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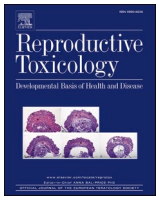

Investigating the significance of the transgenerational impact of high and repeated doses of ivermectin: Effects on paternal testis histopathology, pups' development, and sexual behavior

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ABSTRACT

Paternal exposure to environmental challenges is critical for the offspring's future health, and the transmission of acquired traits through generations increases the risk of offspring developing diseases. Ivermectin (IVM) is widely used in veterinary and human medicine to treat parasitosis. Our previous studies showed that IVM acute administration induced disorganization of the germinal epithelium and could cause damage to sperm production. Thus, this study investigated the effects of paternal exposure to repeated high ivermectin doses on paternal testis histology. After mating, their pups' development and sexual behavior in adult rats were examined. Method: Two groups of male rats were treated with IVM or its vehicle once a week for three weeks. We observed these males' body weight, organs and testis histology, and testosterone levels. These rats were mated with females without any treatment: the reproductive performance, the offspring development, and the male and female sexual behavior observed in adulthood. Relative to controls, the IVM paternal testis histology showed hypertrophy and hyperplasia of Leydig cells and increased diameter of the seminiferous tubules—no impairment in reproductive performance. In males and females, the physical and reflexes were modified. In adult age, female rats of the IVM group showed reduced sexual behavior and sexual preferences for the same sex, while male sexual behavior was not altered. Thus, it is possible that paternal exposure to IVM interfered with pups' hormonal and growth factors during development and in adult age. Further studies are needed to explore IVM transgenerational effects identifying possible mechanisms underpinning behavioral effects.

1. Introduction

Avermectins are macrocyclic lactones discovered in the 1970s, and they are used as antiparasitic and pesticides in agriculture[1]. It is widely used in veterinary medicine to control several endo and ecto-parasites[2–4]. In humans, IVM is also indicated for treating various conditions caused by worms or parasites[5–7].

The mechanism of action of IVM, which consists of enhancing the inhibitory action of gamma-aminobutyric acid (GABA), promotes hyperpolarization of the neuron, favoring the entry of chlorine ions and, thus, inhibiting nervous transmission[8]. GABA and its receptors exist in several non-neural tissues, including the pituitary, pancreas, testis, and endocrine organs. In the case of rat testes, GABA appears to be linked to the regulation of steroid synthesis by Leydig cells through GABA(A) receptors [9]. However, neither the testicular sources of GABA nor the precise nature of testicular GABA receptors is known. fully known[10,11].

Our research group analyzed various aspects of the effects of IVM exposure on the reproductive area. Thus, we showed a reduction in the sexual behavior of male [12] and female rats[13], in penile erection, and decreased testosterone levels and central neurotransmitters[14]. Additionally, Cordeiro et al. [15] observed that acute exposure to IVM reduced testicular volume, tubular diameter, and height of the germinal epithelium, in addition to observing a loss in the cellular organization of

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the seminiferous epithelium. It also promotes cellular disorganization of the germinal epithelium, which can cause damage to sperm production and, consequently, possible damage to fertility and fetal development, thus potentially causing damage to the behavior of the offspring both during the development period and in adulthood. Furthermore, it was found that IVM plasma levels were reduced after 72 hours of acute administration of 1 mg/kg of the drug. Also, we observed that this IVM therapeutical dose 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 by [16]

Given the results reported by Cordeiro et al.[15,16], and the lack of studies about the effects of paternal treatment with IVM, we first evaluated the impact of repeated high IVM doses on rats' testis histology. The reproductive effects of paternal exposure to IVM were analyzed after matin. Then, the pups of F1 generation development and the male and female adult sexual behavior were observed.

2. Material and methods

2.1. Ethics statement

The experimental protocols conducted in this study were approved by the Ethics Committee for Animal Use of Paulista University (protocol number 7690240119, CEUA-UNIP). All experiments were performed following standardized laboratory practice protocols and adherence to quality assurance methods. Every effort was made to minimize animal suffering.

2.2. Animals

Twenty-eight, fourteen males and fourteen females Wistar rats (specific-pathogen-free), aged 90 days (F0 generation), weighting 398.10 ± 8.10 g and 280.50 ± 9.23 g at the start of treatments, respectively, from the vivarium of the Institute of Biomedical Sciences of the University of São Paulo, were used. Seven other females and seven males of the same origin were used to perform sexual behavior. The animals (4/cage, 40 ×34 x 20 cm, 1.530 cm2) were housed in microisolators (Tecniplast S.P.A. 20020 Buguggiate (VA), Italy, with a 2i̊C/83, 75 A.CH,-20 %, Muliticages, adapted to 4 rats), 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.

2.3. Drugs and treatment

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, being chosen considering a previous study from our laboratory, in which it was observed that acutely treatment with this dose promoted impairment in the sexual behavior of male rats [17] and prejudices in spermatogenesis and spermiogenesis [15,16]. The commercial preparation of estradiol valerate (Primogyna®) was diluted in saline and administered, SC, at a dose of 0.5 mg/kg to induce female sexual behavior in the offspring study of male sexual behavior [18]. All solutions were administered in a volume of 1.0 ml/kg.

2.4. Experimental procedures

Before starting any procedure, the male and female F0 rats remained in the vivarium for ten days to adapt to the laboratory conditions. Fourteen male rats were used and distributed into two groups of 7 animals/group, one experimental group, and one control group. The experimental group's male rats received 1.0 mg/kg of ivermectin via SC once a week for three weeks, and the control group received the IVM vehicle. The body weight of both groups was measured weekly measures. Twenty-four hours after the last treatment, these rats were mated with untreated female rats (N=7/group). For this, after detecting the estrous phase, the female rats were introduced into the cage of a male rat (this one was treated with ivermectin or control solution). Vaginal washings were collected in the morning, between 8:30 a.m. and 10:30 a.m. The presence of sperm on the slide indicates day zero of gestation (DG 0). Then, the F0 males were euthanized, and the right testis and fragments of the epididymis, liver, and adrenal were submitted to histopathological studies. The testis was submitted to histopathological and microscopic morphometry analyses.

The pregnant females were housed individually in cages throughout their gestation, which lasted 21 days. The day of delivery occurred at postnatal (PND)1, and on PND2, the reproductive performance was observed based on the litter weight, the number of live/dead pups, the number of female/male pups, and the weight of the group. After this procedure, and on the same day, the litter size was randomly standardized to 8 pups (four of each sex per litter). The pups were weighed again at PND2, PND9, and PND21 when weaned and housed according to sex and treatments. The male and female pups' anogenital distance (PND2 and PND21), body length (PND2 and PND21), and physical and reflexes development were observed from PND2 to PND 30. At PND 60, females (F1 generation) had their estrous cycle monitored for eight consecutive days. At PND 75–80, male and female offspring were evaluated for sexual behavior. Immediately after this procedure, the animals were euthanized by deep anesthesia (Ketamine,75 mg/kg + xylazine,2,5 mg/kg, i.p), and the liver, kidneys, adrenal glands, epididymis, ventral prostate, seminal vesicle (full and empty), uterus, and ovaries were collected, weighed, and sent for histopathological analysis. Fig. 1 shows this experimental design.

2.5. Methods

2.5.1. Testes evaluations

2.5.1.1. Method. Immediately after euthanasia, the testes were fixed by immersion in Bouin's liquid fixative for 48 hours. 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[19]. Six μm-thick sections were stained with the Hematoxilin-Eosin (HE) method to identify all spermatogenesis phases better. A periodic acid-Schiff method, counterstained with Harris'Hematoxylin (PAS + H), was also processed as a histochemical method to determine interstitial cells. The analysis involved tracking the blades containing the histological cuts observed in the optical light microscope at 400 and 1000 times increases.

2.5.1.2. Tubular diameter and germinal epithelium height. To determine tubular diameter sizing and the height of the germinal epithelium, ten fields were selected from the histological testicular sections of each animal, and photomicrographs were taken using a 40x objective. Five tubules were measured for each field, and the distance of tubular diameter and the height of the germinal epithelium were automatically calculated in pixels using ImageJ software, totaling 50 tubules per animal.

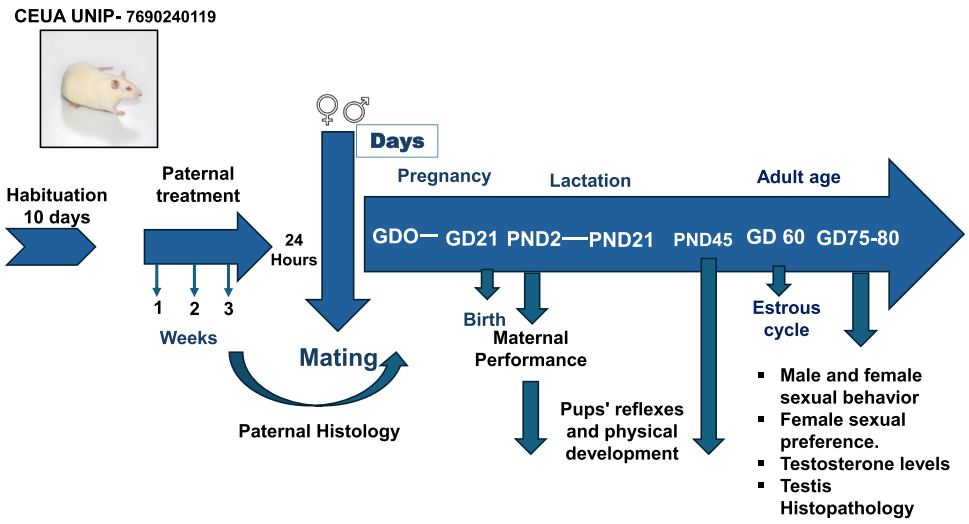

Fig. 1. Experimental design. GD- gestation day, PND- postnatal day.

2.5.1.3. Interstitial cells frequency. The frequency of interstitial cells per field was estimated in ten fields from the histological testicular sections of each animal, which were photographed using a 1000x objective at the microscope.

2.5.2. Testosterone levels

The rats of the F0 generation had their trunk blood collected and plasma samples obtained using standard protocols[15]. Plasmatic testosterone levels were assessed using commercially available enzyme-linked immunosorbent assays (testosterone kit, Cayman Chemical, Ann Arbor, MI, USA; catalog no. 582701). The procedure was performed according to the manufacturer's instructions.

2.5.3. Physical and reflexological development of male and female offspring during childhood

On the day of birth, no procedure was carried out to avoid maternal cannibalism (Camargo, 2010). On PND 2, after observing the female's body weight gain, eight pups, four males and four females from each litter, had their paws carefully marked with India ink. These pups were weighed, and the anogenital and head-to-tail distance were evaluated. When the anogenital and head-tail distances were measured again on PND 21 (weaning), these animals were weighed again.

The physical development was monitored in the identical pups used to measure body weight. The evaluation of each animal, starting on PND 2, was carried out until the occurrence of each of the following parameters: Ear unfolding, the appearance of hair, incisor teeth eruption, eye-opening, adult walking day, testis descent, and vaginal opening. The reflexological development assessment was conducted with identical pups submitted to the physical development assessment. The pups were observed daily until 100 % of the marked pups from each litter showed the following reflexes and behaviors: palmar grasp reflex, righting reflex, negative geotaxis reflex, and startle reflex. The day the criterion examined appeared was observed and noted for each physical and reflexological development parameter. All data obtained on physical and reflexological development with the offspring were analyzed. Supplementary Table 1 describes these procedures.

2.5.4. Assessment of the estrous cycle of female offspring in adulthood

The frequency of estrous phase:pro-estrus, estrus, diestrus, and metestrus were evaluated daily (8:00 – 9:00 a.m.) for eight consecutive days between 60 and 70 years of age [20].

2.5.5. Assessment of the sexual behavior

The evaluation of sexual behavior in males and females during the dark period of the animals' light cycle, starting three hours after the lights were turned off. A red light (25 W) was used to allow observation of behavior. Therefore, observations took place between 1:00 p.m. and 5:00 p.m.

2.5.6. Male sexual behavior

Sexual behavior was assessed in a glass box (56 ×32 x 32 cm); the box floor will be lined with wood shavings. For evaluations of male sexual behavior, we used sexually receptive females. The sexual receptivity of the female rats was induced by administering 0.5 mg/kg of estradiol valerate, SC, 24 hours before the day of evaluating the male's behavior[13]. The male's sexual behavior was assessed by placing him individually in the observation box. After 5 minutes, the bait female was introduced into this observation box, beginning the observation of the male's sexual behavior for 10 minutes. The parameters evaluated were the following: Latency to first mount: time elapsed from the introduction of the female into the observation box until the first mount with vaginal intromission; Latency for first intromission: time elapsed from the introduction of the female into the observation box until the first mount with vaginal intromission. Intrusion will be inferred by observing the characteristic motor pattern, that is, mounting with the deep movement of the pelvis (thrusting) and rapid dismounting, accompanied by lordosis of the female; after dismounting, the male's genital cleaning is observed. Latencies were recorded using a stopwatch and expressed in minutes. The assessment of the males' sexual behavior was carried out in the dark period of the animals' light cycle, from 1:00 p.m. to 5:00 p.m.

2.5.7. Female sexual behavior

The device for observing sexual behavior was the same used for male sexual behavior. To evaluate the females' sexual behavior, vaginal washing was performed daily in the morning when the female was in estrus, and the sexual behavior was assessed in the afternoon paired with a sexually experienced male rat. The male was initially introduced into the observation box for habituation (5 min.) to impregnate its scent on the wood shavings. Then, the female was introduced into the box, beginning the observation of sexual behavior for ten months with or not with lordosis. This behavior was evaluated in two ways: by the lordosis coefficient and intensity. Lordosis was characterized by the concave flexion of the female's spinal column, elevating the tail and thus exposing the genitalia [21]. The lordosis coefficient (in %) was calculated by the number of lordosis in 10 mounts multiplied by 100. The intensity of lordosis was assessed by assigning scores to the behavior exhibited by the female in each of the ten mounts[22].After observing each female's sexual behavior, both the test rat and the bait rat were

removed from the box, and the wood shavings were exchanged. Next, the same bait rat was placed back in the box, and the same experimental procedure described was followed; each male was used three times on the same day. Interspersing animals made observations from the experimental and control groups.

2.5.8. Female sexual preference

the device for observing sexual preference was based on Crawley's three-chamber device proposed for the sociability test[23]. In summary, testing was performed in a room illuminated only by red light during the dark cycle between 9:00 and 14:00. The arena is a rectangular box with 121x40x38 cm divided into three cameras by partitions, each measuring 27 cm by 38 cm. Two small cameras with wire division measuring 20 cm×40 cm x 383 cm (length x width x height) were in opposite corners of the left and right cameras. In the first (habituation) session, the female rats of control or IVM groups were placed in the center compartment of the arena alone and allowed to explore all chambers. In the second session (test session) performed 24 hr after habituation, one *bait* male and one female in estrus (induced by 0.5 mg/kg of estradiol valerate, SC, 24 hours before the day of the test) were introduced in the corners of the left or right chambers. Then, one female IVM or control group was introduced to the central compartments. The time of sniffing (sec) of either the male or female baits and the number of transitions between the two compartments were annotated.

2.5.9. Assessment of the relative weight of organs and histopathological study of male and female offspring

To evaluate the relative weight of organs and histopathological study of the F1 male and female generation, the rats were weighed individually and subjected to euthanasia (deep anesthesia with 1.0 mg/kg acepromazine + 10 mg/kg xylazine + 60 mg/kg ketamine, via IP). Then, the testicles, epididymis, seminal vesicle (with and without secretion), prostate, liver, kidneys, adrenal glands, uterus, and ovaries were removed and weighed. The organs' relative weight (PR) was calculated as PR = PO/BW x 100, where PO = organ weight and BW = body weight.

2.5.10. Statistical analysis

Homocedasticity was verified using the F or Bartlet tests, and normality was determined using the Kolmogorov-Smirnov test. Tests for two variables were analyzed using the Student t-test or U- of Mann-Whitney test, depending on the normality of the data. The ANOVA analyzed data with more than two groups in one way and those of two factors in the two-way ANOVA. The results were considered significant when $\alpha < 0.05$.

3. Results

3.1. Male F0 treatment with IVM induces testis hypertrophy and hyperplasia of Leydig cells, increasing the diameter of seminiferous tubules

Treatment with IVM with 1.0 mg/kg of ivermectin once a week for three weeks did not affect the body weight gain relative to control group in the same week (Fig. 2, Interaction - F (2, 36) = 0.002, P=0.99; weeks - F (2, 36) = 4.883, P=0.01 and treatment-F (1, 54) = 13.73 P=0.0005), but the body weight increased in IVM group at the third week of treatment relative to first day of treatment (P< 0.05).

Fig. 3 shows the testis morphometric results of controls and IVM-treated rats. There was an increase in the epithelium height (Fig. 3a, t=2.423, df=12, P = 0.032), in the tubular diameter (Fig. 3b—t=2.317, df=12, P = 0.039), and the frequency of Leydig cells (Fig. 3c—t=2.148, df=12, P= 0.05).

As for stereological analysis, the histological cuts of the control group testicles have exhibited typical characteristics (Fig. 4). Seminiferous tubules, stained in H.E., were organized in well-defined cellular associations, containing support cells (Sertoli) and various characteristic

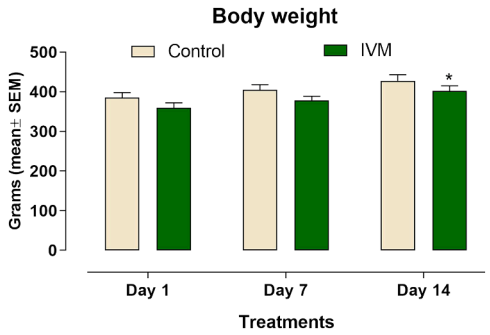

Fig. 2. Paternal body weight during treatment with 1.0 mg/kg of IVM or its vehicle, SC, once a week for three weeks. Two-way ANOVA followed by Sidak's multiple comparisons test.

cellular types of spermatogenesis germ cells. Also, they presented the 14 stages of the seminiferous epithelium cycle, tubular sections containing 4–6 concentric layers and an average of 210 pixels in diameter (Fig. 4A). The interstitial tissue, located between the seminiferous tubules, subjected to the PAS method, was composed of connective tissue cells, which encompassed fibroblasts, fibrocytes, macrophages, mastocytes, lymphocytes, mesenchymal cells, and interstitial cells (Leydig cells (Leydig cells) characteristics of the testis. Leydig cells were in isolation or "islets" around blood vessels. They exhibited rounded or oval nuclei with prominent nucleoli and lightweight cytoplasmic acidophilic compatible with normality (Fig. 4B) and had an average amount per field of 7.675 cells (Fig. 4C).

The seminiferous tubules of the experimental group's animal testicles showed characteristics close to those of the control group. However, the diameters increased significantly compared to those of the control group, with an average of 216 pixels in diameter. In the interstitial tissue, the presence of eosinophilia and vacuoles was verified in some testicular sections (Fig. 5A). Also, in the interstitial tissue, Leydig cells with hypertrophy characteristics present heterochromatin rejected to the cellular periphery and a more rounded morphology than those of the control group (Fig. 5B). Fig. 5C shows Leydig cells with a medium of 11,125 field cells, demonstrating cell hyperplasia compared to the control group.

No differences were observed in the plasmatic testosterone levels (Control- 0.232 ± 0.0123; IVM −0.233 ± 0.0115 pg/ml, t=0.01920, df=13, P=0.985).

3.2. No effects of paternal IVM treatment on reproductive performance and pups' body weight during lactation after mating with females without any treatment

In the reproductive evaluation, the total number of pups born, the litter weight, and the male and female weight during lactation (PND2, PND,9, and PND21) did not differ. No pup deaths occurred during this period (Supplementary Table 2).

3.3. Paternal treatment with IVM dysregulated some physical and reflexological development of male and female offspring during childhood

Fig. 6-I show the body length and anogenital distance of male pups of the control and IVM groups observed at PND2 and PND21. There were no significant differences between the body length of male pups of IVM and control groups at PND2 (Fig. 6a) and PND21(Fig. 6b). Concerning the anogenital distance also, this parameter did not differ at PND2 between groups (Fig. 6c) but at PND21 male pups of IVM group showed increased anogenital distance relative to control group (Fig. 6d, p<0.05).

In female pups (Fig. 6 -II), only the anogenital distance was increased in the IVM group relative to the control at 21 PND21 (Fig. 6-d, p< 0.05).

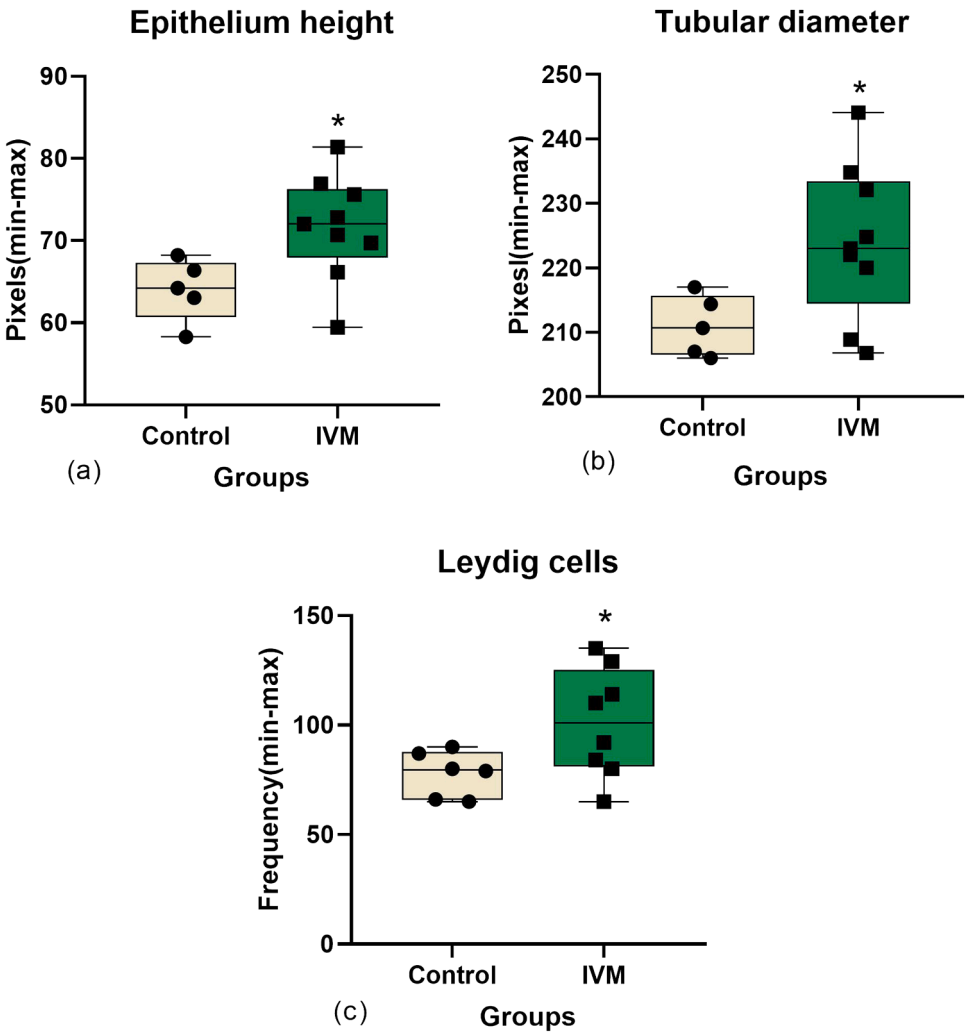

Fig. 3. Seminiferous tubular diameter (A), height of germinal tubular epithelium (B), and frequency of Leydig cells (C) of male rats treated once a week for three weeks with 1.0 mg/kg of IVM, observed 24 h after the last treatments. N =5–9/group. The data are presented as means ± SEM. *p < 0.05, Student t-test relative to the control group.

The remaining parameters did not differ between groups.

Fig. 7. shows the physical (I) and reflexes (II) development of male rats from paternal rats treated with one dose every three weeks with 1.0 mg/kg IVM or its vehicle. The days of incisor eruption, eye-opening, and the testis descent advanced relative to the control group, while the day of adult walking retarded. Only the day of negative geotaxis was advanced in rats of IVM groups relative to its control rats. In the remaining reflexes, neither group differed. In the remaining physical and reflexes, the development of both groups did not differ. In female rats, the hair growth was advanced, and the day of the adult walking day was retarded in the IVM group relative to the control group (Fig. 8). The remaining physical parameters of both groups did not differ.

All statistical data of male and female pups' physical and reflex development are shown in Supplementary Table 3.

3.4. Paternal treatment with IVM did not interfere with the female F1 estrous cycle:

The female estrous cycle of the IVM group was the same as that of the control group (Supplementary Fig. 1).

3.5. Paternal treatment with IVM did not modify the male F1 sexual behavior

No differences occurred between the sexual parameters of IVM male rats relative to controls (Table 1).

3.6. Paternal treatment with IVM reduced female F1 sexual behavior

Female rats of the IVM group presented a reduced percentage of lordosis (U= 0.0017) and in the frequencies of exaggerated (U= 0.0017) and normal lordosis (t=2.950, df=12, P = 0.012) relative to control females. No differences were detected between groups in the frequencies of low lordosis (t=1.393, df=12, P =0.19).

3.7. Paternal treatment with IVM-induced female F1 sexual preference for the same sex

Female rats of the IVM group remained more time on the female side than on the male side (Interaction- F (1, 22) = 57.40, P<0,0001, preference-F (1, 22) = 181.60, P<0.0001 and treatment-F (1, 22) = 8.438e-005, P=0.992). Otherwise, the treated female visited more times the male side than the female side (Interaction-F (1, 22) = 0.56, P=0.4621; preference-F (1, 22) = 0.103, P=0.751) and treatment (F (1, 22) = 8.882, P=0.007).

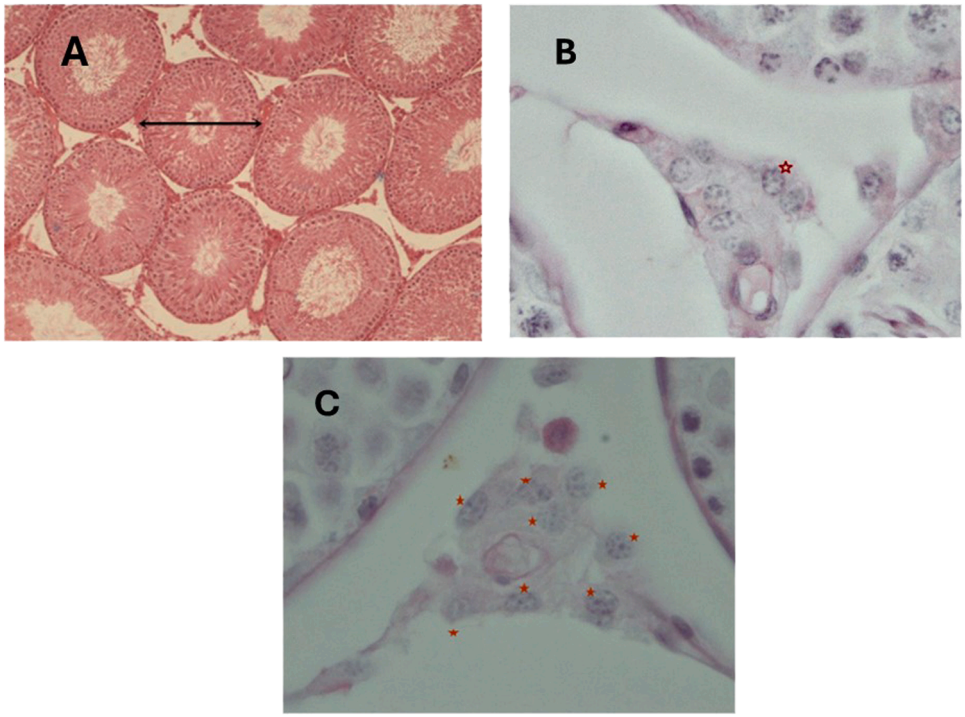

Fig. 4. Histology of the control group. A—seminiferous tubes with normal morphology and mean diameter of 210 pixels de diameter (set) (H.E. 400x). B—interstitial cells (Leydig cells) (PAS 1000x) with normal cellular morphology (star). C-Interstitial cells (Leydig cells), PAS 1000x, mean of 7675 cells/ field in five rats (stars).

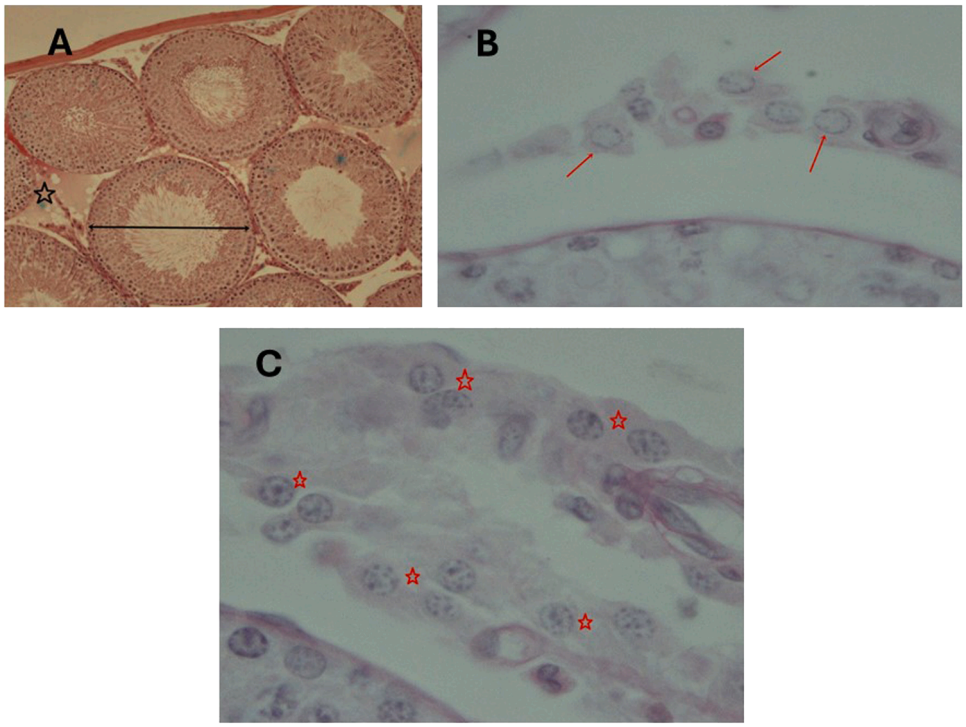

Fig. 5. Histology of the IVM group—A- Seminiferous tubules showing an increase in diameter compared to the control group—216 pixels (arrow), presence of eosinophilia and vacuoles in the interstitial space (star) (H.E. 400x). B- Interstitial cells (Leydig) with altered morphology showing hypertrophy (arrows) (PAS 1000x). C- interstitial cells (Leydig) PAS 1000x, an average of 11,125 cells per field in five rats - showing hyperplasia (stars).

3.8. Lack of difference on relative weight of organs and histopathological study of male and female offspring from paternal treatment with IVM or its vehicle

No differences were observed between the relative weight of organs and the histopathological study of male and female offspring (data not shown).

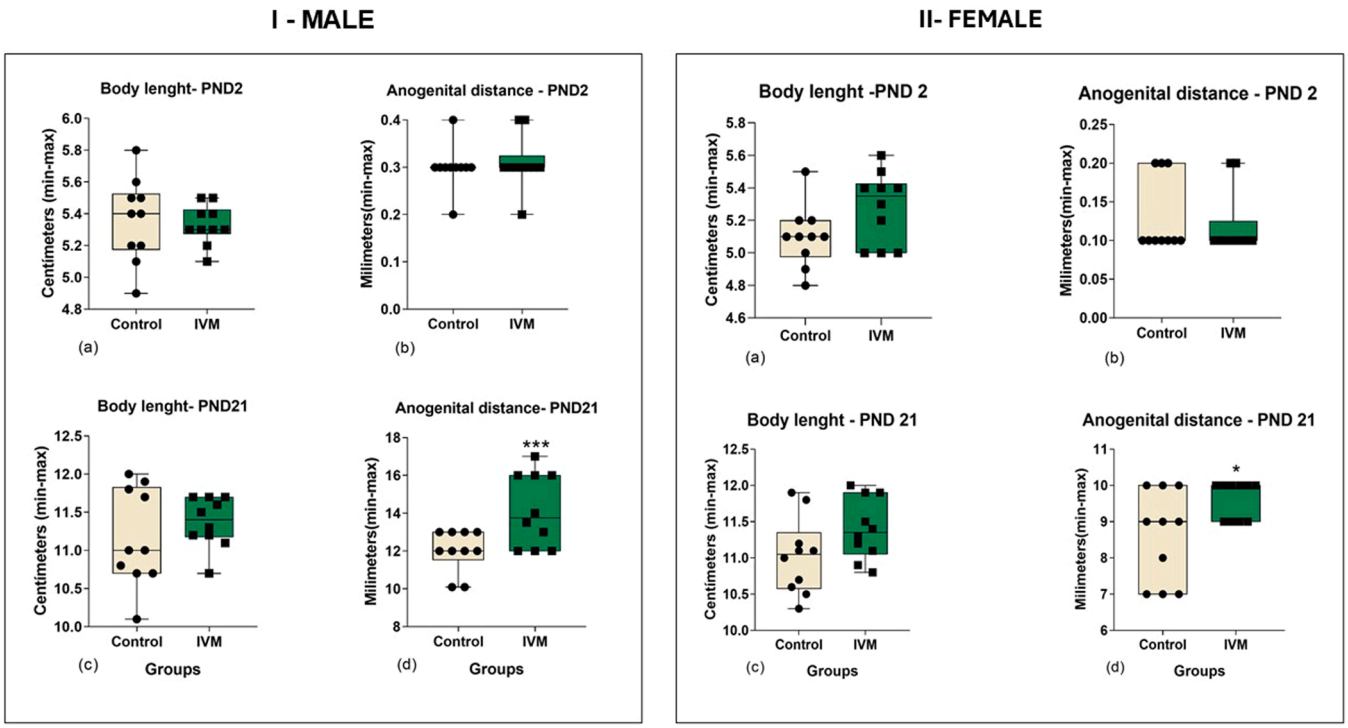

Fig. 6. I- Male body length and anogenital distance of rats from paternal rats treated with one dose every three weeks with 1.0 mg/kg IVM or its vehicle measured at PND2 and PND21. * p< 0.05 relative to the control group. Student t-test. II- Female body length and anogenital distance of rats from paternal rats treated with one dose every three weeks with 1.0 mg/kg IVM or its vehicle measured at PND2 and PND21. * p< 0.05 relative to the control group. Student t-test.

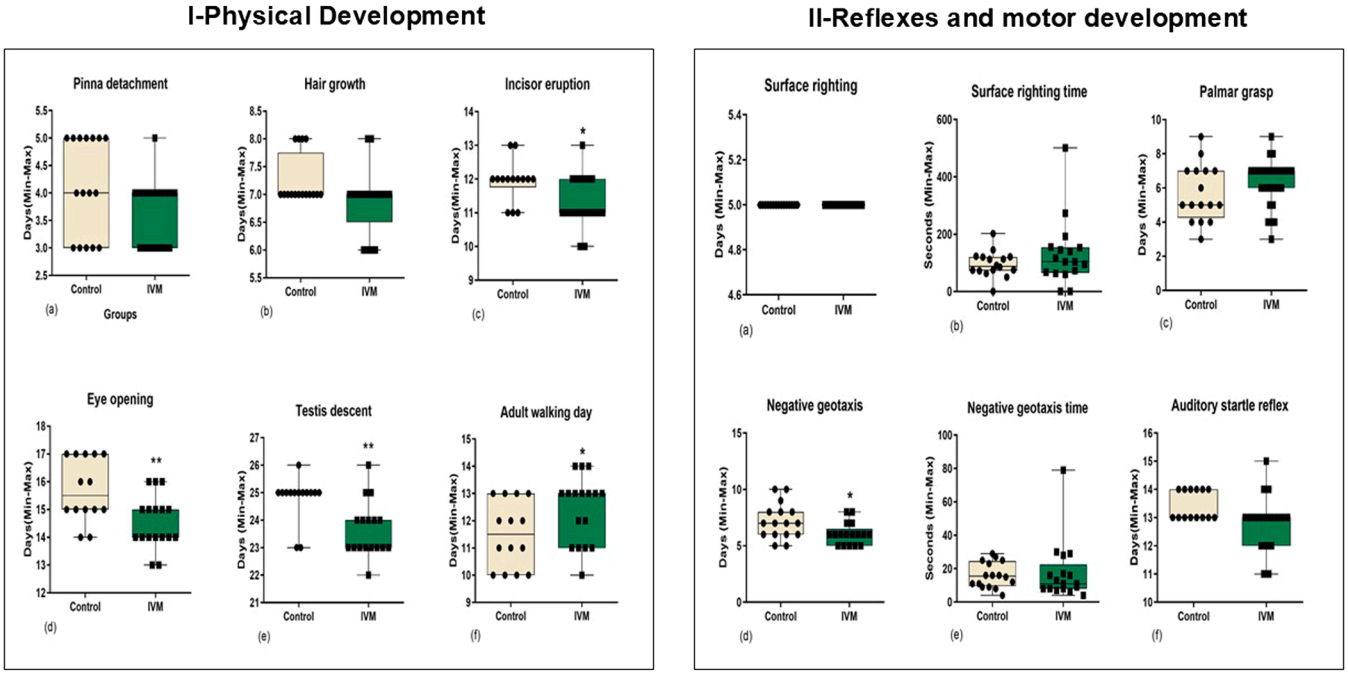

Fig. 7. Physical (I) and reflexes development of male rats from paternal rats treated with one dose every three weeks with 1.0 mg/kg IVM or its vehicle * p< 0.05, ** p< 0.01 relative to the control group. Student t-test.

4. Discussion

Repeated treatment with IVM did not interfere with paternal body weight gain. Thus, at least in body weight, repeated high doses of IVM did not show toxicity. Then, we examined how our repeated treatment affects the testis histopathology and morphometry. The histological studies show that tubular sections containing several histological changes indicate spermatogenesis interruption, such as disorganization of germinal epithelium, vacuolar degeneration of the germ cells, and sloughing of cells into the tubular lumen, no differences in testosterone levels. These results were similar to our previous study, showing that 1.0 mg/kg SC IVM acute exposure reduced the testicular volume, the

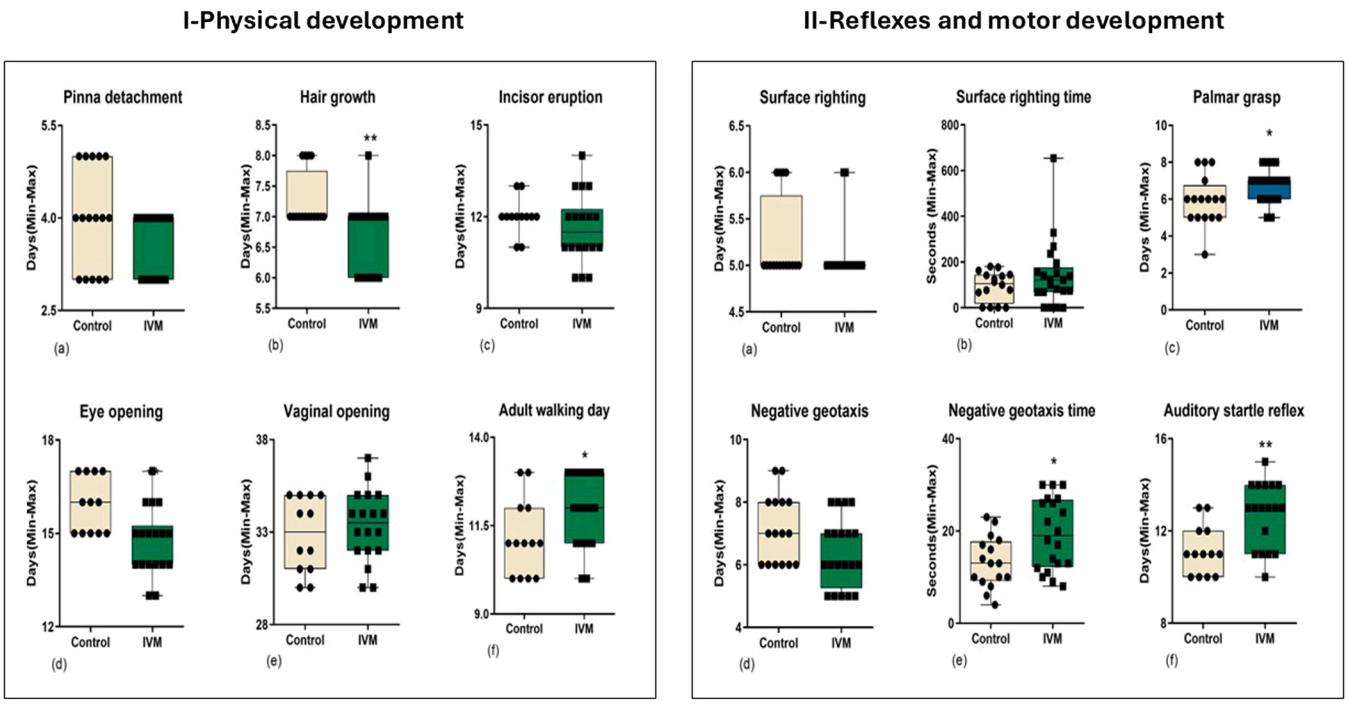

Fig. 8. Physical (I) and reflexes (II) development of female rats from paternal rats treated with one dose every three weeks with 1.0 mg/kg IVM or its vehicle * p< 0.05, ** p< 0.01 relative to the control group. Student t-test.

tubular diameter, and the germinal epithelium height without interferences on Leydig cells frequency. Thus, our repeated treatment sensitized the testis microscopic morphometric and stereological IVM effects compared to the acute dose without altering testosterone levels. The lack of differences in the Leydig cell frequency is related to the absence of testosterone levels in our study since the principal role of Leydig cells is the synthesis and secretion of testosterone in response to stimulation by LH[24].

After the F0 males were mated with females, no differences were observed in all parameters measured regarding maternal reproductive performance. Previous studies in a multigenerational study about long-term exposure to ivermectin did not show female and male reproductive toxicity[25]. In this respect, more recently, Salman et al.[26] showed several reproductive toxicities of avermectins related to germ cells and sexual behavior due to impaired hormone production. Also, avermectins target the female reproductive organs, leading to deleterious effects. However, among these avermectins, the authors cited that IVM induces testicular toxicity in rabbits[27] and Holstein calves[28] and toxic effects on the reproductive performance of exposed females. Our novelty experiment shows paternal exposure did not affect maternal reproductive performance despite the testis histopathological data observed.

During development, IVM paternal exposure dysregulated the day of appearance of several male and female physical and reflex landmarks.

In this respect, AGD, the distance from the center of the anus to the genitals, is considered a sensitive biomarker of initial prenatal androgen exposure during the critical window of genital development in rodents and humans in infancy[29,30]. AGD has been linked in males and females to reproductive problems, becoming a well-accepted proxy for reproductive function in future life. Male AGD is 50–100 % longer than females [31]). While the phenomenon is much less studied than male AGD, a longer female AGD is likely to result from higher androgen levels in fetal life. In the present study, male and female IVM pups showed longer anogenital index at PND 21 but not at PND2, while no differences occurred in the body length distance. In this respect, males have a markedly longer anogenital distance than females[30], and perinatal castration of males reduces anogenital distance. In addition, perinatally, testosterone administration to females increases their anogenital distance[32].

Therefore, AGD, a life-long indicator of androgen action during pregnancy, could affect the male masculinization programming window and could influence testis descent in puberty and sexual behavior in adult age[32,33]. In the present study, the time of testis descent was only increased at PND21 but not at PND2. Studies reported that gestational days 14–16 are the critical window for determining sensitivity to changes in AGD.In this respect, shorter AGD correlates with decreased fertility, reduced spermatogenesis, and azoospermia[34–36]. However why, in our study, paternal exposure to IVM increased male pups' AGD at PND21 and advanced the day of puberty remained to be investigated. In addition, no effects were observed in male sexual behavior in adult age.

Puberty in the male rat is a complex process involving maturational changes in the hypothalamus, pituitary, testes, and secondary sexual organs and their interrelationships. In the sexual maturation process, the negative feedback control systems for the gonadotropins become less responsive to testosterone, while the testes become more responsive to LH. In immature rats, testosterone potentiates the effect of LH–RH on pituitary LH release, but this response is lost with sexual maturation. LH–RH sensitizes the mature male pituitary glands for subsequent LH–RH administration. In addition, the responsiveness of the secondary organs to testosterone is also modified with age related to the relative proportion of testosterone *versus* androstenedione secreted by the testes [28]. Thus, the improvement of AGD and advanced puberty in males and females could be related to interferences in this window pups' programming caused by paternal IVM exposure. Additional studies will also be necessary to clarify these changes.

Paternal exposure to IVM advanced the incisor eruption and eye-opening time in male rats, delaying the day of adult walking. Female rats only showed advanced time in hair growth. In the reflexes development, only the day of geotaxis was advanced in males, while in females, this reflex, the palmar grasp, and the auditory startle reflex were retarded.

Polypeptide Growth Factors(EGF) are protein molecules that regulate cell proliferation and differentiation. Growth Factors (GFs) have been identified and characterized in recent years, and they have been

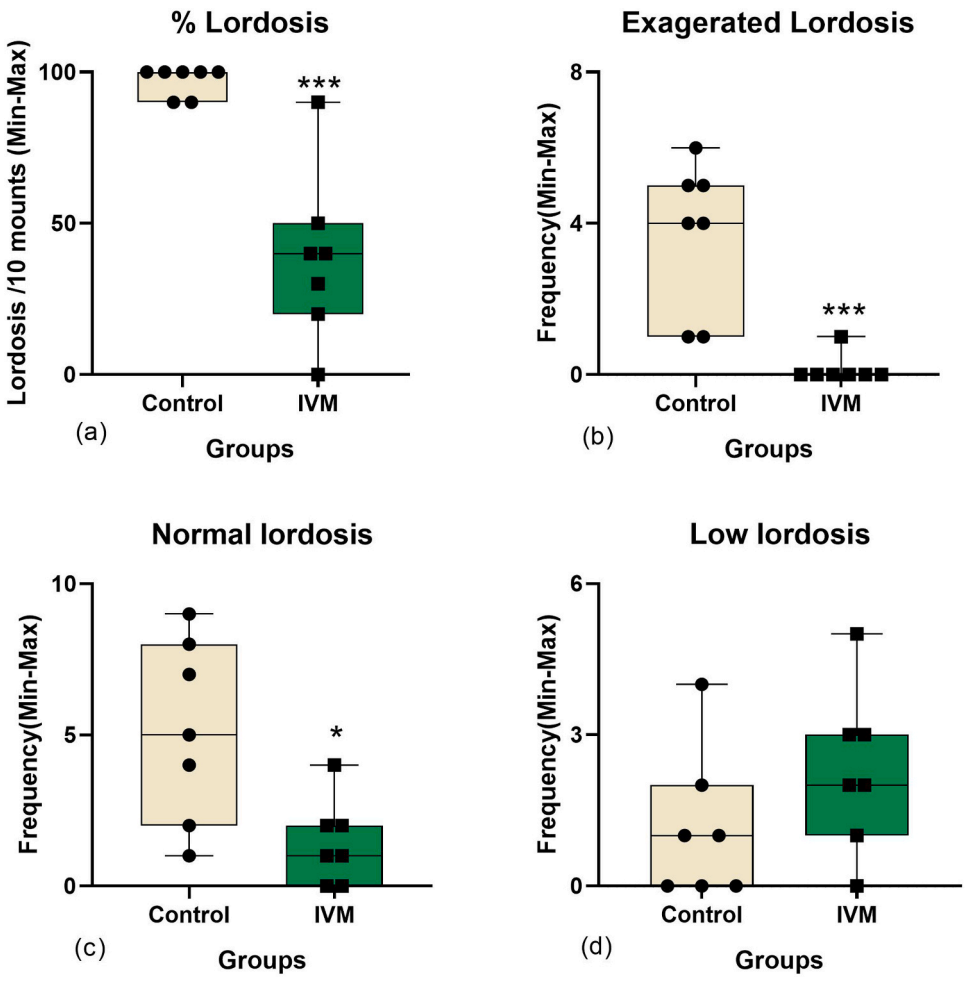

Fig. 9. Percentage of lordosis (a), exaggerated (b), normal (c), and low lordosis frequencies of female rats from F1 generation whose male of F0 generation received 1.0 mg/kg of IVM or its vehicle.*** P< 0.001-Mann-Whiteny test; * P< 0.05- Student t test.

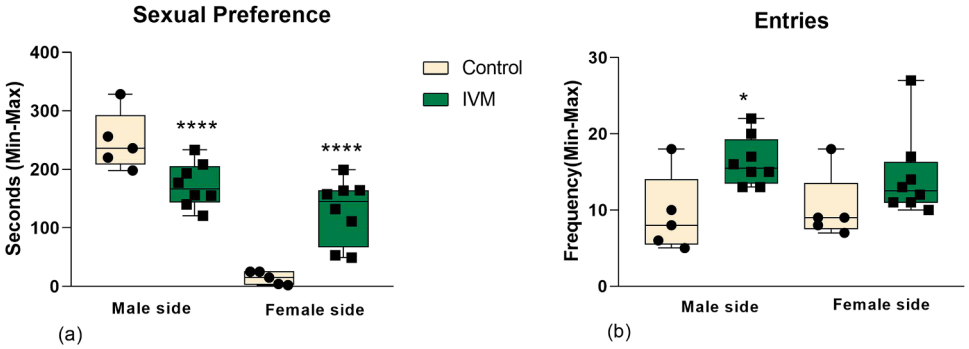

Fig. 10. Sexual preference (a) and number of entries in each compartment (b) of female rats from F1 generation whose male of F0 generation received 1.0 mg/kg of IVM or its vehicle. * p< 0.05, **** p< 0.0001; Two-way ANOVA followed by Sidak's multiple comparisons test.

shown to control several physiological processes, such as growth, repair, differentiation, and development of specific cell populations. Various forms of EGF administered to neonatal rodents produce both accelerating and retarding effects on somatic and behavioral development[37, 38]. Subcutaneous injection of mouse epidermal EGF in neonatal rats and mice produces alterations in craniofacial development characterized by precocious eye-opening and incisor eruption. However, retardation of somatic growth, inhibition of hair growth, and delayed development of some reflexes and behaviors have also been observed in the same animals[37–40]. No reports exist on the effects and mechanism of paternal exposure to avermectins on physical and reflexed development in rats. Therefore, it is necessary to investigate how the paternal administration of the IVM promotes changes in the development of its offspring. These changes could be reflected in the pup's survival in the environment.

Paternal exposure to IVM unaffected the F1 female estrous cycle but increased the anogenital index, unaffected the day of the vaginal opening, reduced female sexual behavior, and modified sexual preference. These females showed a diminished lordosis ratio and reduced exaggerated and normal lordosis relative to controls. Moreover, in the

Table 1
Male sexual behavior of rats from the F1 generation whose males from the F0 generation received repeated IVM doses (1.0 mg/kg) or its vehicle. N=7/group. Data are presented as means± SEM. Student t-test.

Parameters	Control (n=7)	IVM (n=7)	Statistics
Latency to 1st mount (sec)	191.0 ± 76.05	82.50 ± 12.02	t=1409, df=14, P=0.18
Mounts frequency	34.25 ± 9.45	48.50 ± 9.28	t=1076, df=14, P=0.30
Intromissions frequency	36.88 ± 6.86	40.75 ± 7.89	t=0,3705, df=14, P=0.72
Latency to 1st ejaculation (sec)	1069.0 ± 145.10	864.30 ± 104.50	t=1115, df=14, P=0.29
Number of ejaculations	0,38 ± 0,18	0,75 ± 0,25	t=1210, df=14, P=0.25

sexual preference test, the IVM female interacts more with females than males. These data are based on a masculinizing effect of paternal exposure of females to IVM suggested by early increased anogenital distance.

Our study's limitation is that we only described the transgenerational effects of paternal exposure to IVM. Future studies must investigate the mechanisms underlying these effects.

5. Conclusions

It is possible that paternal exposure to IVM interfered with pups' hormonal and growth factors during development and adulthood. Therefore, further studies are urgently needed to explore IVM transgenerational effects better and identify possible mechanisms underpinning behavioral effects.

CRediT authorship contribution statement

Laurade Macedo Amado: Software, Methodology, Investigation, Formal analysis, Data curation. **Lais Coelho Cortez:** Software, Methodology, Investigation, Formal analysis, Data curation. **Gabriel Aur Borges:** Software, Methodology, Investigation, Formal analysis, Data curation. **Kassia de Carvalho:** Methodology, Investigation, Formal analysis, Data curation. **Natalia Moreira:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria Martha Bernardi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Flora Cordeiro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rodrigo Augusto Foganholi da Silva:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this work, the authors did not use any AI tool/service. The authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maria Martha Bernardi reports financial support was provided by Paulista University. If there are other authors, they 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2024.108743.

Data availability

Data will be made available on request.

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