Zn (50 mg/L Cd + 60 mg/L Zn) in their drinking water during only gestation (Prenatal study) or during gestation and lactation periods (Postnatal study). Cd and Zn doses and manner of administration were chosen on the basis of our previous study [15] and of available literature [16, 17]. The 24-h consumption of drinking water and body weight were monitored during the whole experiment. Drinking water consumption and daily Cd and Zn intake were investigated according to the method described by Brzoska and Moniuszko-Jakoniuk [18]. Two experiments were conducted using the protocol described above. In experiment 1 (n=4 per groups), the dams were sacrificed on GD20 to evaluate the early effects of maternal exposure to Cd and / or Zn on male fetuses. In experiment 2, the dams (n = 8 per group) were allowed to deliver. The day of parturition was designated as post-natal day zero (PND 0) and male pups were sacrificed at PND 12 and 21. Some offspring were weaned at PND 21 and housed in unisexual groups on litter bases with access to laboratory diet and drinking water and sacrificed at PND 35.

Experiment 1: Prenatal study

Pregnant rats were treated from GD0 to GD20. Their water consumption and body weight was monitored throughout pregnancy. On GD20, the dams were killed under ether anesthesia and uterus and fetuses were removed. Maternal blood was sampled by cardiac puncture in heparinized tube. Plasma was separated from the blood cells by centrifugation at 5000 rpm for 10 min and stored at -20 °C for determination of plasmatic contents of Cd and Zn. The number and body weight of male fetuses were recorded. Placenta, fetal liver and testis were then removed and treated as described below.

Experiment 2: postnatal study

Pregnant rats were treated from GD0 to PND21; their water consumption was monitored throughout pregnancy and lactation. The numbers of live and stillborn pups were recorded for

each litter after delivery on PND0 (live birth index), and the viability from PND0 to 4 and PND0 to 7 (survival index) was determined in each litter. The anogenital distance (AGD) of male pups was recorded on PND4 and PND21. Male pups were weighed and sacrificed under anesthesia at PND12, 21 and 35. Their testes were removed, weighed and treated as described below.

2. Analytical procedures

2.1 Measurement of cadmium and zinc concentrations

Testis and liver destined for Cd and Zn analyses were oven-dried (60 °C) to constant weight. Dried tissues and plasma samples were digested with concentrated nitric acid (Merck, 65%) at 120 °C. When fumes were white and the solution was completely clear, the samples were cooled to room temperature and the tubes were filled to 5 ml with ultra pure water [11]. All samples were analyzed to determine Zn and Cd concentrations using flame atomic absorption spectrometry. These measures were implemented using a ZEE nit 700-Analytik-Jena, Germany equipped with deuterium and Zeeman background correction. Samples were analyzed in triplicate and the variation coefficient was usually less than 10%.

2.2 Histology

Testes were immersed and fixed in Bouin's fixative (75% saturated picric acid, 20% formalin, and 5% acetic acid) for 1 h at GD20, for 6h at PND12 and for 24h at PND21 and 35. They were after that dehydrated in ascending grades of **ethanol** before paraffin inclusion. Testicular sections (5 µm) were obtained, stained with hematoxylin and eosin, and examined under a PrimoStar light microscope (Zeiss) for histopathological evaluation. Photographs were taken using the AxioCam ERc5s camera and the Zen software (Blue Edition 2012).

2.3- Immunohistochemistry:

Testicular sections of 5 μm thickness were taken and processed for immunohistochemistry using the avidin-biotin-peroxidase method provided by the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) as previously described [19]. Primary antibodies were used with the following dilution: 1:200 for the Anti-müllerian Hormone (AMH/MIS, SC-6886, Santa Cruz Biotechnology (Dallas, TX)), 1:100 for the P450 side chain cleavage (P450scc, ABS235, Millipore) and 1:100 for the Heat shock protein 90 (HSP 90, #610419, BD Biosciences, (Mississauga, ON, Canada)). Briefly, sections were incubated with 3% H₂O₂ in distilled water for 10 min to inactivate endogenous peroxidases. The slides were then washed, blocked for 1 hour with 5% normal mouse serum for P450scc and HSP 90 or with 5% bovine serum albumin (BSA) for AMH/MIS followed by an overnight incubation with the primary antibody at 4°C. These primary antibodies were then detected using avidin-biotin-peroxidase complex (Vectastain Elite ABC mice; Vector Laboratories, Burlingame, CA) for P450scc and HSP90 immunostaining or an anti-goat-HRP (SC-2020, Santa Cruz, 1:200) for AMH/MIS. Peroxidase activity was visualized using 3,3-diaminobenzidine as a substrate (DAB Substrate Kit, SK4100, Vector Laboratories) and sections were counterstained with hematoxylin. For all immunohistochemical staining, negative controls were done by omitting the primary antibody.

2.4 Stereological analysis

2.4.1 Seminiferous tubules diameters measure

Tubular diameter was obtained by the random measurement of 50 cross-sections of seminiferous tubules having the most circular contour possible. This was done using the Blue Edition of the Zen software (Zeiss).

2.4.2 Identification and counting of the Leydig cells

Leydig cells were identified by detection of P450scc as previously described. Positive cells were counted in randomly chosen sections through whole testes. At least three different sections were counted per testis, at PND12, 21 and 35 and the number of Leydig cells per section was divided by the corresponding section area measured using the Blue Edition of the Zen software (Zeiss). Results are the average density of Leydig cells per testis surface unit.

2.5. Testosterone extraction and measurement

Each testis was placed in a 12 × 75 mm glass test tube with 100µl of distilled water. One ml (0.5 ml, 2×) of ethyl ether (99% pure) was added and the testis was crushed using a plastic pestle. The homogenized sample was placed in an acetone/dry ice bath until the aqueous portion was frozen. The ethyl ether fraction was poured off into a clean 12 × 75 mm glass test tube and the 2 extractions were pooled and evaporated to dryness in a fume hood overnight. Tubes were parafilm-sealed and kept no longer than 2 weeks at room temperature until quantification of testosterone by Elisa (BioVendor, RTC001R, Medicine laboratories, Czech Republic). Briefly, dried testes extracts were resuspended in 250 µl of extraction buffer provided with the Testosterone Elisa kit. An aliquot (50 µl) of each testis sample was analyzed according to the kit recommendations instructions.

2.6. Statistical analysis

All the data were expressed as mean ± SEM. Differences among the experimental groups were assessed by one-way ANOVA followed by Least Significant Difference test (PLSD Fisher). Values were considered statistically significant when $p < 0.05$.

Results

1. Level of exposure

1.1 Maternal water consumption and Cd/Zn daily intake

Results presented in **Fig. 1** show that overall water consumption of pregnant rats from GD0 to GD21 were significantly lower when water was supplemented with Zn, alone or in combination to Cd. During the lactation period, no difference was observed in water consumption between controls and females exposed to Cd or Zn. However, pregnant rats exposed orally to the combination of Cd and Zn showed significant lower water consumption when compared to the Cd group. Despite the difference in water consumption, daily Cd intakes, during gestation, were within the same ranges of values independently of whether this metal was administered alone (7.88 ± 0.87 mg/kg/24h) or in combination with Zn (5.58 ± 1.01 mg/kg/24h). Also, Zn intakes were within the same ranges of values independently of whether this trace element was administered alone (6.88 ± 0.90 mg/kg/24h) or with Cd (6.70 ± 1.20 mg/kg/24h). The same refers to Cd and Zn intakes during lactation.

1.2 Cadmium and Zinc levels

1.2.1. Cd and Zn levels in maternal plasma, placenta and in male fetal liver

A significant ($p < 0.01$) accumulation of Cd was measured in maternal plasma, placenta and in the male fetal liver of Cd-treated animals in comparison to control rats (**Table 1**). Interestingly, under Cd exposure, the circulating levels of Zn were significantly decreased in pregnant females as well as in male fetal liver when compared to controls. Co-exposure to Cd and Zn resulted in a significant decrease of Cd levels in all tissues but no change in Zn levels when compared to controls. Co-treatment with Cd and Zn resulted in lower levels of plasmatic Cd, but an accumulation of Cd in the placenta and decreased transfer of Cd to the male fetal liver when compared to Cd-exposed animals. As well, the Cd-induced depletion of Zn concentrations in the maternal plasma was reversed in the co-exposure group.

1.2.2. In testis at different ages of development

➤ At GD 20

Fig. 2 shows that Cd is undetectable in the fetal testis at GD 20 of all groups. Interestingly, though, Cd gestational exposure decreased significantly ($p<0.05$) the Zn levels measured in fetal testis. Zn treatment alone or in combination with Cd entirely reversed the Cd induced depletion of Zn concentrations in fetal testes ($p<0.05$).

➤ At PND 12, PND 21 and PND 35

Cd-exposure during gestation and lactation resulted in detectable levels of Cd in the testis at PND12 (Fig. 2). These levels increased from PND12 to PND21 and stayed constant at PND35. Cd treatment also decreased significantly ($p<0.05$) the Zn levels in the testis at different postnatal age except PND 35. When mother rats were concomitantly exposed to Cd and Zn during gestation and lactation, a significant decrease of Cd level in the testis of their pups at PND 12, PND 21 and PND 35 were detected compared with Cd-exposed animals. This treatment also partially reversed the Cd induced depletion of Zn concentrations in the testes at PND 12 ($p<0.05$)

2. Effects on reproductive outcome and offspring development

2.1 Effects on the body weight gain and reproductive outcome

During pregnancy, no significant differences were observed in maternal body weight gain between controls and treated groups. No exposed dams to Cd and/or Zn during gestation presented dystocia or late delivery. The length of pregnancy, litter size, sex ratio, live birth index (LBI) at PND0 and pups survival index (SI) at PND 4 and PND7 were unaffected by exposure to Cd and/or Zn (**Table 2**).

2.2 Effects on male offspring development

2.2.1 Body weight, body size and relative testes weight

As shown in **Table 3**, exposure to Cd did not affect body weights (BW) or size measured in GD20 fetuses or pups at PND12, but significantly decreased ($p < 0.05$) BW and cranio-caudal length (CCL) of the male offspring at PND 21 when compared to the control animals. This difference could not be observed in pre-pubertal animals at PND35. Interestingly, whereas exposure to Zn alone had no effect on weight or size of the progeny, it entirely reversed the Cd-induced weight and length decrease observed at PND 21 in the co-treatment group (Cd+Zn). Indeed, BW and CCL of animals exposed to the combination of Zn and Cd were almost similar to control values at PND 21 and were significantly higher ($p < 0.05$) than in Cd group.

The relative testis weight (RTW) were significantly decreased ($p < 0.05$) in the Cd-exposed group but only at PND 35 when compared to controls. As observed in BW and CCL, exposure to Zn alone had no effect on RTW but entirely reversed the Cd-induced decrease in RTW at PND 35 in the co-exposure group.

2.2.2 AGD and testosterone level

To assess whether androgen production was affected by the gestational exposure to Cd and /or Zn, we measured fetal testicular testosterone content as well as the ano-genital distance (AGD) in male pups at PND4. Measurement of testicular testosterone content was highly variable and did not show any significant difference among groups. Similarly, there was no difference in the AGD measure between the four groups (**Table 2**).

To test if lactation exposure to Cd and /or Zn had an impact on testosterone production in **pre-pubertal** age, the testicular testosterone content as well as the plasmatic concentration of testosterone were evaluated at PND 35. Similarly to what was observed in male **fetus**, no significant differences were observed in the testicular testosterone content at PND 35. Interestingly though, plasmatic testosterone was significantly decreased after exposure to Cd

when compared to control animals (**Fig. 3**). Importantly, whereas exposure to Zn alone had no effect on plasmatic testosterone at PND35, it reversed the Cd-induced decrease in testosterone concentration observed at PND 35 (**Fig. 3**).

3. Histological and immunohistochemical analysis

3.1 Histological and immunohistochemical analysis of fetal testis

Testis morphology at GD20 remained normal and very similar between the four groups (**Fig. 4A**). AMH staining was as expected, in the cytoplasm of Sertoli cells in the four groups (**Fig. 4B**). The signal intensity was not different in any group. HSP90 immunostaining showed the expected immunoreactivity in the cytoplasm of gonocytes in all groups (**Fig. 4C**). Interestingly, we systematically observed a higher HSP90 signal in the cytoplasm of gonocytes from the three treatment groups (Cd, Zn and Cd+Zn)

3.2 Histological and immunohistochemical analysis of testicular postnatal development

Testicular histology, at all postnatal ages analyzed (PND12, 21 and 35), was similar in all groups (**Fig. 5**) and the tubule diameters were unaffected by any exposures (**Table 4**). Moreover, after specific staining of the Leydig cells by P450scc, a normal distribution was observed in the **interstitium**. As well, the number of Leydig cells determined per surface area was not affected by any treatment at any age (**Table 4**).

At PND12, testis morphology remained normal and very similar between the four groups; the tubules are lined by numerous Sertoli cells and spermatogonia, forming a pseudostratified layer (**Fig. 5A**). At PND21, rat testicular sections from control animals showed the expected histological structure of seminiferous tubules with maturation of spermatogenic layers. But, interestingly, a significant increase in the percentage of seminiferous tubules containing a

lumen was noted in the testis of animals exposed to Zn alone or in combination with Cd (**Fig. 5B**). Moreover, at PND35, a significant increase in the percentage of abnormal seminiferous tubules was observed in the testis of animals exposed to Cd or Zn alone (**Fig. 5C**). Seminiferous tubules were considered abnormal when complete or partial loss of germ cells was observed or when the presences of multinuclear giant cells were seen. It is interesting to note that when animals were exposed to both Cd and Zn, the percentage of abnormal tubule was similar to control animal.

Immunohistochemical analysis of HSP90 in control animals showed expression in maturing germ cells. Interestingly, in testes exposed to Cd, a stronger immunoreactivity was observed in the cytoplasm of spermatogonia at PND12 (**Fig. 6 A**) and in spermatocyte at PND21 (**Fig. 6 B**) when compare to controls. Exposure to Zn alone did not affect the expression of HSP90 at any age but importantly, when given with Cd, it reversed the Cd-induced increase in HSP90 quantity at PND 12 and PND 21(**Fig 6**).

Discussion

The hypothesis that Zn can protect testicular tissue against Cd-induced testicular damage in adult rats was established by our and several other previous studies [8, 11, 20]. In addition, studies demonstrated that gestational and lactational exposure to Cd may produce adverse effects in the offspring, resulting in developmental, reproductive and behavioural deficits [21-24]. However, consequences of Cd exposure and protective effect of Zn during pregnancy and lactation on the testicular development and function of the pups are still poorly understood. To our knowledge, the current study is the first to investigate the effect of Cd and/or Zn exposure during gestation and lactation on the development of testis in rat.

In agreement with previous study of García and González [25], our investigation demonstrated that Cd exposure exerted no marked effect on maternal weight gain during gestation or lactation periods. However, Zn exposed animals exhibited lower water consumption in comparison to control and Cd-exposed animals during gestation or lactation period without affecting the daily Cd and Zn intake. We also observed that no maternal death was induced by the treatment, nor did we observe any difference in the gestational length, the number of pups per litter, and sex ratio in the offspring.

It was postulated that the mammalian testis was more sensitive to Cd than other organs and that low doses with no detectable effects on general health can interfere with testis function [26, 27]. In the present study, we first investigated the effects of maternal Cd exposure during gestation and lactation on fetal and early postnatal testis development. We found that Cd exposure did not impair absolute and relative fetal testis weight, which is in contradiction with the results reported by Ji et al. [6] who administered Cd intraperitoneally with CdCl₂ (0.5 mg/kg) daily during late pregnant period (from GD 13 to GD 17). This contradiction can be explained by the difference in the exposure period, route and dosage. Moreover, except the increased expression of HSP 90, no abnormal morphology was observed in fetal testes of Cd-exposed animals. In parallel, we could not measure detectable amount of Cd in fetal testis. In the absence of detectable Cd, the increased expression of HSP 90 may be explained, at least in part, by an indirect mechanism of Cd toxicity such as interference with placental transport of essential element like Zn to the fetus [18, 28, 29]. Indeed, the observed decrease of Zn contents in fetal liver and testis, measured in the present study, could be caused by Cd altered maternal Zn metabolism causing less transfer of this elements through the maternal side of placenta to the fetus by the inhibition of transport proteins [30, 31]. Also, our results show that Cd was accumulated in the placenta and in fetal liver but it was not detected in the fetal testis of Cd exposed-animals. These results suggest

that placenta could deter most of Cd to passing from dams to fetus [32-34]. Importantly, exposure to Cd in utero did not impair fetus growth since the fetal weights and the cranio-caudal length at the end of gestation (GD20) of the control and Cd group were not statistically different. This is in accordance with the study of Salvatori et al. [35], who administered Cd at 20 mg/kg of body weight from GD 6 to GD 14. The Cd dose administered in the present study, considered as low [36], may explain the absence of toxicity in male fetus.

The transfer of Cd through maternal milk represents the primary route of offspring exposure when rodents are exposed during both gestational and lactational periods [33, 37]. Given that at PND 17 is the moment when the offspring for the first time consume the solid food together with milk and at PND 19 the first day of intake of drinking water occurs [38], the present study, in agreement with previous investigations [39, 40], confirms the importance of lactational transfer of Cd. In fact, in Cd and Cd+Zn-exposed animals, Cd was accumulated in the testis at different postnatal ages and reached conspicuous concentrations at PND 21, whereas no Cd could be detected in the control and Zn-exposed animals.

Despite the accumulation of Cd in testes during postnatal development, there is no quantitative changes in rat seminiferous tubules with respect to an eventual seminiferous tubule atrophy (there were no changes in the tubular diameter) or in relation to the amount of Leydig cells (similar to the amount observed in control testes). Excepting the increase in the percentage of tubes with lumen, under Cd and Zn influences, no histological alterations were observed at PND12 or PND21. However, the morphology and structure of the testes were significantly altered at pre-pubertal age (PND35) as evidenced by a decrease in the relative testis weight and in plasma testosterone level, indicating interference of Cd with spermatogenesis and steroidogenesis. Similar results were obtained by Pillai et al. [5] after gestational and lactational exposure to Cd. Reduction in testicular weight has been, partly, attributed to the necrotic and degenerative Cd-induced changes [41,42]. Lower serum

testosterone levels noted in Cd-exposed animals might be due to the direct effect of Cd on Leydig cells [43-45] or to an indirect effect by altering gonadotrophin from pituitary, because Cd have been shown to accumulate in the hypothalamus and the pituitary [46]. Histological alteration at PND 35 and the increased expression of HSP 90 under Cd exposure during gestation and lactation may be attributed not only to the accumulation of the toxic metal in the male gonad during postnatal development, but also to the indirect effect of Cd by interference with Zn metabolism during lactation which leads to Zn depletion in testicular tissue as shown in the present study. In fact, during the lactation period, Cd is thought to be transported from maternal plasma to mammary gland and secreted into breast milk as well as essential element like Zn. Our previous study [15] imply that the downregulation of ZnT as well as the overexpression of ZIP transporters in the mammary gland of lactating rat and in the intestine of their offspring, play a major role in Cd accumulation and Zn redistribution in tissues of suckling rats. Zn testicular depletion during lactation seems also to be due to its sequestration by Cd-induced hepatic metallothioneins (MTs) from the plasma in the organism of offspring rats, thereby increasing its concentration in the liver and kidneys and probably restricting Zn supply to other tissues [18]. Furthermore, the present study showed that Cd-exposure during pregnancy and lactation has important effects on male offspring growth by reducing the body weight and the cranio-caudal length at the end of lactation (PND 21). Accordingly, Luo et al [21] indicated that the increases in body weight and size were delayed by prenatal and lactation exposure to 5 or 10 ppm Cd in male and female offspring. The endocrine and biochemical mechanisms underlying the growth suppression produced by gestational and lactational Cd exposure can be related to decrease in the level of circulating insulin-like growth factor I (IGF-I) [47] due to alterations in the bioavailability of essential metals such as Zn [28]. In contrast, others studies showed that gestational [16] or lactational [37] Cd exposure did not affect the offspring growth. According to available data, it seems that Cd can

reduce [21] or not [16, 37] male offspring growth depending on the experimental protocol. The possible consequences of Cd exposure during pregnancy and lactation on reproductive parameters of the pups at adulthood are still poorly understood. A few studies showed that the exposure to Cd in utero and through lactation adversely affected reproductive parameters of adult male rats and mice as evidenced by compromised sperm morphology and motility and increased rate of cell death in testis as well as by decreased serum and testicular testosterone [4, 6, 7].

In the second part of the present study we investigated the possible protective effect of Zn against Cd toxicity during testis development. Zn is one of the most important nutritional factors influencing the metabolism and toxicity of Cd [18]. The protective effect of Zn in condition of Cd exposure in animals has been demonstrated for many years by our and others research groups [11, 17, 48]. As shown in the present work, the concomitant treatment with Cd and Zn reduced Cd transfer from dams to the fetus during pregnancy and its accumulation in the testis during postnatal development. The obtained results also show that prevention of Cd accumulation and Zn deficiency under Zn supplementation was accompanied by a total protection against Cd damage in the testis at different ages of development. The protective effect of Zn against Cd-induced testicular damage may be due to interaction between the two metals at three major sites: small intestine, liver and testis. In the small intestine, enhanced consumption of Zn may decrease Cd absorption from the digestive tract and its accumulation in the offspring rat's organism, and as a result, it may protect against the toxic effects of Cd [15]. In the liver, interaction between Cd and Zn to induce MTs which sequesters Cd had been suggested as the most likely mechanisms [18]. Pre-treatment with Zn leads to rapid immobilization of Cd ions in the liver (in the form bound to the Zn-induced MTs) resulting in fewer of the ions present in the bloodstream for transport into testes [49]. Alternatively, testes produce less MTs in response to Cd accumulation than liver. Therefore, this might account for

the higher susceptibility of testes to Cd toxicity [50, 51]. The protective effect of Zn may be due to interaction with Cd in the testis itself, where uptake of the two metals across endothelial cells occurs to gain access to the parenchyma. Earlier studies utilizing genetic approaches have shed light on the role of Zn transporters, such as zinc-iron related transporter proteins (ZIP8), in the uptake of Cd to the testis [52]. Moreover, Liu et al. [53] demonstrated that Zn was the best inhibitor of ZIP8-mediated Cd uptake into cells and reported that this influx increased under condition of hypozincemia. In addition, the competitive mechanism of interaction is another possible mechanism of protection of Zn in relation to Cd toxicity. Indeed, radio-labelled Zn had been reported to be incorporated into elongated spermatids and to display competitive interaction with heavy metals for incorporation [54].

The current study shows that Zn treatment, in combination with Cd, induced a partial or total protection against Cd toxicity for several developmental parameters (Body weight and size evolution, tubular lumen formation and plasmatic testosterone level). It has been shown that Zn deficiency especially during pregnancy may impair fetal development or postnatal growth [55, 56] and Zn supplementation increases the growth rate [57].

Conclusion,

Our results imply that Cd exposure during gestation and lactation has adverse effect on testis development. The toxic effect of Cd are, partly, caused by the disruption of Zn metabolism, which is established in mothers during pregnancy causing Zn deficiency in fetus and continues to become more pronounced during lactation when Cd is being transferred through the milk. Furthermore, our study is one of the few data that explored the protective effect of dietary Zn supplementation against Cd toxicity during early development in rat and is the first data highlighting the interactions between the two metals in fetal and postnatal rat testis. The protective effect of Zn is mediated, partly, by the increase of Zn availability and by the decrease in Cd accumulation in offspring rat organism. Other additional studies at the

molecular level are required to elucidate the exact mechanisms of Cd toxicity and its interaction with Zn in testis during development in rats. Also, it would be interesting to investigate if the effects of exposure to Cd in utero and through lactation would persist later in adulthood.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1

Cadmium and Zinc concentrations at GD 20 in the mother's plasma, placenta and liver of male fetus

Groups		C	Cd	Zn	Cd+Zn
Plasma	Cd ($\mu\text{g/L}$)	nd	8.1 \pm 2.21 ^{ac}	nd	6.2 \pm 3.26 ^{abc}
	Zn ($\mu\text{g/mL}$)	1.9 \pm 1.55	1.2 \pm 1.74 ^a	2.1 \pm 0.89 ^b	1.8 \pm 1.32 ^b
Placenta	Cd (ng/g dry weight)	2.2 \pm 0.94	158.3 \pm 5.94 ^{ac}	nd	109.4 \pm 6.75 ^{abc}
	Zn ($\mu\text{g/g}$ dry weight)	25.0 \pm 4.81	24.9 \pm 4.26	22.1 \pm 4.52	24.6 \pm 6.05
Fetal liver	Cd ($\mu\text{g/g}$ dry weight)	nd	24.8 \pm 3.99 ^{ac}	nd	16.0 \pm 4.09 ^{abc}
	Zn ($\mu\text{g/g}$ dry weight)	62.3 \pm 6.53	33.2 \pm 7.27 ^a	67.8 \pm 9.27 ^b	48.4 \pm 7.86 ^{abc}

Values are expressed as mean \pm SEM from 6 litters in each group. nd not detected. **a:** $p < 0.05$ when compared to Controls (C) animals; **b:** $p < 0.05$ when compared to Cd-exposed animals; **c** $p < 0.05$ when compared to Zn-exposed animals

Table 2

Reproductive outcome of mother rats and anogenital distance (AGD) of their offspring

Findings	Control (C)	Cd	Zn	Cd+Zn
Number of dams	8	9	8	6
Initial mother weight (g)	169.29±6.51	160.39±3.17	162.21±4.74	170.7±5.61
Mother weight gain GD0-20 (% of initial body weight)	47.92± 6.42	45.59 ±5.68	43.95± 4.81	34.7 ±5.34
Length of gestation (day)	21.5±0.53	21.86±0.38	21.875±0.83	21.67±0.82
Litter size	9.125±1.46	7.22±1.96	9.25±2.19	8.67±1.03
Sexe ratio (M/F)	0.89±0.58	1.64±1.75	1.15±1.20	1.05±0.41
LBI %	100±0	100±0	100±0	100±0
SI % Pnd1-4	100±0	100±0	100±0	96.48±5.46
SI % Pnd1-7	94.32±10.8	95.62±9.04	93.35±10.01	94.81±5.69
Male AGD PND 4 (mm)*	3.65±0.39	3.75±0.42	3.60±0.58	3.87±0.12
Male AGD PND 21 (mm)*	10.04±0.84	9.31±0.68	9.72±0.58	9.58±1.01

Values are expressed as mean ± SEM from 6 litters in each group. *No significant differences have been observed between the four groups. LBI live birth index, SI Survival index, AGD Anogenital-distance.* AGD of 2-4 male per dams from each group was measured*

Table 3

Developmental parameters of male pups from mother's rats treated with Cd and/or Zn during gestation and lactation periods

Finding	Age	Control (C)	Cd	Zn	Cd+Zn
Body weight (g)	GD 20	3.45±0.16	3.33±0.05	3.50±0.12	3.31±0.14
	PND 12	14.29±1.05	13.61±1.07	15.12±0.44	15.48±1.06
	PND 21	25.69±2.71	18.75±1.17 ^a	24.16±3.05 ^b	23.17±2.71 ^b
	PND 35	54.92±6.72	49.31±4.01	58.75±5.68	53.19±5.34
Cranio-caudal length (cm)	GD 20	5.03±0.29	4.89±0.33	4.78±0.34	4.82±0.25
	PND 12	9.80±0.75	8.97±1.11	9.80±0.68	9.72±0.59
	PND 21	13.90±0.62	8.06±0.43 ^a	14.30±0.57 ^b	13.42±0.80 ^b
	PND 35	21.15±2.12	19.50±1.37	20.12±1.03	19.67±1.50
Relative testis weight (g/100g b.w)	PND 12	0.19±0.04	0.20±0.03	0.19±0.04	0.16±0.04
	PND 21	0.28±0.04	0.31±0.04	0.28±0.02	0.26±0.06
	PND 35	0.40±0.08	0.31±0.08 ^a	0.40±0.09 ^b	0.38±0.09 ^b

Values are expressed as mean ± SEM from 16-24 animals from each group (2-3 male per litter was measured). *a*: $p < 0.05$ when compared to Controls (C) animals; *b* $p < 0.05$ when compared to Cd-exposed animals.

Table 4

Stereological parameters

	Age	Control (C)	Cd	Zn	Cd+Zn
Seminiferous tubules	PND 12	67,48±2.50	65,21±2.21	62,49±1.35	62,73±1.69
Diameter (µm)	PND 21	102.93±2.70	106.04±5.50	106.25±1.50	102.63±5.60
	PND 35	151.64±8.50	147.58±8.50	163.74±3.10	151.44±8.70
Leydig cells number/um ²	PND 12	36.64±4.42	35.83±2.90	36.15±3.78	42.11±6.65
	PND 21	29.67±1.23	39.62±2.46	29.80±3.23	33.15±1.38
	PND 35	43.17±4.41	35.56±1.12	45±8.13	56.66±5.13
% of tubules with lumen	PND 21	2.43±0.75	11.03±3.20 ^a	13.49±1.55 ^a	11±2.11 ^a
% of abnormal tubules*	PND 35	3.31±1.56	18.76±3.86 ^a	12.95±8.25	4.99±0.51 ^b

Values are expressed as mean ± SEM from 4 litters in each group. *a*: $p < 0.05$ when compared to Controls (C) animals; *b* $p < 0.05$ when compared to Cd-exposed animals.

*Seminiferous tubules were considered abnormal when complete or partial loss of germ cells was observed or when the presences of multinuclear giant cells were seen.

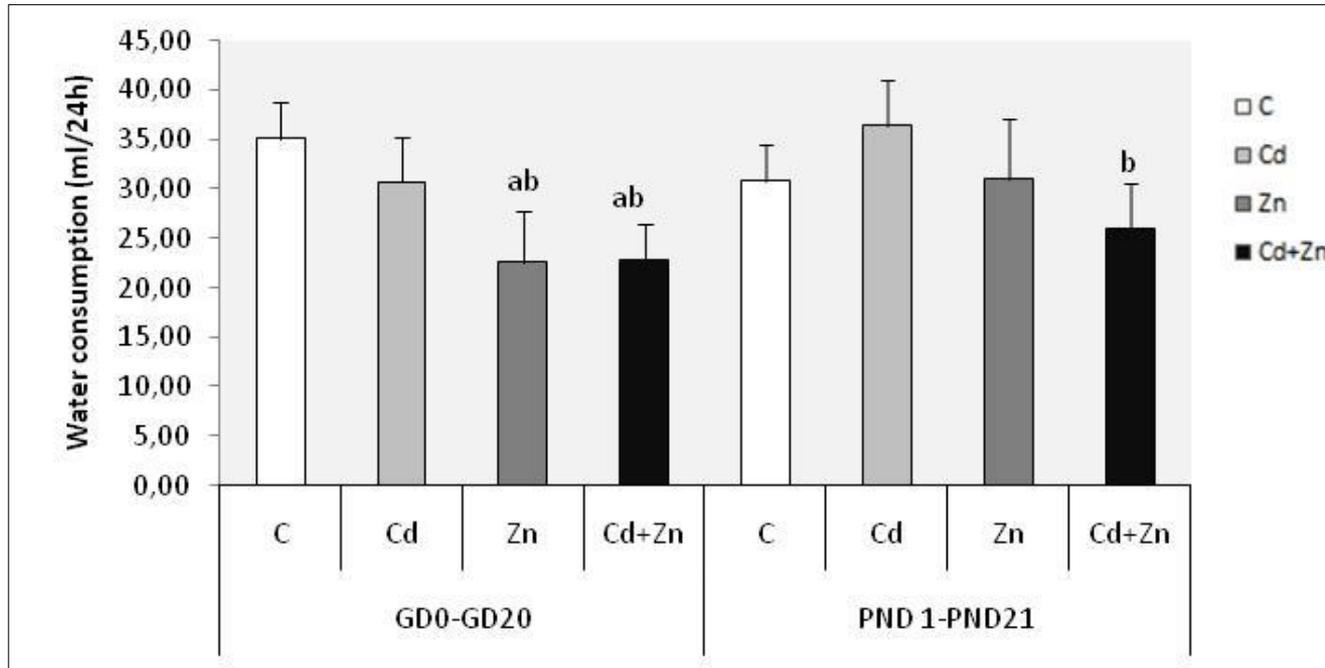


Figure 1

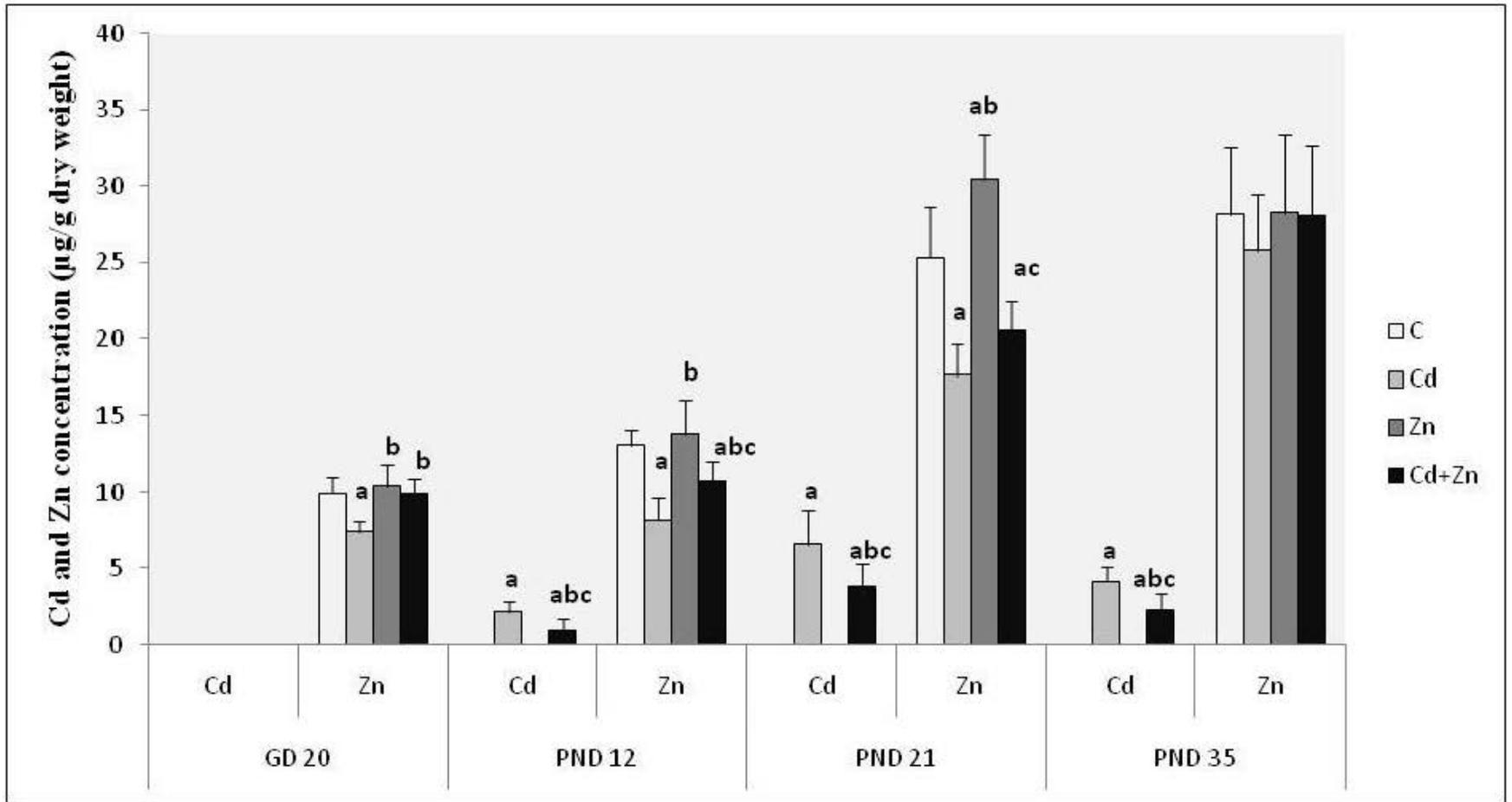


Figure 2

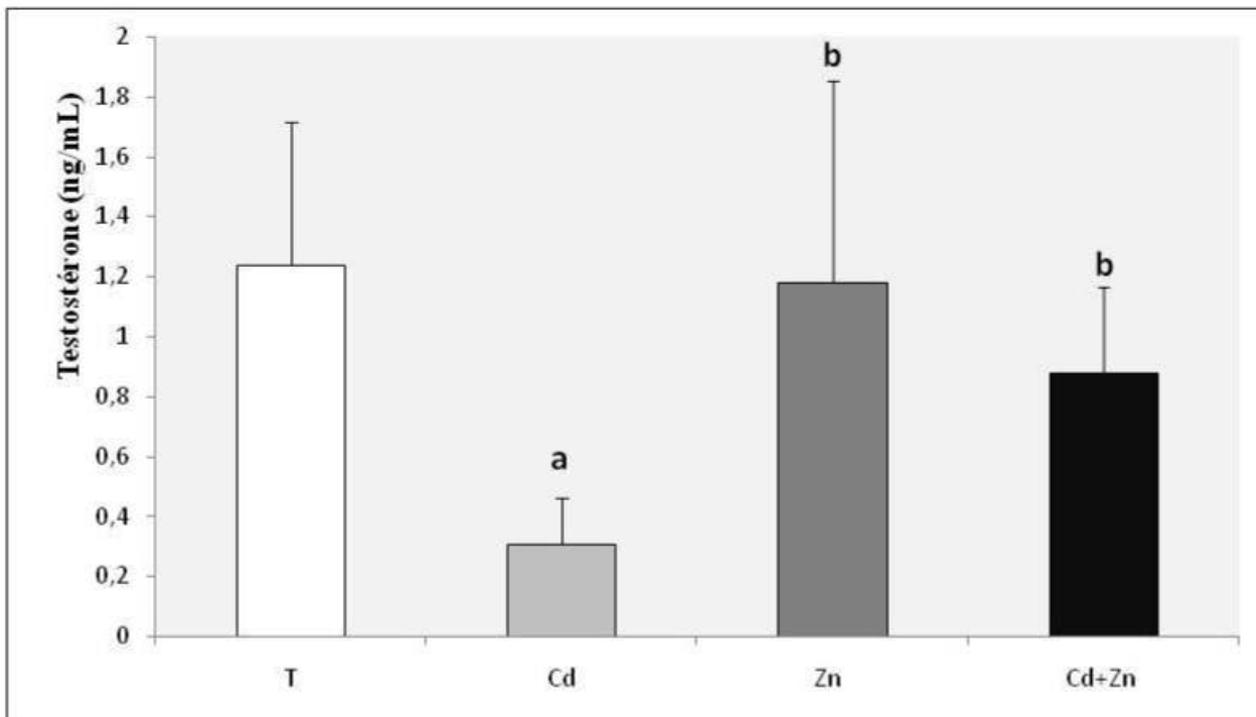


Figure 3

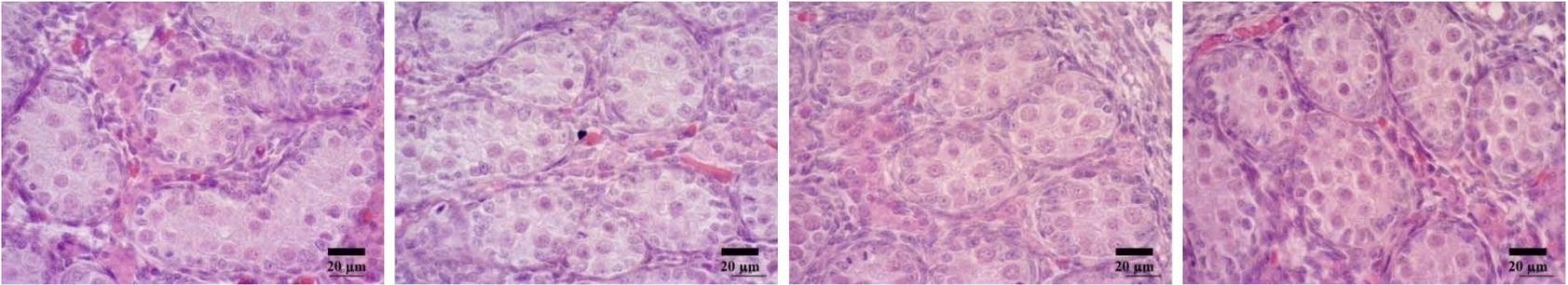
Control (C)

Cd

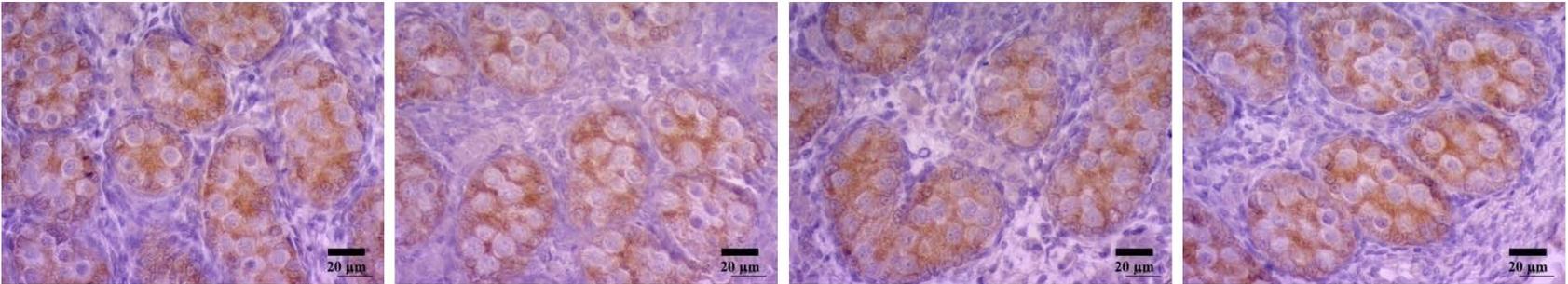
Zn

Cd+Zn

A



B



C

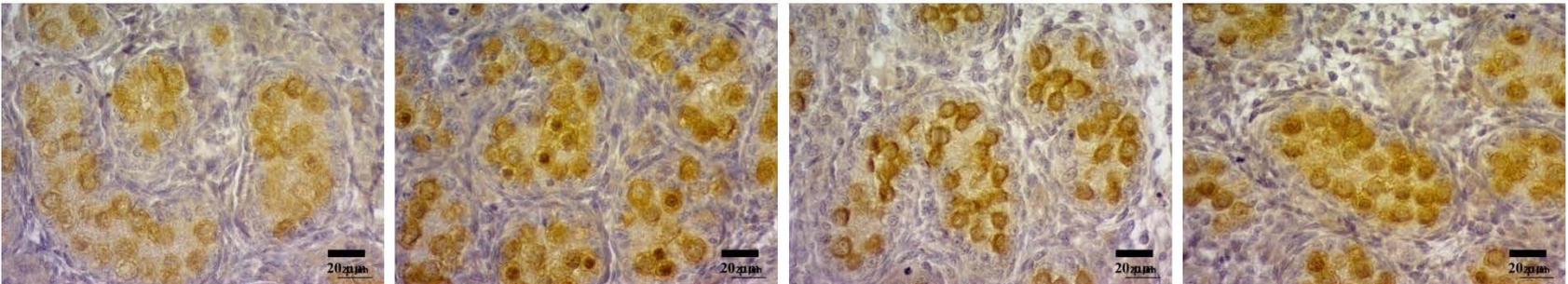


Figure 4

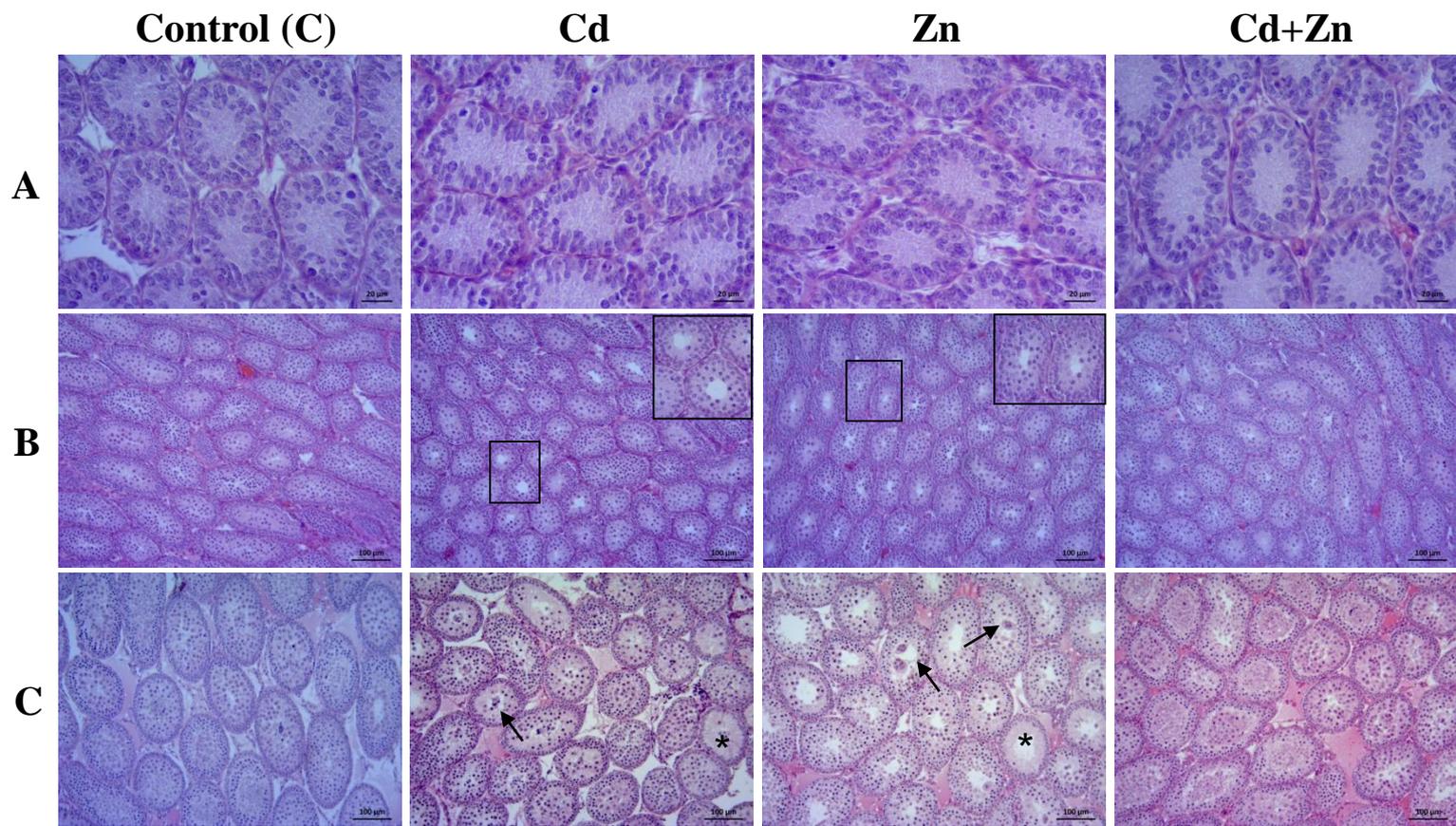


Figure 5

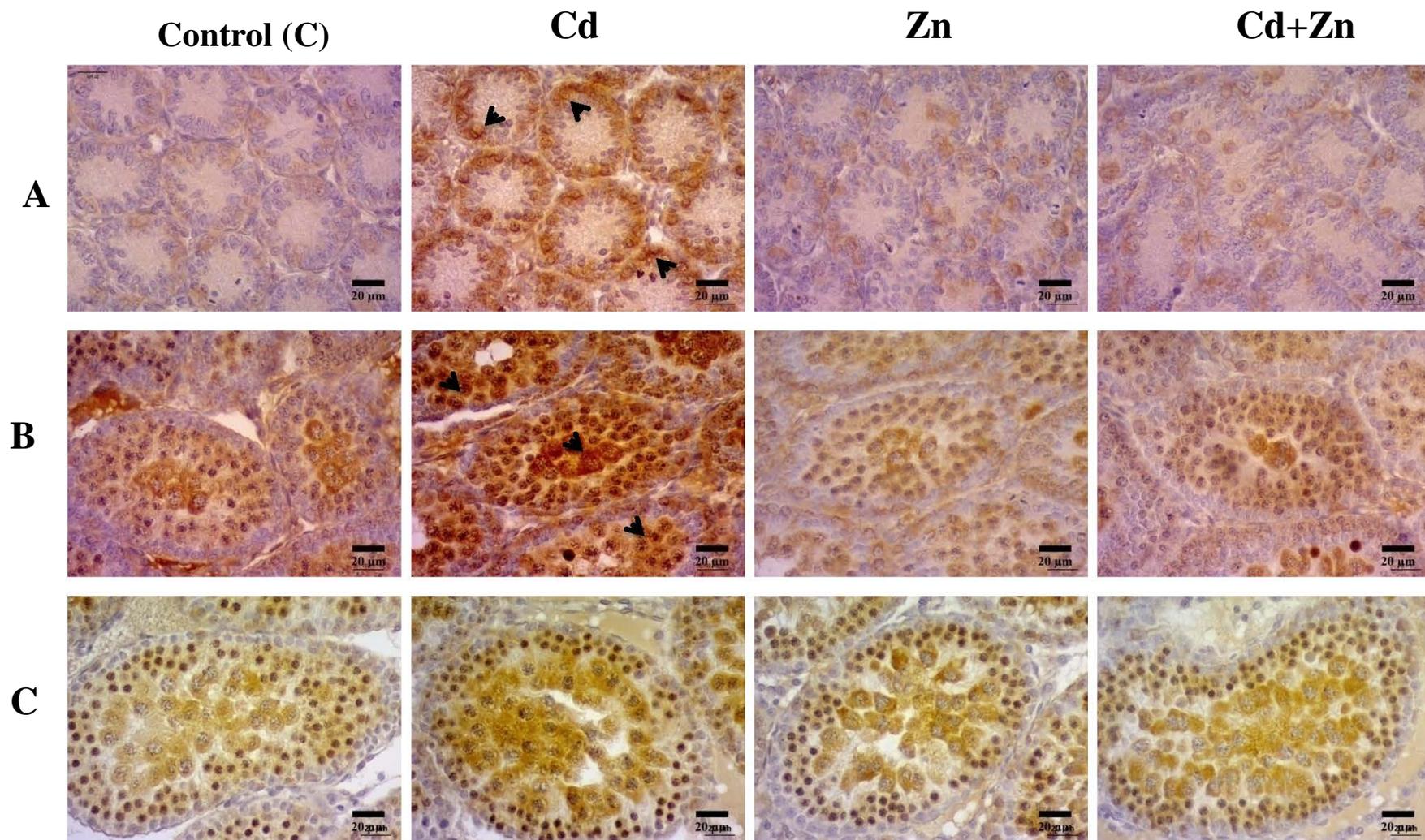


Figure 6

Figure 1: Water consumption of Control (C), Cadmium (Cd) and/or Zinc (Zn) exposed female rat during gestation and lactation periods.

Water volume was measured every couple of days during exposure and averaged as ml per 24h. Values are expressed as mean \pm SEM from 6 animals in each group. *a: p < 0.05 when compared to Controls (C) animals;* *b p < 0.05 when compared to Cd-exposed animals.*

Figure 1: Cadmium (Cd) and Zinc (Zn) concentrations ($\mu\text{g} / \text{g}$ dry weight) in testis (at GD20, PND 12, PND 21 and PND 35) from animals exposed to Cd and/or Zn during gestation and lactation periods

Values are expressed as mean \pm SEM from 6 animals in each group. *a: p < 0.05 when compared to Controls (C) animals;* *b: p < 0.05 when compared to Cd-exposed animals;* *c p < 0.05 when compared to Zn-exposed animals*

Figure 2: Plasmatic testosterone concentration at PND 35 after gestation and lactation exposure to Cadmium (Cd) and/or Zinc (Zn).

Values are expressed as Means \pm SEM from 6 animals in each group. *a: p < 0.05 when compared to Controls (C) animals;* *b: p < 0.05 when compared to Cd-exposed animals;* *c p < 0.05 when compared to Zn-exposed animals*

Figure 3: Representative photomicrographs of fetal rat testis tissues at GD20, from control and mothers treated with Cadmium (Cd) and/or Zinc (Zn) during gestation. 5 μm section of paraffin-embedded tissues were obtained and stained with hematoxylin and eosin H&E (A) or immunostained for the anti-mullerian hormone (AMH) (B) or the Heat Shock Protein 90 (HSP90) (C) (scale bar=20 μm).

Figure 5: Representative photomicrographs of rat testis from control (C) or mothers treated with Cadmium (Cd) and/or Zinc (Zn). 5 μm section of paraffin-embedded tissues were

obtained at PND12 (A) (Scale bar=20µm), PND 21 (B) (Scale bar=100µm) and at PND 35 (C) (Scale bar=100µm) and stained with hematoxylin and eosin H&E.

Seminiferous tubules with lumen were observed at PND21 in the Cd and Zn-treated groups (see picture inserts). Abnormal seminiferous tubules were observed at PND35 with germ cells loss (asterix) and multinuclear giant cells in the lumen (arrow). For higher magnifications, see Supplemental Figure 1.

Figure 4: Representative photomicrographs of rat testis from control (C) or mothers treated with Cadmium (Cd) and/or Zinc (Zn). 5µm section of paraffin-embedded tissues were obtained at PND12 (A), PND 21 (B) and at PND 35 (C) and stained with hematoxylin and immunostained for the Heat Shock Protein 90 (HSP90) (Scale bar=20µm).

Note the strong immunoreactivity in the cytoplasm of spermatogonia (PND12, arrowhead) and spermatocyte (PND 21, arrowhead) in the Cd group. The immunoreactivity for HSP 90 in Zn and Cd+Zn groups at PND 12 and PND 21 was similar to the control. At PND 35 the staining intensity was similar in all groups.