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Multigenerational analysis of the functional status of male reproductive system in mice after exposure to realistic doses of manganese

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ABSTRACT

The present study aimed to analyze the effect of multigenerational exposure to Mn at realistic doses on the functional quality of the male reproductive system of mice. Females and males (generation F0) were treated for 60 days with $MnCl_2$, via gavage, at the doses of 0, 0.013, 0.13, and 1.3 mg/kg/day. Treatment of F0 dams continued throughout gestation and lactation periods. At the time of weaning, the offspring (F1 generation) was divided into: animals that were not exposed after weaning – parental exposure (PE); and those exposed via parental generation and directly (PDE) for additional 60 days, at the same dose of F0 generation. F0 and F1 males were euthanized for assessment of sperm parameters and redox changes in the reproductive system. There was a decrease in the sperm concentration of the F0 generation. In addition, the sperm parameters of F1 generation were drastically affected. The activity of antioxidant enzymes was significantly reduced in PE animals. It was possible to verify that the biochemical damages were higher in the PE individuals, as demonstrated by the integrated biomarker response index. Our results show that Mn, even at low doses, is able to promote a reduction in sperm quality over a generation.

1. Introduction

Due to increased exploration and manufacture of numerous industrial compounds, a series of toxic components are released into the environment, increasing the risk of exposure of various organisms and the human population to such agents. Among them, manganese (Mn), an essential trace metal found in the lithosphere, hydrosphere, and biological systems, stands out (Howe et al., 2004; WHO, 2011). In its inorganic form, this element is widely used in the textile industry and in the production of fertilizers, glass, steel, and its derivatives. Its organic forms, such as methylcyclopentadienyl manganese tricarbonyl (MMT), can be used as additives in fuels and fungicides (ATSDR, 2012; Gerber et al., 2002).

Naturally, this element is found in water and in foods such as vegetables and meats. According to the Institute of Medicine (IOM) (2001) and WHO (2011), the appropriate doses of daily Mn intake are 2.3 and 1.8 mg/day for men and women, respectively, and the maximum amount of 11 mg/day is considered tolerable. However, human activities, such as mining and discharge of urban and industrial waste, increase the environmental concentrations of this element, as well as the chances of exposure to higher doses of Mn through water and air (ATSDR, 2012; Gerber et al., 2002; Howe et al., 2004; Signorelli et al., 2019; WHO, 2011). According to WHO (2011), the maximum Mn dose established for drinking water, which is 0.4 mg/L, is higher than what is normally found in drinking water samples. However, a review study conducted by Frisbie et al. (2015) reveals that, in several localities around the world, there are populations using water with Mn concentrations above this limit.

In spite of being an essential metal, Mn in excessive quantities can cause deleterious and pathological effects to organic systems (Ferrante et al., 2017; Gerber et al., 2002; Martinez- Finley et al., 2013; Okada et al., 2016; Sanders et al., 2014). It is an element known for its neurotoxic potential, which can cause cognitive problems and a condition similar to Parkinson's disease, called manganism (Erikson et al., 2007; Ferrante and Conti, 2017; Nascimento et al., 2016; Roels et al., 2012). Nonetheless, the damage promoted by Mn is not limited to the nervous system and can also affect the embryonic development of animal and human (Grazuleviciene et al., 2009; Rudge et al., 2009; Sánchez et al., 1993). Therefore, there is a great concern regarding the daily intake of Mn by pregnant women, since some metals are permeable to the

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placental barrier and can affect intrauterine development (Agrawal, 2012; Fraser et al., 2014; Liu et al., 2013; Rudge et al., 2009; Zota et al., 2009). Population studies have shown that Mn interferes with the weight of newborns and the growth of children whose mothers have been exposed to water containing high concentrations of this element (Grazuleviciene et al., 2009; Rahman et al., 2015). Also, research in animal models had shown the adverse effects of Mn during development (Hernandez et al., 2015; Tu et al., 2017).

Adult animals exposed directly to Mn present testicular and spermatic damages, such as motility loss and decreased sperm concentration (Adedara et al., 2017a, 2017b; Ponnapakkam et al., 2003), histopathological changes and increased testicular apoptosis (Liu et al., 2013a), reduction of sex hormone levels (Adedara et al., 2017a, 2017b; Liu et al., 2013b), and loss of homeostasis of the antioxidant defense system (Adedara et al., 2017a, 2017b; Liu et al., 2013a). In addition, concentrations of Mn and other metals in human blood and urine have been correlated with sperm apoptosis and decreased motility (Wang et al., 2016, 2017; Wirth et al., 2007). Therefore, it is likely that a multigenerational ingestion of inadequate levels of Mn during critical periods of development may impair the male reproductive system, altering its reproductive parameters.

Despite numerous studies on Mn, its effects on the reproductive parameters of animals exposed during intrauterine development are poorly understood. To our knowledge, the study by McGough and Jardine (2017), who used inhalation exposure and showed no alteration in reproductive parameters, is the only multigenerational study on Mn. The present study examined the effects of multigenerational Mn exposure at environmentally realistic doses, via oral exposure, encompassing various developmental stages of the organism, on functional parameters and redox conditions of the male reproductive system.

2. Materials and methods

2.1. Experimental design

The mice used in the experiments were obtained from the animal facility of the Biological Sciences Sector at the Federal University of Paraná (UFPR). The study was approved by the Animal Use Ethics Committee (CEUA), number 1015/2016. The experiment was divided into three stages:

1) F0 generation: exposure of the breeding mice and mating

Sixty adult Swiss mice, 40 females and 20 males, aged approximately 49 days were used. The animals were kept separated by sex in polypropylene cages. The temperature (23 ± 2 °C) and the light-dark cycle (12:12 h) were controlled, with food and water supplied *ad libitum*.

The animals were weighed and then separated into four groups, with 10 females and 5 males each. The groups were exposed to MnCl2 (Sigma-Aldrich) at doses of 0 (control group), 0.013, 0.13, and 1.3 mg/kg/day for 60 days, via oral gavage. The intermediate dose (realistic) was based on: 1) the daily exposure for adult humans weighing 70 kg and consuming 1.2 mg, that is 0.017 mg/kg/day; 2) accounting for the.

maximum Mn water intake allowed by WHO (2011) of 0.4 mg/L; 3) the assumption that an adult weighing 70 kg drinks on average 2 L of water daily, which is the equivalent to a dose of 0.8 mg/L/day of Mn; and 4) the assumption that there is a 0.4 mg/day ingestion of Mn from other sources, resulting in a total Mn dose of 0.017 mg/kg/day. An allometric calculation was carried out for extrapolating the equivalent value for mice, considering the metabolism of these animals, obtaining a final dose of 0.13 mg/kg/day (Okada et al., 2016).

After 60 days of exposure, the animals were placed into cages in a 1:2 male-female ratio for mating, according to their group, with continuous exposure to MnCl2. The gestation day 0 (GD0) was considered when the vaginal plug was observed on the females. After this step, females that did not mate and males were euthanized by cervical dislocation.

2) Gestation and lactation

Pregnant females continued to be exposed to MnCl2 throughout gestation (21 days) and lactation (21 days). After the lactation period, the pups were weaned and sexed.

3) F1 generation: offspring treatment

After weaning, the offspring of each group was divided into two groups, resulting in two postnatal experimental conditions. Each pup was used as a statistical unit, according to the "within-litter design" method (Festing, 2006), considering that the offspring individuals received different treatments. At weaning, F1 males from the each parental treatment group were randomly selected from different litters to compose the postnatal experimental groups used in this study:

Group 1 - Parental exposure (PE): the animals were exposed to MnCl2 only during intrauterine development and lactation periods, remaining untreated after weaning for 60 days (n = 10 mice/dose level).

Group 2 - Parental and direct exposure (PDE): after weaning, the animals were continuously exposed for 60 days to MnCl2, by oral gavage, at the same dosage of their progenitors (n = 9 mice/dose level).

2.2. Gestation and fertility analysis - F0 generation

Females' weight was monitored throughout the pre-gestational, gestational, and post-gestational periods. After the gestation period, the number of copulated females, the copulation rate [(number of copulated females/total number of females) x 100], the litter size per female, and the total number of pups per group were evaluated.

2.3. Dissection and collection of organs

At the end of the experimental period, all animals were weighed and euthanized by cervical dislocation. The testes and seminal vesicles were collected for weighing and the epididymis for sperm analysis. Each material was treated specifically for the different methods of analysis used.

2.4. Analysis of sperm parameters

The epididymis tail of the F0 (n = 5) and F1 (n = 9–10) animals were sectioned and placed in 400 µl of Gamete Buffer (COOK Medical®), at 37 °C for 5 min, for dispersion of spermatozoa. Subsequently, aliquots were withdrawn for analysis of sperm parameters, based on WHO (2010), described below.

2.4.1. Sperm motility

Two hundred sperm cells per animal were evaluated and classified for motility in three categories: progressive motile, non-progressive motile, and non-motile. For this, 30 µl of sperm samples were placed on a slide, covered by coverslip and observed under a light microscope with a 40x objective lens.

2.4.2. Sperm concentration

The spermatic concentration was estimated in millions/mL by way of cell counting in Neubauer hemocytometer chamber. For this count, 20 µl of the sperm sample was diluted 10x in distilled water. The sperm cells were counted in five quadrants and, for the calculation of the concentration, the following equation was used: [concentration] = number of sperm cells x 5 x dilution factor x 10000.

2.4.3. Sperm vitality

The hypoosmotic test aims to evaluate the sperm vitality through the osmotic differential between the cell and the medium. Sample aliquots of 50 µl were added in 100 µl of distilled water for 5 min and then observed under a light microscope with a 40x objective lens. Only spermatozoa with coiled tail were considered alive.

2.5. Biochemical analysis – redox status

To evaluate whether the treatments induced redox changes on F1 generation, the testes and seminal vesicles (n = 9–10) were analyzed by different tests. The organs were preserved at −80 °C, homogenized in 20 mM Tris-HCl buffer, 1.0 mM EDTA, 1 mM (pH 7.6) PMSF (Phenylmethylsulfonyl fluoride), followed by centrifugation at 12,000 *g* for 20 min at 4 °C. The protein content of the supernatants was measured by the Bradford (1976), in triplicate, with readings at 595 nm. For the analysis of antioxidant defense system enzymes, the activities of catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Kono, 1978), and glutathione S-transferase (GST) (Keen et al., 1976) were measured. For the assessment of antioxidant molecules, non-protein thiols (NPT) were measured according to Sedlak and Lindsay (1968), while oxidative damages were evaluated through the protocol of Jiang et al. (1991, 1992) for lipid peroxidation (LPO).

2.6. Integrated biomarker response (IBR)

The methodology described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013) was employed to assess the integrated biomarker response (IBR). The results obtained through the biomarker analysis of each dose used were divided by the corresponding control group, followed by logarithmic transformations to reduce the variance. From the logarithmized data (Yi), the mean (µ) and the standard deviation (sd) were calculated for all analyzes. Then, the equation Zi = (Yi - µ)/sd was applied to each treatment, where the difference between the treated and the control group (Zi - Z0) was used to obtain the value of (A) - corresponding to the integrated result for each biomarker analyzed. The results were plotted on a radar chart, where values above or below zero (control) indicate the stimulus or inhibition of a given biomarker. Finally, for the calculation of the IBR index of each group, the values of (A) were converted to absolute numbers (S) and summed.

2.7. Statistical analysis

The data were tested for normality and analyzed by one-way (F0 generation data) or two-way (F1 generation data, factors: exposure type and dose) ANOVA tests, when applicable, followed by Dunnet or SNK (Student Newman Keuls) *post hoc* tests, respectively. F1 generation data, in each type of exposure adopted, treated groups were compared with its corresponding control (0 mg/kg/day). All data were presented as mean ± standard deviation or mean ± standard error and p values < 0.05 were considered significant.

3. Results

3.1. Gestation and fertility – F0 generation

The weight of the females during the pre-gestational, gestational, and post-gestational (lactation) periods did not differ significantly between the doses tested. In addition, none of the fecundity and fertility parameters were statistically significant in the F0 generation (Supplementary Table 1).

3.2. General development and organs weight

Breeding males (F0 generation) did not present significant differences in weight gain over the 60-day treatment period nor any toxicity signs, such as piloerection, altered respiratory rate, and locomotor activity loss. In addition, there was no change in absolute and relative weight of the testes (Supplementary Table 2).

Table 1
General data of offspring (F1 generation).

Parental Exposure (PE) - $^{MnCl}2$ (mg/kg/day)				
Parameter	0 (control)	0.013	0.13	1.3
Body weight (g)				
Initial (PND 21)	32.2 ± 1.22	31.8 ± 3.85	30.3 ± 2.79	30.1 ± 1.4
Final (PND 81)	38.3 ± 3.31	39.4 ± 3.08	36.4 ± 2.35	36.4 ± 2.16
Absolute weight (g)				
Testis	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Seminal Vesicle	0.34 ± 0.09	0.33 ± 0.05	0.38 ± 0.04	0.41 ± 0.05
Relative weight (%)				
Testis	0.31 ± 0.04	0.30 ± 0.05	0.29 ± 0.04	0.31 ± 0.05
Seminal Vesicle	0.98 ± 0.11	0.75 ± 0.31	0.70 ± 0.12	0.92 ± 0.17
Parental and Direct Exposure (PDE) - MnCl2 (mg/kg/day)				
Parameter	0 (control)	0.013	0.13	1.3
Body weight (g)				
Initial (PND 21)	32.1 ± 2.33	29.6 ± 1.50	30.7 ± 1.70	31.0 ± 2.00
Final (PND 81)	39.4 ± 2.36	38.1 ± 2.23	39.9 ± 2.18	37.6 ± 2.29
Absolute weight (g)				
Testis	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Seminal Vesicle	0.37 ± 0.04	0.29 ± 0.11	0.25 ± 0.05*	0.33 ± 0.05
Relative weight (%)				
Testis	0.30 ± 0.04	0.28 ± 0.4	0.28 ± 0.02	0.31 ± 0.04
Seminal Vesicle	0.87 ± 0.23	0.88 ± 0.13	0.71 ± 0.13*	1.09 ± 0.14*

Mean ± Standard deviation. * Values differ significantly from control (0 mg/kg/day). ANOVA two-way – *post hoc* test: Student Newman Keuls. Parental Exposure: n = 10; Parental and Direct Exposure: n = 9. PND: postnatal day.

The offspring, corresponding to the F1 generation, also did not show changes in the initial and final weight, for both exposures, parental (PE) and parental and direct (PDE) (Table 1). However, the seminal vesicle of animals exposed to the 0.013 mg/kg/day dose showed a reduction in absolute weight and the ones exposed to the 1.3 mg/kg/day dose showed an increase in relative weight, both in the PDE group (dose factor: p < 0.05) (Table 1).

3.3. Sperm parameters

The motility and vitality parameters of the F0 generation, at all doses tested, did not change significantly (Fig. 1A–D). However, the sperm cell concentration was reduced by approximately 50% in all Mn-treated groups (Fig. 1E). In turn, the F1 generation presented drastic changes in sperm motility, especially a dose-dependent effect in progressive motility (Fig. 2A–C), both in PE and PDE exposure. This reduction, approximately 40% in relation to the control group, was significant (p < 0.05) at doses of 0.13 and 1.3 mg/kg/day. Vitality was significantly reduced only in the 1.3 mg/kg/day PE group (Fig. 2D). Sperm concentration was also altered in F1 generation at doses of 0.13 and 1.3 mg/kg/day (Fig. 2E).

In general, among the effects observed in F1 generation, it was determined, through two-way ANOVA, that the dose was the main factor responsible for the variation of the data obtained in the present study (Table 2).

3.4. Redox status

The results obtained for the redox biomarkers in the testis and seminal vesicles are shown in Figs. 3 and 4. The catalase (CAT) activity was significantly reduced only in the testis of the animals from the PE group at the dose of 1.3 mg/kg/day (Fig. 3A–B).

Superoxide dismutase (SOD) activity was not altered in the testis in

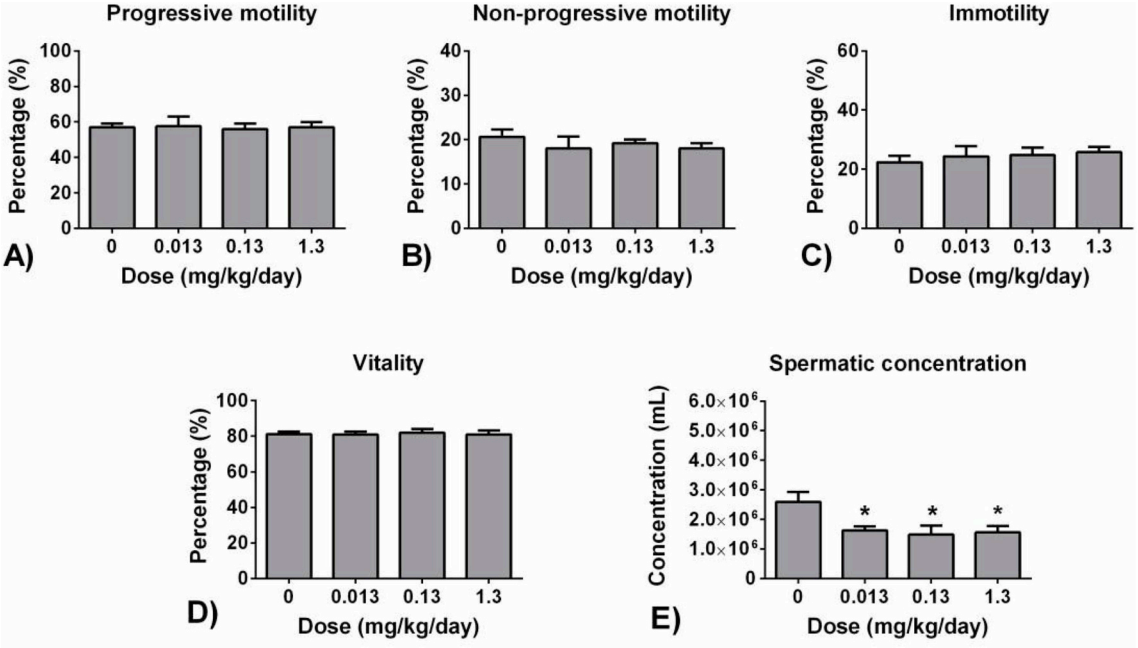

Fig. 1. Sperm parameters of F0 generation. A) Sperm with progressive motility; B) Sperm with non-progressive motility; C) Immotile sperm; D) Vitality; E) Sperm concentration. n = 5. * = p < 0.05 (ANOVA - *post hoc* teste: Dunnet). Bar: SEM.

any of the forms of exposure to Mn (Fig. 3C). However, a dose-dependent reduction was observed in the seminal vesicle of the PE group animals, being significant at the 1.3 mg/kg/day dose (Fig. 3D). For glutathione S-transferase (GST), there were no significant results in most of the treatments used, as well as in the different organs evaluated (Fig. 3E–F). Exceptions occurred in the testis, in treatments 1.3 mg/kg/day PE and 0.013 mg/kg/day PDE, which presented a reduction and an increase, respectively, in the activity of this enzyme (Fig. 3E).

For the non-enzymatic biomarkers, some changes were noticed on the amount of non-protein thiols (NPT) and an increase in the

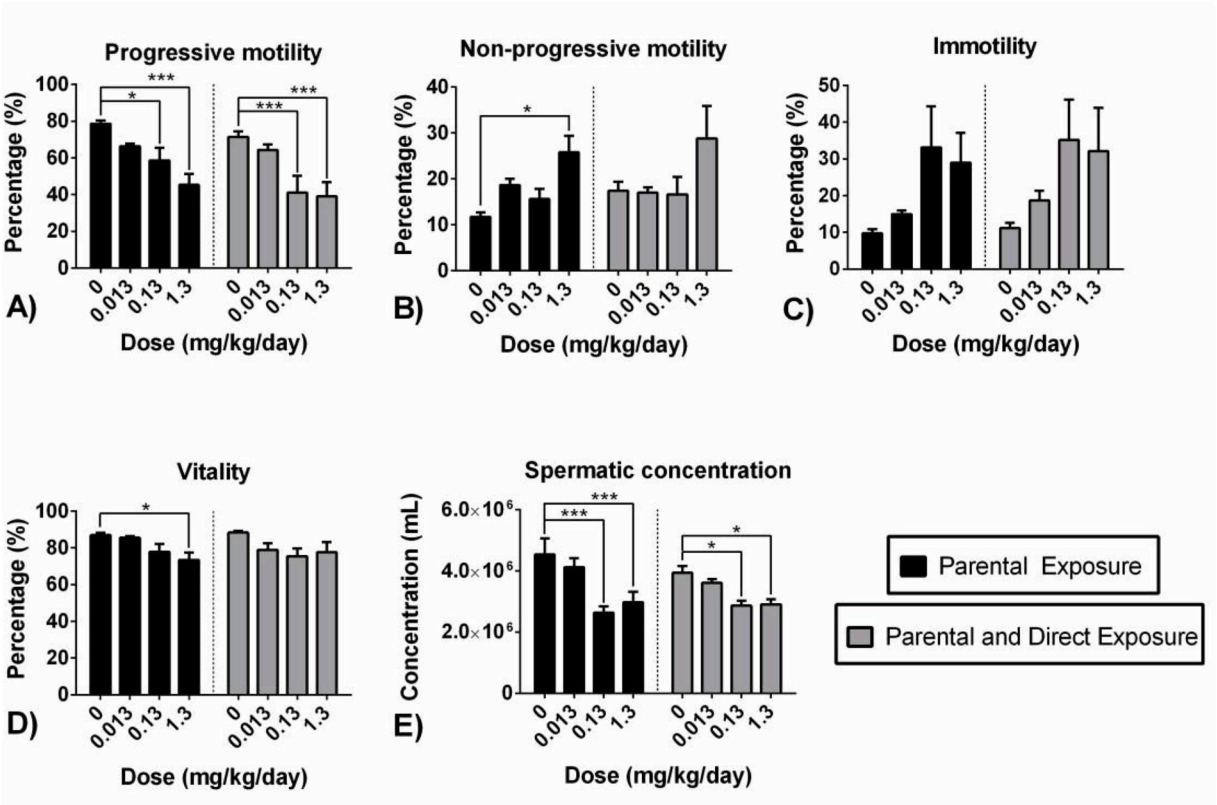

Fig. 2. Sperm parameters of F1 generation. A) Sperm with progressive motility; B) Sperm with non-progressive motility; C) Immotile sperm; D) Vitality; E) Sperm concentration. Parental exposure (PE): n = 10; Parental and direct exposure (PDE): n = 9. * = p < 0.05; *** = p < 0.0001. (ANOVA – *post hoc* test: Student Newman Keuls). Bar: SEM.

Table 2
Two-way ANOVA – Spermatic parameters.

Parameter	Exposure Type	Dose	Interaction
Progressive motility	0.0408*	< 0.0001*	0.5589
Non-progressive motility	0.394	0.0012*	0.7251
Immotility	0.6287	0.007*	0.9988
Vitality	0.7455	0.0031*	0.4423
Concentration	0.4322	0.0001*	0.2427

*statistical significance ($p < 0.05$ – two-way ANOVA).

hydroperoxide concentration, derived from lipid peroxidation (LPO), in the seminal vesicle of the animals that received doses of 0.013 and 1.3 mg/kg/day PE, respectively, differently from that observed for the testis (Fig. 4A–D). Although the level of testicular LPO did not show any significant difference between the PE groups, there was an increase in the average production of hydroperoxides at doses of 0.013 and 1.3 mg/kg/day of the PDE group (Fig. 4C). Among the effects found through biomarker analysis, it was established, through statistical analysis, that the dose was the main factor responsible for the variation of the data (Table 3), as observed for the sperm parameters.

3.5. Analysis of the integrated biomarker response (IBR)

The IBR data are shown in Table 4. When evaluating the testicular response of the PE animals, it was observed that the 1.3 mg/kg/day dose caused greater alterations in the biomarkers and, consequently, a higher IBR index (10.74) when compared to PDE animals (7.96). For the seminal vesicle, in this same exposure condition (PE), IBR values are expressive at doses of 0.013 and 1.3 mg/kg/day, being 8.05 and 8.15, respectively.

The PDE individuals presented similar indexes to both organs evaluated, with the doses of 0.013 and 1.3 mg/kg/day being the most representative. The IBR values for the testes were 7.48 and 7.46, while for the seminal vesicle they were 8.78 and 6.59, to the lowest and highest dose, respectively. Performing a global comparison of the different groups, involving the sum of the indexes obtained for the seminal vesicle and the testis, it was noted that the PE animals have a higher index in almost all the doses tested, when compared with those of the PDE group, except in 0.013 mg/kg/day. Thus, possibly these animals are more susceptible to biochemical changes when compared to the PDE group. The way the treatments and doses used in the study modified the dynamics of increase and decrease of biomarkers is shown in Fig. 5.

4. Discussion

Even though Mn is considered an essential element, little is known about its effect on the male reproductive system, especially during a multigenerational exposure. The present study used doses and routes relevant for human exposure, seeking to identify possible reproductive effects. None of the doses used in this study was able to change the weight of the animals in F0 and F1 generations, different from that observed for the animals treated with arsenic compounds (Bourguignon et al., 2017) or chrome (Cr) (Chandra et al., 2007). Despite the few changes observed in relation to body mass gain and organ weight, it was found that the reproductive system, in functional terms, was strongly affected. Relevant multigenerational reductions in sperm quality and redox conditions were found in the reproductive system of F1 generation.

Sperm motility was not affected in males of the F0 generation. However, the offspring (F1 generation) in both exposure conditions (PE and PDE), presented severe alterations on parameters evaluating sperm quality. The decrease in sperm motility may be explained by the ability of Mn to block motor proteins and cytoskeletal filaments (Towler et al., 2000). In addition, Mn affects mitochondria function, altering mitochondrial membrane potential, homeostasis of Ca^{2+} levels, and depletion of ATP production (Cheng et al., 2003, 2005; Tarale et al., 2016). Consequently, given the drastic effects on cytoskeleton and mitochondrial physiology, sperm motility was affected by Mn exposure. The results demonstrated that the use of low and environmental realistic doses are able to significantly modify sperm motility, an essential characteristic for male fertility.

During pre-natal development, spermatogenic process occurs concomitantly with cell maturation. Thereby, pre-natal exposure to stressing agents can interfere in the development and differentiation of Sertoli cells or spermatogenic stem cells, directly impairing spermatogenesis during adulthood (Estill and Krawetz, 2016; Sharpe, 2010) and reducing sperm concentration. Leydig cells, responsible for androgen synthesis, may also be affected after exposure to metals such as Cd (Tian et al., 2018) and Mn (Cheng et al., 2003, 2005), compromising testicular physiology. In this context, it is plausible that animals exposed only during gestation and lactation presented a reduction in sperm parameters in adulthood, and suggests that Mn can affect several cell types and promote testicular dysfunction. Therefore, it is necessary to consider the ability of metals, either essential or not, to be transferred to the offspring via the placental barrier or lactation and potentially affect the gonadal development (Agrawal, 2012; Gravwé and Oskarsson, 2000; Liu et al., 2013; Rudge et al., 2009; Tian et al., 2018).

Based on the sperm analysis of the F0 generation, the only parameter significantly altered was the sperm concentration. However, the fecundity and fertility characteristics of these animals were not affected. These findings corroborate our argument on the negative impact of mainly pre-natal and lactation Mn exposure to espermatogenesis for F1 generation. The F0 generation started to be exposed at 49 days old – when the reproductive germ cells and supporting cells were already formed and matured. In addition, it is important to consider that rodent models can be resistant to reproductive alterations, since a drastic reduction in sperm parameters, such as daily sperm production, does not alter the fertility of these individuals (Andrade et al., 2002). Nonetheless, changes with a low degree of severity in animal models may be represented as harmful effects to humans (Andrade et al., 2002; Zenick and Clegg, 1989). Elbetieha et al. (2001) demonstrated that Mn intake at high concentrations (1000–8000 mg/L) negatively affects the fertilization rate and the number of viable fetuses in mice. Furthermore, spermatozoa exposed *in vitro* to Cd have a lower chance of successful fertilization and viable blastocyst formation (Zhao et al., 2017). Although the fertility parameters of the F1 generation animals were not tested, the sperm analysis indicated that there could be some change in the fecundity or fertility of these animals.

The deleterious effects observed on the sperm parameters of the F1 generation may have resulted from alterations in the oxidative state of the reproductive system, since seminal and spermatic characteristics are also biomarkers of redox changes (Bergamo et al., 2016). Although no significant difference was observed between many biochemical parameters analyzed in the PE and PDE groups, in relation to the control, changes in some enzymatic activities, such as CAT and GST, could be observed. There are no results reported in the literature on the influence of Mn on the redox changes in the seminal vesicle. However, our results demonstrated a dose-dependent reduction of SOD activity in PE animals, resulting in a deficit of elimination of superoxide radicals. According to Adedara et al. (2017a), animals treated with a mixture of As and Mn showed a reduction of CAT and an increase of H2O2 in the testes and epididymis, indicating loss of ROS elimination capacity. Additionally, according to Goodrich and Basu (2012), metals such as Pb, Cd, Se, As and Mn can inhibit the enzymatic activity of several variants of GST.

The levels of other biochemical biomarkers measured in the testes, NPT and LPO did not vary significantly between groups. However, even at low doses, it was observed that in the testis of PDE animals there is higher concentration of hydroperoxides when compared with the control group. This may indicate that, although the results are not

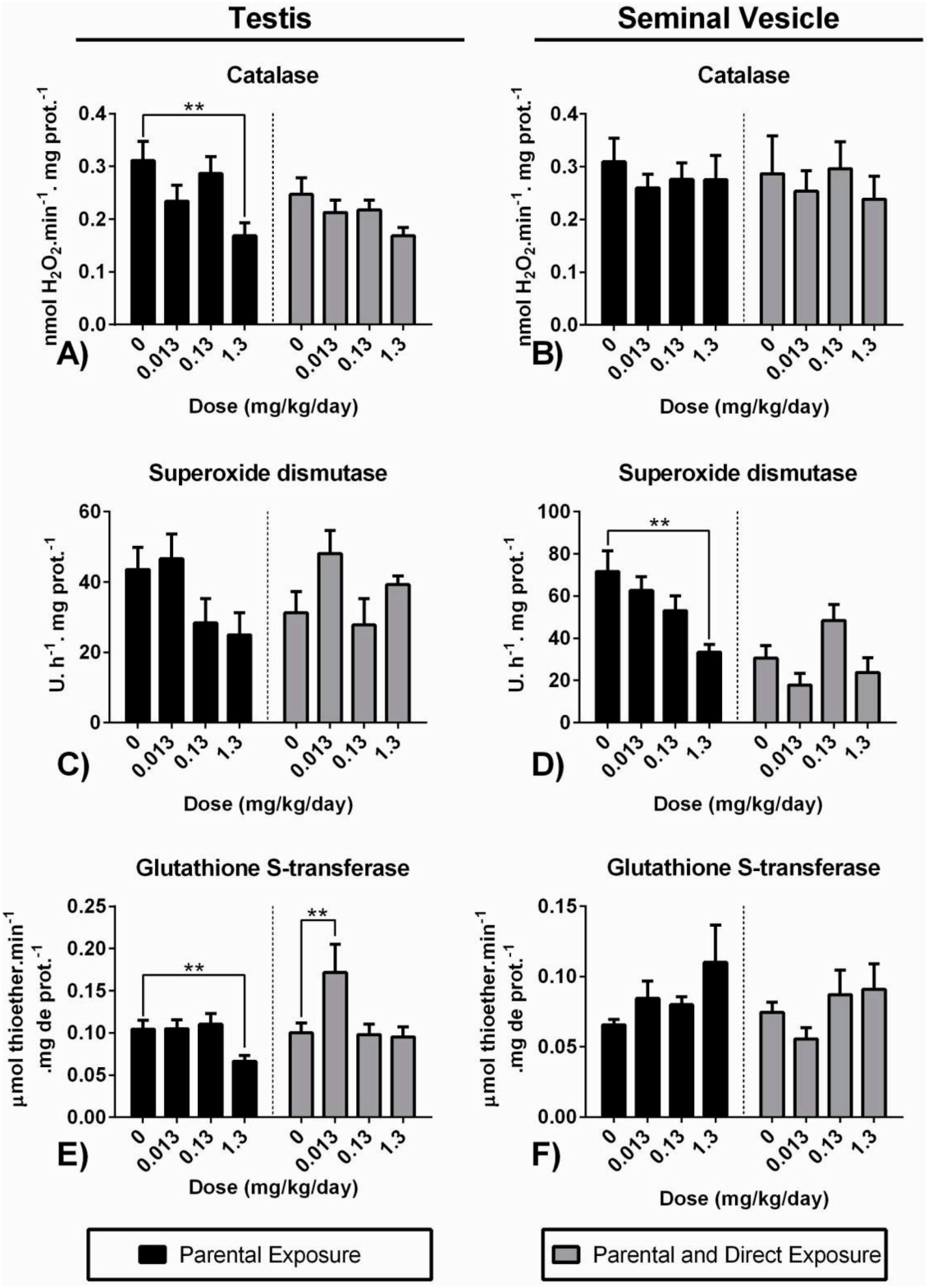

Fig. 3. Enzymatic biomarkers – redox status. A-B) Catalase (CAT); C-D) Superoxide dismutase (SOD); E-F) Glutathione S-transferase (GST). Parental exposure (PE): n = 10; Parental and direct exposure (PDE): n = 9. * = $p < 0.05$; ** = $p < 0.01$. (ANOVA – *post hoc* test: Student Newman Keuls). Bar: SEM.

significant, damage to the testis cell lipids may occur and possibly indicate that long-term exposure to low levels of Mn can be as harmful as overexposure cases. The opposite effect is observed in the reproductive system of organisms exposed to high Mn doses (range: 15–1800 mg/kg) (Adedara et al., 2017a, 2017b; Liu et al., 2013a). Overall, these data suggest that the alterations of biochemical biomarkers indicate loss of redox homeostasis and can negatively influence sperm quality.

The IBR index is a widely used method for ecotoxicological studies (Baudou et al., 2019; Marins et al., 2018), as it evaluates the behavior of increase and reduction of biochemical biomarkers. Through this index, it was possible to evaluate how the different doses and exposure methods affected the oxide-reducing conditions of the male

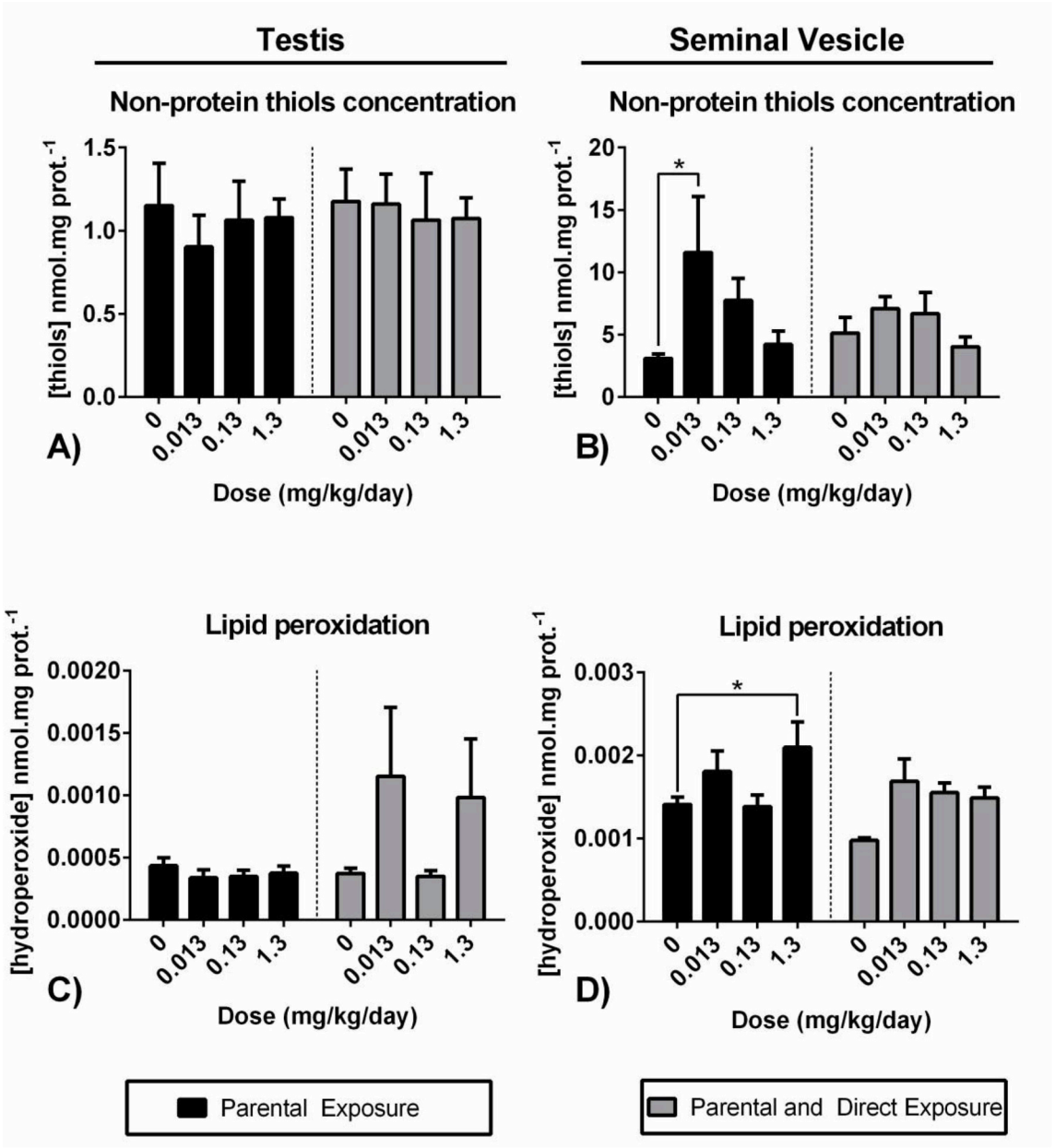

Fig. 4. Non-enzymatic biomarkers – redox status. A-B) Non-thiols protein concentration (NPT); C-D) Lipid peroxidation (LPO). Parental exposure (PE): n = 10; Parental and direct exposure (PDE): n = 9. * = $p < 0.05$. (ANOVA – *post hoc* test: Student Newman Keuls). Bar: SEM.

Table 3
Two-way ANOVA – Redox biomarkers.

Parameter	Testis			Seminal Vesicle		
	Exposure	Dose	Interaction	Exposure	Dose	Interaction
CAT	0.0556	0.0013*	0.5439	0.7274	0.7409	0.9344
SOD	0.8793	0.0311*	0.269	< 0.0001*	0.0051*	0.0061*
GST	0.0713	0.0037*	0.0468*	0.4402	0.1264	0.47
GSH	0.6314	0.9268	0.9041	0.5318	0.037*	0.4818
LPO	0.0675	0.3385	0.2501	0.0734	0.0092*	0.1978

*statistical significance ($p < 0.05$ – two-way ANOVA).

reproductive system in the present study. The results showed that the testis, at 1.3 mg/kg/day in PE and PDE groups, presented the highest IBR values, suggesting a greater susceptibility of the organ to oxidative variations, which may be detrimental to spermatogenesis. In addition, comparing the indexes between the different types of exposure, it was noticed that the animals from the PE treatment group had higher IBR values. Consequently, these individuals are predisposed to a greater disorder and stress in the antioxidant defense system than those from the PDE group, considering the present Mn treatments.

According to the results obtained in the present study, it is

Table 4
Integrated Biomarker Response (IBR) index.

Parental Exposure (PE)			
Dose (mg/kg/day)	Testis	Seminal Vesicle	Σ
0	0	0	0
0.013	6.45	8.05	14.5
0.13	6.63	6.77	13.4
1.3	10.74	8.15	18.89
Parental and Direct Exposure (PDE)			
Dose (mg/kg/day)	**Testis**	**Seminal Vesicle**	**Σ**
0	0	0	0
0.013	7.48	8.78	16.26
0.13	2.06	5.87	7.93
1.3	7.96	6.59	14.55

important to note that, when comparing both F1 exposure conditions, the animals that received Mn only through the parental route presented a higher number of significant changes in sperm and biochemical parameters than those exposed through parental and direct route, indicating two possible explanatory hypotheses. It is known that Mn is capable of promoting epigenetic changes in the nervous system (Tarale et al., 2016), while other compounds, such as phthalates (Manikkam et al., 2013) and Cd (Angelis et al., 2017; Tian et al., 2014), also cause this effect in the reproductive system. During the development of the organism, a series of epigenetic reprogramming occurs (McSwiggin and O'Doherty, 2018) and, during the spermatogenic process, there is the reorganization of the chromatin (Estill and Krawetz, 2016), which could be considered a hot spot for these modifications. Thus, it is possible to propose that PDE animals acquired, through epigenetic inheritance, a higher resistance to Mn than those belonging to the PE group, especially when considering the multigenerational exposure. On the other hand, it is also possible that the PE animals are more sensitive to changes due to exposure only in the intrauterine period, with the changes being maintained until adult life. Also, the animals exposed directly (PDE) might present a higher resistance to damages due to an attempt of the organism to recover homeostasis in the face of the stress continuously caused by Mn.

In addition, there is a great concern regarding the risk of exposure to Mn and its effects on the reproductive system and alteration of seminal parameters in humans. The present study used realistic doses, referring to the recommended values of daily intake and to the maximum limit tolerated by humans (IOM, 2001; WHO, 2011). Even so, toxic effects were found in the reproductive system, during oral exposure over a generation, corroborating the neurotoxicity study performed by Okada et al. (2016). In contrast, McGough and Jardine (2017) did not find reproductive alterations when exposing rats to Mn through the airway for two generations, demonstrating that the oral route can be an important route of exposure. In the present study, the dose was the main factor responsible for the variation of the data obtained; suggesting that oral and prolonged exposure to low doses of Mn may cause damage to the reproductive system and potential risks to human health.

In humans, semen is used as a biomarker for polluted environments (Bergamo et al., 2016; Li et al., 2015, 2016; Wang et al., 2017) and

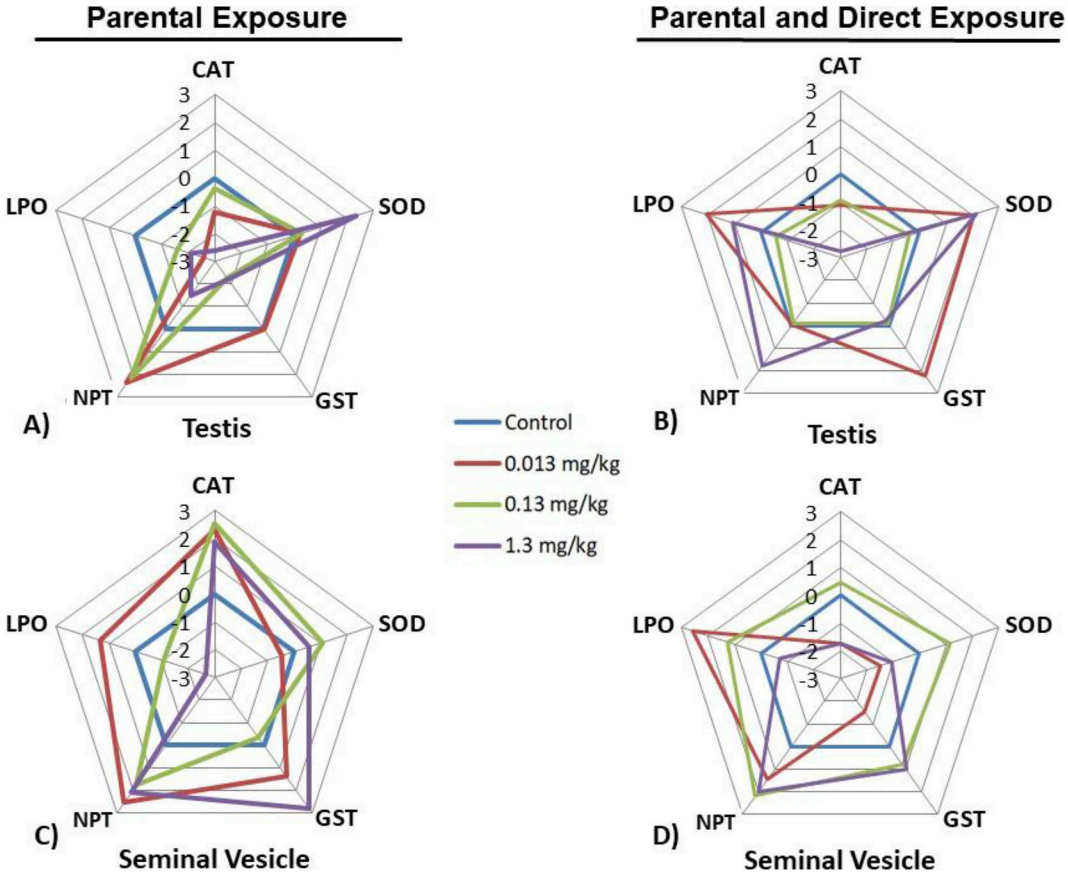

Fig. 5. Graphical representation of integrated biomarker response (IBR). A-B) Testis; C-D) Seminal Vesicle. Parental exposure (EP): n = 10; Parental and direct exposure (PDE): n = 9. The graph shows the oscillation of the biomarkers in the treated groups compared to the control group (Baseline - blue). Area below zero indicates reduction of the biomarker, while values above zero indicate induction of the biomarker. Red line: 0.013 mg/kg/dia; Green line: 0.13 mg/kg/dia; Purple line: 1.3 mg/kg/dia; CAT: Catalase; SOD: Superoxide dismutase; GST: Glutathione s-transferase; NPT: Non-thiols protein; LPO: Lipid peroxidation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

parameters such as sperm motility, volume, and concentration may indicate the effect of environmental contaminants on seminal quality. Based on analyzes of population-based studies, the effect of Mn on seminal quality has been related to markers of apoptosis in spermatozoa and reported as detrimental to sperm motility (Li et al., 2012; Wang et al., 2016; Wirth et al., 2007). Furthermore, there may be a relationship between Mn levels and newborn weight and length reduction in human populations (Rahman et al., 2015; Xia et al., 2016). These findings demonstrate that Mn may be harmful to reproductive aspects in both animal and human models.

5. Conclusion

It is possible to conclude that Mn, even at environmentally realistic doses, may promote reproductive changes in a multigenerational exposure, modifying the sperm parameters and oxidative status of the testes and seminal vesicle. In addition, considering the forms of exposure used in the experiments - parental and parental and direct - it is possible to suggest that epigenetic mechanisms may be involved in the toxicity process, so further studies are needed on this subject.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110763.

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