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RESEARCH ARTICLE

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Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss mice

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Abstract

Children and adults with frequent and severe episodes of epilepsy that do not respond to standard treatments (such as carbamazepine, phenytoin and valproate) have long been prescribed cannabidiol (CBD) as an anticonvulsant drug. However, the safety of its chronic use in relation to reproduction has not been fully examined. This study aimed to assess the effects of chronic CBD exposure on the male reproductive system. CBD was orally administered to 21-day-old male Swiss mice at doses of 15 and 30 mg kg^{-1} daily (CBD 15 and 30 groups, respectively), with a control group receiving sunflower oil, for 34 consecutive days. After a 35 day recovery period, the following parameters were evaluated: weight of reproductive organs, testosterone concentration, spermatogenesis, histomorphometry, daily sperm production and its morphology. The CBD 30 group had a 76% decrease in total circulating testosterone, but it remained within the physiological normal range (240–1100 ng dl^{-1}). CBD treatment induced a significant increase in the frequency of stages I–IV and V–VI of spermatogenesis, and a decrease in the frequency of stages VII–VIII and XII. A significant decrease in the number of Sertoli cells was observed only in the CBD 30 group. In both CBD groups the number of spermatozoa in the epididymis tail was reduced by 38%, sperm had head abnormalities, and cytoplasmic droplets were observed in the medial region of flagellum. These results indicated that chronic CBD exposure was associated with changes in the male reproductive system, suggesting its reproductive toxicity.

KEYWORDS

cannabinoids, endocannabinoids, spermatogenesis, spermatozoa, testosterone

1 | INTRODUCTION

Cannabis sativa L. (marijuana) is a plant species that contains more than 400 compounds, and approximately 100 of them are phytocannabinoids. Cannabidiol (CBD) is the major phytocannabinoid, accounting for up to 40% of marijuana extract (Crippa et al., 2009).

Advances in knowledge of the pharmacological properties of *Cannabis* in recent decades (Gallant, Odei-Addo, Frost, & Levendal, 2009; Gustafsson et al., 2013) have led to the identification of the endocannabinoid system (ECS), a biochemical component of endogenous signaling. The ECS acts physiologically in body regulation and comprises cannabinoid G-protein-coupled receptors CB1 and CB2, endogenous cannabinoids (such as anandamide), enzymes involved in endocannabinoid metabolism and membrane transporters (Fonseca, Costa, Almada, Correia-da-Silva, & Teixeira, 2013). Clinical and experimental evidence has suggested that the ECS participates in the modulation of several physiological responses (Campos et al., 2013; Mlost, Kostrzewa, Malek, & Starowicz, 2018).

Previous studies indicated that most actions of the cannabimimetic compound may result from their interaction with

cannabinoid receptors or through inhibition of the enzymatic metabolism of endogenous ligands such as anandamide (Koller, Zlabinger, Auwärter, Fuchs, & Knasmueller, 2013; Leweke et al., 2012). G-protein-coupled receptors have been found in reproductive tissues, such as the testicles and epididymides (Pertwee, Ross, Craib, & Thomas, 2002; Rossi et al., 2007), where they inhibit adenylyl cyclase and Ca^{2+} currents and stimulate K^+ currents (Mechoulam & Hanus, 2002).

The results of several preclinical and clinical studies point to the pharmacological effects of CBD in psychiatric disorders and in the control of some pathophysiological effects, such as pain, anxiety, dysphoria, emesis, insomnia and inflammation (Lehmann et al., 2016; Parker, Rock, Sticht, Wills, & Limebeer, 2015; Welty, Luebke, & Gidal, 2014). Based on this evidence describing the pharmacological properties of CBD, public and political pressure for the legalization of the medical use of marijuana has been increasing recently in several countries. The use of CBD in Brazil, at doses of 2.5–25 mg kg^{-1} day^{-1}, has recently been authorized for use with children and adolescents with epilepsy refractory to conventional treatments. This decision was based on evidence that CBD alone is not associated with dependence (Brucki et al., 2015).

However, cannabinoid consumption has been reported to cause disorders in mammalian reproductive systems. In males, these include inhibition of spermatogenesis; reduced concentrations of hypothalamic, pituitary and gonadal hormones, and changes in sperm morphology (Dalterio, Steger, Mayfield, & Bartke, 1984; Pacey et al., 2014; Thompson, 1993). Despite the scientific evidence supporting the therapeutic potential of CBD, the impact and effects of its long-term use on the mammalian reproductive system have not yet been fully elucidated. Therefore, this study aimed to evaluate the effects of chronic CBD exposure, from weaning to sexual maturity, on testicular and epididymal parameters in mice.

2 | MATERIALS AND METHODS

2.1 | Test compound

CBD powder (approximately 99.9% purity), purchased from THC Pharm GmbH (Frankfurt, Germany), was dissolved in sunflower oil prior to use; chemical name: (1′R,2′R)-5′-methyl-4-pentyl-2′-(prop-1-en-2-yl)-1′,2′,3′,4′-tetrahydro-[1,1′-biphenyl]-2,6-diol; formula: $C_{21}H_{30}O_2$; molecular weight: 314.46 g mol^{-1}; CAS no. 13956–29-1; batch no. CBD11–001.

2.2 | Animals and experimental groups

All experimental protocols were approved by the Ethics Committee on the Use of Animals of the Universidade Federal de Goiás (protocol no. 088-CEUA/UFG) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Healthy 21-day-old male Swiss mice (*Mus musculus*), weighing 13–15 g, obtained from the Central Animal House of the Universidade Federal de Goiás were housed in standard polypropylene cages (40 × 30 × 16 cm) (10 mice per cage) at 23°C, with a light/dark cycle of 12 hours (lights on at 06.00 hours). Food (commercial rodent diet Presence®; Presence Alimentos, Paulínia, SP, Brazil) and filtered tap water were available ad libitum.

Animals were randomly allocated to three experimental groups (n = 10 each) and treated daily by oral gavage for 34 consecutive days, as follows: (1) CBD 15 group, received 15 mg kg^{-1} body weight (bw) CBD dissolved in sunflower oil; (2) CBD 30 group, received 30 mg kg^{-1} bw CBD dissolved in sunflower oil; and (3) control group, received sunflower oil. The volume administered was corrected daily based on animal bw. The doses used in this study were selected in accordance with previous research (Campos et al., 2013; Uribe-Mari o et al., 2012).

The treatment regimen was established to include four spermatogenic cycles. This period allowed us to have full evaluation of the behavioral and hormonal aims of the current study. In mice, each cycle lasts 8.6 days (Clermont & Trott, 1969). Thus, the complete treatment is adequate for the reproductive purposes of the reproductive toxicity evaluation. A washout period was included due to the behavioral protocol and fertility evaluation (data not shown).

2.3 | Organ weights

After a 35 day recovery period, the mice were killed by decapitation. The testes, epididymides and seminal vesicles with secretion were removed and weighed to determine absolute and relative weight.

2.4 | Testosterone level

Blood samples were collected to evaluate plasma concentrations of testosterone. The samples were collected in Eppendorf tubes and centrifuged (907 *g* for 20 minutes) at 4°C. Total testosterone was measured in the plasma using a chemiluminescence assay (ARCHITECT 2nd Generation Testosterone Assay; Abbott Laboratories, Abbott Park, IL, USA) in an ADVIA Centaur®CP Immunoassay System (Siemens Healthineers, Erlangen, Germany). Intra-assay sensitivity was 2.30 ng dl^{-1}.

2.5 | Spermatogenesis and histomorphometry

The right testes were fixed in methacarn fixative solution (methanol, chloroform and acetic acid, 6:3:1, v/v/v) by immersion for 4 hours. After embedding in paraffin (Histosec®; Merck KGaA, Darmstadt, Germany), 5 μm sections were stained with hematoxylin and eosin. Histopathological assessment and digital imaging were performed using a light microscope (Olympus Biological Microscope Model CH30; Olympus Optical Co., LTD., Tokyo, Japan) associated with a Moticam 230 digital camera and the Motic Images Plus 2.0 software (Motic Asia, Hong Kong).

The dynamics of spermatogenesis were evaluated by estimating stage frequency: I–IV and V–VI (two generations of spermatids), VII–VIII (mature spermatids), IX (only one generation of spermatids), X–XI (two generations of spermatids), XII (secondary spermatocyte) in 105 transverse sections of seminiferous tubules per animal (Hess & Franca, 2008).

Histomorphometric analyses were carried out evaluating the lumen, epithelium and seminiferous tubules at stages VII–VIII in 15 random cross sections of tubules per animal (n = 6). The following

parameters were determined: area (S_0) and perimeter (P_0) of the tubule; area (S_1) and perimeter (P_1) of the lumen; height (h) and area (S_2) of the epithelium. Furthermore, the number of Sertoli cells with evident nucleoli was determined in histological sections in 10 seminiferous tubules per testis (n = 6) at stages I–VIII and XII of spermatogenesis.

2.6 | Daily sperm production

The left testes were weighed, stored at −20°C and posteriorly used to estimate the number of mature spermatids and daily sperm production (DSP) in tissue homogenates (Robb, Amann, & Killian, 1978). Testicular spermatids resistant to homogenization (step 16) were counted. DSP was calculated by dividing the total number of spermatids resistant to homogenization found per testis by 4.8 (Robb et al., 1978).

2.7 | Sperm morphology

Spermatozoa morphology was evaluated in spermatic smears obtained from the vas deferens. Approximately 200 cells were analyzed per animal (Filler, 1993). Frequencies of normal or deformed sperm, as heads (isolated, "banana" shaped, round, flat or macrocephalic), tails (short, bifurcated or rolled) and presence (proximal, medial or distal) of cytoplasmic droplets were determined.

2.8 | Statistical analysis

Data were evaluated using analysis of variance and Tukey's post-hoc test, and homogeneity was assessed using the Levene's test. Non-parametric data (sperm morphology) were analyzed using generalized linear model, the distribution and link function were Poisson regression and identity, respectively. The level of significance used was $P < .05$.

TABLE 1 Absolute and relative weight of reproductive organs of 90-day-old Swiss mice orally treated for 34 days with 15 and 30 mg kg^{-1} body weight of CBD 15 and 30 groups, respectively, and control group

Parameter	Weight		
	Control (n = 10)	CBD 15 (n = 10)	CBD 30 (n = 10)
Absolute			
Body (g)	46.22 ± 3.48	45.66 ± 3.95	44.76 ± 3.93
Testis (mg)	133.15 ± 17.23	130.38 ± 15.81	127.24 ± 14.52
Epididymis caput/corpus (mg)	27.54 ± 5.12	23.99 ± 6.04	24.56 ± 4.74
Epididymis tail (mg)	15.32 ± 8.42	17.39 ± 7.07	14.30 ± 3.26
Seminal vesicle with fluid (mg)	116.06 ± 32.12	122.28 ± 16.03	121.88 ± 33.47
Relative			
Testis (g 100 g^{-1})	0.29 ± 0.03	0.29 ± 0.03	0.28 ± 0.02
Epididymis caput/corpus (g 100 g^{-1})	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Epididymis tail (g 100 g^{-1})	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01
Seminal vesicle with fluid (g 100 g^{-1})	0.25 ± 0.07	0.27 ± 0.03	0.27 ± 0.06

CBD, cannabidiol.

Values are mean ± standard deviation ($P < .05$). No significant differences using ANOVA.

3 | RESULTS

3.1 | Somatic parameters

Neither dose of CBD significantly affected body weight or the absolute and relative organ weights (testis, epididymis or seminal vesicle with fluid) of treated mice (Table 1). During the experimental period, no mortality or adverse effects were observed.

3.2 | Testosterone level

The hormone profile of the two groups of mice treated for 34 days with different doses of CBD followed by a 35 day washout period is depicted in Figure 1. The CBD 30 group had a significant 76% decrease in plasma testosterone level compared to the control group, while no significant difference was observed for the CDB 15 group ($F_{(2,27)}$ = 4.243, $P < .05$; Figure 1).

3.3 | Spermatogenesis

Significant differences were found among the germinal epithelium stages of the control, CBD 15, and CBD 30 groups (Table 2). Larger numbers of stages I–IV ($F_{(2,27)}$ = 7.234, $P < .05$) and V–VI ($F_{(2,27)}$ = 9.893, $P < .001$) and smaller numbers of stages VII–VIII ($F_{(2,27)}$ = 16.114, $P < .001$) and XII ($F_{(2,27)}$ = 8.322, $P < .05$) were observed in the CBD 15 and 30 groups compared to the control group.

3.4 | Sertoli cells

Owing to the increase in the frequency of stages I–IV and V–VI, and the decrease in the frequency of stages VII–VIII and XII in the

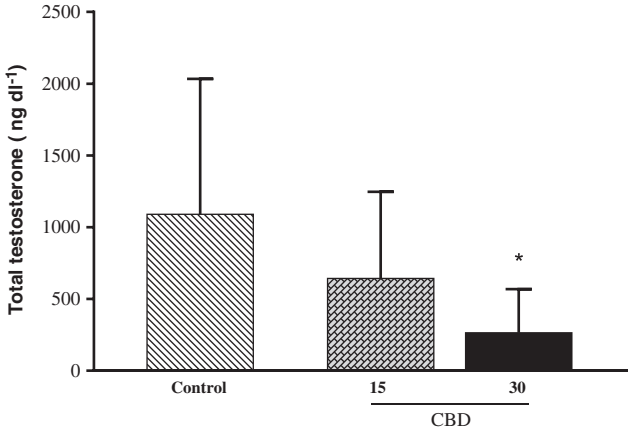

FIGURE 1 Total plasmatic testosterone levels (ng dl^{-1}) of CBD 15 (n = 10), CBD 30 (n = 10) and control group (n = 10). Values are mean ± standard deviation (*$P < .05$). ANOVA followed by Tukey's post-hoc test. CBD, cannabidiol

TABLE 2 Frequency of germinal epithelium stages obtained from cross-sections of seminiferous tubules of 90-day-old Swiss mice orally treated for 34 days with 15 and 30 mg kg^{-1} body weight of CBD 15 and 30 groups, respectively, and control group

Germinal epithelium stage	Frequency		
	Control (n = 10)	CBD 15 (n = 10)	CBD 30 (n = 10)
I–IV	31.0 ± 5.0[a]	36.0 ± 4.0[b*]	37.0 ± 4.0[b*]
V–VI	13.0 ± 5.0[a]	19.0 ± 4.0[b*]	21.0 ± 4.0[b*]
VII–VIII	25.0 ± 4.0[a]	15.0 ± 4.0[b*]	17.0 ± 3.0[b*]
IX	16.0 ± 5.0	17.0 ± 4.0	14.0 ± 4.0
X–XI	11.0 ± 3.0	13.0 ± 3.0	10.0 ± 3.0
XII	9.0 ± 4.0[a]	5.0 ± 1.0[b]	6.0 ± 2.0[b]

CBD, cannabidiol.

Values are mean ± standard deviation (P < .05). ANOVA followed by Tukey's post-hoc test (*P < .001).

[a]Values similar to the control group.

[b]Significant differences between treated and control groups.

experimental groups, the Sertoli cell count was performed at these stages (Table 3). A significant increase in Sertoli cell number was observed at stages I–IV ($F_{(2,15)}$ = 6.607, P < .05) and V–VI ($F_{(2,15)}$ = 11.47, P < .001) in the CBD 15 group, whereas a significant decrease was found at stage XII in both the CBD 30 and 15 groups ($F_{(2,15)}$ = 104.816, P < .001) compared to the control group. The total number of Sertoli cells at all stages significantly decreased in the CBD 30 group ($F_{(2,15)}$ = 29.62, P < .001).

3.5 | Histomorphometric analysis

Based on histomorphometric analyses, the number of seminiferous tubules decreased at stages VII–VIII in both experimental groups (Figure 2). No significant differences were found in terms of tubule area among groups (Figure 2A). A significant increase in the perimeter ($F_{(2,15)}$ = 3.843, P < .05; Figure 2b) of tubules was observed in the CBD 15 group. There were no significant differences in seminiferous lumen area (Figure 2c) and perimeter (Figure 2d) among groups. The epithelium area significantly increased only in the CBD 15 group ($F_{(2,15)}$ = 5.575, P < .05; Figure 2e). Regarding the seminiferous epithelium, both the CBD 15 and 30 groups showed a significant increase in height ($F_{(2,15)}$ = 28.055, P < .001; Figure 2f).

TABLE 3 Number of Sertoli cells per section of seminiferous tubules at stages I–IV, V–VI, VII–VIII and XII of 90-day-old Swiss mice orally treated for 34 days with 15 and 30 mg kg^{-1} body weight of CBD 15 and 30 groups, respectively, and control group

Germinal epithelium stage	Sertoli cells (no.)		
	Control (n = 10)	CBD 15 (n = 10)	CBD 30 (n = 10)
I–IV	116.2 ± 7.1[a]	132.3 ± 9.9[b]	117.8 ± 8.2[a]
V–VI	117.0 ± 3.0[a]	126.8 ± 6.8[b*]	114.3 ± 3.5[a]
VII–VIII	119.3 ± 4.8[a]	124.3 ± 4.4[a]	112.3 ± 6.9[a, c]
XII	131.7 ± 6.1[a]	100.2 ± 5.3[b*]	99.0 ± 4.0[b*]
Total	484.2 ± 12.6[a]	483.7 ± 8.6[a]	443.5 ± 13.4[b*]

CBD, cannabidiol.

Values are mean ± standard deviation (P < .05). ANOVA followed by Tukey's post-hoc test (*P < .001).

[a]Values similar to the control group.

[b]Significant differences between treated and control groups.

[c]Significant differences between CBD 15 and 30 groups.

3.6 | Daily sperm production

No significant alterations were observed in testicular sperm counts (number of mature spermatids and relative number of mature spermatids) or in DSP and relative DSP between the treated groups (CBD 15 and 30) and the control group (Table 4).

3.7 | Sperm morphology

In the evaluation of the total number of spermatozoa (approximately 200 per animal), the CBD 15 and 30 groups had a higher number with head abnormalities (Wald χ_2^2 = 12.329, P < .05; Table 5) than the control group (Figure 3b–e). The CBD 15 group had a smaller number of decapitated spermatozoa (Wald χ_2^2 = 61.796, P < .001; Figure 3b). The number of spermatozoa with tail abnormalities was similar in all groups (Figure 3f–i).

The CBD 15 and 30 groups had a larger number of spermatozoa with cytoplasmic droplets (Wald χ_2^2 = 39.233; P < .001; Table 5) compared to the control group. The CBD 30 group did not have spermatozoa with proximal cytoplasmic droplets. The CBD 15 and 30 groups displayed a significant increase in the number of spermatozoa with medial cytoplasmic droplets (Wald χ_2^2 = 27.811, P < .001; Figure 3j). The number of spermatozoa with distal cytoplasmic droplets was similar in all groups.

4 | DISCUSSION

The effects of chronic exposure to CBD, from weaning to sexual maturity, on the reproductive system of male adult mice were investigated in this study. The results revealed that the administration of CBD at doses of 15 and 30 mg kg^{-1} bw for 34 consecutive days, followed by a 35 day recovery period caused significant changes in the testes such as an increase or decrease in the frequency of spermatogenesis stages, and a decrease in testosterone levels at the higher dose.

It is necessary to highlight that, until this moment, there is no pharmacokinetics study on CBD plasma levels using the administration schedule employed in the present work. However, based on the study of Deiana et al. (2011), it is possible to speculate that CBD blood levels at the steady state for these doses (15 and 30 mg kg^{-1} p.o.) would be about 80–160 ng ml^{-1}.

Cannabinoids are known to affect reproductive functions in mammals. More specifically, studies have reported decreases in gonadotropin-releasing hormone secretion by the adenohypophysis, and testosterone secretion suppression in mice induced by cannabinoids (Bloch, Thysen, Morrill, Gardner, & Fujimoto, 1978; Dalterio, Bartke, & Burstein, 1977). Cannabinoid receptors and the

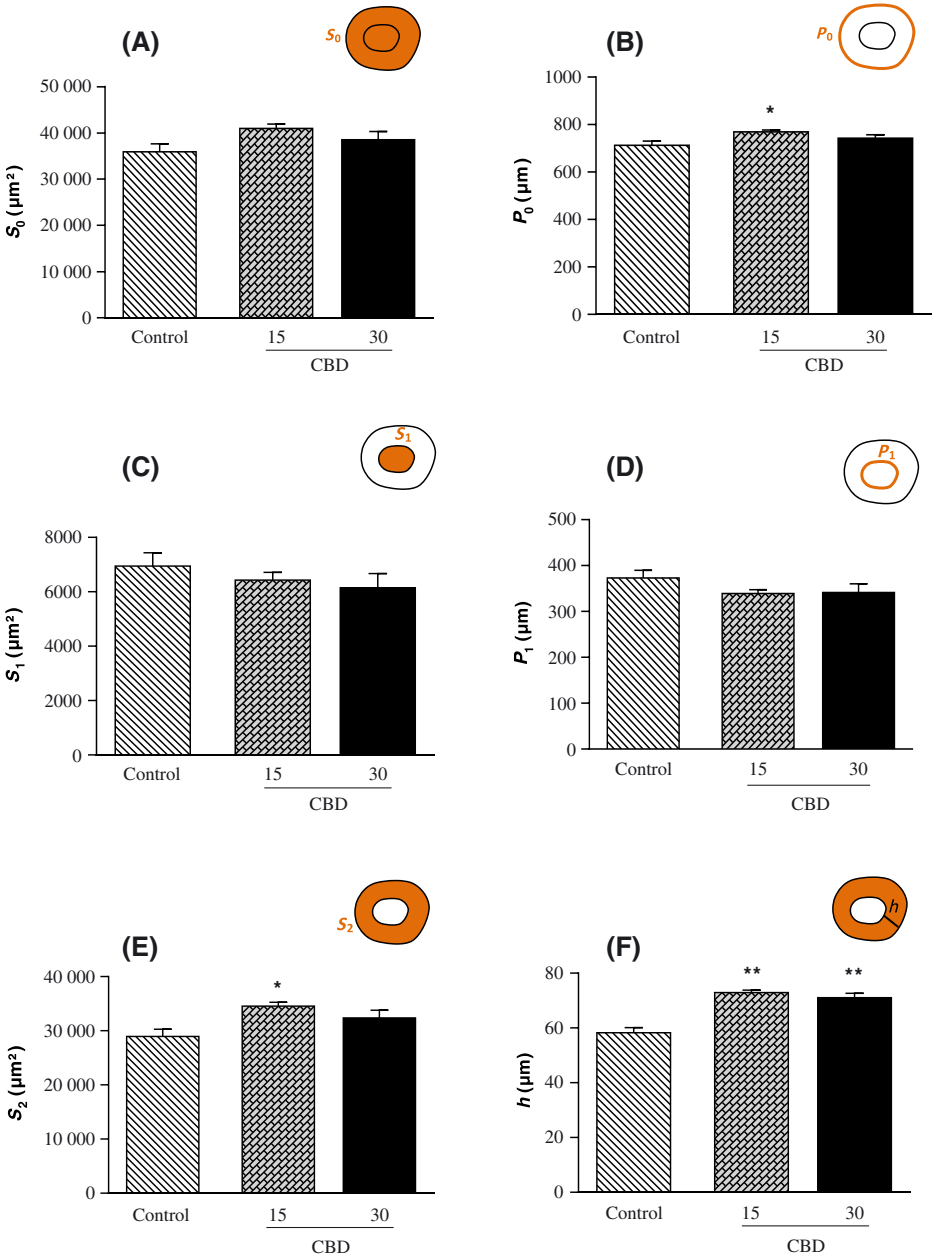

FIGURE 2 Histomorphometry of seminiferous tubules at stages VII–VIII of CBD 15 (n = 6), CBD 30 (n = 6) and control group (n = 6): (A) tubular area (S_0); (B) tubular perimeter (P_0); (C) lumen area (S_1); (D) lumen perimeter (P_1); (E) epithelium area (S_2); (F) epithelium height (h). Values are mean ± standard deviation (P < .05). ANOVA followed by Tukey's post-hoc test (**P < .001). CBD, cannabidiol

enzymes responsible for endocannabinoid synthesis/metabolism are present in the hypothalamic–pituitary–gonadal axis, raising the possibility of an interaction of cannabinoids with reproductive parameters (Gammon, Freeman, Xie, Petersen, & Wetsel, 2005; Gye, Kang, & Kang, 2005) and their direct interference in this system (Leweke et al., 2012). Despite a significant decrease in total testosterone levels in the CBD 30 group, no significant alterations occurred in the absolute and relative weight of the reproductive tissues in all groups, as this decrease in androgen levels was within the normal reference range (240–1100 ng dl^{-1}) (Wang, Catlin, Demers, Starcevic, & Swerdloff, 2004). Similar values were found in other experiments with male mice (Bo et al., 2015; Xiong, Zhong, & Xu, 2014).

It must be taken into consideration that the blood collection for hormonal analyses occurred 35 days after the end of the CBD treatment, thus establishing a washout period of 5 weeks. Owing to the length of this period, testosterone synthesis and secretion in the testes may have returned to normal concentrations. The differentiation of sexual organs is regulated by testosterone action (Spritzer & Reis, 2013), and during puberty testicular androgen concentrations increase, leading to the development of the primary sex organs (penis, testicles, seminal vesicles and epididymides). Considering the washout period and the androgen levels, the recovery of testicular steroidogenic function may have been responsible for the lack of differences in the mass of the androgen-dependent tissues among the groups. Additional studies into the effects of chronic exposure to CBD on

TABLE 4 Sperm count and DSP on testis of 90-day-old Swiss mice orally treated for 34 days with 15 and 30 mg kg^{-1} body weight of CBD 15 and 30 groups, respectively, and control group

Parameter	No.		
	Control (n = 10)	CBD 15 (n = 10)	CBD 30 (n = 10)
Mature spermatids ($\times 10^6$ per testis)	31.0 ± 5.0	36.0 ± 4.0	37.0 ± 4.0
Relative mature spermatids ($\times 10^6$ g^{-1} $testis^{-1}$)	13.0 ± 5.0	19.0 ± 4.0	21.0 ± 4.0
DSP ($\times 10^6$ testis day^{-1})	25.0 ± 4.0	15.0 ± 4.0	17.0 ± 3.0
Relative DSP ($\times 10^6$ testis g^{-1} day^{-1})	16.0 ± 5.0	17.0 ± 4.0	14.0 ± 4.0

CBD, cannabidiol; DSP, daily sperm production.

Values are mean ± standard deviation (P < .05). No significant differences using ANOVA.

TABLE 5 Sperm morphology (approximately 200 per animal) in vas deferens and presence/position of the cytoplasmic droplet in 90-day-old Swiss mice orally treated for 34 days with 15 and 30 mg kg^{-1} body weight of CBD 15 and 30 groups, respectively, and control group

Parameter	No.		
	Control (n = 10)	CBD 15 (n = 10)	CBD 30 (n = 10)
Sperm morphology			
Normal	102.0 ± 26.0	100.0 ± 15.0	95.0 ± 10.0
Head abnormalities	49.0 ± 18.0[a]	60.0 ± 14.0[b]	57.0 ± 16.0[b]
Tail abnormalities	35.0 ± 18.0	36.0 ± 9.0	37.0 ± 18.0
Isolated head	14.0 ± 18.0[a]	4.0 ± 4.0[b*]	11.0 ± 10.0[a]
Cytoplasmic droplet			
Absent	91.0 ± 35.0[a]	67.0 ± 20.0[b*]	70.0 ± 21.0[b*]
Proximal	1.0 ± 1.0[a]	1.0 ± 1.0[a]	0.0 ± 0.0[b*]
Medial	104.0 ± 33.0[a]	127.0 ± 22.0[b*]	125.0 ± 22.0[b*]
Distal	4.0 ± 4.0	5.0 ± 5.0	5.0 ± 5.0

Values are mean ± standard deviation (P < .05). Generalized linear model (Poisson regression, identity) (*P < .001).

[a]Values similar to the control group.

[b]Significant differences between treated and control groups.

the gonadal hormones and sex organs of male mice are required to elucidate properly these results.

Although a drop in sperm count induced by cannabinoids has previously been reported (Hembree, Nahas, Zeidenberg, & Huang, 1979), in the present study no marked changes were observed in this parameter or in DSP in either experimental group. We speculate that this was due to testosterone recovery during the washout period. Significant changes in spermatogenic dynamics were observed in both experimental groups. During the spermatogenic cycle, six successive peaks of mitosis occur and correspond to stages IX (initial mitotic stage), XI, I, II, IV and VI (Clermont & Leblond, 1953; Monesi, 1962). In the present study, stages IX–XI were similar in all groups. However, both experimental groups showed an increase in subsequent mitotic stages (I–VI) and a decrease in stages VII–VIII (spermiation) and XII (meiotic). CBD involves the inhibition of the reuptake and degradation of endocannabinoids, particularly the inhibition of the hydrolytic enzyme fatty acid amide hydrolase (FAAH). This leads to an increase in the concentrations of these substances at their binding sites (Leweke et al., 2012; Sadock, Sadock, & Ruiz, 2016). Anandamide might promote different effects on spermatogenesis, depending on the receptor target that is activated. In addition to the cannabinoid receptors (CB1 and CB2), another molecular target of anandamide is the transient receptor potential cation channel subfamily V member 1 (TRPV1). The activation of TRPV1 seems to play a role in the stabilization of the effects caused by this endocannabinoid (Maccarrone et al., 2005).

The interaction between endocannabinoids and CB2 receptors evidenced by the increase in mitotic stages (I–VI) may act as an autocrine and/or paracrine mediator during spermatogenesis (Grimaldi et al., 2009). A high expression of TRPV1 has been observed during the meiotic stage (XII), suggesting that this receptor participates in the control of this stage (Grimaldi et al., 2009).

Given the fact that endocannabinoids bind to CB2 receptors during the mitotic stages, our hypothesis is that the CBD used in this study blocked FAAH, which induced an increase in anandamide at the binding site and, consequently, enhanced the frequencies of mitotic stages I–II and IV–VI. From a quantitative point of view, the higher the number of mitotic divisions, the higher the number of sperm produced per unit of mass of testicles. This would explain, at least in part, the decrease in sperm stages (VII–VIII), which may have been accelerated due to the high amount of sperm produced. It would also account for the fact that no significant differences were found in the number of testicular spermatids and DSP of the experimental groups, even after the reduction in stages VII–VIII. Assuming that the increase in stages I–VI promotes paracrine regulation and decreases meiosis, it is possible that the reduction in stage XII is a response to this chemical control. As meiosis can be regulated by TRPV1 ion channels, the decrease in stage XII observed in this study could have been promoted by the anandamide TRPV1-mediated action following its accumulation as a result of FAAH inhibition induced by the chronic treatment with CBD.

In spite of a 5 week period without exposure to CBD, changes were observed in the spermatogenic dynamics, particularly in the stages, tubular morphometry and number of Sertoli cells, which may have occurred as result of the chronic treatment. Thus, it is necessary to compare the acute effects of CBD treatment on the dynamics of the germinal epithelium with those obtained after chronic CBD exposure, as a new experimental proposal.

Both CBD doses promoted similar alterations in the tubular structure. The measurement of the tubular diameter and height of the germinal epithelium has been classically used as an indicator of spermatogenic activity (França & Russel, 1998). Such evidence indicates that the increase in epithelium height might have compensated for the decrease in stages VII–VIII, contributing to an increase in the number of germ cells. The CBD 15 group had an increase in the number of Sertoli cells at stages I–IV (14%) and V–VI (8%), but at stage XII it decreased by 24%. The CBD 30 group showed no significant changes in the number of Sertoli cells at the mitotic stages, but displayed a 25% decrease at stage XII. However, this reduction did

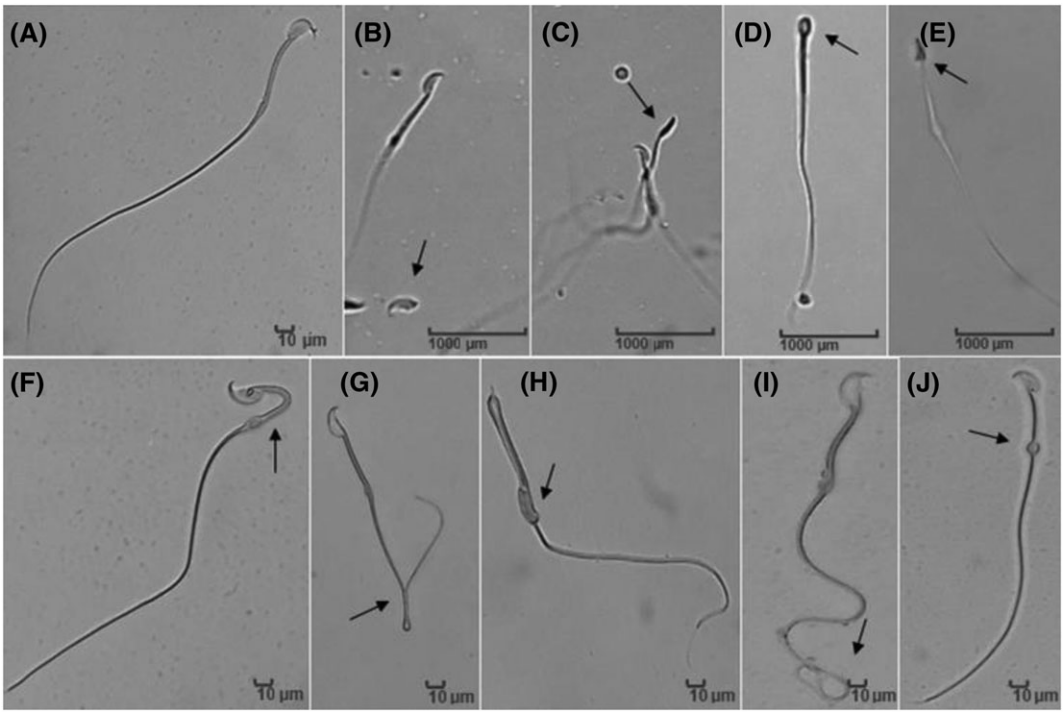

FIGURE 3 Sperm morphology (approximately 200 per animal) in the vas deferens of CBD 15 (n = 10) and CBD 30 (n = 10) group, observed using light microscopy. (a) Normal sperm; (b) isolated head (arrow); (c–e) head abnormalities: (c) sperm with "banana" head (arrow); (d) sperm with round head; (e) sperm with flattened head; (f–i) tail abnormalities, sperm with folded tail: (f) proximal region; (g) distal region; (h) medial region and presence of head with no hook (arrow); (i) sperm with coiled tail; (j) sperm with medial cytoplasmic droplet (arrow)

not affect sperm production, possibly due to testicular androgen levels balancing this cellular decrease.

We hypothesized that chronic treatment with CBD would reduce the expression of FAAH and, consequently, promote the accumulation of anandamide in the testes. This accumulation would stimulate the cannabinoid and/or TRPV1 receptors present in Sertoli cells, triggering the activation of an apoptotic cascade. This effect could explain the lower number of Sertoli cells with evident nucleoli at stage XII. However, the fact that there was a decrease in the frequency of stages XII in the CBD-treated groups must be taken into consideration.

In the CBD-treated groups, spermatozoa collected in the vas deferens showed significantly more head abnormalities. This finding suggests a possible anandamide action facilitated by the chronic exposure to CBD during the long process of sperm formation and maturation. The morphological changes observed in spermatozoa (development of the acrosome, nuclear formation and chromatin condensation) appear to be related to the activity of endocannabinoids and CB1 receptors (Cobellis et al., 2010; Ricci et al., 2007).

Another parameter evaluated in the present study was the presence and position of the cytoplasmic droplet. Our results showed that most of the sperm recovered from the vas deferens had the cytoplasmic droplet in the flagella medial region, regardless of the experimental group. The migration starting sites differ among species and the precise mechanisms controlling this process are not fully understood (Yeung & Cooper, 2003).

The occurrence of over 5% of proximal cytoplasmic droplet in ejaculated spermatozoa indicates defects of a testicular origin and may reduce fertility potential in several species (Leidl, Stolla, Schefls, & Schad, 1999). Our results indicate that most droplets were in the medial region of flagella. As the material was collected in the vas deferens, we assume that, by the time it reached the urethra for ejaculation, the droplet could be displaced to the flagella distal region.

In conclusion, chronic exposure of male mice to 15 and 30 mg kg^{-1} bw CBD for 34 days, from weaning to sexual maturity, induced significant changes in the reproductive system in adulthood. Even though CBD has a short half-life, persistent negative effects were found in the male reproductive system, despite the washout period.

AUTHORS' RELATIVE CONTRIBUTIONS

R.K.C. contributed to the conception and design, acquisition, analysis and interpretation of data and drafting the article; M.L.S., M.R.S. and T.L.R. contributed to the acquisition, analysis, interpretation of data and drafting the article; F.S.G. contributed to the drafting, analysis and interpretation of the article, revising it critically for important intellectual content; J.A.A.-F. contributed to the analysis and interpretation of data, revising it critically for important intellectual content; R.M.-C. contributed to study conception, design and acquisition, drafting the article, analysis and interpretation of data, revising it critically for important intellectual content. All authors gave final approval of the version to be published.

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CONFLICT OF INTEREST

F.S.G. is a co-inventor of the patent "Fluorinated CBD compounds, compositions and uses thereof" (publication no. WO/2014/108899 and international application no. PCT/IL2014/050023). All other authors: none to declare.

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