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Impact of increased expression of testicular caspase-3 in early ages of rats exposed to Ivermectin

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Abstract: Studies from our group have revealed that treatment with a therapeutic dose of ivermectin in adult rats increases the expression of caspase-3 and the number of tubules with labeled cells in the tubular lumen, thereby affecting the testicular natural homeostasis process. These effects resulted in impairments of spermatogenesis and spermiogenesis. The present study investigated whether exposure to a therapeutic dose of ivermectin at young and peripubertal ages affected spermatogenesis, spermiogenesis, and the expression of caspase-3 in the testes of rats. Methods: Wistar rats received two doses of 1.0 mg/kg IVM or 0.9% saline solution at 29 and 44 days of age. At 45 days of age, these groups were euthanized, and the rats and testicles were weighed. We conducted a macroscopic evaluation of the animals and a microscopic morphometry evaluation of the seminiferous epithelium. The testis tissue apoptosis was made using Caspase's methodology. The rat's body weight, testicular volume, relative weight, the major and minor axes, and the frequency of Leydig cells did not differ between groups. The epithelium tubular height increased in ivermectin-treated rats compared to the control group. In the IVM group, histopathological analysis revealed disorganization of the germinal epithelium, vacuolation, desquamation of cells into the tubular lumen, and cell nuclei with pyknotic aspects. Ivermectin increased the expression of caspase-3 in median and strongly labeled tubules, suggesting possible testicular damage at young and peripubertal ages in male rats.
Keywords: Avermectins; Morphometry; Testis histological analysis; Seminiferous epithelium; Apoptosis

1. Introduction

Ivermectin (IVM) is a macrocyclic lactone used to treat parasitic diseases and is widely used in veterinary medicine as an insecticide, as well as in humans to treat parasitic infections. IVM interacts with GABAergic receptors on vertebrate (mammal) neurons. However, it has a greater affinity (approximately 100 times more) for invertebrate receptors; in invertebrates, IVM also acts on glutamate receptors (Sears and Hewett 2021). Currently, three classes of GABAergic receptors are known: GABAA(Kim and Hibbs 2021), GABAB(Xie et al. 2025), and GABAC (Johnston et al. 2010). The GABA A receptor in the central nervous system is responsible for the behavioral effects producing central depression(Hu et al. 2023; Michałowski et al. 2025).

In addition, GABA and its receptors are also present in several non-neural tissues, including the endocrine organs of the pituitary, pancreas, and testis(Gajić Bojić et al. 2025). In the case of rat testis, GABA appears to be linked to the regulation of steroid synthesis by Leydig cells through GABA(A) receptors. Still, neither the testicular sources of GABA nor the precise nature of testicular GABA receptors is fully known. In male gonads and accessory reproductive organs, it was reported that GABA could mimic and potentiate the action of progesterone in initiating the acrosome reaction of mammalian sperm, indicating that sperm contain receptors for GABA(Gürsoy Gürgen et al. 2021). In this respect, the GABAA (Geigerseder et al. 2003), GABAB (He et al. 2003), and GABA C (Li et al. 2008) receptors occur in rat testis and sperm. In addition, the GABAergic system plays a modulatory role in spermiogenesis, as the expression of glutamate decarboxylase mRNAs, the enzymes responsible for GABA synthesis, has been observed in both round and elongated spermatids (Kanbara et al., 2005, 2010). Moreover, the GABAergic system has been found in adult Leydig cells in rodents and humans. These data suggest that the regulation of steroid synthesis by Leydig cells is linked via local GABAA receptors(Geigerseder et al. 2004; Hauet et al. 2005).

Cordeiro et al.(2018) showed that acute IVM therapeutic doses impair spermatogenesis and spermiogenesis in adult rats, but they have no effects on Leydig cells and testosterone levels. Despite negatively affecting the adult rat testis, evaluations of the temporal effects of the low and high therapeutic doses of IVM were reversible and correlated with the IVM plasmatic levels. More recently, these authors revealed that ivermectin treatment increased the expression of caspase-3 (labeled seminiferous tubules and strongly labeled tubules), as well as increased the number of tubules that presented labeled cells in the tubular lumen, compared to the control group (Cordeiro et al. 2023). Thus, the impairments in spermatogenesis and spermiogenesis affecting testicular homeostasis were attributed to the expressed apoptosis in cells of the seminiferous tubules of rats.

The histologic appearance in the male reproductive tract in response to exposure to several drugs and toxicants is affected by age. However, testing the testis during maturation is hampered because, in immature testis, these effects are superimposed by normal growth and development changes. In this respect, postnatal development of the rat testes can be divided into four recognized stages: neonatal (birth to postnatal day, PND 7), infantile (PND 8–20), young (PND 21–32), and peripubertal (PND 33–55). Although IVM is indicated to treat children parasitosis, it is contraindicated in children who are younger than five years old or who weigh <15 kg because the use of this type of drug during brain periods of maturation can lead to several adverse disorders(Wilkins et al. 2018; Jittamala et al. 2021). IVM exposure in the early stages of life has been shown to have several adverse effects, including acute vision changes and ataxia(Bhardwaj et al., 2023), mildly elevated creatine kinase levels, eczema flare-ups, diarrhea, vomiting, irritability, pruritus, and pustular skin reactions(Lobo and Wheller 2021), nephrotoxicity with histological damages, and ultrastructure

examination showed an alteration in cell architecture(Tawfeek et al. 2021). In this respect, Moreira et al. (2020) showed that 0.2 mg/kg IVM administered during the prepubertal and peripubertal periods of rats does not alter the parameters of sexual development or sexual behavior in adult males. However, it decreases the relative weight of the entire seminal vesicle. More recently, Moreira et al. (2024) demonstrated that repeated IVM doses of 1.0 mg/kg induce testis hypertrophy and hyperplasia of Leydig cells, thereby increasing the diameter of the seminiferous tubules. The present study investigated whether exposure to a therapeutic dose of ivermectin at young and peripubertal ages affected spermatogenesis, spermiogenesis, and the expression of caspase-3 in the testes of rats.

2. Materials e Methods

2.1. Animals

Male Wistar rats, 29 days old (mean weight 88.11 ± 3.29 g), were used at the beginning of the experiments, purchased from the Institute of Biomedical Sciences of the University of São Paulo (São Paulo, SP). The animals were housed in polypropylene cages (40 × 50 × 20 cm) at a controlled temperature of (20 ± 21 °C) and humidity of (60 ± 5%) under a controlled light/dark schedule (12 h light/12 h dark), with lights on at 10:00 AM for at least seven days before the experiments. Food (Nuvilab CR1, species-specific ration; Sogorb Ind & Com Ltd, São Paulo, Brazil) and water (filtered through porcelain) were freely available throughout the study. All procedures were reviewed and approved by the Committee of Ethics of Animal Experiments at Paulista University, Brazil, which approved this protocol (CEUA-UNIP permit number 414/15) and confirmed compliance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.2. Drugs

Ivermectin 1% Ivomec injectable, Merial, Paulinia, São Paulo, Brazil) was dissolved in Tween 80 (1 drop/1 ml of 1% Ivomec) and administered subcutaneously (s.c.) at a 1.0 mg/kg dose. Tween 80 was also administered in the same form as a control solution (1 drop/1 ml of 0.9% NaCl). All solutions were prepared immediately before use and administered at a volume of 1 mL/kg body weight.

2.3. Groups formation

Twelve male rats, 29 days old, were divided into two equal groups of six rats each. One group was injected with the control solution (Control group), and the experimental male group was injected with 1.0 mg/kg of IVM. At 44 days old, these treatments were repeated, and 24 hours later, the rats were euthanized.

2.4. Procedures

2.4.1. Testicular weight and testis volume

Twenty-four hours after the last treatments, the rats were weighed before euthanizing (decapitation), and their testis were collected. The testis was weighed and calculated as the proportion of the gonad mass relative to total body mass: Testicular weight = (gonad weight/body weight) × 100. The volume of each testis (V) was calculated: $V = 4/3 \cdot pi \cdot a \cdot b2$, where a is the semi-prolate axis, and b is the semi-oblate axis (Miraglia and Hayashi 1993).

2.4.2. Histological examination

The testes were 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 (Gundersen et al., 1988). 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).

2.4.3. Morphometric examination

2.4.3.1. 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 tubular lumen's beginning 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.

2.4.3.2. Interstitial cell frequency

Ten fields were selected from the histological testicular sections of each animal. The frequency of interstitial cells per field was estimated using a 40× objective directly at the microscope.

2.4.4. Immunohistochemistry

One testicular section (5 µm thick) per rat was made, and caspase-3 immunohistochemistry was performed using the chain polymer-conjugated staining method. Polyclonal rabbit anti-caspase 3 immunoglobulin (1:100; ab4051, Abcam, Cambridge, United

Kingdom) was used as the primary antibody, followed by the EnVision+ Kit for detection (HRP. Rabbit. DAB+, K4011, Dako/Agilent, Santa Clara, CA, USA). Antigen retrieval was achieved by heating the slides in citrate buffer (pH 6.0) at 95º C for 15 min. in a steamer. PBS solution was used as the negative control instead of the primary antibody during immunohistochemical staining. The sections were counterstained with Harris hematoxylin and mounted with DPX (06522, Sigma Aldrich, St. Louis, MO, USA). Each testicular immunohistochemistry section of each animal was scanned and photographed (40x objective, Nikon E200 microscope, equipped with a Nikon Coolpix digital camera, Kanagawa, Japan). The following parameters were evaluated for the total testicular section of each animal: (A) total number of tubules (labeled or not with caspase 3 (B) % of tubules labeled weakly; (C) % of tubules labeled strongly; and (D) % of tubules that presented labeled cells in the tubular lumen. Parameters B, C, and D were calculated as a percentage relative to the total tubules.

2.5. Statistical analysis

Homogeneity was verified using Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. The Student t-test was used for data analysis. The results are expressed as the mean ± SEM or percentage. In all cases, the results were considered significant at $\alpha < 0.05$.

3. Results

Figure 1 shows the body weight and testis parameters of male rats at a young age treated with saline or IVM. The Student's t-test did not reveal significant differences between groups in any of the observed parameters.

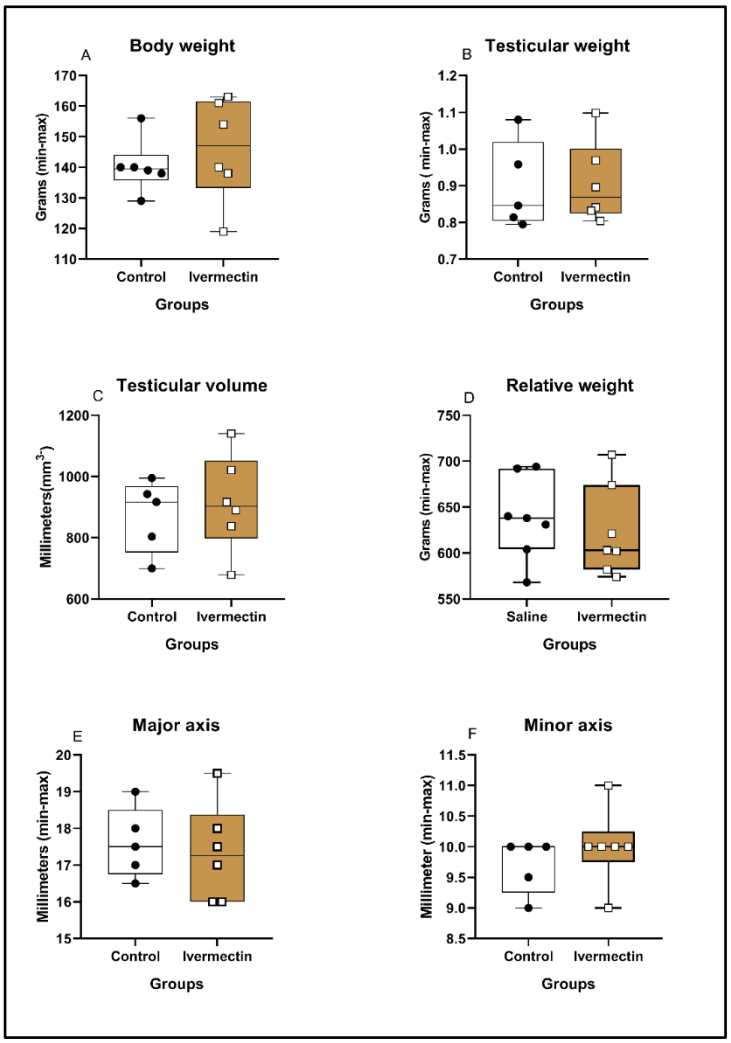

Figure 1 – Macroscopic morphometry. Effects of a single dose of Ivermectin or saline solution on macroscopic morphometry parameters in the testis of young and peripubertal rats, treated at 29 and 44 days of age, observed 24 h later (n=6 rats/group). A Student t-test was used to compare the groups. Data are expressed as box and whisker plots (min to max).

The epithelium tubular height increased in IVM-treated rats compared to the control group (Fig.2A, $p<0.024$) but did not affect the number of Leydig cells (Fig.2B, $p = 0.91$).

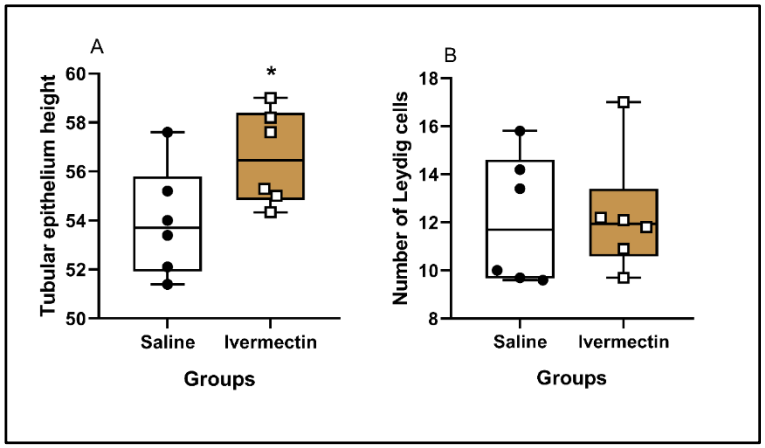

Figure 2 – Microscopic and stereological morphometry. Effects of Ivermectin or saline solution on microscopic and stereological morphometry parameters in the testis of young and prepubertal rats treated at 29 and 44 days of age and observed 24 h later (n=6 rats/group). *p< 0.05 - Student t-test. Data are expressed as box and whisker plots (min to max) graphs.

Concerning the testicular caspase-3 immunohistochemistry (Fig.3), ivermectin treatment increased the number of the median labeled tubules (B, p <0.02 but not the % of strongly labeled tubules (A) compared to the control group. Data are expressed as the mean ± SEM in box and whiskers (min to max) graphs.

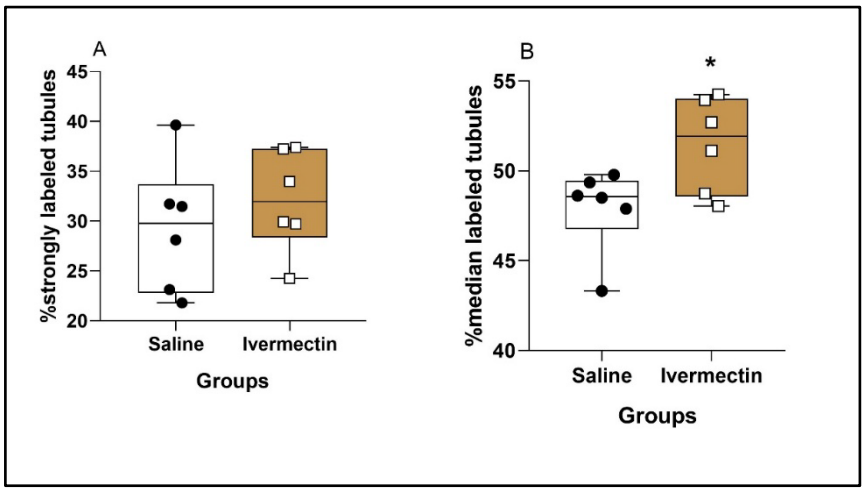

Figure 3 – Testicular caspase-3 immunohistochemistry of rats treated with Ivermectin or saline solution in young and prepubertal rats treated at 29 and 44 days of age and observed 24 h later (n=6 rats/group). *p< 0.05 - Student t-test. Data are expressed as box and whisker plots (min to max) graphs.

3.1. Histological evaluation

Regarding histology, the testicles of animals in the control group, treated with saline solutions alone, exhibited typical characteristics. The tubules of seminiferous cells were organized into well-defined cellular associations, representing the distinct stages of the seminiferous epithelium cycle. Tubular sections contained 4 to 6 concentric layers, characteristic of spermatogenesis germ cells (Fig. 4A). In the Ivermectin experimental group, however, sections of tubular cells containing several histological changes characteristic of quantitative interruption of spermatogenesis, such as disorganization of the germinal epithelium, vacuolation, desquamation of cells into the tubular lumen and cell nuclei containing pyknotic aspects (Fig. 4B).

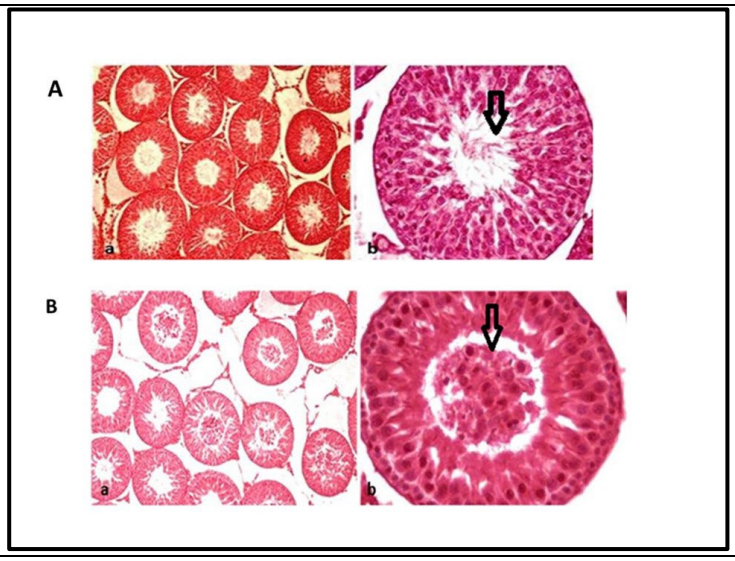

Figure 4 – (A) Photomicrographs of testis from 45-day-old rats in the saline group (H.E. staining).
a – seminiferous tubules (100x); **b** – presence of sperm in the tubular lumen (arrow) (400x). **(4B)** Photomicrographs of the testis of 45-day-old rats from the animals treated with IVM (H.E. staining). **A**: tubules containing cells in the light (100x); **b**: presence of pyknotic nuclei in the tubular lumen (arrow) (400x).

The expression of caspase-3 is visualized in brown in the seminiferous tubules of testes of both control (Fig. 5A) and ivermectin-treated rats (Fig. 5B) in low—and high-magnification photomicrographs. The seminiferous tubules of the ivermectin group (Fig. 5B, c, and d) presented increased expression of caspase three and the presence of cells in apoptosis in the tubular lumen.

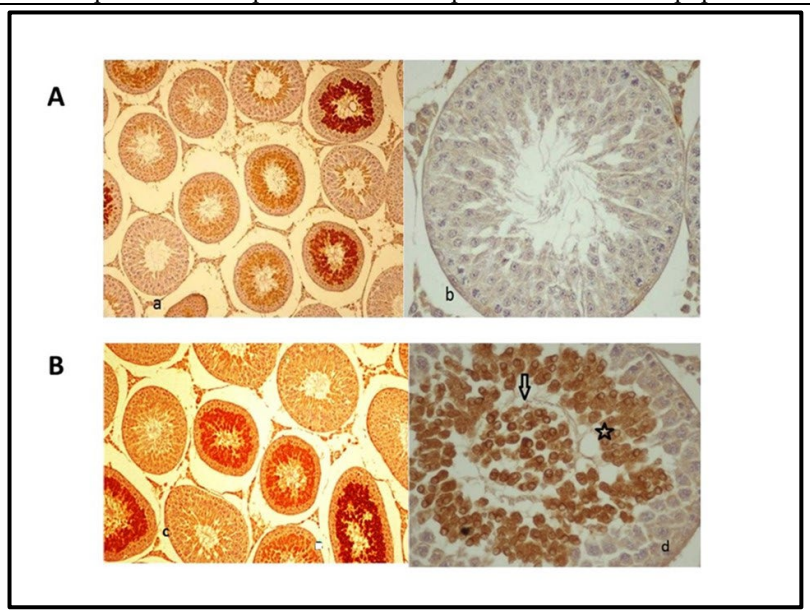

Figure 5 – A: Photomicrographs of testis from 45-day-old rats in the saline group (Caspase).
a - General view of tubules marked by caspase in brown -100x.; **b** – Tubule without labeling by caspase – 400x. **B**: Photomicrographs of the testis of 45-day-old rats from the animals treated with IVM (Caspase); **c** - General view of tubules marked by caspase in brown -100x.; **d** - Tubule with desquamation of germinal epithelium cells, presence of vacuoles (star), and presence of cells in the tubular lumen labeled in brown (arrow) – 400x.

4. Discussion

Several therapeutic agents may have varied toxic effects on the testes, leading to changes in reproductive function, lesions in the seminiferous epithelium, and the induction of cell death by apoptosis in germ-line cells, thereby compromising fertility. Our group has been studying for several years the actions of IVM in the most diverse reproductive functions, such as changes in the sexual behavior of males (Bernardi et al. 2011; Moreira et al. 2020) and female rats (Moreira et al. 2014), reduction of testosterone levels and sexual motivation in male rats(Moreira et al. 2017) and, at a young age, disrupt the sexual dimorphic behaviors of rats exposed

to stress. Moreover, we also examined testicular histological damage after the acute administration of therapeutic doses in adult rats (Cordeiro et al., 2018) and, more recently, evaluated apoptosis in the seminiferous epithelium of adult rats(Cordeiro et al. 2023). In the present study, we investigated the effects of IVM on the structure of the testicular parenchyma and the efficiency of the spermatogenic process in young and peripubertal Wistar rats, given the importance of evaluating different reproductive ages when considering the drug action in animals and children of young reproductive age.

Morphometric and stereological assessments, such as tubular diameter, seminiferous epithelium height, cellular quantifications, body weight, and testicular volume, as well as histopathological evaluation, are crucial for reproductive testicular assessment and diagnosis of anomalies. In this study, the morphometric review of young animals revealed no significant differences between groups in any of the observed parameters. However, in the cellular and histological analysis, tubular sections were found to contain several alterations characteristic of a quantitative interruption of spermatogenesis, such as disorganization of the germinal epithelium, vacuolization, desquamation of cells into the tubular lumen, and cell nuclei containing pycnotic aspects, suggesting cellular apoptosis, as shown in the images presented.

Previous studies on the temporal effects of therapeutic doses of IVM in the morphometric and histological assessment of the testis showed that acute administration of IVM impaired spermatogenesis and spermiogenesis in adult rats, presenting the same alterations found in the present study in young animals (Cordeiro et al., 2018). This leads us to conclude that the probable deleterious effects of Ivermectin on the testicular germ epithelium are independent of reproductive age. Since testicular histological changes are common due to the action of Ivermectin, we have sought to evaluate whether germ cell apoptosis has intensified as a result of a local and reversible toxicity action, given its widespread therapeutic use in the prevention and treatment of animals and humans.

Apoptosis maintains the body's equilibrium, ensuring the structural and functional homeostasis of tissues and removing damaged cells that can pose a danger to the organism. The condensation of chromatin occurs in the DNA, and its digestion is mediated by the action of proteolytic enzymes (Shaha et al. 2010. Zhang et al.(2001) . Lirdi et al. (2008) have already demonstrated that agents such as cisplatin and etoposide promote the death of spermatogonia, spermatocytes, and spermatids through apoptosis, and that this death is related to the dose and duration of exposure to the drug and can be induced and increased. Apoptosis occurs physiologically in testicular parenchyma, specifically during spermatogenesis(Santos, 1999; Jurjen et al., 2000). It is considered a physiological phenomenon in the testicular parenchyma, being suggested to control the number of germinative cells and the maturation of defective cells, which could cause spermiation (Yin et al., 1998). According to Holstein et al. (2003), approximately 25 to 75% of spermatozoa in formation degenerate and die in the testicular parenchyma of adult mammals.

Du et al. (2013) showed that GABA is a negative regulator of stem cell proliferation to maintain testicular spermatogenesis homeostasis. A previous study in adult animals administered a therapeutic dose of 1.0 mg/kg aimed to evaluate whether cells in the seminiferous tubules of adult male rats exhibit excessive apoptosis due to the use of Ivermectin. The results showed that Ivermectin treatment increased the expression of caspase-3 by increasing the number of tubules that presented labeled cells in the tubular lumen. In the present study, the same analyses were applied concerning immunohistochemistry using Caspase-3 in young animals. These data suggest that the apoptosis process had already begun, although there was not yet a significant increase in the percentage of strongly labeled tubules. There was no significant increase in the presence of caspase-marked cells in the tubular lumen of the group treated with IVM relative to the control group. This effect suggests the final stage of cellular desquamation and apoptosis of germ cells, which have not been previously phagocytosed, due to the process of local toxicity and disorganization of the germinal epithelium.

These findings in the Caspase-3 labeling of germ cells and the histological changes presented in the various photomicrograph images are in agreement with studies by other researchers in the testicular reproductive area, who, when studying the action of toxic agents such as cisplatin in prepubertal rats exhibited alterations similar to those found in this study such as germ cell depletion, vacuolization and germinal epithelial cell disorganization, in addition to cell death by apoptosis(Lirdi et al. 2008). Histological analysis also revealed the presence of pycnotic nuclei and depletion of the germinal epithelium, indicating a certain degree of cell degeneration in the seminiferous epithelium and apoptosis-mediated cell death.

However, the mechanism underlying the increased IVM-induced testicular apoptosis has not been determined until now. Based on the IVM studies about the molecular mechanisms underlying the antitumor effects of IVM, it was shown that it induces apoptosis through two pathways: 1- the intracellular pathway, mitochondrial-mediated, which is the collapse of the mitochondrial membrane potential and the release of cytochrome c and, 2- the extracellular pathway, death receptor-mediated(Liu et al., 2020; Zhang et al., 2016). Thus, studies about the mechanism underlying the apoptosis at the testicular level after IVM need to be further investigated. Such damage may seem subtle, but it is essential to verify the continuity of the animals' lives and whether these changes are permanent, as the administration of IVM in adult animals is attenuated after 72 hours of administration at a dose of 1.0 mg/kg of IVM.

5. Conclusions

In conclusion, although the morphometry data did not indicate significant testicular damage, the histological analysis revealed that IVM promoted testicular damage in rats when administered at 29 and 44 days of age.

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

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Conflict of interest: The authors declare that there is no conflict of interest.

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Data Accessibility Statement: All data underlying the findings described in the manuscript are fully available without restriction. All relevant data are included in the paper and its supporting information files.

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