ARTIGO ORIGINAL

TRANSGENERATIONAL IMPACT OF HIGH AND REPEATED DOSES OF IVERMECTIN: STUDIES IN PUPS' TESTIS HISTOPATHOLOGY AT ADULT AGE

Impacto transgeracional de doses altas e repetidas de ivermectina: estudos histopatológicos dos testículos da prole na idade adulta

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ABSTRACT

Objective: We previously observed that repeated high doses of ivermectin (IVM) affect the histology of the paternal testis. Currently, we are analyzing the possible transgenerational effects of paternal exposure to IVM on the histology of their offspring's testes. Methods: Male rats were treated with IVM or its vehicle once a week for three weeks and mated with females without any treatment. Their male pups were evaluated by their body weight and the day of puberty. At adult age, these pups were divided into new groups and treated with saline or IVM, forming the following groups: saline +saline (SAL/SAL group), saline + 1.0 mg/kg of IVM (SAL/IVM group), ivermectin +saline (IVM/SAL), and IVM plus 1.0 mg/kg (IVM/IVM group). They were euthanized, and the testis, epididymis, prostate, and seminal vesicle were weighed. The testis morphometry and histopathology were evaluated. Results: In the IVM/SAL group, histological analysis revealed the same histological alterations observed in animals of the paternal group that received IVM, including signs of cellular disorganization. The presence of eosinophilia with vacuoles in the interstitium and the tubular lumen was also noticeable, indicating possible edema. In the IVM/IVM group, we observed that administering an additional dose of Ivermectin to the pups resulted in a significant reduction in both the height of the reproductive epithelium and the number of Leydig cells compared to the control. Conclusions: We propose that epigenetic mechanisms at the paternal sperm level, induced by IVM treatment, could be involved in these transgenerational effects. In addition, a challenge dose of IVM exacerbates these changes.

Keywords: Avermectins, offspring, transgenerational, histopathology, testis.

RESUMO

Objetivo: Anteriormente observamos que altas doses repetidas de ivermectina (IVM) afetam a histologia paterna dos testículos. Presentemente, analisamos os efeitos transgeracionais da exposição paterna à IVM na histologia dos testículos de seus descendentes. Métodos: Ratos machos foram tratados com MIV ou seu veículo uma vez por semana durante três semanas e acasalados com fêmeas sem qualquer tratamento. Seus filhotes machos foram avaliados quanto ao peso corporal e ao dia da puberdade. Na idade adulta, esses filhotes foram divididos em novos grupos e tratados com solução salina ou MIV, formando os seguintes grupos: solução salina + solução salina (grupo SAL/SAL), solução salina + 1,0 mg/kg de MIV (grupo SAL/MIV), ivermectina + solução salina (MIV/SAL) e MIV mais 1,0 mg/kg (grupo MIV/MIV). Vinte e quatro horas após esses tratamentos, eles foram eutanasiados, e os testículos, epidídimos, próstata e vesícula seminal foram pesados. A morfometria e a histopatologia dos testículos foram avaliadas. Resultados: No grupo IVM/SAL, uma geração F0 foi exposta à IVM, e sua prole não recebeu nenhum tratamento,a análise histológica revelou as mesmas alterações histológicas observadas nos animais do grupo paterno que receberam IVM, incluindo sinais de desorganização celular. A presença de eosinofilia com vacúolos no interstício e no lúmen tubular também foi perceptível, indicando possível edema. No grupo IVM/IVM, observamos que o desafio de dose adicional de IVM resultou em redução significativa tanto na altura do epitélio reprodutivo quanto no número de células de Leydig em comparação ao controle. Conclusões: Propomos que mecanismos epigenéticos no nível do esperma paterno, induzidos pelo tratamento IVM, podem estar envolvidos nesses efeitos transgeracionais. Além disso, uma dose de desafio de IVM exacerba essas alterações.

Palavras-chave: Avermectinas, prole, transgeracional, histopatologia, testículo.

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INTRODUCTION

Ivermectin (IVM) is a macrocyclic lactone used widely in veterinary medicine as an insecticide and in humans to treat parasitosis. IVM interacts with GABAergic receptors on neurons in vertebrates. GABAA, GABAB, and GABAC are three classes of GABAergic receptors[1]. Besides its action in the central nervous system, GABA and its receptors are present in several non-neural tissues, including the endocrine organs of the pituitary, pancreas, and testis[2]. In the testis, GABA is linked to the regulation of steroid synthesis by Leydig cells through GABA(A) receptors, despite the testicular sources of GABA, nor the precise nature of testicular GABA receptors is fully known. In male gonads and accessory reproductive organs, GABA could mimic and potentiate the action of progesterone in initiating the acrosome reaction of mammalian sperm, indicating that sperm contain receptors for GABA[3,4]. Also, the GABAA[5], GABAB[6], and GABA C[7] receptors occur in rat testis and sperm. It is reported that the GABAergic system plays a modulatory role in spermiogenesis because the expression of glutamate decarboxylase mRNAs, the GABA synthetic enzymes, was observed in both round and elongated spermatids[8]. Moreover, the GABAergic system in adult Leydig cells in rodents and human testis was found, and it was suggested that the regulation of steroid synthesis by Leydig cells is linked via local GABAA receptors[9,10].

Cordeiro et al.[11] demonstrated that acute IVM therapeutic doses impair spermatogenesis and spermiogenesis in adult rats, without affecting Leydig cells or testosterone levels, increasing the expression of caspase-3 (labeled seminiferous tubules and strongly labeled tubules), as well as the number of tubules presenting labeled cells in the tubular lumen, compared to the control group[12]. Thus, the impairments in spermatogenesis and spermiogenesis affecting testicular homeostasis were attributed to the expressed apoptosis in cells of the rats' seminiferous tubules.

In addition, our group showed that high and repeated doses of IVM induced paternal testis histopathology and prejudiced the pups' development, as well as their sexual behavior. In comparison to controls, the IVM paternal testis histology revealed hypertrophy and hyperplasia of Leydig cells, as well as an increased diameter of the seminiferous tubules, without any impairment in reproductive performance. These results suggest that epigenetic mechanisms could be responsible for these effects[13]. In the present study, we investigated whether paternal exposure to high IVM doses has transgenerational effects on the testicular histology of their offspring. Additionally, the effects of a challenged IVM dose were investigated.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations of the

Guide for the Care and Use of Laboratory Animals (GCULA) of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Paulista University, Brazil (Permit number 7690240119, CEUA-UNIP). All efforts were made to minimize suffering, reduce the number of animals used, and utilize alternatives to in vivo techniques when available. The experiments were also performed in accordance with good laboratory practice protocols and quality assurance methods.

Animal Conditions and Experimental Design

The rats used in this experiment were the F1 generation previously described by Moreira et al.[14] presented here in Fig. 1. After birth, the litters were reduced to 8 pups (4 males and four females (F1 generation), maintained with their mother in 1/cage, 40 ×34 x 20 cm, 1.530 cm2) in microisolators (Tecniplast S.P.A. 20020 Buguggiate (VA), Italy, with a 21ºC/ 83, 75 A.CH,-20 %, Multiage's), with air in rooms with controlled temperature through air conditioning (22±4ºC), humidity (50–65 %) and controlled light cycle 12:12 h (light on at 07:00 h). Filtered water and food (BioBase, Aguas Frias, Brazil) were provided ad libitum to the animals throughout the experimental procedure. The feed provided was irradiated, and the wood shavings were autoclaved. The pup's body weight and the day of preputial detachment were measured. Between 75-80 days of age, these pups were divided into new groups: saline +saline (SAL/SAL), saline + 1.0 mg/kg of IVM (SAL/IVM group), ivermectin +saline (IVM/SAL), and IVM plus 1.0 mg/kg (IVM/IVM group). Twenty-four hours after these treatments, they were euthanized, and the testis, epididymis, prostate, and seminal vesicle were weighed. For macroscopic evaluation the microscopic morphometry, and the histological evaluation of the seminiferous tubules were performed. Fig.1. shows this experimental design.

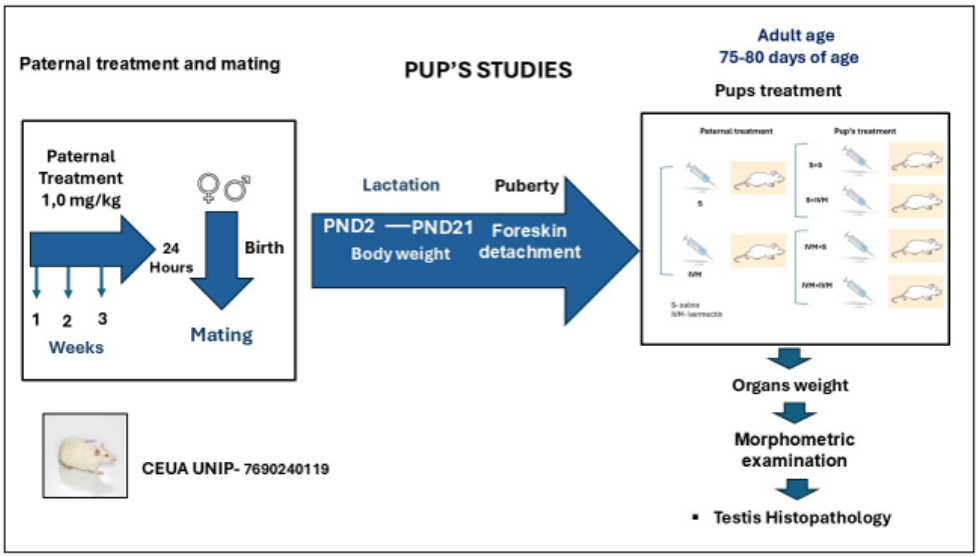


Fig.1.Experimental design.

Drugs

Ivermectin (injectable Ivomec® – Merial Saúde Animal Ltda., Paulínia/SP);• Tween 80 (Labsynth Produtos para laboratory Ltda., Diadema/SP) were used. The ivermectin solution administered to the animals was prepared by adding one drop of Tween 80 for each ml of Ivomec®; after vigorous homogenization, the saline solution (0.9% NaCl) was added gradually until a concentration of 1.0 mg/ml of ivermectin was obtained. Saline solution plus Tween 80 (1 drop for each ml of 0.9% NaCl) was used as a control solution. All solutions were administered subcutaneously (SC). The dose of ivermectin administered to the rats was 1.0 mg/kg, based on a previous study from our laboratory, in which it was observed that acute treatment with this dose promoted impairment in the sexual behavior of male rats[13] and prejudices in spermatogenesis and spermiogenesis[14]. All solutions were administered at a volume of 1.0 ml/kg.

Methods

Pups' body weight and puberty

The pup's body weight was measured at 2-, 9-, and 21-days during lactation. The preputial separation was observed daily until it occurred.

Organ weights and testicular volume

After the euthanasia, the testis, epididymis, prostate, and seminal vesicle (right and left) were weighed.

The rats' body weights and testes were measured and converted to relative weight, i.e., testicular weight/100 g of body weight. The transverse (laterolateral) and longitudinal (cephalocaudal) axes of each testis were measured using a Vernier caliper, allowing for 0.1 mm discrimination. The total volume of each testis was calculated using the ellipsoid formula: $V = 4/3.\pi.a.b2$, where a is the longitudinal semi-axis and b is the transverse semi-axis.

Testis evaluation

Morphometric examination: Tubular diameter and germinal epithelium height

The linear morphology of seminiferous tubules was analyzed to determine tubular diameter sizing by measuring the distance between the basal lamina of a tubule and the basal lamina on the opposite side. To determine the height of the germinal epithelium, the distance from the tubule's basal lamina to the beginning of the tubular lumen was measured using a computerized image analysis program, ImageJ software. Ten fields were selected from the histological testicular sections of each animal, and photomicrographs were taken using a 40× objective. Five tubules were measured for each field, and the distance of tubular diameter and the germinal epithelium height were automatically calculated in pixels using ImageJ software, totaling 50 tubules per animal.

Histological examination

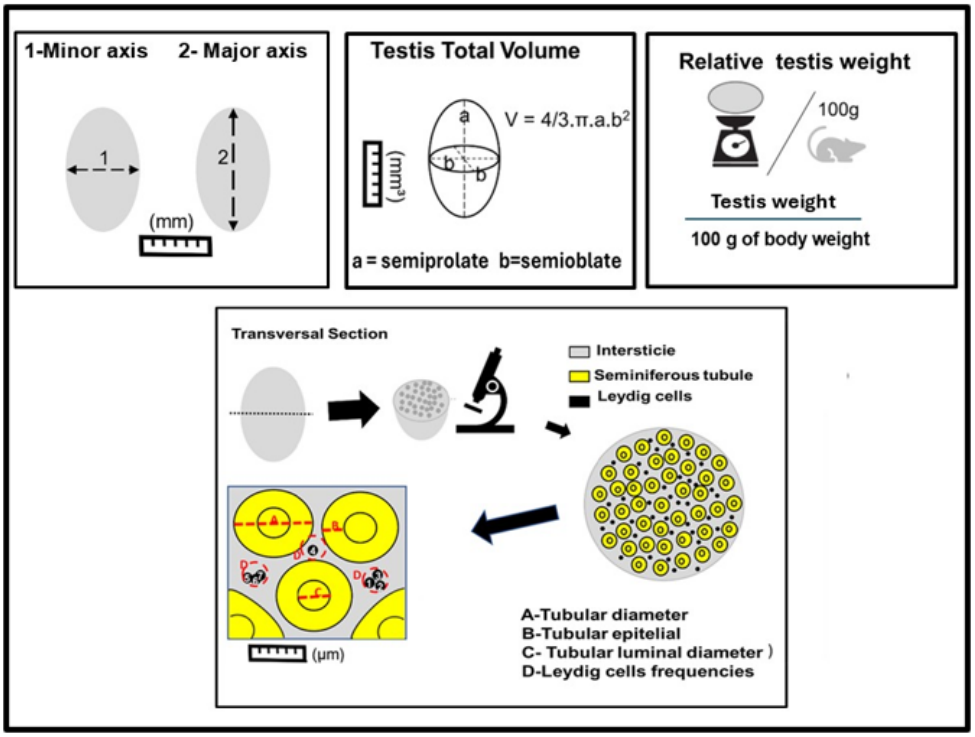
The testis was fixed by immersion in Bouin's liquid fixative for 48 h. The specimens

were processed and embedded in Paraplast Plus (Sigma Chemical Co., St. Louis, MO). Cross sections were obtained from the fragments of the gonad, allowing an adequate morphometric analysis[15]. Six µm-thick sections were stained with the Hematoxilin-Eosin (HE) method (right testis) to identify all phases of spermatogenesis. A periodic acid-Schiff method, counterstained with Harris'Hematoxylin (PAS +H), was also processed as a histochemical method to determine interstitial cells and elongate spermatids (left testis).

Interstitial cell frequency

Ten fields were selected from the histological testicular sections of each animal. Photomicrographs were taken using a 100× objective, and the frequency of interstitial cells per field was estimated by Image J software.

Fig.2 summarizes these methods



Based inGalvão et al. [16]

Pups' body weight and puberty

The pup's body weight was measured at 2-, 9-, and 21- days during lactation. The preputial separation was observed daily until it occurred.

Statistical analysis

Homoscedasticity was verified using the F or Bartlett tests, and normality was determined using the Kolmogorov-Smirnov test. The ANOVA, followed by Tukey's multiple comparisons test,

analyzed data with more than two groups in one way, and Sidak's multiple comparisons test followed those of two factors in the two-way ANOVA. As recommended by our CEUA -UNIP, the effect size needs to be calculated based on some parameter factors, considering a test power of 80%. The sample size of minimal N=6/7per group was estimated based on the formula n=4C*(s/d)2], C=(Zα/2+zβ)2, considering a confidence interval of 0.95/2 (0.475), z=1.96, and test power of 80% (zβ=0.842), the maximum deviation of 0.2 (20%) and the difference between groups of 0.5 (50%) [17].The total number of animals required for the experiments was also estimated by a less accurate method named "resource equation method," which stipulates an amount between 10 and 20 as the adequate number for planning the experiments [18]. Thus, we employed 7 animals/groups of different litters to avoid the litter effect[19]. The results were considered significant when $\alpha < 0.05$.

RESULTS

Body weight and puberty

Fig.3a shows the pup's body weight during lactation. No differences occurred in each day (Interaction- F=0.006, P=0.978; days-F=90.50, treatments-F=0.008, P=0.809) but differences between the days (days-F=90.50, P<0.0001).In the saline group, puberty occurred at 24.75 ± 0.25 and 23.92 ± 0.31, respectively, in the SAL and IVM groups (Fig.3b). The Student t-test indicates that the IVM group's puberty day was delayed relative to the Saline group (t=4.474, df=26, P=0.0001).

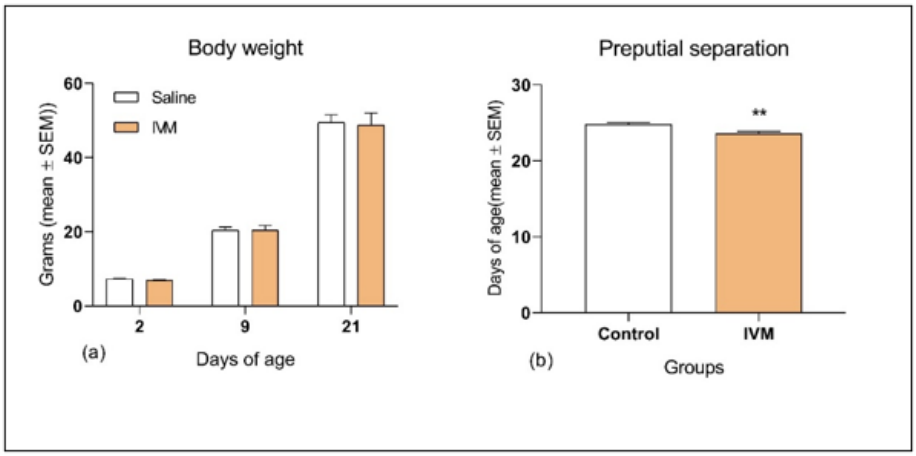


Fig. 3. Body weight (a) and preputial separation (b) of pups from paternal treatment with saline or IVM observed during lactation. N=12/group. Two-way ANOVA followed by Sidak's multiple comparisons test.**p< 0.01 relative to control groups

Organs, body, testis, and epididymis weight

The body weight, testis, and epididymis weights in adult age groups of pups from paternal treatment after the IVM or saline challenge did not reveal differences between groups (Fig. 4). Statistical data are shown in Supplementary Table 1.

Supplementary Table. Statistical data of organ weight of pup rats from paternal treatment after the IVM or saline challenge.

Organs weight	One -way ANOVA
Testis right	F=0.4424,P=0.0486
Testis left	F=0.8467,P=0.089
Epididymis right	F-0.2548, P=0.029
Epididymis left	F= 0.6247, P=0.072
Prostate	F=1.120,P=0.1145
Seminal vesicle empty	F=1.247, P=0.126

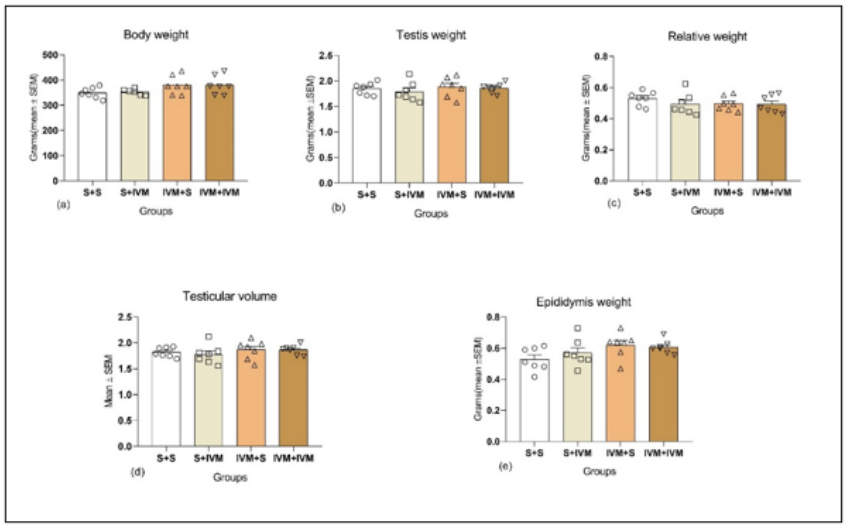
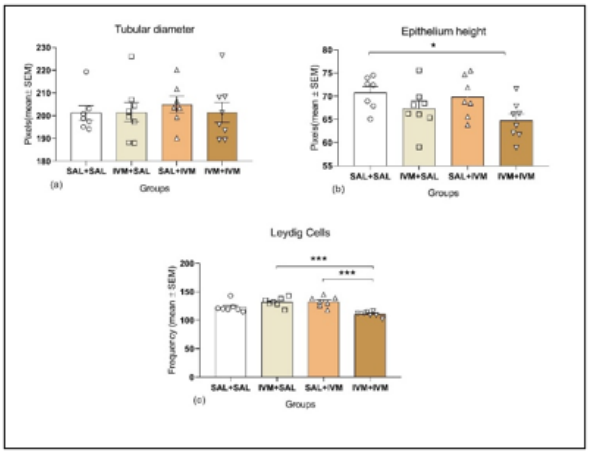


Fig.4. Body weight (a), testis weight (b), relative weight (c), testicular weight (d), and epididymis weight of pups fat adult age from paternal treatment challenged with saline (1 ml/kg) or IVM (1.0 mg/kg). N= 7/group. One-way ANOVA

Testis evaluation

Morphometric evaluation

Fig. 5 depicts the morphometric evaluation of pups at adult age from paternal treatment with saline or IVM after a challenge dose of IVM or saline. No differences were reported in the tubular diameter of all groups(Fig.5a; interaction-F (1, 26) = 0.239, P=0.628); paternal treatment (F (1, 26) = 0.168, P=0.685) and pups treatment (F (1, 26) = 0.266, P=0.610). Relative to epithelium height (Fig.5b), the two-way ANOVA shows an interaction between groups[F (1, 26) = 0.0396, P = 0.8438] and differences induced by paternal treatment [F (1, 26) = 6,002, P=0,0213] but not pups treatment [F (1, 26) = 2.145, P=0.1550]. Tukey's multiple comparisons test indicates that the epithelium height of the IVM/IVM group was reduced compared to the SAL/ SAL group (p=0.0305). Concerning the Leydig cells frequencies (Fig.5c), the two-way ANOVA shows an interaction between groups {F (1, 24) = 27.16, P<0.0001], no differences induced by paternal treatment [F (1, 24) = 2.735, P=0.111] and by pups treatment [F (1, 24) = 2.434, P=0.131]. Sidak's multiple comparisons test shows that the frequency of Leydig cells was reduced relative to IVM/SAL and IVM/IVM groups (P= 0.0004).



Fig,5 Morphometric data of pups from paternal treatment with saline or IVM at adult age challenged with saline (1 ml/kg) or IVM (1.0 mg/kg). (a)-tubular diameter; (b)-epithelium height; (c)-Leydig cell frequencies. N= 7/group. Two-way ANOVA followed by Sidak's multiple comparisons test. *p= 0.0305; ***p=0.0004.

Histological analysis

Histopathological analysis of the testicles in the SAL/SAL group revealed typical normal characteristics (Fig. 6). The seminiferous tubules, stained with H.E., were organized into well-defined cellular associations, containing supporting cells (Sertoli) and several cell types characteristic of spermatogenesis, including germ cells. In addition, they presented the 14 stages of the seminiferous epithelium cycle and tubular sections containing 4 to 6 concentric layers.

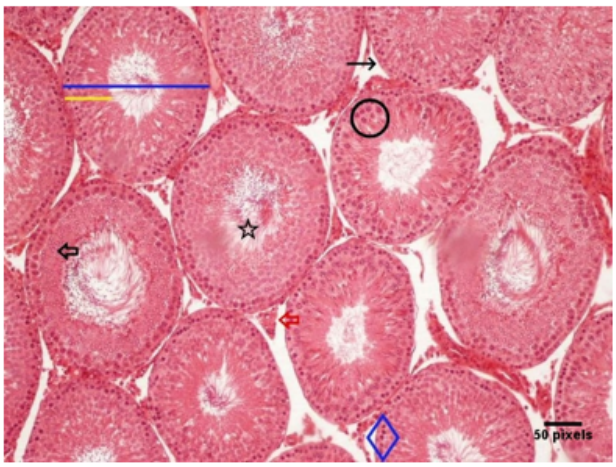


Fig.6. - Histology of the SAL/SAL group. Seminiferous tubules with normal morphology, diameter (blue line), and epithelial height (yellow line). Tubular lumen containing mature spermatozoa (star), presence of a group of spermatids (thick arrow), Sertoli cells (thin arrow), spermatocytes (circle), interstitial tissue (red arrow), and spermatogonia (blue diamond) (H.E. 400x).

The seminiferous tubules of the testicles of animals in the SAL/IVM experimental group presented characteristics like those of the control group, such as their diameter and epithelial height. However, their structure showed signs of cellular disorganization, with the detachment of epithelial cells towards the tubular lumen (Fig. 7A) and fissures in the epithelium (Fig. 7B) being possible. The presence of eosinophilia with vacuoles in the interstitium (Fig. 7A) and within the tubular lumen (Fig. 7C) was also noticeable, indicating possible edema. In addition, pyknotic nuclei were identified in the tubular lumen (Fig. 7C), a sign that may indicate an apoptotic process at the site.

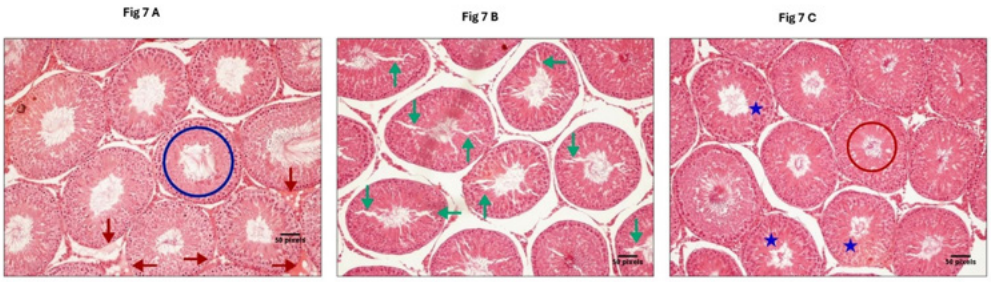


Fig.7 – Histological analysis of the SAL/IVM experimental group. A – Detachment of epithelial cells towards the tubular lumen (blue circle) and eosinophilia with vacuoles in the interstitium (red arrows). B – Fissures in the tubular epithelium (green arrows). C – Vacuoles within the tubular lumen (blue stars) and pyknotic nuclei are also found in the tubular lumen (red circle). (H.E. 400x)

The seminiferous tubules of the testes of animals in the IVM/SAL experimental group presented characteristics like those of the control group (SAL/SAL) with their diameter and epithelial height (Fig. 8A), but their structure showed signs of cellular disorganization (Fig. 8B). The presence of eosinophilia with vacuoles in the interstitium and within the tubular lumen was also noticeable (Fig. 8C), indicating possible edema. In addition, pyknotic nuclei were identified in the tubular lumen (Fig. 8D).

The seminiferous tubules of the testes of the animals in the IVM/IVM group presented a tubular diameter like that of the control group and decreased epithelial height (Fig. 9A). Their structure showed signs of cellular disorganization, with numerous tubules having fissures and vacuoles in the epithelium (Fig. 9B). It is also possible to note the presence of eosinophilia with vacuoles in the interstitium and within the tubular lumen (Fig. 9A). This sign may indicate tissue edema.

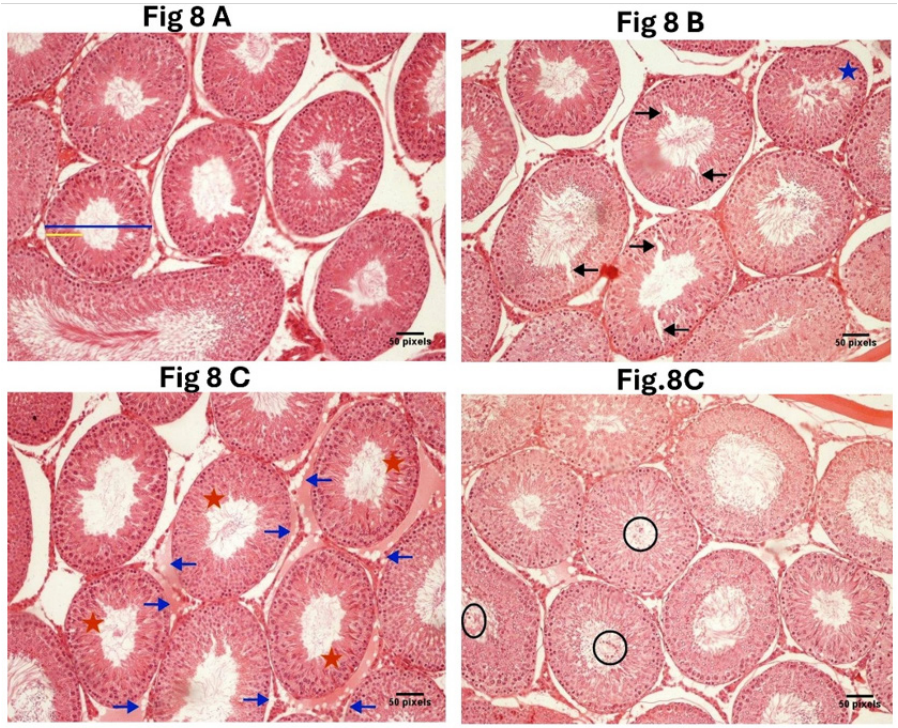


Fig. 8 – Histological analysis of the IVM/SAL experimental group. A - Diameter (blue line) and epithelial height (yellow line) are similar to the control group, indicating normality. B - Fissures in the tubular epithelium (black arrows) and detachment of epithelial cells towards the tubular lumen (blue star). C - Eosinophilia and the presence of vacuoles in the interstitium (blue arrows) and vacuoles within the tubular lumen (red stars). D - Pyknotic nuclei found within the tubular lumen (black circles) (H.E. 400x).

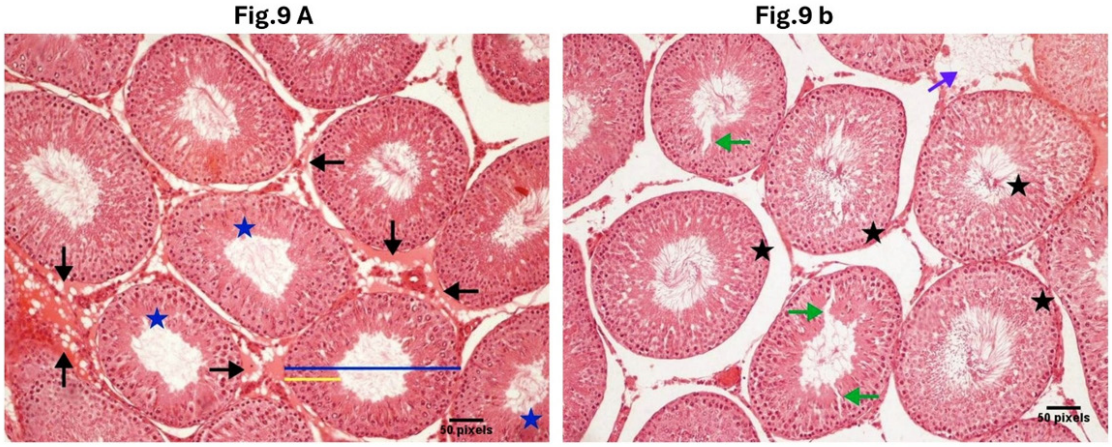


Fig.9 – Histological analysis of the IVM/IVM experimental group. A – Tubule with a diameter like the control (blue line) and decreased epithelial height (yellow line), eosinophilia with vacuoles in the interstitium (black arrows), and vacuoles present in the tubular lumen (blue stars). B – Fissures in the epithelium (green arrows), vacuoles between the epithelial cells of the tubule (black stars), and vacuoles in the interstitium (purple arrow) (H.E. 400x).

Interstitial tissue

Analysis of the interstitial tissue of the SAL/SAL group (fig. 10A), located between the seminiferous tubules, stained with PAS., showed that it was composed of connective tissue cells, including fibroblasts, fibrocytes, macrophages, mast cells, lymphocytes, mesenchymal cells, and characteristic interstitial cells (Leydig). Leydig cells were isolated or in "islets" around the blood vessels. They exhibited rounded or oval nuclei with prominent nucleoli and light acidophilic cytoplasm compatible with normality.

The interstitial tissue of the IVM/SAL group (fig. 10B) did not present significant changes in the count and morphology of Leydig cells.

The interstitial tissue of the IVM/IVM group (Fig. 10 C) exhibited significant changes in Leydig cell count, with a relatively smaller number of cells.

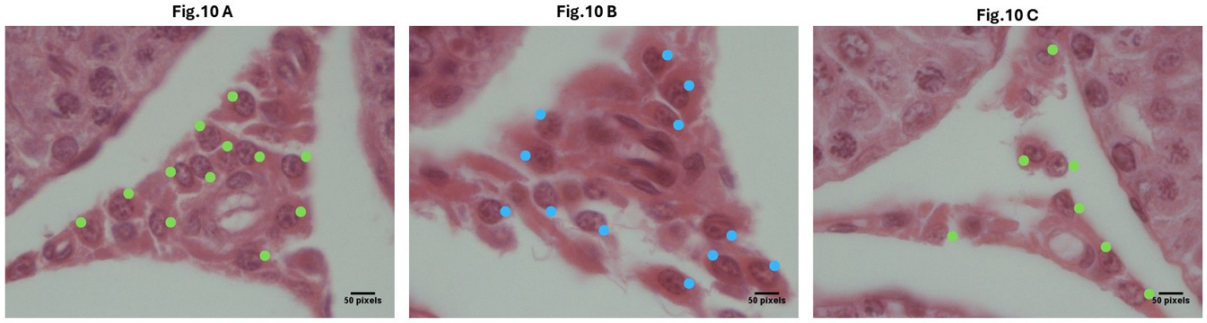


Fig.10 - Analysis of interstitial tissue of the SAL/SAL group (A) and the IVM/SAL group (B). The Leydig cell count (blue circles) showed no significant difference between the two groups The Leydig cell count (green circles) showed a significant difference between IVM/IVM and SAL/IVM groups (C), with fewer cells in IVM/IVM group. (PAS 1000x).

DISCUSSION

Repeated paternal exposure to IVM did not affect the body weight or organ weight of the male pups; however, it advanced the day of preputial separation relative to the saline group. The phenomenon of the prepuce separation from the glans penis (Balanus), because of the cornification of the balanopreputial epithelium, occurred around the time of puberty. The preputial separation is a hormone-dependent event that occurs during periods of low androgen levels. It happens at the beginning of puberty, immediately before the appearance of mature sperm in the cauda epididymis, and the significant increase in circulating androgens[20]. Preputial separation also precedes the development of complete copulatory behavior. We observed the first sign of preputial separation earlier in the IVM group compared to the saline group. Thus, it is possible that paternal repeated exposure to ivermectin at high levels affected the levels of androgens of pups, advancing the start of puberty. Interestingly, IVM acute exposure to 1.0 mg/kg reduced testosterone levels, while paternal repeated exposure to this same dose did not.

In addition to the studies by Cordeiro et al[11] showing that single therapeutic IVM doses impaired the gametogenesis of adult rats, other studies from our group demonstrate that the same treatment affects animals at different

ages, such as 45-day-old animals, considered pubescent, and 30-day-old animals, prepubertal, which present the same histological changes observed in adult animals. This suggests that different ages can also be affected by a treatment considered therapeutic[12,16]. Following our line of research, we analysed that treatment with three doses repeated weekly, affected the paternal testis histology exhibiting hypertrophy and hyperplasia of Leydig cells, along with an increased diameter of the seminiferous tubules, without affecting testosterone levels.

The present results revealed that paternal exposure to repeated and high doses of IVM affects the testicular histology of the offspring thereby increasing the risk of disease development in the offspring.

Studies carried out by parents on their offspring in a non-genetic manner are of a different type and may seem beneficial or not to the offspring. The mechanisms underlying this process are poorly understood and subject to controversy[13].

The results demonstrated that the SAL/SAL group functioned adequately as a control group. As in previous studies, the macroscopic morphometric changes, including animal weight and reproductive organs, did not exhibit any changes. The microscopic morphometric changes agreed with the histological changes respective to each group studied. For the SAL/IVM group, we observed the same histological effects characteristic of acute IVM treatment, with signs of edema in the interstitium; however, there were no changes in the number of Leydig cells or testosterone levels as described by[11]. These effects were reversible, despite negatively affecting the adult rat testis. Evaluations of the temporal effects of the low and high therapeutic doses of IVM were also reversible and correlated with the IVM plasma levels.

In the IVM/SAL group, an F0 generation was exposed to IVM, and their offspring did not receive treatment. However, histological analysis of these offspring revealed the same histological alterations observed in animals that received IVM, including signs of cellular disorganization. The presence of eosinophilia with vacuoles in the interstitium and the tubular lumen was also noticeable, indicating possible edema. In addition, pyknotic nuclei were identified in the tubular lumen. These results reflect a transgenerational effect of paternal exposure to IVM since the F1 generation was only treated with saline.

Studies in humans[21–24] and rodents[23,25,26] on epigenetics has highlighted paternal exposure to environmental changes and challenges that can affect the future health of generations, particularly in terms of disease transmission. Furthermore, epigenetic information has been referred to as heritable changes in gene expression that occur without modifications at the DNA sequence level and suggest the transmission of epigenetic information from parents to their offspring via spermatozoa[27]. Based on epigenetic studies, we aim to continue investigating the possibility

of generational transmission via spermatozoa following exposure to ivermectin.

Finally, in the IVM/IVM group, we found that the challenge with an additional dose of Ivermectin in the pups demonstrated a significant reduction in both the height of the reproductive epithelium and the number of Leydig cells relative to controls. This result is in accordance with the testicular histological evaluation of the animals in this same group, presenting alterations such as signs of cellular disorganization, with numerous tubules having fissures and vacuoles in the epithelium, presence of eosinophilia with vacuoles in the interstitium and within the tubular lumen, which may indicate tissue edema.

CONCLUSIONS

The present results showed transgenerational effects of paternal exposure to repeated and high doses of IVM at testicular levels. We propose that epigenetic mechanisms at the level of paternal sperm, induced by IVM treatment, may be involved in these transgenerational effects. In addition, a challenge dose of IVM exacerbates these changes.

Credit authorship contribution statement

MM Bernardi, N. Moreira-Conceptualization, project administration, supervision, resources. F. Cordeiro: testis studies and revised the text. N. Moreira,GA Borges,K. Carvalho, L M Delapena-Silva; L. Macedo Amado performed experiments. F. Cordeiro: testis studies and revised the text. MM Bernardi and RA Silva - wrote the original draft, review@editing.

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Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Conflict of interest

The authors declare no conflict of interest.

AI statement

While preparing this work, the authors did not utilize any AI tools or services. The authors reviewed and edited the content as needed and took full responsibility for the publication's

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Observação: os/(as) autores/(as) declaram não existir conflitos de interesses de qualquer natureza.