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Acute reproductive toxicology after intratesticular injection of Silver nanoparticles (AgNPs) in Wistar rats

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Abstract

This study aimed to evaluate the effects of an intratesticular injection of silver nanoparticles (AgNPs) on reproductive parameters and health of rats, and to evaluate the AgNPs biodistribution in order to developing a nanotechnological contraceptive agent for male animals. Treated animals received 220 µL of AgNPs solution (0.46µg-Ag/mL) in each testicle and were euthanized: 7, 14, 28 and 56 days after injection. A significant decrease ($P<0.05$) in the percentage of motile sperm in D7 (8.8%)

was observed, comparing to the control (73.3%), D14 (86.0%), D28 (68.2%) and D56 (90.0%) groups. D7 group also presented a decrease ($P<0.05$) in the percentage of normal spermatozoa. Additionally, D7 group showed an increase ($P<0.05$) in abnormal midpiece and sperm head morphology compared to the Control group. Seminiferous tubules presented all germline cell types and spermatozoa for all groups. However, D7 group did not present spermatozoa in the epididymis, whereas some spermatozoa and cellular debris were visible in D14 and D28 groups. All animals presented haematological parameters, creatinine and ALT values within the normal limits for Wistar rats. The percentage of silver found in the liver was always higher than in the other organs analyzed. A pioneering mathematical model is proposed, from which the half-life time of silver in the liver (17 days), spleen (23 days), lungs (30 days) and kidneys (35 days) was extracted. In conclusion, some acute and severe toxic effects were observed in sperm cells following intratesticular injection of AgNPs, although these effects were reversible. No adverse effects to general animal health were observed.

Keywords: reproductive toxicity, sperm morphology, sperm motility, seminiferous tubules, nanosilver, rats.

Introduction

It is well documented that many nanoparticles (NPs) are capable of crossing the hemato-testicular barrier, leading to toxic effects on male reproductive functions such as reducing sperm count and altering sperm cell function and cell viability (Braydich-Stolle et al. 2010; Ema et al. 2010; Kim et al. 2010). Profiting from advances in the nanobiotechnology field, it is possible to make use of the already known toxicity of some nanomaterials to develop alternative methods to neuter stray animals.

The overpopulation of stray animals (cats and dogs) is a major public health problem (Slater, 2001; Amaku et al. 2010) particularly because stray animals can transmit diseases to domestic animals and humans (Slater, 2002; Slater and Shain, 2005; FAO, 2011), not to mention preying on wild animals. In the same way, exotic invasive animals, as cats in Australia, rabbits in New Zealand and feral pigs in Brazil, cause ecological and economic problems. The major concern regarding these animal populations is their high reproductive potential (Olson and Johnston, 1993). It is well established that the most effective way to control and reduce the number of street animals is their sterilization (Jana and Samanta, 2007). Nevertheless, surgical castration programs currently available are not sufficiently effective on big populations. This way, there is a huge need for alternative methods for castration/contraception for animals.

Since the 1960' researchers are looking for simple methods to neuter male animals, requiring for example a single injection of a neutering drug. Many attempts to find effective drugs to produce the so called chemical castration have been carried out: using calcium chloride (Jana and Samanta, 2006; 2007; 2011; Leoci et al., 2019), cadmium chloride (Kar, 1961), ferric chloride and ferrous sulfate (Kar et al., 1965), glycerol (Wiebe et al., 1989), lactic acid (Fordyce et al., 1989) and zinc gluconate by intratesticular injection. In fact, the intratesticular treatment with an injectable zinc gluconate (neutralized in arginine) has been

approved in 2003 by the Food and Drug Administration (FDA) for use in dogs (ACC&D, 2016). However, it has not been widely used due to side effects and researches are still being performed. WE saw in the nanotechnology a potential tool to develop an alternative castration/contraception method, an aspect that only a few research groups have addressed until now (Liu et al., 2015a; 2015b; Yostawonkul et al., 2017).

Silver nanoparticles (AgNPs) are known to induce toxic effects on spermatogenesis. Studies have reported that AgNPs cause: DNA damage in germ cells, decrease sperm count and promote morphometric changes in seminiferous tubules (Dziendzikowska et al. 2012; Gromadzka-Ostrowska et al. 2012); decrease spermatogonia proliferation (Braydich-Stolle et al. 2005, 2010), and increase germ cell apoptosis (Garcia et al. 2014). In an elegant study, Zhang et al. (2015) demonstrated that AgNPs induced massive cell death in TM3 (Leydig cell line) and TM4 (Sertoli cell line) cells, and inhibited proliferation and self-renewal of spermatogonial stem cells (SSCs). In this study AgNPs induced expression of autophagy-related genes and activated signaling molecules involved in apoptosis. The mRNA levels of proteins that play important roles in self-renewal, proliferation, meiosis and differentiation of SSCs were also significantly lower in AgNPs-treated cells, suggesting that AgNPs could affect the development of germ cells in the testes. Finally, they proved that AgNPs toxicity in those cells was due to ROS generation and accumulation.

Given the toxic effects of AgNPs in the reproductive system following systemic injection, we decided to explore the potential of AgNPs as a sterilizing agent, maximizing its activity by a local intratesticular injection. This approach could be used as a strategy to reduce fertility/reproduction in stray animals. Therefore, the aims of this study were to (i) evaluate the effects of an AgNP intratesticular injection on reproductive parameters and health, and (ii) evaluate the AgNP biodistribution after intratesticular administration.

Material and methods

Silver nanoparticle preparation and characterization

Silver nanopowder containing PVP as dispersant was obtained from Sigma-Aldrich (CAS number 7440-22-4). According to the AgNPs Safety Data Sheet provided by Sigma-Aldrich, the particle size was <100 nm. An AgNPs suspension was prepared according to Cáceres-Vélez et al. (2018). Briefly, the powder was diluted in ultrapure water at 0.46mg-Ag/mL concentration. The suspension was sonicated (Vibra-Cell™ 75042, 20 kHz, 750 W) on ice for 40 min, with pulsing for 5/3 (ON/OFF) seconds and with the amplitude of 20%.

The stock solution (pH 7.49) was diluted (1:4) in ultrapure water while particle size and surface charge were measured in triplicate with 20 sub-runs by DLS (Dynamic Light Scattering), using a Zetasizer Nano-ZS90 (Malvern, Malvern Hills, UK). Samples of this diluted solution were dripped onto a 300-mesh copper grid, dried overnight at room temperature (RT), and analyzed in a Transmission Electron Microscope (TEM) system (JEM-2100F, JEOL, Tokyo, Japan). Images were taken at 20,000× and 50,000× magnification and the sizes of 1000 particles captured. Other characteristics of AgNPs are described in Cáceres-Vélez et al. (2018).

Experimental design

Animal experimentation was approved by the Ethics Committee on Animal Use of the University of Brasilia (CEUA-UnB - n° 102854/2015). A total of 28 male Wistar rats (8-9 weeks old and weighting 154±25 g) were kept in polypropylene cages with woodchips as bedding, in a ventilated room with temperature ~24 °C and under a 12h/12h light/dark cycle with ad libitum access to commercial food (Nutrina - Average nutrient content/Kg: protein-minimum 230g, total fat- minimum 25g, crude fibre-maximum 90g, crude ash-maximum 80g, Calcium-maximum 18g, Phosphorus-minimum 8g) and tap water. Initially, animals were

randomly divided into 3 groups: 1) animals without any manipulation (Control group: N=4), 2) animals receiving an intratesticular injection of saline solution (Sham group: N=4), and 3) animals receiving an intratesticular injection of the AgNPs solution (AgNPs group: N = 20). All animals from the Sham and AgNPs groups were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) (Syntec®) prior to intratesticular injection. Each animal from the AgNPs group received 220 µL of AgNPs solution in each testicle (corresponding to 100 µg Ag/testicle, which is equivalent to 50 µg Ag/g testicular tissue), in 2 different spots. The chosen dose was based on in vitro studies that calculated the EC50 of AgNPs to produce toxic effects in male reproductive cells was 8.75 µg/ml (Braydich-Stolle et al. 2005) and that concentrations ≥ 10 µg/ml, Ag-NPs induced a significant decline in spermatogonial stem cells (SSC) proliferation (Braydich-Stolle et al. 2010). To maximize the effects in vivo we used ~5 times the dose used in vitro. Animals from the Sham group received 0.9% sterile saline solution in the same volume and manner. Animals from the AgNPs group were subdivided into 4 subgroups, according to the day of euthanasia (D7, D14, D28 and D56; N = 5 in each subgroup). Animals from the Control group were euthanized after 14 days, whereas animals from the Sham group were euthanized 7 days after the start of the experiment.

The animals were weighed weekly, and their behaviour was observed daily for signs of pain and/or inflammatory response. The animals were euthanized by an anaesthetic overdose (ketamine and xylazine) and cardiac puncture. Following euthanasia, blood, both testicles and epididymis, liver, spleen, lungs and kidneys were collected for further evaluation.

Blood analysis

Blood was collected for haematological and biochemical analysis. The following haematological parameters were evaluated: white blood cell count (WBC), red blood cell count (RBC), haemoglobin concentration (HB), hematocrit (HTC), mean corpuscular volume

(MCV) and mean corpuscular haemoglobin (MCH) using an automated haematology analyzer (Sysmex pocH – 110 iV Diff TM, Japan). The biochemical parameters evaluated were: alanine aminotransferase (ALT), aspartate aminotransferase (AST) for liver function, and urea and creatinine for kidney function using specific assay kits (Labmax 100 - Labtest[®], Brazil).

Organs Morphometrics

The testicles and epididymis were removed, separated, measured and weighed. The liver, spleen, lungs and kidneys were also weighed. The absolute weight of each organ was transformed into relative weight (organ weight/body weight ×100).

Testicular length and width measurements were used to determine testicular volume (VOL). For this, the average of 2 mathematical formulae was used: the cylinder ($VOLC = 2 \times [\pi(WIDTH/2)^2 \times (LENGTH)]$) and the prolate spheroid ($VOLP = 2 \times [(4/3)\pi(WIDTH/2)^2 \times (LENGTH/2)]$), which together form the closest value to the true testicle volume (Louvandini et al. 2008).

Testicles and epididymis processing

The left epididymis was minced in 2 mL of warmed (37 °C) saline solution to remove the spermatozoa. One drop was immediately placed on a slide, covered with a cover slip and analyzed for sperm motility. In order to evaluate sperm morphology, 1 mL of sperm suspension was added to 1 mL of 10% formalin, with 100 sperm cells per sample classified according to their morphology under a phase contrast microscope (Nikon Eclipse Ci, Tokyo, Japan).

Both testicles and the right epididymis were fixed in Bouin's fixative solution for at least 24 h, dehydrated in a graded ethanol series, and embedded in Paraplast[®] (Sigma Aldrich, USA). Five non-consecutive sections (5 µm thick) from each organ were mounted on

slides, stained with hematoxylin and eosin, and evaluated under a light microscope (Nikon Eclipse Ci, Tokyo, Japan).

Testicular sections were investigated for the presence of spermatogenic lineage, Sertoli and Leydig cells. Histomorphometric parameters were obtained from 25 seminiferous tubule cross-sections for each testicle. Diameter, tubule and tubular lumen area, together with seminiferous epithelium height, were measured using the ImageJ software Version 1.52a (USA).

Determination of silver concentration in organs

One kidney and one half of each of the other organs were frozen for silver quantification and biodistribution analyses.

Total silver concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS), with a two-step digestion protocol (Ashoka et al. 2009). Briefly, 2 mL of 70% nitric acid reacted with the organs for 3 days at RT, under airflow. All samples were diluted to reach a concentration of 1.94%. Finally, 285 μ L of each sample was diluted in 10 mL of ultrapure water and promptly analyzed by ICP-MS (iCAPTM Q ICP-MS Thermo ScientificTM) at the CIC biomaGUNE Mass facility (San Sebastián, Spain).

Statistical analysis

Results were analysed using the SPSS 22.0 (IBM[®] Statistics) package software. Data were tested for normality by the Shapiro-Wilk test. The variables were compared among groups using Analysis of Variance (ANOVA) and Tukey's test. Differences were considered significant when $P < 0.05$.

Results

AgNP characterization

The morphological characteristics of the AgNPs are presented in Figure 1. [Figure 1 near here] According to the TEM micrographs, the AgNPs are approximately spherical, with a mean diameter of 23 ± 8 nm (varying from 3 nm to 46 nm). According to the DSL measurements, the mean hydrodynamic diameter of the AgNPs is 215 nm (PDI of 0.41), with a mean zeta potential of -28.

Animal observation (body, epididymis, testicles, and organ weight)

In general, all animals exhibited normal weight gain (Figure 2) [Figure 2 near here] and a healthy state throughout the study. In addition, no sign of inflammatory process or pain was observed.

The relative weight of testicles and epididymides show no significant differences among groups (Table 1). [Table 1 near here] The volume of the left testicle in the groups treated with AgNPs euthanized at D14, D28 and D56 was significantly higher ($P<0.05$) than in AgNP-treated group euthanized at D7 and the Sham group.

The general aspect of the liver, spleen, lungs and kidneys was normal during euthanasia. No differences in terms of aspect, color or format were observed. The relative weight of the spleen presents a higher value for the AgNPs-D28 group in comparison with other groups, except in the Sham group. The relative weight of the liver also showed a higher value in the AgNPs-D7 group when compared with the other groups, with the exception of the Sham group (Table 2). [Table 2 near here]

Sperm motility and morphology

A significant effect of AgNPs intratesticular injection was observed on sperm motility, with a significant decrease ($P < 0.05$) in the percentage of motile sperm in the AgNPs-D7

group, in comparison to all other groups, with the exception of the Sham group (Figure 3).
[Figure 3 near here]

A significant decrease ($P < 0.05$) in the percentage of normal spermatozoa was also observed in the AgNPs-D7 group in comparison to all other groups (Figure 3). The AgNPs-D7 group also demonstrated a significant increase ($P < 0.05$) in abnormal morphology of the midpiece when compared to all other groups, and a significant increase ($P < 0.05$) in abnormal sperm head morphology compared to the control group (Figure 4). [Figure 4 near here]

Haematological analysis

Mean ($\pm$ SD) values, together with minimum and maximum values, for the haematological analysis of each group are presented in Table 3. Data from each animal is analyzed individually. In general, all animals presented haematological parameters within the normal limits for Wistar rats (last column in Table 3). [Table 3 near here] In a few cases, the specific parameters were altered, but without correlation with the treatments.

Mean ($\pm$ SD) values, together with minimum and maximum values, for the biochemical analysis for each group are presented in Table 4, with the normal limits for Wistar rats in the final column. [Table 4 near here] Individual analysis of each animal showed all animals presented creatinine and ALT values within the normal limits for Wistar rats. However, all animals (treated and controls) presented urea values higher than the normal limit for Wistar rats. Concerning AST, all animals (treated and controls) showed values higher than the normal limits.

Histomorphometry of seminiferous and epididimal tubules

Histological analysis of the testicles allowed the identification of all germline cells in the seminiferous tubules, as well as spermatozoa in the lumen of the tubules, and normal architecture of the seminiferous tubule with the germinal epithelium at different stages of development for the animals of the Control, Sham, and AgNPs-D56 groups. In the AgNPs-D7, AgNPs-D14 and AgNPs-D28 groups, it was possible to visualize that the architecture of the seminiferous tubule is normal, despite the presence of some debris in the lumen and few spermatozoa (Figure 5). [Figure 5 near here]

The histology of the epididymis showed spermatozoa in the lumen in the Control and Sham groups, as well as in the AgNP-D56 group. In the AgNP-D7 group, it was not possible to visualize any spermatozoa, whereas in the AgNPs-D14 and AgNPs-D28 groups some spermatozoa were visible together with some debris (Figure 6). [Figure 6 near here]

Compared to the Control group, all tubular parameters evaluated are significantly smaller ($P < 0.05$) in the AgNPs-D7 and Sham groups (Table 5). [Table 5 near here] Furthermore, a significantly lower epithelium height in the AgNPs-D28 group and a significant smaller lumen diameter in the AgNPs-D14 group were noted in comparison to the Control group values ($P < 0.05$).

Inductively coupled plasma mass spectrometry (ICP-MS)

The total amount of silver (C) in the organs (liver, spleen, lungs, and kidneys) as a function of time (t), determined by ICP-MS, is shown in Figure 7. [Figure 7 near here] Following intratesticular injection, silver was found mainly in the liver (0.82-2.64 $\mu\text{g-Ag}$), whereas the amount of silver in the spleen (0.006-0.220 $\mu\text{g-Ag}$), lungs (0.10-0.87 $\mu\text{g-Ag}$) and kidneys (0.04-0.36 $\mu\text{g-Ag}$) was considerably lower. The data were fitted using a mathematical model that enabled extraction of the half-life time (T_h) of the injected AgNPs in each of the evaluated organs individually. In Figure 6, solid symbols represent the best curve fitting to the

experimental data (solid circles) using the equation described in the Material and Methods section. The mathematical model determined a lower half-life time in the liver (17 days), followed by the spleen (23 days), lungs (30 days) and kidneys (35 days). It is worth mentioning that the mathematical model also suggested that after the intratesticular injection of AgNPs, the NPs take longer to reach the analyzed organs (liver, spleen, lungs, and kidneys). In the testicle, the mean amount of silver found decreased over time (from 0.74 $\mu\text{g-Ag}$ on D7 to 0.19 $\mu\text{g-Ag}$ on D56), but no significant differences were evidenced ($P > 0.05$). The amount of silver found in the epididymis was negligible (0.002-0.004 $\mu\text{g-Ag}$).

While injected into the testicles, the data analyzed allows us to conclude that there is a tendency of AgNPs to accumulate preferably in the liver, although the organ is capable of eliminating the injected silver in a rather fast manner.

The percentage of silver found in each systemic organ (liver, spleen, lungs, and kidneys) is shown in Figure 8. The percentage of silver found in the liver was significantly higher on D7 than D56 ($P < 0.05$). Conversely, the percentage of silver found in the spleen was significantly higher ($P < 0.05$) on D56 than on D7 (Figure 8), although the total silver amount was lower (0.22 $\mu\text{g-Ag}$). [Figure 8 near here]

Discussion

For many years, researchers have looked for an alternative neutering/contraceptive method for animals, especially stray dogs and cats. A single intratesticular injection that could cause the interruption of spermatogenesis would be particularly interesting for its simplicity and speed, as it could be applied to many animals in a single day without the need for specific equipment or environment. In general, zinc gluconate and calcium chloride, the most used substances for chemical castration by intratesticular injection, cause atrophy in the seminiferous tubules, disruption in spermatogenesis, fibrosis and calcification of testicular

parenchyma and reduction of testicular volume, together with reduced sperm motility and low sperm count or azoospermia, leading to subfertility or infertility in male animals (Fagundes et al. 2013, Vanucchi et al. 2015, Silva et al. 2017, Leoci et al. 2019, Rafatmah et al. 2019). However, many studies report side effects in the treated animals, such as swelling, inflammation, pain, hemorrhage, necrosis and scrotal ulcerations (Levy et al. 2008; Oliveira et al. 2013; Forzán et al. 2014; Rafatmah et al. 2019). In this sense, researches are still needed to optimize these methods or find new approaches to impair male animal reproduction.

This study intended to evaluate the effects of AgNPs injected directly in the testicles, with the view to developing a contraceptive method for animals based on a single injection. We observed a severe reduction in sperm motility and the number of sperm with normal morphology 7 days after the treatment, although these findings were not long lasting. Sperm parameters start to recover after 14 days and return back to normal 56 days after injection. It has already been shown that AgNPs cause sperm damage. Ahmed et al. (2016) observed a significant reduction in sperm count and motility together with an increase in sperm abnormality in animals that received intraperitoneal injections of AgNPs (100 or 1000 mg/Kg) daily for a total of 7 or 28 days. Baki et al. (2014) also observed a significant decrease in sperm motility and the number of sperm with normal morphology in Wistar rats that were fed AgNPs (25, 50, 100 or 200 mg/Kg) daily for a total of 45 days. Gromadzka-Ostrowska et al. (2012) observed a decrease in the number of epididymal spermatozoa and an increase in the number of dead sperm cells, together with significantly higher DNA damage in germ cells, after a single intravenous injection of AgNPs (5 or 10 mg/Kg). Although the doses and administration route greatly varies among studies, all of the aforementioned authors pointed out that the nanotoxicity of AgNPs in sperm cells was enhanced with increasing dosage and nanoparticle exposure (Gromadzka-Ostrowska et al. 2012; Baki et al. 2014; Ahmed et al. 2016). In our study we used a relatively low dose (equivalent to 1.25 mg/kg)

compared with these previous studies; however, we used a local administration (~50 µg/g of testicular tissue) instead of the systemic administration used by the cited authors, where only a part (not known) of the dose would reach the gonad. We envisaged that a local administration would potentialize the toxic effects on the spermatogenesis with minimal effects in systemic organs. Moreover, as these studies did not evaluate the results over time, there is no mention of the effects being permanent or temporary.

The local administration used in this work (intratesticular injection) was chosen to take advantage of a direct effect of the AgNPs on the sperm cell lineage. In an in vitro study with C18-4 cell line (a type A spermatogonia isolated from mouse testes) it was shown that AgNPs drastically reduced mitochondrial function and cell viability, leading to necrosis and apoptosis of the cells (Braydich-Stolle et al. 2005). These toxic effect of AgNPs started between 5 and 10 µg/ml, with an EC50 calculated at 8.75 µg/ml (Braydich-Stolle et al. 2005). In the present study, the dose used was ~50 µg/g of testicular tissue, which is 5.7 times the EC50 reported by these authors. Moreover, at concentrations ≥ 10 µg/ml, Ag-NPs induced a significant decline in spermatogonial stem cells (SSC) proliferation by disrupting components of the GDNF signaling pathway, which is essential for SSC self-renewal in vivo and in vitro (Braydich-Stolle et al. 2010). AgNPs was proven to be toxic for the cells involved in spermatogenesis, such as Leydig and Sertoli cells and spermatogonial stem cells (SSCs), and this toxicity was linked to a rapid ROS production and accumulation (Zhang et al. 2015). High ROS levels in seminal plasma have been correlated with poor sperm morphology and is associated with the inhibition of sperm function and viability (Aziz et al. 2004). However, in our study, we found the negative effects on male germinal cells are not long lasting, suggesting that spermatogonia were not lethally impacted. In fact, most of the AgNP effects in the present study seem to be on spermied spermatozoa, which presented reduced motility and abnormal morphology. This may explain the normal parameters observed 56 days post-

treatment, which correspond to a complete spermatogenesis process in rats (Russell et al. 1990; McCarrey, 1993; Creasy, 1997). Gromadzka-Ostrowska et al. (2012) also suggested that the genotoxicity effects of systemic AgNPs observed in their study is pronounced in mature sperm cells in the epididymis. Another in vitro study with ejaculated sperm suggests AgNPs interfere with membrane receptors or cell signaling involved in sperm motility (Terzuoli et al. 2012).

Regarding testicle histology, no damage or absence of any germ cell type was observed in the present study. Only a reduction in the amount of spermatozoa and the presence of debris within the lumen of seminiferous tubules in the AgNPs-D7 group were observed. Moreover, no spermatozoa were observed in the epididymis lumen of animals in the AgNPs-D7 group, whereas some spermatozoa and debris were found in the epididymis of the AgNPs-D14 and AgNPs-D28 groups. Similarly, Lafuente et al. (2016) reported an increased number of epididymal sperm morphological abnormalities but no significant changes in testicle and epididymis morphology of rats treated orally with PVP-AgNPs (50, 100 or 200 mg/kg) daily for 90 days. In contrast, Gromadzka-Ostrowska et al. (2012) observed damage to the seminiferous epithelium, wider intercellular spaces and vacuolization after a single intravenous injection of AgNPs (5 or 10 mg/kg). Considering the higher dose used by Gromadzka-Ostrowska et al. (2012), even if the AgNPs were equally distributed through the various body tissues/organs, which is probably not the case, the maximum amount of AgNPs reaching each testicle would be 20 µg. Considering the local injection and the dose (100 µg/testicle) of AgNPs used, we expected to observe more pronounced changes in testicular morphology. Our findings suggest that only spermied spermatozoa are affected, either within the seminiferous tubules or in the epididymis. Some reproductive toxicology studies classified numerous chemicals that are toxic to the testis with the ability to alter the quantity/quality of the sperm produced via spermatogenesis, together with the quantity and quality of sperm that

enter the epididymis, thus identifying the epididymis as a testis-independent target. Additionally, there are numerous chemicals that produce epididymis-specific reduction in cauda epididymal sperm number, without altering testicular sperm numbers (Kempinas and Klinefelter, 2010, 2014).

The seminiferous tubule morphometric analysis showed that all of the tubular parameters evaluated significantly reduced in the AgNPs-D7 group, whereas no difference was found between AgNPs-D14, D28 and D56 and Control groups. Similar results were reported by Fathi et al. (2018), who found that an intraperitoneal injection of AgNPs (125 mg/kg) caused a significant reduction in the diameter, area and circumference of seminiferous tubules. In contrast, increased diameter, area and circumference of seminiferous tubules in animals treated with AgNPs were observed by Gromadzka-Ostrowska et al. (2012). Yet, Miresmaeili et al. (2013) found no significant changes in seminiferous tubule diameter in animals daily treated by the oral administration of AgNPs at doses varying from 25 to 200 mg/kg, after 48 days. These differences may be due to the different administration routes, treatment period and AgNP formulation employed. Moreover, in the present study, no differences between the relative weight of the testicles and epididymis in animals treated with the AgNPs were observed. Similarly, other studies using different AgNP administration routes reported no significant relative changes in body, testicle or other organ weights in male rats (Sung et al. 2009; Kim et al. 2010; Gromadzka-Ostrowska et al. 2012; Garcia et al. 2014).

Regardless of the route of administration, NPs can cross body barriers and enter the bloodstream through the circulatory and lymphatic systems, translocating from the blood circulation to the main organs, such as liver, spleen, lungs and kidneys (De Jong et al. 2008; Singh and Lillard, 2009; Dziendzikowska et al. 2012). Thus, we evaluated the animals for systemic functions and did not observe any alterations in water or food consumption. All animals gained weight as expected. Previous reports showed that food and water

consumption, together with body and organ weight did not differ between animals treated with AgNPs and control groups (Sung et al. 2009; Kim et al. 2010).

Moreover, all animals presented haematological parameters within the normal limits for Wistar rats (Cubas et al. 2014). Some specific parameters were altered, but without correlation to the treatments. In the same way, other authors did not observe any significant changes in the haematological parameters of rats exposed to AgNPs (Sung et al. 2009; Kim et al. 2010; Yang et al. 2017). In this study, the biochemical parameters of ALT and creatinine were within the normal limits for Wistar rats (Cubas et al. 2014) for all animals, while increased urea and AST values were observed for all animals in the control, as well as in the treated groups, which are not correlated with the treatment. It is important to note that the literature presents quite discrepant data for biochemical parameter limits in Wistar rats. For example, the AST limits are described as 63 – 175 IU/L (Quesenberry and Carpenter, 2012) or 39 – 92 IU/L (Cubas et al. 2014). Similarly, for urea, the limits are described as 26 – 58 mg/dl (Lima et al. 2014) or 15 – 21 mg/dl (Cubas et al. 2014). Other studies also related no significant alterations in blood biochemical parameters in animals treated with AgNPs (Sung et al. 2009; Yang et al. 2017), although most of the reported studies did not use the same analytical approach.

We also used ICP-MS to analyze the presence of silver in the liver, spleen, lungs and kidneys after the intratesticular injection of AgNPs. In general, the amount of Ag found in these organs (less than 200 ng/g of wet weight in any of the analyzed organs) was lower than the described by other authors (Kim et al. 2010; Van Der Zande et al. 2012; Yang et al. 2017) when administering AgNP to animals by other via (oral or IV), which might be due to the very low dose we used (~1.25 mg/kg) or a result of choosing an intratesticular administration. In this work, minimal amounts of AgNPs were found in all of the organs analyzed (liver, spleen, lungs, and kidneys), but the percentage of silver found was consistently higher in the

liver than in all of other organs examined. In fact, other authors described the liver as the target organ in AgNP tissue distribution (Kim et al. 2010; Lankveld et al. 2010; Van Der Zande et al. 2012; Yang et al. 2017; Gan et al. 2019), independent of the administration route. When administrated orally, silver is mostly excreted in faeces, and the analysis of total silver showed accumulation in the liver (Jiménez-Lamana et al. 2014). AgNPs are also excreted in faeces when injected intravenously, and the authors suggested they were secreted by bile (Park et al. 2011). Pathological changes in the liver together with significant changes in serum AST and ALT were observed in mice receiving AgNP orally (250 mg/Kg) for 28 days, indicating that high doses of AgNPs had a subacute toxicity on mice liver (Gan et al. 2019). Kim et al. (2010) also found damage to the liver, including histopathological findings (bile-duct hyperplasia and increased foci) and alkaline phosphatase increase, when rats received 125 mg/kg of AgNP by gavage for 13 weeks. In the present study, ALS and AST levels did not suggest pathological alterations in the liver of animals that received the intratesticular injection of AgNPs. It is important to note that the doses administered in the studies of Kim et al. (2010) and Gan et al. (2019) were much higher than the one used in the present study, and that the animals received AgNP for several days. In fact, no sign of any adverse effects was observed in the animals in the present study.

The intratesticular AgNP injection caused some acute and severe toxic effects in sperm cells, although not permanent, revealing reversibility while not being able to neuter the treated animals. Despite that, no adverse effects are caused in the vital organs (liver, spleen, lungs, and kidneys) relating to the intratesticular injection of AgNPs. It is still possible that the intended animal neutering effect could be achieved by increasing the dose used. Using ICP-MS, it is possible to track the time-dependence (t) of the silver content (C) in key organs (liver, spleen, lungs, and kidneys) up to 56 days. Moreover, this study proposes a pioneering mathematical model describing C versus t , from which the half-life time (T_h) of the injected

silver (via AgNPs) in the liver ($T_h = 17$ days), spleen ($T_h = 23$ days), lungs ($T_h = 30$ days) and kidneys ($T_h = 35$ days) are successfully extracted. Considering the temporary effect of the AgNP intratesticular injection and the absence of adverse effects, further investigations on testicular injections of AgNPs must be done in the attempt to develop a contraceptive method for male animals.

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DISCLOSURE OF INTEREST

The authors report no conflict of interest.

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Figure 1: Electron micrography of the AgNPs (upper left-hand side panel), size distribution of the AgNPs by TEM measurement (upper right-hand side panel). Table (lower panel) presents the morphological parameters (TEM and DSL) and zeta potential of the AgNPs.

Figure 2: Weekly weight gain in each study group during the experimental period.

Figure 3: Mean percentage of motile spermatozoa in each study group (A, B - different letters indicate significant difference, $P < 0.05$).

Figure 4: Mean percentage of normal and abnormal sperm morphology by spermatogenic region (A, B - different letters indicate significant difference in the same category, $P < 0.05$).

Figure 5: Micrographs of seminiferous tubules from each group. Control group (a); Sham group (b); AgNPs-D7 group (c); AgNPs-D14 group (d); AgNPs-D28 group (e); AgNPs-D56 group (f). (Bars = 10 μ m).

Figure 6: Micrographs of epididymis from each group. Control group (a); Sham group (b); AgNPs-D7 group (c); AgNPs-D14 group (d); AgNPs-D28 group (e); AgNPs-D56 group (f). (Bars = 10 μ m).

Figure 7: Mean amount (C) of silver (μ g-Ag) in the liver (a), spleen (b), lungs (c) and kidneys (d) of animals treated with AgNPs versus time (t), in the range of 7 to 56 days. Solid lines represent the best curve fitting to the experimental data (solid symbols). The half-life time (T_h) of the injected AgNPs in the organs are included in the panels.

Figure 8: Distribution of the total measured silver by the organs analyzed (liver, spleen, lungs and kidneys) from animals treated with AgNPs on days 7, 14, 28 and 56 after intratesticular injection.