Does a therapeutical dose of ivermectin impairs testicular homeostasis of rats via excessive apoptosis?

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Summary
Ivermectin is a medication used to treat parasite infestations in humans and in veterinary medicine. Previously we showed that therapeutical doses of ivermectin impaired spermatogenesis and spermiogenesis in adult rats. The present study was proposed to understand the pathophysiological mechanism that triggered these impairments induced by ivermectin. It was a particular objective to study if ivermectin induced excessive apoptosis. Adult rats were treated with a therapeutical dose of ivermectin (subcutaneously). Their testis was evaluated for the expression of caspase-3 (a marker of apoptosis), using immunohistochemistry techniques. Results 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 data of the control group. In conclusion, a therapeutical dose of ivermectin induced expressive apoptosis in cells of the seminiferous tubules of rats, affecting the testicular natural homeostasis process, which resulted in the spermatogenesis and spermiogenesis impairments previously reported.

Introduction

Ivermectin is a medication used to treat parasite infestations in humans and animals, being also used for agricultural practice (Omura, 2008; Gonzalez *et al.*, 2012; Campbell, 2012).

A previous study of our group showed that acute therapeutical doses of ivermectin induces behavioral, brain, and reproductive impairments in rats, including impairments in sexual behavior, penile erection, striatal dopaminergic system, and testosterone levels (Bernardi *et al.*, 2011; Moreira *et al.*, 2014; Moreira *et al.*, 2017). Moreover, ivermectin impaired spermatogenesis and spermiogenesis in adult rats. It was proposed that these effects were not consequence of ivermectin at the Leydig cells, because no effects were observed at this level. It was suggested that some testicular effects are reversible and correlated with the plasmatic levels of ivermectin (Cordeiro *et al.*, 2018).

In vertebrates, ivermectin induces gamma-aminobutyric acid (GABA)-mimetic effects by acting as $GABA_A$ receptor agonist and stimulating GABA release (Shoop *et al.*, 1995; Dawson *et al.*, 2000; De Souza Spinosa *et al.*, 2000; Yang, 2012). Both, GABA and GABA receptors are present in human and rodents testes (Errington *et al.*, 2014), being involved in spermatogonial stem cells proliferation and spermatogenesis (HU *et al.*, 2004; Du *et al.*, 2013).

Cell apoptosis is a normal and natural process of cellular self-destruction in multicellular organisms, allowing the regulation of development, senescence, and homeostasis (Yao *et al.*, 2018). For male reproductive physiology and spermatogenesis, apoptosis is also a crucial process for cell differentiation, sperm

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maturation in the testis, and for the elimination of excessive or abnormal germ cells (Shaha, 2007; Yao *et al.*, 2018). Imbalances in apoptotic processes during spermatogenesis could lead to defective sperm formation and incidentally, increased apoptosis may induce infertility (Xu *et al.*, 2016). Both internal factors involved in the control of homeostasis and external agents, such as testicular toxins, heat stress and chemotherapeutic agents can affect testicular apoptosis (Shaha *et al.*, 2010).

There is *in vitro* evidence of ivermectin causing testicular apoptosis. Therapeutical doses of ivermectin, administered subcutaneously once weekly for four consecutive weeks, induce apoptosis in testicular cells isolated from adult Wistar rats (Ahmed *et al.*, 2020). The detection used the Sulforhodamine-B method, evaluating the viable versus apoptotic cells. Although this study revealed reprotoxicity after ivermectin exposure, the administration protocol included four exposures, not similar as to the clinical protocol. Moreover, the findings of testicular apoptosis were reported only *in vitro*.

Considering the negative effects of ivermectin on spermatogenesis, particularly on spermiogenesis, the present study was proposed to understand the pathophysiological mechanism that triggered these reproductive impairments. It was a particular objective to study if ivermectin induced excessive apoptosis.

Materials and methods

Ethics and animals

All experimental procedures were approved by the Animal Care Committee of Paulista University (permit no. 333/15) in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

Ten male Wistar rats (90 day-old) from the School of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ/USP), were used. The rats were housed and maintained under standard conditions according to previously described (Cordeiro *et al.*, 2018).

Experimental groups

Rats were divided into two groups of five animals each. One group received 1.0 mg/kg of ivermectin (Ivomec, Merial Animal Health Ltda., Paulínia, SP, Brazil, dissolved in a saline solution, NaCl 0.9%, plus a drop of Tween 80) and other group received the ivermectin vehicle solution, subcutaneously, according to previously described (Cordeiro *et al.*, 2018). All solutions were administered in a volume of 1.0 mL/kg. The ivermectin dose was chosen because it is considered a therapeutic dose to rats (Campbell *et al.*, 1989) but was previously reported as responsible for reproductive impairments (Bernardi *et al.*, 2011; Moreira *et al.*, 2017; Cordeiro *et al.*, 2018).

Immunohistochemistry

Twenty-four hours after the ivermectin (or vehicle) administration, rats were euthanized, and testes were fixed by immersion in Bouin's liquid fixative for 48 h. After 2 hours, a transverse cut was performed in the median testicular region for greater fixative absorption. The testes were then processed and paraffin-embedded to obtaining blocks for histological sections.

One testicular section (5 µm-thick) per rat was obtained 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 95°C for 15 min in a steamer. PBS solution instead of the primary antibody was used as the negative control during immunohistochemical staining. The sections were counterstained with Harris's hematoxylin and mounted with DPX (06522, Sigma Aldrich, St. Louis, MO, USA).

The testicular immunolabeled section of each animal was totally scrutinized and photographed (40x objective, Nikon E200 microscope, equipped with a Nikon Coolpix digital camera, Kanagawa, Japan).

The following parameters were quantitatively evaluated for the total testicular section of each animal: (A) total number of seminiferous tubules (labeled or not with caspase-3); (B) number of labeled tubules; (C) number of non-labeled tubules; (D) number of tubules labeled weakly; (E) number of tubules labeled strongly; and (F) number of tubules that presented labeled cells in the tubular lumen. Parameters B to F were all calculated as a frequency in relation to the total tubules.

Statistical analysis

Homogeneity was verified using the F test or Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. The Student *t* test was used to analyze the parametric data and the Mann-Whitney test to non-parametric data. In all cases, the results were considered significant at $p < 0.05$.

Results

The expression of caspase-3 is visualized in brown in the seminiferous tubules of testes of both control (Fig 1A, C, and E) and ivermectin-treated rats (Fig 1B, D, and F).

Comparing photomicrographs in low magnification, seminiferous tubules of ivermectin group (Fig 1B) presented increased expression of caspase-3 (vs. control group, Fig 1A), as well as presence of cells in apoptosis in the lumen. In greater magnification, the scenario is the same (Figs 1 C and D). A detailed seminiferous tubule not labeled with caspase-3 is shown in Fig 1E, whereas a seminiferous tubule strongly labeled with caspase-3 is shown in Fig 1F. Figure 1 (A-F) also differentiates seminiferous tubules non-labeled, weakly labeled, and strongly labeled.

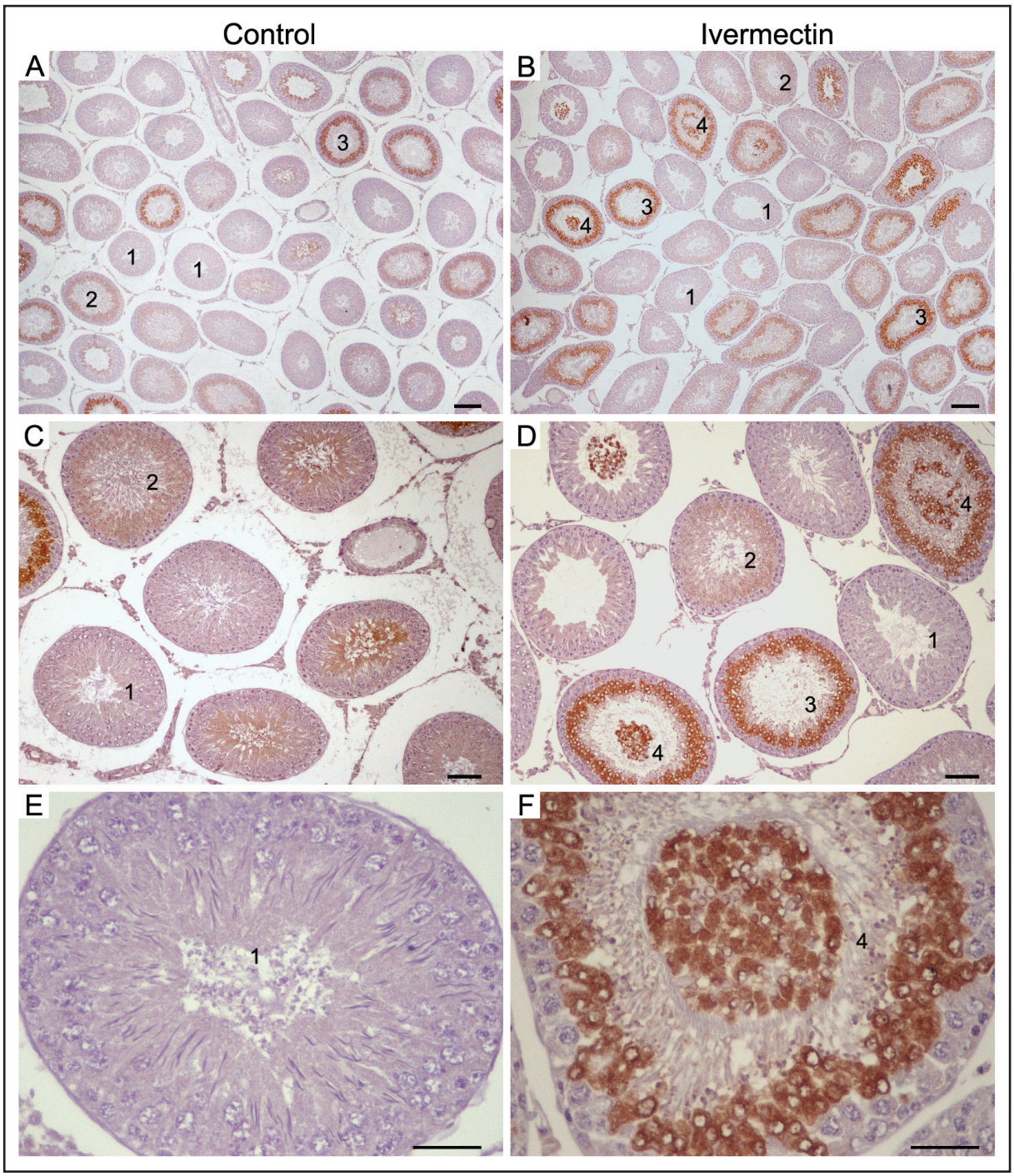

Figure 1. *Effects of a therapeutical dose of ivermectin (1.0 mg/kg, subcutaneously) on the caspase-3 expression in testicular tissue of rats. Photomicrographs of seminiferous tubules immunolabeled sections. Bar = (A and B) 200 µm, (C and D) 100 µm, and (E and F) 50 µm. (E) Tubule in stage XII of the seminiferous epithelial cycle, (F) tubule in stage VII of the seminiferous epithelial cycle. (1) non-labeled seminiferous tubules, (2) weakly labeled tubules, (3) strongly labeled tubules, and (4) strongly labeled tubules in lumen.*

Statistical analyses are presented in Fig. 2.

Ivermectin treatment reduced the total number of tubules (labeled or not with caspase-3), compared to the data of the control group (t=3.76, df=8, p=0.0056, η^2=0.6381, Fig2A).

Ivermectin increased the expression of caspase-3 (labeled tubules in relation to total tubules), compared to the data of the control group (U=0.0079, Fig 2B).

Likewise, the number of tubules labeled strongly (t=2.55, df=8, p=0.0341, η^2=0.4488, Fig 2E) and the number of tubules that presented labeled cells in the tubular lumen (t=2.43, df=8, p=0.0414, η^2=0.4242, Fig 2F) were increased in the ivermectin group compared to the data of the control group. However, the number of non-labeled tubules (t=0.49, df=8, p=0.6396, η^2=0.0287, Fig 2C) and the number of tubules labeled weakly (t=1.53, df=8, p=0.1637, η^2=0.2272, Fig 2D) did not differ statistically between groups.

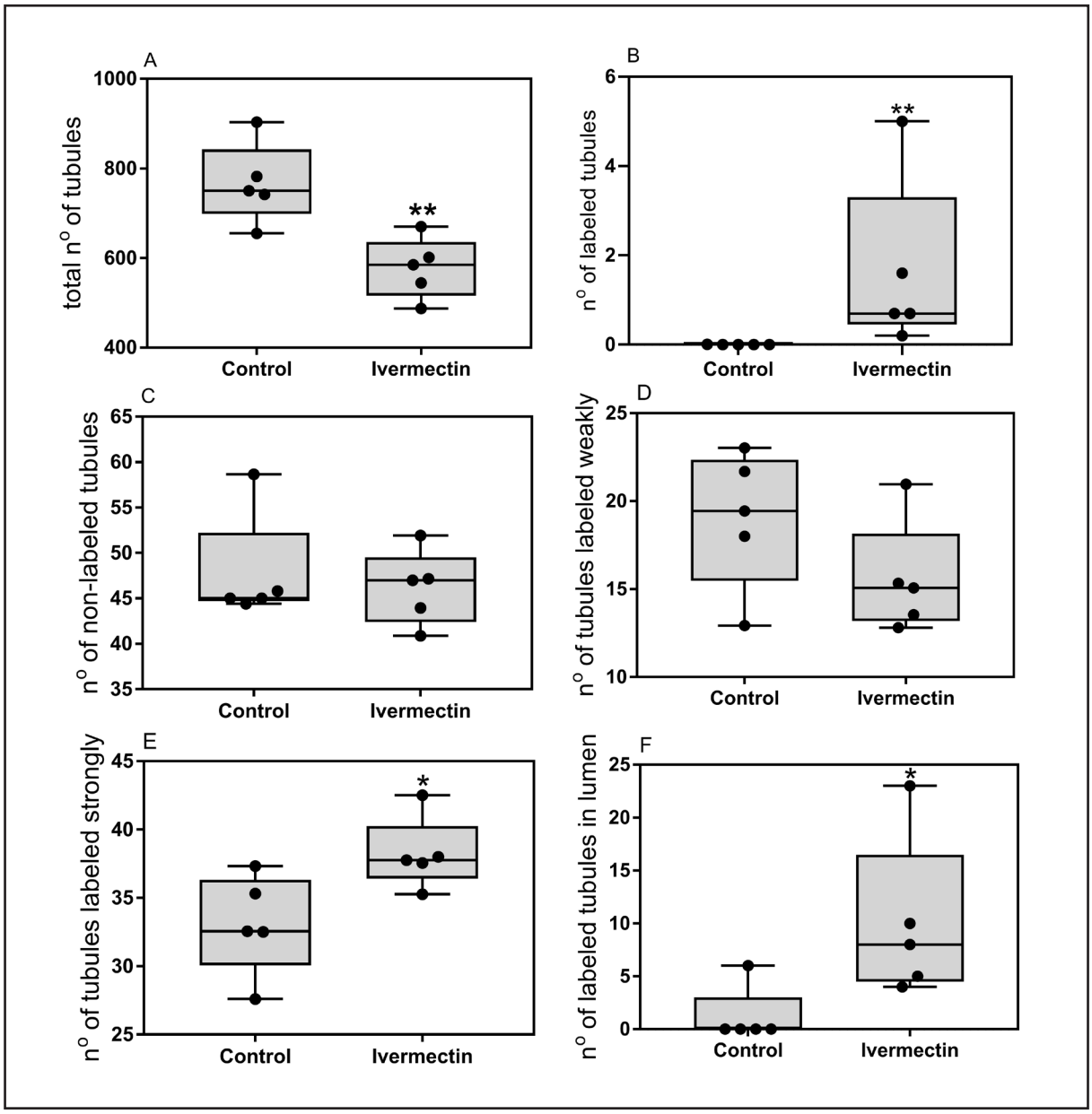

Figure 1. *Effects of a therapeutical dose of ivermectin (1.0 mg/kg, subcutaneously) on the caspase-3 expression in testicular tissue of rats (n = 5 rats/group). *p < 0.05 and **p < 0.01 (Student t test or Mann Whitney tests). Data are expressed as the mean ± SD, using box plots with individual data points.*

Discussion

It is known the role of ivermectin in the release and interaction in the GABAergic system. GABA in gonads and accessory reproductive organs, directly affects the steroidogenesis, sperm viability, and motility (Erdö *et al*., 1983; Frungieri *et al*., 1996). GABA mimics and potentiates the action of progesterone in initiating the acrosome reaction of mammalian sperm, indicating that sperm contain receptors for GABA. Moreover, the $GABA_A$ (HE *et al*., 2003), $GABA_B$ (He *et al*., 2001) and $GABA_C$ (Li *et al*., 2008) receptors were identified in rat testis and sperm. The GABAergic systems may play modulatory roles in spermiogenesis because expression of glutamate decarboxylase mRNAs, which is GABA synthetic enzymes, has been observed in both round and elongated spermatids (Kanbara *et al*., 2005). Furthermore, the GABAergic system located in adult Leydig cells in rodent and human testis appears to be linked to regulation of steroid synthesis by Leydig cells via local GABAA receptors (Hu *et al*., 2002; Geigerseder *et al*., 2004; Hauet *et al*., 2005).

Previous study about the temporal effects of therapeutic doses of ivermectin in the morphometric and histological assessment of testis showed that ivermectin acute administration impaired the spermatogenesis and spermiogenesis of adult rats (Cordeiro *et al*., 2018). Despite no differences on testosterone levels and on Leydig cells frequencies levels, ivermectin acute exposure, mainly in the higher dose, reduced the testicular volume, the tubular diameter, and the germinal epithelium height. Moreover, tubular sections presented several histological changes, such as disorganization of germinal epithelium, vacuolar degeneration of the germ cells, and sloughing of cells into the tubular lumen, i.e., evidences of spermatogenesis damages (Cordeiro *et al*., 2018). Thus, the reduced mean diameter of the tubules here observed could be explained by the decrease in the testicular volume.

Apoptosis acts on the body's equilibrium, ensuring the structural and functional homeostasis of the tissues, removes damaged cells that can cause some danger to our organism (Elmore, 2007). In the testicular parenchyma, apoptosis controls the amount of germinative cells and maturation of the defective cells in the spermiation process, leading to death 25% to 75% of the spermatozoa in adult mammals (Dunkel *et al*., 1997; Shaha *et al*., 2010).

It was observed in the present study the presence of tubules marked by caspase-3 containing apoptotic round cells in the tubular lumen, both in the control group and in the experimental group. This was an expected finding because of the process of natural homeostasis in testicles.

However, there was a significant increase in labeled cells in the tubular lumen of the rats from ivermectin group, suggesting an extension of the apoptotic process as comparing with the control group. Thus, there was an extension of the apoptotic process in these rats probably due to the similar effect of GABA present in ivermectin.

Fig 1F revealed seminiferous tubule strongly labeled with caspase-3 after ivermectin exposure. This pattern was associated with the stage VII of the seminiferous epithelium cycle. Premature and round spermatids are predominantly found at this stage, characterized by stage 6, according to the classification by Leblond and Clermont (1952). On the other hand, the negative labeling shown in the Fig 1E (control group), is associated with stage XII, where premature spermatids are not found. These findings may initiate a novel investigation, in which the systematic study of this correlation in a representative number of tubule sections could be carried out in the experimental and control groups. The objective would be to associate each stage of the germinal cycle with the volumetric density of the caspase 3-positive germinal epithelium, and further confirmation of this hypothesis.

Numerous toxic agents,such as cisplatin (Soni *et al*., 2016), etoposide (Stumpp *et al*., 2004), and doxorubicin (Ujah *et al*., 2021) can interfere in the cellular microenvironment of the germinative epithelium resulting in histopathological processes, with consequent changes in the development of gametogenesis.

The action of ivermectin in the cellular microenvironment of the seminiferous epithelium may also have led to a histopathological process resulting in cell disorganization and sloughing of germ cells into the tubular lumen. In addition, the cellular toxicity may have led to disruption of the Sertoli cells occlusive junctions causing a germ cell sloughing toward to the seminiferous tubule lumen (Cheng, 2014; Johnson, 2014; Cordeiro *et al*., 2018). These cells can be released from Sertoli cells by rupture of junctional complexes and being dislayered, individually or in groups, into the tubular lumen. This condition often occurs when germ cell degeneration is massive but has also been seen when only minor changes in the testicle occurred (Russell *et al*., 1993). However, more studies are needed to be conducted to show that cellular junctions were compromised.

The study of the role of Sertoli cells in regulating the impact of ivermectin on spermiogenesis can be complemented by the comparison of immunohistochemical techniques for caspase-3 and TUNEL. The caspase-3 assay identifies cells at an early stage of pro-apoptosis signaling, before presenting morphological changes observable under optical microscopy. The TUNEL assay identifies

chromatin fragmentation at a later stage of the apoptosis process or even necrosis (Gavrieli *et al.*, 1992; Sasso-Cerri *et al.*, 2002). The finding of the cell type involved in the desquamation process using the TUNEL method would bring another element to elucidate the pathogenesis of the toxic effects of ivermectin on tubular cell constituents.

GABA acts as a negative regulator of stem cells proliferation to maintain the testicular spermatogenesis homeostasis and the in vivo testicular microenvironment undoubtedly provides an inhibitory signal for spermatogonial stem cells proliferation to maintain spermatogenesis homeostasis (Du *et al.*, 2013). GABA signaling is such a proliferation inhibitor allowing the maintenance of spermatogonial stem cells homeostasis. They proposed that physiological levels of GABA synthesized by spermatogonial stem cells or interstitial cells through $GABA_A$ receptors result in a decreased proliferation of spermatogonial stem cells.

Finally, further studies are planned to investigate the immunohistochemical expression of caspase-3, both in the germinal epithelium and in Sertoli cells. The investigation will possibly confirm whether the degenerative action of ivermectin affects early stages of spermiogenesis and, in parallel, verify a possible protective role of Sertoli cells, limiting and/or reversing these injuries. This study may be predictive for the degree of functional impairment of the reproductive system in animals exposed to therapeutical doses of ivermectin at reproductive age.

Considering the present and past studies of our group that therapeutical doses of ivermectin induces reproductive and behavioral impairments, its uses should be revised in human and veterinary medicine, both during reproductive phases and for juveniles.

Conclusions

Ivermectin treatment increased the expression of caspase-3, as well as increased the number of seminiferous tubules that presented labeled cells into the tubular lumen. Therefore, a therapeutical dose of ivermectin induced expressive apoptosis in cells of the seminiferous tubules of rats, affecting the testicular natural homeostasis process, which resulted in the spermatogenesis (particularly on spermiogenesis) impairments previously reported.

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