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Ascorbic acid co-administered with rosuvastatin reduces reproductive impairment in the male offspring from male rats exposed to the statin at pre-puberty

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ABSTRACT

Obesity during childhood and adolescence is closely related to dysfunctions on lipid profile in children. Rosuvastatin is a statin that decreases serum total cholesterol. Ascorbic acid is an important antioxidant compound for male reproduction. Pre-pubertal male rats were distributed into six experimental groups that received saline solution 0.9% (vehicle), 3 or 10 mg/kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 3 or 10 mg/kg/day of rosuvastatin co-administered with 150 mg/day of ascorbic acid by gavage from post-natal day (PND) 23 until PND53. Rats were maintained until adulthood and mated with nulliparous females to obtain the male offspring, whose animals were evaluated at adulthood in relation to reproductive parameters. This study is a follow up of a previous paper addressing potential effects on F0 generation only (Leite et al., 2017). Male offspring from rosuvastatin-exposed groups showed increased sperm DNA fragmentation, androgen depletion and impairment on the testicular and epididymal structure. Ascorbic acid coadministered to the fathers ameliorated the reproductive damage in the offspring. In summary, paternal exposure to rosuvastatin may affect the reproduction in the male offspring; however, paternal supplementation with ascorbic acid was able to reduce the reproductive impairment in the male offspring caused by statin treatment to the fathers.

1. Introduction

Children and adolescents have been affected earlier by dysfunctions such as dyslipidemias, thus they have presented increased concentrations of serum LDL-cholesterol, VLDL-cholesterol and triglycerides, as well as diminished concentrations of HDL-cholesterol (Jiménez and Ferre, 2011; Kwiterovich, 2008a, 2008b). Currently, more attention has been paid to the pediatric population in relation to the dysfunctions associated with dyslipidemias, such as metabolic syndrome, obesity, glucose intolerance, insulin resistance and higher blood pressure (Kwiterovich, 2008b).

Pediatric dyslipidemias may have a genetic component leading to changes in lipid profile or may be related to the lack of physical exercises (Cook and Kavey, 2011; Ross, 2016), bad eating and sedentary habits (Izar et al., 2011; Ross, 2016).

Statins are the main drug class used in the treatment of dyslipidemias, thus they are lipid-lowering medications that act inhibiting the limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (Istvan and Deisenhofer, 2001; Jiménez and Ferre, 2011).

Rosuvastatin is one of the last generation statins and was approved by regulatory agencies in 2001 (McTaggart, 2003) and furthermore, it presents superior inhibitory effects on cholesterol biosynthesis and higher capacity to diminish total cholesterol concentrations (Holdgate et al., 2003; McTaggart, 2003; Vaughan and Gotto, 2004).

Statins reduce androgen biosynthesis by decreasing expression and isoprenylation of CYP17A1 (Ortega et al., 2014, 2012). A previous study has shown that statin exposure may increase reactive oxygen species and augment oxidative stress (Qi et al., 2013). Moreover, androgen depletion may increase oxidative stress and provoke an augmentation of DNA damage and fragmentation, leading to germ cell death (Leite et al., 2017a; Stanton et al., 2012).

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Children and adolescents are exposed to many chemical substances and some of these chemicals may compromise puberty timing, acquisition and development of reproductive capability (Stoker et al., 2000). Previous studies showed that rosuvastatin administration during pre-puberty provoked delayed puberty installation and impaired sperm quality and testicular and epididymal morphology on puberty and adulthood (Leite et al., 2017a, 2017b, 2014).

Several studies have proposed the use of substances that act as an antioxidant or present an effect on diminishing the toxic adverse effects of a medication (Corsetti et al., 2011; Fernandes et al., 2011a, 2011b; Mukhopadhyay et al., 2013; Pandir et al., 2014). Several medications are necessary for the treatment of diseases, however, they may show adverse effects during the period when people are been exposed to them (Pandir et al., 2014; Sooriyaarachchi et al., 2012).

Ascorbic acid is a vitamin required for many biochemical reactions in the organism (Fernandes et al., 2011a, 2011b; Sönmez et al., 2005). On male reproductive system, ascorbic acid acts as an antioxidant compound against the oxidative stress and has an essential role in fertility and sperm integrity (Agarwal et al., 2005; Eskenazi et al., 2005; Fernandes et al., 2011a; Shrilatha and Muralidhara, 2007).

Ascorbic acid improve testosterone levels (Sönmez et al., 2005) by augmenting LH receptors expression in Leydig cells and stimulating the steroidogenic enzymes 17β-HSD, 3β-HSD and StAR protein in testes, leading to increased androgen biosynthesis (Harikrishnan et al., 2013; Radhakrishnakartha et al., 2014).

Moreover, various studies have shown that parental exposure to an endocrine disruptor may compromise reproduction of the exposed animals, as well as, affect reproductive function of the male or female offspring from the exposed animals even for two subsequent generations (Favareto et al., 2011; Schagdarsurengin and Steger, 2016; Silva et al., 2016; Zhao et al., 2015).

Previous study have insinuated possible epigenetic changes induced by statins (Allen and Mamotte, 2017). Bustan and Jawad (2017) suggested that rosuvastatin or atorvastatin exposure to both male and females may affect the fertility of their offspring by epigenetics changes. Additionally, it was shown increased sperm DNA damage in pre-pubertal rosuvastatin-exposed rats at adulthood and augmented post-implantation loss in females mated with these males (Leite et al., 2017a).

Considering the increased post-implantation loss in females mated with statin-treated males (Leite et al., 2017a) and the evidence of transgenerational effects following the exposure to statins (Bustan and Jawad, 2017), the present study aimed to assess the reproductive parameters on male offspring from rosuvastatin-treated rats and evaluate the possible preventive role of paternal supplementation with ascorbic acid for male offspring reproduction, since previous studies reported that ascorbic acid prevented the reproductive adverse effects in the F0 generation by improving testosterone levels and reducing oxidative stress (Leite et al., 2017b, 2017a).

2. Material and methods

2.1. Animals

2.1.1. Obtainment of pups and reduction of litters

Nonpathogenic free *Wistar* rats, both males and females with 45 days of age, were supplied from Central Biotherium of São Paulo State University (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu during the experiment.

Male and female *Wistar* rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled conditions for temperature (23 ± 1 °C) and lighting conditions (12:12 h photoperiod). The health condition of the animals was monitored throughout the experiment. Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During sexual maturity two nulliparous female rats (75 days of age) were mated with one male (90 days of age) during the dark phase of the lighting cycle; the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating female rats were maintained in individual cages.

After birth, the number of pups per litter was reduced to eight on postnatal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not included in the experimental protocol and were subsequently euthanized by cervical dislocation.

2.1.2. Experimental design

Non-obese immature male rats were distributed into six experimental groups on PND 23 (n = 10 per group, with one pup per litter for each group), that received vehicle (saline solution 0.9%, control group), supplementation with 150 mg/day of ascorbic acid (AA), 3 or 10 mg/kg/day of rosuvastatin diluted in saline solution 0.9% (3 mg or 10 mg) or 150 mg/day of ascorbic acid associated with 3 or 10 mg/kg/day of rosuvastatin (3 mg + AA or 10 mg + AA). L-ascorbic acid (98,04% of purity) and rosuvastatin calcium (95,24% of purity) were purchased from a commercial pharmacy (Farmácia Botica Oficinal, Botucatu/Brazil) in the form of active principle. The drugs were administered orally by gavage from PND 23 to PND 53, following male pubertal assay of 31 days recommended by U.S. Environmental Protection Agency (EPA) (Stoker et al., 2000). This period of treatment represents a critical period for development and maturation of reproductive system (Leite et al., 2017b; Stoker et al., 2000).

The doses of rosuvastatin between 5 and 40 mg/day are frequently used by humans to decrease total cholesterol and LDL-cholesterol (Vaughan and Gotto, 2004), thus the doses used in this study were based on body surface area correction from children available doses of rosuvastatin to prepubertal rats equivalent doses (Reagan-Shaw et al., 2008). The doses of ascorbic acid supplementation were based on previous studies (Fernandes et al., 2011b, 2011a).

Male rats (generation F0) were maintained until postnatal day (PND) 110 when were mated with nulliparous female rats to obtain their male offspring (generation F1). After the birth of pups, litters were reduced to eight pups on PND 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not included in the experiment and thereafter, were euthanized. This paper is a follow up of a previous study addressing effects on F_0 generation only (Leite et al. Food Chem Toxicol 2017, 109(Pt 1):272–283).

Male offspring were maintained until PND 110, after performing the sexual behavior test on PND 100, in order to evaluate the intergenerational reproductive effects due to paternal exposure to rosuvastatin and/or ascorbic acid supplementation during pre-puberty. Thereby, the following parameters were obtained: final body weight, reproductive and vital organ weights, testicular and epididymal histopathology and spermatogenesis kinetics, as wells as, sperm counts, motility and morphology, sperm DNA damage and hormonal concentrations. Furthermore, male rats were assessed in relation to their reproductive performance and fertility after sexual behavior test.

Rats were monitored in relation to the indications of distress, such as the presence of bristling hair and the ingestion of food and water during the whole experiment. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

2.2. Evaluation of male sexual behavior

Male rats were placed individually in clear polycarbonate cages, measuring 44 × 31 × 16 cm, 5 min before the introduction of one sexually receptive nulliparous adult female in natural proestrus or estrus determined by vaginal smear. Behavioral testing was assessed in

the dark phase of the cycle between 8:00 am and 12:00 pm in a separate room under dim red illumination. If the male rat did not mount within the next 10 min, a second try was allowed on the next day.

The following measures were recorded (Ahlenius and Larsson, 1984): intromission latency, defined as the time from introduction of the female in the cage to the first intromission; the number of mounts and intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to the first ejaculation; latency to the first post-ejaculatory intromission, the time to the first intromission after the first ejaculation; the number of intromissions after the first ejaculation and total number of ejaculations. During the second chance, if the male did not mount in the first 10 min following the introduction of one receptive adult female into the cage, it was considered sexually inactive.

2.3. Euthanasia of the rats, body weight and organ weights

Male offspring (one male per litter) were weighed on PND 110 and euthanized following narcosis by CO_2 asphyxiation and thereafter, blood was collected by inferior vena cava, between 9:00 and 11:30 a.m. Reproductive organs, such as left testis, epididymis and vas deferens, seminal gland (full and empty, without the coagulating gland) and ventral prostate, from the rats were collected and weighed. Vital organs that have an important role for toxicological parameters, such as kidneys, adrenal glands, liver, thyroid, pituitary and brain were also obtained and weighed.

2.4. Hormonal concentrations

Serum was obtained by centrifugation (2400 rpm, 20 min, 4 °C) in a refrigerated device and was frozen at −20 °C until the moment of hormonal dosages. Furthermore, testicular fluid was obtained by centrifugation (10000 × g, 5 min, 4 °C) of the right testis (without tunica albuginea) in a refrigerated device and was frozen at – 80 °C until the moment of hormonal determination. Testosterone, FSH and LH were determined by the double-antibody radioimmunoassay. Plasma LH and FSH concentrations were determined using specific kits provided by the National Hormone and Peptide Program (Harbor-UCLA, USA). The primary antibodies for LH and FSH were anti-rat LH-S10 and FSH-S11 and the references were LH-RP3 and FSH-RP2, respectively. The lower limit of detection for LH was 0.04 ng/mL and for FSH, 0.2 ng/mL. The intra-assay coefficients of variation were 3.4% for LH and 3.0% for FSH. Plasma levels of testosterone were determined using specific kits provided by MP Biomedicals (Orangeburgh, NY, USA). The lower limit of detection was 0.07 pg/mL and the intra-assay coefficient of variation was 4.0%. All samples were measured in duplicates and included in the same assay to avoid inter-assay errors.

2.5. Sperm motility and morphology

Sperm motility was assessed soon after euthanasia. The right vas deferens was collected, sperm were obtained and a sample was diluted in 2 mL of the modified HTF medium (Spectrun 90126), then a 10 μL aliquot was transferred to a Mackler chamber. Under a light microscope (20x magnification), 100 spermatozoa were analyzed and classified as: type A, motile with regular and fast progressive movement; type B, motile with non-progressive movement or type C, immotile. Sperm motility was expressed as the percentage of total sperm (Perobelli et al., 2012).

Sperm diluted in modified HTF medium were also used for sperm morphological evaluation and were added to 1.0 mL of saline formol. For the analysis, smears were prepared on histological slides that were left to dry for 90 min and observed in a phase-contrast microscope (400× magnification); 200 spermatozoa were analyzed per animal (Seed et al., 1996). Morphological abnormalities were classified into general categories pertaining to head morphology (with curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken, isolated, i.e., no head attached, or rolled into a spiral). Moreover, the presence and position (proximal, medial or distal) of the cytoplasmic droplet were evaluated in the same sperm (Filler, 1993).

2.6. Sperm counts in the testis and epididymis and sperm transit time

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as previously reported (Robb et al., 1978), with the following adaptations: the right testis from one male rat of each litter were decapsulated and weighed immediately after collection, were homogenized in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 s. After a 10-fold dilution, a sample was transferred to Neubauer chambers (4 fields per animal), where mature spermatids were enumerated. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

To obtain the number of mature spermatids per gram of testis and the relative DSP, the number of mature spermatids and the DSP were divided by the weight of the testicular parenchyma. In the same manner, caput/corpus and cauda epididymis parts were cut into small fragments with scissors and homogenized, and sperm enumerated as described for the testis. The sperm transit time throughout the epididymis was determined by dividing the number of sperm in each part of the organ by the DSP.

2.7. Sperm DNA damage (comet assay)

The comet assay was used for detecting primary DNA damage (single- and double-strand breaks and alkali-labile sites) in sperm isolated from the cauda epididymis that were maintained in modified HTF medium stored at −80 °C. The alkaline version of the assay was carried out according to a previous study (Tice et al., 2000). Briefly, the samples were thawed and 5 μL of the HTF medium with sperm sample was mixed with 75 μL of low-melting-point agarose (0.5%) and then placed onto slides that had previously been covered with a thin layer of normal-melting-point agarose (1.5%). The slides were covered with lysis solution (100 mM Na_2-EDTA, 10 mM Tris-HCl, 2,5 M NaCl, pH 11, containing 40 mM DTT and 2% Triton X-100) and incubated for 1 h at 4 °C. Thereafter, the slides were submitted to a second lysis solution containing proteinase K (100 μg/mL) for 2 h and 30 min at 37 °C and were subsequently incubated in a horizontal electrophoresis tank containing freshly prepared cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH > 13) for 45 min to allow the DNA to unwind and for alkali-labile site expression in an air conditioned room (18 °C). Electrophoresis was conducted in the same alkaline buffer for 20 min at 3 V/cm and 270 mA at 4 °C. Then, the slides were washed in PBS solution, rinsed in water, fixed in absolute ethanol and stored at room temperature until analysis. The slides were stained with SYBR® Gold (1:10,000; Invitrogen; Grand Island, NY, USA) immediately before analysis. A total of 100 randomly selected nucleoids per animal was analyzed under 400 × magnification with a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill, UK). Tail intensity (% DNA in the tail) was used to estimate DNA damage. The slides were prepared in duplicate and all steps were conducted in the dark to prevent additional DNA damage.

2.8. Histological procedures

The left testis and epididymis from one male per litter were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 μm cuts (transversal sections of the testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE)

to evaluate testicular and epididymal histology under light microscope. The evaluation was conducted in a blind assay and the photomicrographs were obtained using a Leica light microscope, coupled to a digital camera and a personal computer with the software Leica Qwin Version 3 for Windows.

Seminiferous tubule cross-sections were randomly chosen in three non-serial testicular sections per animal obtained with a distance of 50 µm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium).

Histopathological evaluation of interstitial tissue and peritubular myoid cells was qualitative; the interstitial analysis aimed to assess Leydig cells morphology and the appearance of blood vessels. The epididymal histopathological analysis was also qualitative to evaluate each region of the organ according to the epithelium, lumen and interstitial tissue morphology.

2.9. Spermatogenesis kinetics

To evaluate spermatogenesis kinetics, one hundred random tubular sections per animal (n = 9 or 10 animals/ group) in three non-consecutive testis cross-sections were classified into four categories: stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelium cycle (Leblond and Clermont, 1952), under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at ×200 magnification.

2.10. TUNEL assay

For evaluation of *in situ* DNA fragmentation associated with cell death, TUNEL assay was performed using a commercial kit (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit S7100, Chemicon, Temecula, CA). The manufacturer's instructions were properly followed. Testicular sections from adult male rats were sectioned in 4 µm cuts, were allocated in silanized slides, dewaxed using xylene (Sigma-Aldrich®, USA), hydrated using decreasing concentrations of ethanol and were washed in PBS buffer. Sections were incubated with proteinase K (20 µg/mL) for 15 min, washed with PBS buffer and then, endogenous peroxidase was quenched with 3% of hydrogen peroxide in PBS buffer for 5 min. After washing, the sections were exposed to equilibration buffer followed by incubation with TdT enzyme for 1 h in an incubator at 37 °C. The reaction was blocked with stop buffer, followed by washing of the sections with PBS buffer and incubation with anti-digoxigenin-peroxidase for 30 min at room temperature. The slides were washed with PBS buffer, and then, submitted to diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich®, MO, USA) for 3 min. After this step, testicular sections were counterstained with hematoxylin, dehydrated in crescent concentrations of ethanol and immersed in xylene. Coverslips were mounted on the slides with Entellan. Negative controls were performed without TdT enzyme. Testicular sections were evaluated in the relation of presence of marked cells in different categories of stages of seminiferous epithelium (stages I-VI, VII-VIII, IX-XIII and XIV) and the results were expressed as a percentage of seminiferous tubules with TUNEL-marked germ cells in each category of stages. All analyses were carried out using a Leica light microscope, coupled to a digital camera and a personal computer with the software Leica Qwin Version 3 for Windows.

2.11. Fertility and reproductive performance

This analysis was performed following natural mating. In the case of rats that ejaculated during the evaluation of sexual behavior, couples stayed together for an additional 4 h, allowing a greater number of ejaculations. Animals that had been deemed inactive were tested again daily for the next 5 consecutive days during which different receptive females were placed in their boxes during the dark phase of the cycle.

Every morning, males considered inactive on sexual behavior testing were separated from the females, and vaginal smears of each female were examined for the presence of sperm. The day on which sperm were found in the vaginal smear was considered day 0 of gestation (GD 0). On the 20th day of gestation (GD 20), females were weighed and afterward euthanized following narcosis by CO_2 asphyxiation and decapitation. After collection of the uterus and ovaries, gravid uterus weight was obtained; corpora lutea, implantation sites, resorptions and live fetuses were enumerated and fetal and placental weights were determined.

The following rates were determined based on these results: gestation rate = number of pregnant females/number of inseminated females x 100; fertility potential (efficiency of implantation) = implantation sites/corpora lutea x 100; rate of post-implantation loss = number of implantations - number of live fetuses/number of implantations x 100; sex ratio = number of male fetuses/number of female fetuses x 100.

2.12. Statistical analysis

The results from the different parameters were compared and analyzed among the experimental groups using two-way ANOVA, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were carried out on GraphPad Prism (version 5.00).

3. Results

3.1. Testicular and epididymal morphology and spermatogenesis kinetics

Male offspring from rosuvastatin-treated groups showed lower

Table 1
Te.sticular histopathological evaluation and spermatogenesis kinetics in the male offspring from experimental groups on post-natal day (PND) 110.

	Experimental Groups (n = 9 or 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
[1]Normal seminiferous tubules (%)	97.00 (96.00–98.50) [a]	95.25 (94.50–97.00) [b]	94.50 (94.00–95.50) [b]	97.50 (97.00–98.50) [a]	96.25 (95.50–98.50) [a]	96.50 (96.00–98.00) [a]
[2]Degeneration of germ cells (% of tubules)	1.75 (0.50–3.00) [a]	4.00 (2.50–5.50) [b]	4.50 (3.50–5.00) [b]	1.75 (1.50–3.00) [a]	1.50 (3.00–4.50) [a]	2.50 (1.50–3.50) [a]
[3]Stages I – VI (%)	34.00 (30.00–42.00) [a]	36.00 (33.00–41.00) [a]	33.00 (29.00–39.00) [a]	34.50 (32.00–43.00) [a]	35.50 (28.00–37.00) [a]	35.00 (30.00–36.00) [a]
[4]Stages VII – VIII (%)	31.00 (26.00–36.00) [a]	29.00 (26.00–31.00) [a]	32.50 (25.00–35.00) [a]	30.50 (25.00–34.00) [a]	29.00 (26.00–35.00) [a]	32.00 (28.00–38.00) [a]
[5]Stages IX – XIII (%)	29.50 (25.00–36.00) [a]	29.50 (21.00–35.00) [a]	30.50 (25.00–33.00) [a]	29.00 (27.00–32.00) [a]	29.50 (24.00–38.00) [a]	30.00 (23.00–35.00) [a]
[6]Stage XIV (%)	5.00 (2.00–8.00) [a]	6.00 (2.00–7.00) [a]	6.00 (3.00–7.00) [a]	5.00 (3.00–8.00) [a]	6.00 (3.00–7.00) [a]	5.00 (2.00–8.00) [a]

Values expressed as median and interquartile intervals, [1]$p < 0.001$, [2]$p < 0.05$ e [3, 4, 5, 6]$p > 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

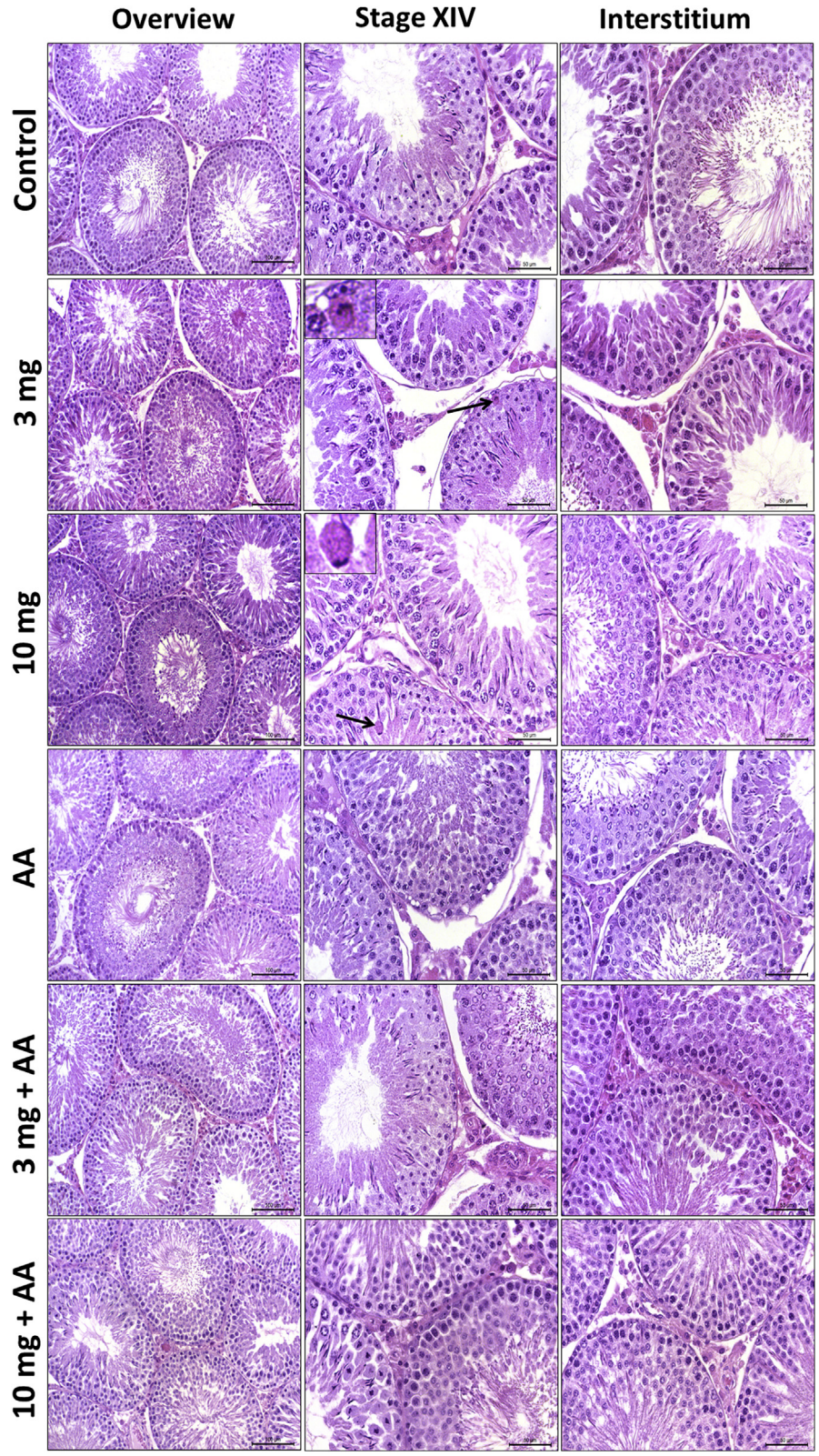

Fig. 1. Photomicrography of testicular sections in the male offspring from experimental groups on postnatal day (PND) 110. Observe the presence of acidophilic germ cells (arrows) in the seminiferous epithelium of the male offspring from rosuvastatin-exposed groups. Hematoxylin and Eosin (HE). Scale bar = 100 μm or 50 μm.

percentage of normal seminiferous tubules in the testis, followed by an increased rate of acidophilic germ cells in the seminiferous epithelium when compared to the control group ($p < 0.05$) (Table 1 and Fig. 1). Paternal supplementation with ascorbic acid prevented the diminished rate of normal seminiferous tubules and avoided the raised percentage of acidophilic germ cells in the seminiferous epithelium of the progeny (Table 1 and Fig. 1). Spermatogenesis kinetics evaluated using categories of seminiferous epithelium stages showed no differences among the male offspring of the experimental groups (Table 1).

The experimental groups whose fathers were exposed to both doses

Table 2
Rate of seminiferous tubules with TUNEL-positive germ cells in relation to the total of seminiferous tubules in the same categories of stages of seminiferous epithelium in the male offspring from experimental groups on PND 110.

Parameters (%)	Experimental Groups (n = 5)					
	Control	3 mg	10 mg	AA	3 mg + AA	10 mg + AA
[1]Total of seminiferous tubules with TUNEL-positive germ cells	3.00 (2.00–4.00) [a]	13.00 (8.00–13.50) [b]	9.00 (7.50–11.50) [b]	4.00 (2.50–4.50) [a]	5.00 (3.00–5.00) [a]	3.00 (2.50–4.50) [a]
[2]Stages I-VI	5.13 (2.57–6.12) [a]	15.00 (12.87–20.77) [b]	11.63 (5.99–13.81) [c]	4.76 (3.39–7.54) [a]	6.12 (3.41–9.10) [a]	6.25 (3.75–6.53) [a]
[3]Stages VII-VIII	0 (0–0) [a]	0 (0–1.52) [a]	0 (0–0) [a]	0 (0–0) [a]	0 (0–0) [a]	0 (0–0) [a]
[4]Stage IX-XIV	3.70 (3.03–5.74) [a]	15.38 (8.04–24.71) [b]	20.00 (20.00–21.29) [b]	4.76 (1.79–8.17) [a]	4.00 (1.67–10.42) [a]	3.70 (1.79–5.93) [a]

Values expressed as median and interquartile intervals, [1], [2], [4]$p < 0.001$ e [3]$p > 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

of rosuvastatin showed an increased rate of TUNEL-positive germ cells, spermatogonias and spermatocytes, in the seminiferous epithelium on stages I-VI and IX-XIV ($p < 0.05$) (Table 2 and Fig. 2). The male offspring from ascorbic-acid-supplemented groups exhibited a rate of seminiferous tubules with TUNEL-marked germ cells similar to the controls (Table 2 and Fig. 2).

Epididymal morphology from the progeny of the rosuvastatin-exposed groups showed apparent hyperplasia of clear cells at proximal cauda of the organ, in comparison with control group (Fig. 3). On the other hand, paternal supplementation with ascorbic acid prevented this histopathological alteration on male offspring (Fig. 3).

3.2. Sperm parameters

Male offspring from the group exposed to the higher dose of rosuvastatin presented lower daily sperm production and a diminished number of mature spermatids in the testis at adulthood, when compared to the control group ($p < 0.05$) (Table 3). Moreover, the progeny of rosuvastatin-treated groups showed lower relative daily sperm production and reduced number of mature spermatids per gram of testis in a dose-dependent manner ($p < 0.05$) (Table 3). Ascorbic acid supplementation for males on generation F0 was able to prevent the decreased sperm production per testis in the offspring (Table 3).

Relative sperm number in the epididymis caput/corpus and cauda was reduced in the male offspring from the group treated with the higher dose of statin, in comparison with the controls ($p < 0.05$) (Table 3). Paternal supplementation with ascorbic acid might avoid this reduced sperm storage in the epididymis of the male offspring from co-exposed groups (Table 3). Sperm transit time throughout the epididymis caput/corpus and cauda was similar among the groups, as well as, total sperm transit time (Table 3).

Sperm motility was lower in the group whose fathers were exposed to 10 mg of rosuvastatin, exhibiting a diminished percentage of progressive and non-progressive sperm and increased frequency of immotile sperm when compared to the control group ($p < 0.05$) (Fig. 4). Ascorbic acid administered to the fathers partially improved the sperm motility on male progeny from co-exposed groups (Fig. 4). On the other hand, the percentage of normally shaped sperm and sperm head and tail abnormalities were similar among the experimental groups (Table 4).

Sperm DNA damage was increased in a dose-dependent manner in the male offspring from statin-treated groups, in comparison with the control group ($p < 0.05$) (Fig. 5). Paternal supplementation with ascorbic acid prevented the increased sperm DNA damage in the male offspring from co-exposed groups (Fig. 5).

3.3. Hormonal concentrations, sexual behavior and reproductive performance

Serum and intratesticular testosterone showed diminished concentrations on male offspring from rosuvastatin-treated groups in a dose-dependent manner when compared to the controls ($p < 0.05$) (Fig. 6). LH concentrations were increased in the group whose fathers were exposed to the higher dose of statin, in comparison with controls ($p < 0.05$) (Fig. 6). Paternal supplementation with ascorbic acid prevented the diminished testosterone concentrations and avoided the increased LH concentrations on male progeny (Fig. 6). FSH concentrations were similar among the offspring of the experimental groups (Fig. 6).

Sexual behavior was similar among the groups for all the parameters evaluated in this study ($p > 0.05$) (Table 5). Reproductive performance and fertility of the males in the generation F1, assessed by natural mating, were not affected by paternal exposure to the statin and/or ascorbic acid supplementation ($p > 0.05$) (Table 6).

3.4. Body weight and organ weights

Final body weight did not exhibit significant differences among the male offspring of the experimental groups ($p > 0.05$) (Table 7). Reproductive organs assessed in this study, such as testis, epididymis, vas deferens, prostate and seminal gland showed similar weights among the groups ($p > 0.05$) (Table 7). In addition, vital organ weights evaluated, such as pituitary, thyroid, liver, adrenal glands, kidneys and brain, also presented no differences among the male offspring of the experimental groups ($p > 0.05$) (Table 8).

4. Discussion

Statins have impaired male reproduction during puberty and on sexual maturity (Leite et al., 2017b, 2017a, 2014); however they are recommended to be used by children and adolescents as lipid-lowering drugs to diminish serum total cholesterol levels. Ascorbic acid has shown a protective role in male reproduction against toxic effects provoked by statin exposure (Leite et al., 2017b, 2017a). The present experimental study aimed to assess the intergenerational reproductive effects in the male offspring of male rats that were exposed to rosuvastatin and/or ascorbic acid during pre-puberty.

This study was performed using non-obese immature Wistar rats; however, it simulates the human situation, since previous studies have reported that pediatric population has exhibited increased frequency of atherosclerosis and also needs to use statins to treat the disease (Ross, 2016; Wiegman et al., 2015); Additionally, more people are taking benefits from the pleiotropic effects of statins (Ludman et al., 2009; Tandon et al., 2005). Furthermore, there is a lack of information about the effects of paternal exposure to rosuvastatin and/or ascorbic acid and the reproductive effects for male offspring.

Androgens are necessary for various biological functions on body, such as the development, maturation and maintenance of male reproductive system, acquisition of secondary sexual characteristics and body growth (Adamopoulos et al., 1990; Mantovani and Fucic, 2014; Marty et al., 2003; Pihlajamaa et al., 2015). Previous study showed that male fetus from pregnant rats exposed to simvastatin, another statin, exhibited lower testosterone production, indicating that reduced

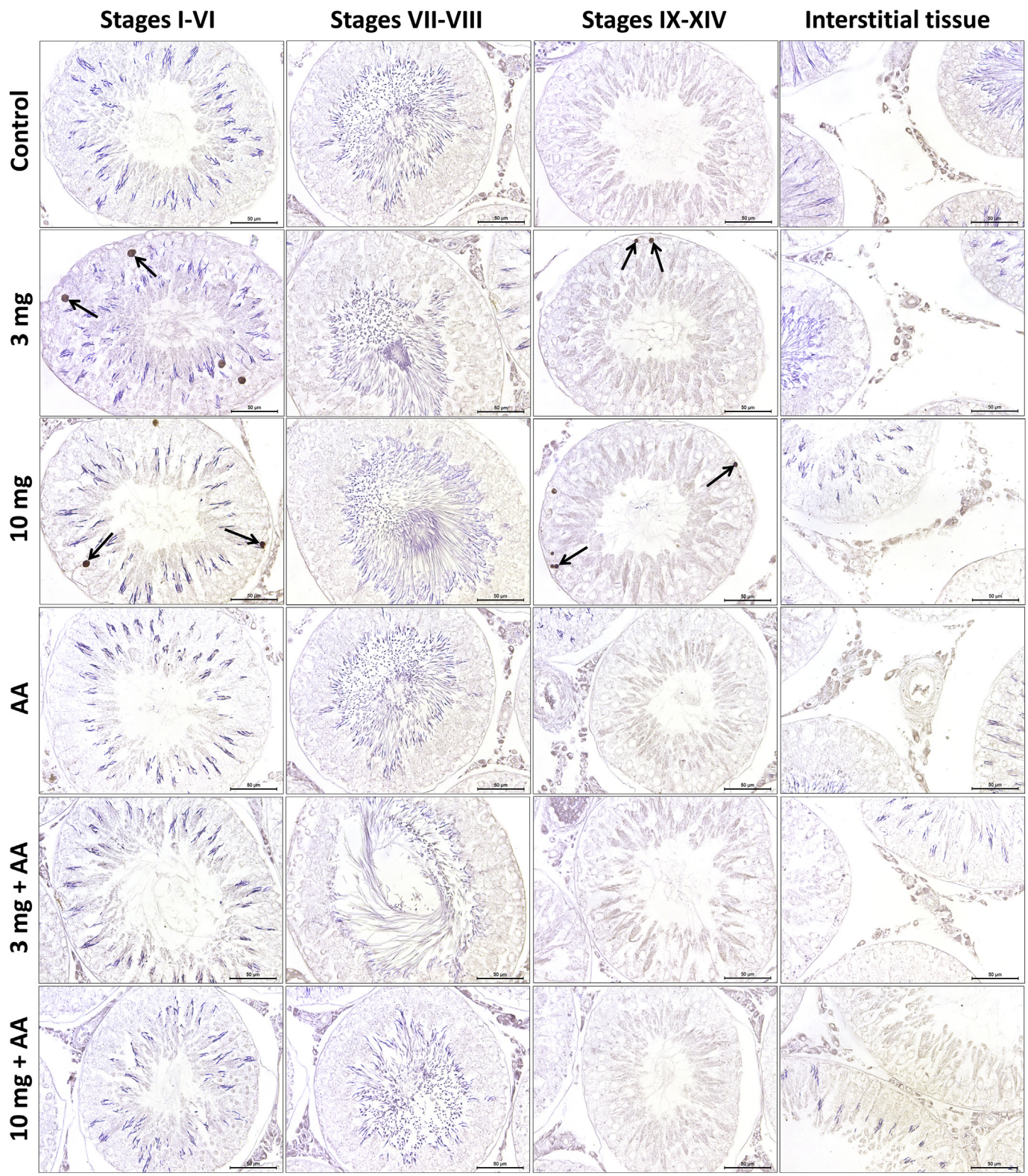

Fig. 2. Photomicrography of TUNEL staining in testicular sections in the male offspring from experimental groups on postnatal day (PND) 110. Observe the presence of TUNEL-marked germ cells (arrows) in the seminiferous epithelium of the male offspring from rosuvastatin-exposed groups. Scale bar = 50 μm.

cholesterol levels, a consequence of statin exposure, are able to decrease androgen synthesis by Leydig cells (Beverly et al., 2014).

Ascorbic acid plays its benefic effects on the prevention of the reproductive impairment by stimulating the steroidogenic enzymes 17β-HSD, 3β-HSD and StAR protein, besides increasing LH receptors expression in Leydig cells, thus stimulating testosterone synthesis (Harikrishnan et al., 2013; Radhakrishnakartha et al., 2014). Additionally, ascorbic acid acts as an antioxidant compound against the testicular oxidative stress and excessive lipid peroxidation (Agarwal et al., 2005; Eskenazi et al., 2005; Fernandes et al., 2011a; Shrilatha and Muralidhara, 2007; Sönmez et al., 2005).

In the present study it was shown that serum and intra-testicular

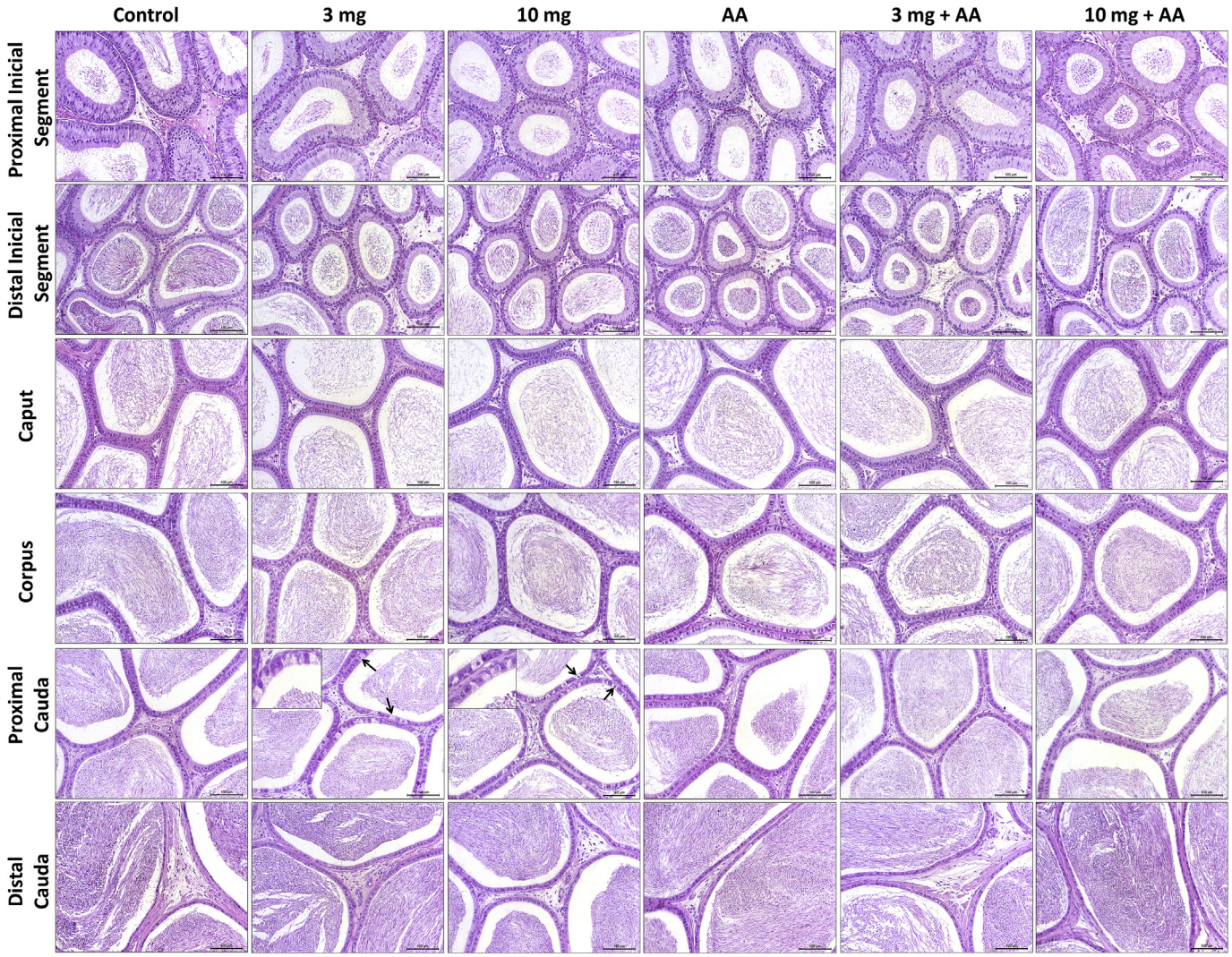

Fig. 3. Photomicrography of epididymal sections in the male offspring from the experimental groups on PND 110. Observe an apparent hyperplasia of clear cells in the epididymal proximal cauda of the male offspring from rosuvastatin-exposed groups (arrows). Hematoxylin and Eosin (HE). Scale bar = 100 μm.

Table 3
Sperm counts in the testis and epididymis in the male offspring from experimental groups on PND 110.

Sperm counts	Experimental Groups (n = 9 or 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Sperm counts in the testis						
[1]Mature spermatid number (10^6/testis)	195.30 ± 2.55 [a]	187.00 ± 4.73 [a]	172.70 ± 3.30 [b]	191.30 ± 4.72 [a]	193.30 ± 3.31 [a]	188.30 ± 2.41 [a]
[2]Mature spermatid number (10^6/g testis)	118.10 ± 2.67 [a]	110.30 ± 3.09 [ab]	100.60 ± 2.89 [b]	114.50 ± 3.46 [a]	112.10 ± 7.74 [a]	111.60 ± 3.62 [a]
[3]Daily sperm production (10^6/testis/day)	32.00 ± 0.42 [a]	30.66 ± 0.74 [a]	28.31 ± 0.54 [b]	31.36 ± 0.77 [a]	31.69 ± 0.54 [a]	30.87 ± 0.40 [a]
[4]Relative sperm production (10^6/g testis/day)	19.36 ± 0.44 [a]	18.08 ± 0.51 [ab]	16.49 ± 0.47 [b]	18.77 ± 0.57 [a]	18.38 ± 0.40 [a]	18.30 ± 0.59 [a]
Sperm counts in the epididymis						
[5]Sperm number in the caput/corpus (x10^6/organ)	126.70 ± 4.32 [a]	127.70 ± 6.80 [a]	117.00 ± 5.38 [a]	127.80 ± 5.31 [a]	123.20 ± 3.72 [a]	124.80 ± 3.66 [a]
[6]Relative sperm number in the caput/corpus (x10^6/g/organ)	448.10 ± 18.38 [a]	412.00 ± 14.44 [a]	379.20 ± 15.28 [b]	457.30 ± 15.85 [a]	439.40 ± 18.12 [a]	430.00 ± 16.10 [a]
[7]Sperm number in the cauda (x10^6/organ)	245.90 ± 4.12 [a]	249.10 ± 9.80 [a]	228.54 ± 5.67 [a]	251.60 ± 6.88 [a]	255.00 ± 8.07 [a]	248.10 ± 5.72 [a]
[8]Relative sperm number in the cauda (x10^6/g/organ)	1265.00 ± 34.10 [a]	1128.00 ± 44.47 [a]	1031.00 ± 31.99 [b]	1138.00 ± 41.59 [a]	1173.00 ± 34.57 [a]	1114.00 ± 54.35 [a]
[9]Sperm transit time in the caput/corpus (days)	3.96 ± 0.11 [a]	4.15 ± 0.14 [a]	4.13 ± 0.16 [a]	4.08 ± 0.12 [a]	3.89 ± 0.11 [a]	4.05 ± 0.12 [a]
[10]Sperm transit time in the cauda (days)	7.69 ± 0.14 [a]	8.11 ± 0.19 [a]	8.08 ± 0.15 [a]	7.82 ± 0.22 [a]	8.03 ± 0.13 [a]	8.04 ± 0.16 [a]
[11]Total sperm transit time (days)	11.65 ± 0.16 [a]	12.27 ± 0.30 [a]	12.20 ± 0.23 [a]	11.89 ± 0.27 [a]	11.92 ± 0.15 [a]	12.09 ± 0.23 [a]

Values expressed as mean ± standard error of mean (SEM), [1], [2], [3], [4],8p < 0.001, [6]p < 0.05 e [5], [7], [9], [10], [11]p > 0.05. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

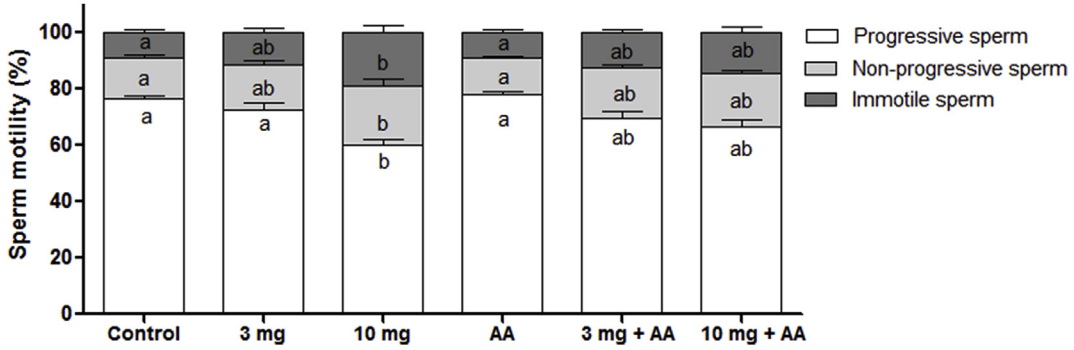

Fig. 4. Sperm motility in rat male offspring from the experimental groups at 110 days of age. Values expressed as median and interquartile intervals. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

testosterone levels of male offspring from rosuvastatin exposed groups were decreased a dose-dependent manner in comparison to control group. Since LH levels showed the opposite profile. i.e., a tendency to increase with 3 mg, and a significant increase with 10 mg of the statin, the data suggest that paternal reduced cholesterol synthesis and decreased androgen levels changed paternal sperm DNA and compromised the testis in the offspring in a transgenerational manner, which is seen by the diminished testosterone secretion by the testis resulting in decreased negative feedback in pituitary gonadotrophs, thus increasing the release of LH. In fact, testosterone produced by Leydig cells is the main modulator of LH secretion while FSH secretion is negatively controlled by inhibin produced by Sertoli cells. Although we did not measure inhibin, we might suggest that the inhibin secretion was not significantly affected by the statin since FSH levels was not modified by any of the statin doses.

The reduced androgens concentrations may be the cause for the reproductive impairment. It was shown that this hormonal deficiency is related to delayed epididymal differentiation, increased rate of germ cell death in the testis (Leite et al., 2017b, 2014) and augmented sperm DNA damage (Leite et al., 2017a).

On testis, Sertoli cells need testosterone to bind their androgen receptors to synthetize proteins related to the survival of germ cells (De Gendt et al., 2004; Stanton et al., 2012). Additionally, when there is testosterone depletion, germ cells undergo cell death (De Gendt et al., 2004; Stanton et al., 2012). Male offspring from rosuvastatin-treated rats, at both doses, showed increased frequency of acidophilic germ cells and TUNEL-positive germ cells on seminiferous epithelium during sexual maturity that represents augmented rate of germ cells undergoing death. On the other hand, male offspring from ascorbic-acid-supplemented groups did not exhibit this augmented rate of germ cells undergoing death, suggesting that paternal supplementation with ascorbic acid was able to prevent this impairment in the offspring. However, spermatogenesis kinetics were similar among the offspring of the experimental groups.

Previous studies reported that prepubertal exposure to rosuvastatin provoked delayed epididymal differentiation (Leite et al., 2017b, 2014) and consequently hyperplasia of clear cells on epididymis cauda at adulthood (Leite et al., 2017a). Clear cells have an important role on the reabsorption of sperm cytoplasmic droplets (Hermo et al., 1992). Furthermore, clear cells are associated with epididymal spermiophagy under normal condition (Kempinas and Klinefelter, 2015), thus increased hyperplasia of clear cells may be related to increased sperm phagocytosis in the epididymis. Epididymal morphology of male offspring from rosuvastatin-exposed rats showed hyperplasia of clear cells on proximal cauda. The groups whose fathers received ascorbic acid did not show this histopathological alteration. The remaining regions of the organ, considering the epithelium, lumen and interstitium showed to be similar among the groups.

Sperm evaluations such as sperm counts, morphology and motility have been used in association as an indicative of sperm quality (Perreault and Cancel, 2001). Lower sperm counts have been associated with diminished male fertility and it is considered as a toxic effect for male reproduction (Perreault and Cancel, 2001). Sperm morphology is used as a parameter to infer the damage that occurred during spermatogenesis on seminiferous epithelium after exposure to a toxicant (Filler, 1993). Furthermore, decreased sperm motility is associated with alterations on the processes of sperm maturation, indicating an adverse effect on epididymis (Perreault, 1998; Perreault and Cancel, 2001). The experimental group whose fathers were exposed to 10 mg of statin exhibited lower sperm motility, diminished sperm production and reduced relative sperm number in the epididymis caput/corpus and cauda. The other experimental groups showed sperm counts and motility similar to the controls. The rate of sperm head and tail abnormalities did not exhibit differences among the groups.

Statins are known to decrease ubiquinone oxidoreductase (coenzyme Q10) concentration at the molecular level, which is related to diminished sperm motility and counts, reduced sperm vitality and increased sperm morphological abnormalities (Littarru and Langsjoen, 2007; Pons-Rejraji et al., 2014). Coenzyme Q10 acts as an important intracellular antioxidant for sperm in the seminal fluid and is positively associated with sperm motility (Pons-Rejraji et al., 2014). Since statins reduce coenzyme Q10 levels, they are able to diminish the efficiency of the antioxidant system (Pons-Rejraji et al., 2014).

Under insufficient androgen stimulation epididymis epithelium regresses, send death signals to the lumen changing the composition of the luminal fluid and activates ubiquitin-proteasome system and

Table 4
Sperm morphology in the male offspring from experimental groups on PND 110.

Sperm morphology (%)	Experimental Groups (n = 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Normal shaped sperm	96.50 (96.00–98.25)	95.00 (94.50–97.88)	96.00 (94.25–96.75)	95.75 (94.38–97.25)	95.50 (94.38–97.25)	95.25 (96.00–97.50)
Sperm head abnormalities	2.00 (0.50–2.50)	1.75 (1.13–2.50)	1.50 (0.75–2.25)	1.75 (0.88–3.13)	2.00 (0.38–2.88)	2.50 (1.00–3.50)
Sperm tail abnormalities	1.50 (1.00–2.25)	2.50 (1.00–4.25)	2.50 (1.50–4.50)	2.25 (0.50–3.13)	2.00 (1.88–3.13)	1.50 (1.00 - 2.25)
Sperm with cytoplasmic droplet	14.00 (10.25–16.25)	13.00 (12.13–14.38)	13.50 (11.25–15.00)	13.00 (12.00–17.00)	13.00 (9.63–14.25)	14.00 (9.50–16.00)

Values expressed as median and interquartile intervals, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

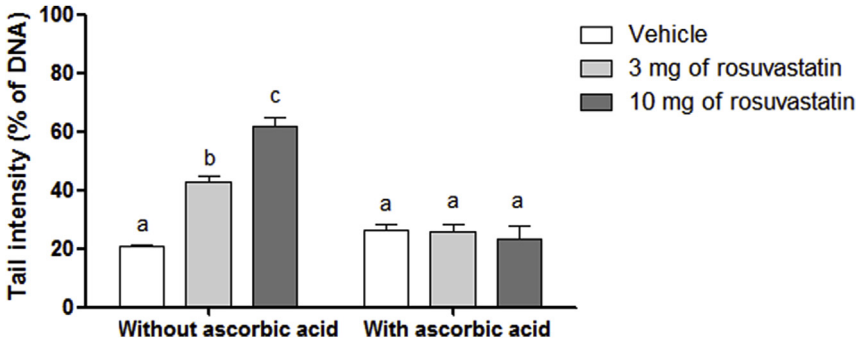

Fig. 5. Sperm DNA fragmentation (Comet assay) in rat male offspring from the experimental groups, observed by percentage of tail intensity. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

DNAses, leading to sperm degradation and sperm disappearing (Jones, 2004). Indeed, reduced androgen levels were accompanied by diminished sperm number in the epididymis from the group whose fathers were treated with the higher dose of statin.

Moreover, a previous study reported a clinical case of transient azoospermia following rosuvastatin medication for hypercholesterolemia, however spermatozoa appeared in the ejaculated semen in two weeks of drug withdrawal (Tada et al., 2015). In this study, rosuvastatin administration for males at pre-puberty compromised reproductive parameters in the male offspring, suggesting a possible reproductive risk for humans that make use of rosuvastatin as a lipid-lowering drug due to the persistence of reproductive damage in the offspring. On the other hand, ascorbic acid supplementation may be useful for clinical purposes due to its actions on improving testosterone levels and reducing oxidative stress (Ahmed et al., 2011; Radhakrishnakartha et al., 2014; Sönmez et al., 2005), like suggested by the restoration of reproductive parameters in the rat male offspring.

The amount of sperm DNA damage is another parameter assessed in toxicological studies on male reproduction (Perreault and Cancel, 2001). Increased sperm DNA damage is related to alterations on embryonic fate and early embryo development (Hales et al., 2005; Marchetti et al., 2003). Previous study has reported that androgen depletion is directly associated with augmented DNA fragmentation (Stanton et al., 2012), and in addition, statin exposure has been also associated with DNA damage (Coetsee et al., 2008; Leite et al., 2017a). Male offspring of rosuvastatin-exposed groups showed decreased testosterone concentrations and increased DNA damage in a dose-dependent manner. These results suggest that rosuvastatin can induce an indirect effect on genome stability, which is transmitted through the germ cell line of exposed parents (Leite et al., 2017a) to their offspring, i.e. epigenetics events. The epigenome is comprised of the modifications made in gene expression by changing DNA and histone structure without changing the DNA sequence itself (Rajender et al., 2011). Epigenetic processes include actions such as DNA methylation, post-translational histone modifications and chromatin remodeling (Rajender et al., 2011). These changes can have short- or long-term effects and might be transgenerational (Rajender et al., 2011).

Several studies reported sperm epigenetic changes compromising embryo development or causing diseases later in life, such as reproductive disorders (Favareto et al., 2011; Ly et al., 2017; Schagdarsurengin and Steger, 2016; Silva et al., 2016; Stuppia et al., 2015). Previous study has associated increased DNA damage caused by oxidative stress with aberrant global methylation (Rajender et al., 2011).

In this context, a previous study has proposed a mechanism wherein a radiation-exposure signal could be inherited through sperm in an epigenetic manner, perhaps by changes in DNA methylation (Dubrova et al., 2000). This epigenetic signal could influence expression patterns

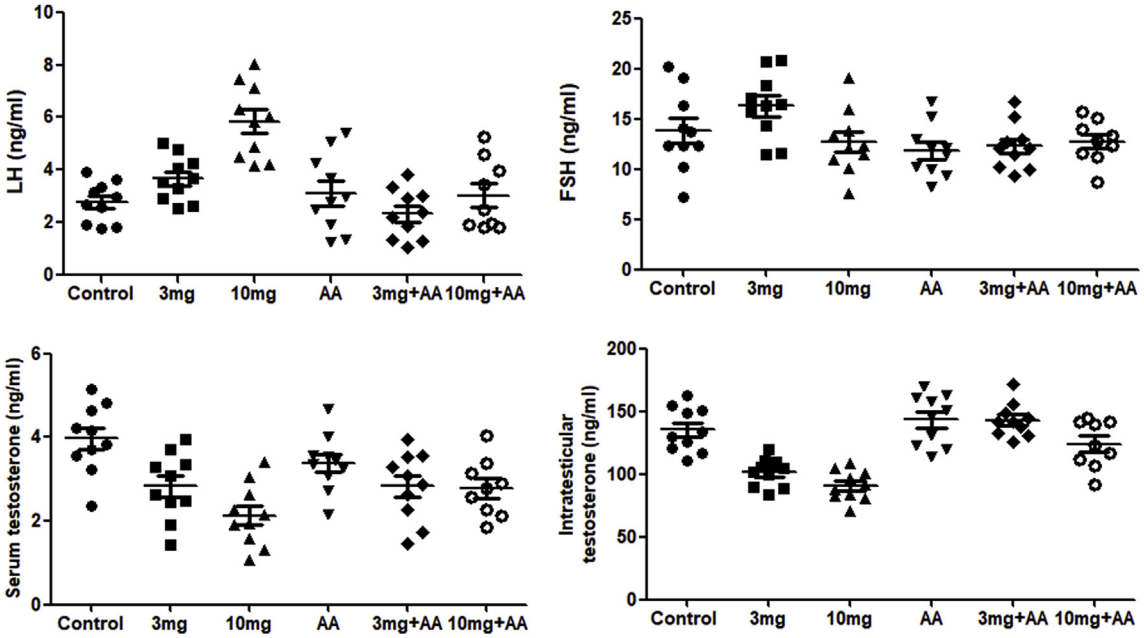

Fig. 6. Hormonal concentrations (ng/ml) in rat male offspring at 110 days of age. Values expressed as mean ± standard error of mean (SEM). Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

Table 5
Sexual behavior test in the male offspring from experimental groups on PND 100.

	Experimental Groups (n = 8)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Number of mounts until de first intromission	2.43 ± 0.61	4.33 ± 1.56	3.86 ± 1.10	3.25 ± 0.49	2.33 ± 0.50	2.25 ± 0.62
Latency to the first intromission (s)	345.78 ± 23.44	318.14 ± 29.58	382.43 ± 26.17	373.71 ± 29.40	349.78 ± 14.12	389.71 ± 23.78
Number of intromissions until the first ejaculation	19.22 ± 2.60	16.00 ± 2.56	17.71 ± 1.82	17.00 ± 3.28	18.80 ± 1.87	18.12 ± 3.00
Latency to the first ejaculation (s)	956.11 ± 91.73	845.00 ± 131.36	960.14 ± 135.08	990.75 ± 143.66	1112.22 ± 132.52	857.86 ± 90.87
Latency to the first post-ejaculatory intromission (s)	183.56 ± 50.30	213.62 ± 53.17	312.00 ± 87.08	189.78 ± 33.84	325.44 ± 57.40	291.62 ± 36.35
Number of post-ejaculatory intromission	7.78 ± 1.29	10.71 ± 1.11	9.57 ± 1.38	11.85 ± 2.63	7.78 ± 0.79	10.00 ± 1.85
Number of ejaculations	2.22 ± 0.22	2.25 ± 0.31	2.57 ± 0.30	2.12 ± 0.35	2.33 ± 0.33	2.57 ± 0.37

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

Table 6
Reproductive performance and fertility test in the male offspring from experimental groups on PND 110.

	Experimental Groups (n = 9 or 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Gestational rate (%)	100%	100%	100%	100%	80%	87.50%
[1]Fertility test (%)	100 (100–100)	100 (94.65–100)	100 (100–100)	100 (96.16–100)	96.43(89.25–100)	100 (91.67–100)
[1]Post-implantation loss (%)	0 (0–8.33)	9.09 (0–22.65)	0 (0–8.33)	0 (0–8.71)	6.90 (0–12.79)	0 (0–7.69)
[1]Sex ratio (M:F)	1.00 (0.53–1.75)	1.00 (1.00–3.50)	0.80 (0.67–1.20)	0.67 (0.35–2.00)	0.84 (0.58–1.25)	0.72 (0.54–1.62)
[2]Body weight (GD20)	342.24 ± 13.62	346.19 ± 11.06	324.36 ± 11.51	336.84 ± 12.38	365.62 ± 8.54	324.79 ± 14.88
[2]Gravid uterus	61.62 ± 3.56	53.58 ± 5.71	57.77 ± 2.22	59.84 ± 2.38	63.99 ± 2.40	56.97 ± 8.76
[2]Male fetus weight	3.42 ± 0.13	3.36 ± 0.11	3.46 ± 0.12	3.49 ± 0.08	3.38 ± 0.09	3.39 ± 0.12
[2] Female fetus weight	3.22 ± 0.12	3.17 ± 0.07	3.21 ± 0.11	3.28 ± 0.09	3.12 ± 0.07	3.09 ± 0.13
[2]Placenta weight from male fetus	0.56 ± 0.02	0.55 ± 0.04	0.55 ± 0.02	0.55 ± 0.01	0.55 ± 0.03	0.63 ± 0.04
[2] Placenta weight from female fetus	0.53 ± 0.02	0.52 ± 0.04	0.55 ± 0.02	0.56 ± 0.03	0.52 ± 0.03	0.60 ± 0.04

Values expressed as median and interquartile intervals[1] or mean ± SEM[2], $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

Table 7
Body weight and reproductive organ weights in the male offspring from experimental groups on PND 110.

	Experimental Groups (n = 9 or 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Final body weight (g)	452.30 ± 7.64	452.90 ± 10.29	429.60 ± 14.58	420.30 ± 8.09	439.80 ± 9.42	435.80 ± 10.93
Testis (g)	1.92 ± 0.05	2.13 ± 0.05	2.13 ± 0.07	2.04 ± 0.06	1.94 ± 0.04	1.99 ± 0.05
Testis (g/100 g BW)	0.39 ± 0.01	0.40 ± 0.01	0.42 ± 0.01	0.42 ± 0.02	0.41 ± 0.01	0.41 ± 0.01
Epididymis (mg)	638.70 ± 15.66	661.80 ± 23.89	653.10 ± 15.16	637.10 ± 17.63	661.30 ± 16.78	642.60 ± 8.97
Epididymis (mg/100 g BW)	141.40 ± 3.64	146.10 ± 4.05	152.80 ± 3.67	152.00 ± 4.80	150.40 ± 2.26	148.00 ± 3.46
Vas deferens (mg)	95.64 ± 3.96	90.16 ± 3.43	102.52 ± 4.12	95.95 ± 3.17	94.20 ± 2.66	91.93 ± 3.89
Vas deferens (mg/100 g BW)	21.29 ± 1.16	19.97 ± 0.85	23.94 ± 0.84	22.86 ± 0.95	21.47 ± 0.66	21.17 ± 0.98
Prostate (mg)	549.70 ± 35.86	634.80 ± 47.44	587.80 ± 44.63	615.40 ± 32.58	609.50 ± 41.60	530.20 ± 36.64
Prostate (mg/100 g BW)	122.70 ± 9.64	140.50 ± 10.74	136.10 ± 7.79	147.40 ± 9.47	137.90 ± 7.58	121.30 ± 6.48
Full seminal gland (mg)	1.48 ± 0.06	1.48 ± 0.06	1.55 ± 0.09	1.37 ± 0.08	1.43 ± 0.10	1.412 ± 0.10
Full seminal gland (mg/100 g BW)	319.30 ± 19.90	328.20 ± 14.69	361.70 ± 17.06	328.20 ± 20.68	324.20 ± 17.30	324.80 ± 25.23
Empty seminal gland (mg)	527.40 ± 14.83	565.70 ± 30.55	550.70 ± 31.97	529.10 ± 39.41	549.70 ± 39.87	494.30 ± 15.84
Empty seminal gland (mg/100 g BW)	117.00 ± 4.34	124.40 ± 4.59	128.70 ± 7.25	126.80 ± 10.52	124.60 ± 7.86	113.90 ± 4.37

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

of genes involved in DNA repair or cell cycle checkpoints in the offspring of irradiated males, such changes could subsequently lead to an accumulation of unrepaired DNA damage and an increase in levels of mutations (Dubrova et al., 2000).

The literature has been suggested that statins can induce epigenetic changes; however, there are scarce available data about rosuvastatin (Allen and Mamotte, 2017). A previous study suggest epigenetics changes affecting the fertility of the offspring from males and females exposed to rosuvastatin or atorvastatin, prolonging the time of gestation in the second generation (Bustan and Jawad, 2017).

Considering the transgenerational DNA damage observed in the offspring from rosuvastatin-exposed groups, we speculated that this lesion triggers an epigenetic signal that influences DNA repair and can survive the reprogramming of DNA methylation during spermatogenesis and early development (Heard and Martienssen, 2014), then subsequent activation of this signal in the germ line could lead to the observed transgenerational mutagenesis, such as was suggested by Dubrova et al. (2000) on radiation studies. In this case, considering germline reprogramming fails, epigenetic marks can be retained, and could be transmitted from one generation to the next (Heard and Martienssen, 2014).

Interestingly, albeit vitamin C was not able to protect F0 from rosuvastin-induced DNA damage by alkaline comet assay (Leite et al., 2017a), its F1 shows low levels of DNA lesions, similar that those from negative control (present study). Considering an epigenetics pathway, the vitamin C could erase epigenetic marks on DNA and enhance reprogramming (Hore et al., 2016). In this way, the methylation induced by rosuvastatin could be demethylated by vitamin C supplementation. In fact, ascorbic acid, named ascorbate in its intracellular form, regulates DNA demethylation, since it is an essential cofactor for Ten-eleven translocation (TET) dioxygenases and regulates histone demethylation because it is an important cofactor for Jumonji C-domain-

Table 8
Vital organ weights in the male offspring from experimental groups on PND 110.

	Experimental Groups (n = 9 or 10)					
	Control	3 mg	10 mg	AA	3 + AA	10 + AA
Pituitary (mg)	10.80 ± 0.84	12.16 ± 0.68	10.46 ± 0.61	11.44 ± 0.50	11.23 ± 0.33	10.70 ± 0.61
Pituitary (mg/100 g BW)	2.38 ± 0.18	2.69 ± 0.14	2.45 ± 0.14	2.72 ± 0.10	2.56 ± 0.08	2.48 ± 0.18
Thyroid (mg)	20.88 ± 1.23	21.71 ± 1.02	19.60 ± 0.79	19.35 ± 0.75	18.69 ± 1.45	19.36 ± 1.64
Thyroid (mg/100 g BW)	4.62 ± 0.27	4.79 ± 0.17	4.60 ± 0.22	4.62 ± 0.19	4.24 ± 0.29	4.42 ± 0.32
Liver (g)	17.08 ± 0.52	16.96 ± 0.50	15.63 ± 0.79	15.41 ± 0.56	15.43 ± 0.70	15.72 ± 0.71
Liver (g/100 g BW)	3.77 ± 0.07	3.75 ± 0.08	3.63 ± 0.12	3.66 ± 0.09	3.50 ± 0.12	3.61 ± 0.12
Adrenal (mg)	36.46 ± 2.25	38.24 ± 0.66	34.78 ± 2.32	33.53 ± 1.24	34.71 ± 1.80	33.94 ± 1.49
Adrenal (mg/100 g BW)	8.03 ± 0.43	8.46 ± 0.17	8.11 ± 0.49	8.01 ± 0.33	7.89 ± 0.36	7.83 ± 0.41
Kidney (g)	1.69 ± 0.04	1.65 ± 0.03	1.60 ± 0.05	1.53 ± 0.04	1.59 ± 0.03	1.65 ± 0.03
Kidney (g/100 g BW)	0.37 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.38 ± 0.01
Brain (g)	2.03 ± 0.03	1.99 ± 0.04	2.01 ± 0.04	1.98 ± 0.03	2.01 ± 0.04	1.99 ± 0.02
Brain (g/100 g BW)	0.45 ± 0.01	0.44 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.46 ± 0.01	0.46 ± 0.01

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

containing histone demethylases (Young et al., 2015).

This is a new study reporting reproductive damage in the male offspring in consequence of paternal exposure to rosuvastatin, besides to report a protecting role of paternal supplementation with ascorbic acid on ameliorating the reproductive impairment on male offspring. The findings in this work suggest that the reproductive adverse effects in the male offspring are related to augmented paternal sperm DNA fragmentation, as previously described (Leite et al., 2017a), besides a possible sperm epigenetic change on parental generation. Additional studies are necessary to clarify the molecular mechanisms involved on reproductive impairment in the male offspring mediated by paternal sperm epigenetics changes.

5. Conclusions

In conclusion, paternal exposure to rosuvastatin induced an impairment in the morphology and physiology of the reproductive system in the male offspring, represented by the increased frequency of germ cells death in the testis, augmented sperm DNA damage, hyperplasia of clear cells on cauda epididymis, reduced sperm counts, lower sperm motility and hormonal changes. The decreased testosterone levels in the plasma and testis, associated with the higher levels of LH in the offspring suggest that paternal exposure to statin led to reduced cholesterol synthesis and diminished androgen levels in the fathers, thus changing paternal sperm DNA and affecting the pituitary-gonadal axis in the male offspring. Paternal supplementation with ascorbic acid played a preventive role in the male offspring against reproductive impairment provoked by paternal exposure to rosuvastatin. Ascorbic acid, when administered to the fathers, was able to protect sperm somehow, thus alleviating the reproductive impairment in their male offspring.

Declaration of interest

The authors declare that there are no conflicts of interest.

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