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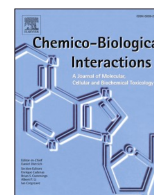

Decreasing sperm quality in mice subjected to chronic cannabidiol exposure: New insights of cannabidiol-mediated male reproductive toxicity

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ABSTRACT

Cannabidiol (CBD) is a natural cannabinoid present in the *Cannabis sativa* plant, widely prescribed as an anticonvulsant drug, especially for pediatric use. However, its effects on male reproduction are still little investigated. Therefore, the present study assessed the effects of CBD on the spermatogenesis and sperm quality. For this, twenty-one-day-old Swiss mice received CBD for 34 consecutive days by gavage at doses of either 15 or 30 mg/kg. Chronic exposure to CBD decreased the frequency of stages VII-VIII and XII of spermatogenesis and an increase in the frequency of stage IX were noted. Furthermore, the seminiferous epithelium height reduced at stage IX and increased at stage XII in both CBD-treated groups. There was a significant rise of sperm DNA damage, while no genotoxic effects were observed in leukocytes. The activities of superoxide dismutase and catalase decreased, while malondialdehyde levels increased in the sperm of mice treated with a higher dose of CBD. Mice exposed to 30 mg/kg of CBD showed a reduction in the mobile spermatozoa percentage and in curvilinear velocity, while straight line and average path velocity decreased in both treated groups. The number of acrosome-intact spermatozoa declined in the CBD 30 group, and the number of abnormal acrosomes raised in both CBD groups. On the other hand, the weight of reproductive organs, sperm count, and hormone levels were not affected by CBD treatment. These findings show that dysregulation of the endocannabinoid system by CBD can reduce sperm quality. The mechanisms responsible may be associated with disorders during spermatogenesis, especially during the final stages of nuclear remodelling and assembly of acrosome. However, changes in mitochondrial function, as well as the reduction on the antioxidant enzyme activities during epididymal transit, at least partly, may also be involved.

1. Introduction

The quality of spermatozoa is important for normal cell function [1]. After fertilization of the female oocyte, this highly specialized cell is responsible for transferring genetic material to future generations [2]. To form mature spermatozoa, mammalian germ cells undergo a remarkable transformation during the phases of spermatogenesis, especially during the final stages of nuclear remodelling (spermiogenesis) [3]. Spermiogenesis is a unique process that includes nuclei condensation and formation of the acrosome [4]. The final maturation status of mammalian spermatozoa occurs during their passage through the epididymis [5]. Finally, capacitation and the acrosome reaction take place in the female genital tract and are fundamental to sperm-oocyte

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Abbreviations

2-AG	2-arachidonoylglycerol
AEA	Arachidonoylethanolamide
ASD	Autism spectrum disorder
BSA	Bovine serum albumin
CAT	Catalase
CB1R and CB2R	Type 1 and 2 cannabinoid receptors
CASA	Computer-assisted sperm analysis
CBD	Cannabidiol
CP	Cyclophosphamide
DNPH	2,4-dinitrophenylhydrazine
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
FAAH	Fatty acid amide hydrolase
FITC	Fluorescein isothiocyanate
HTF	Human tubal fluid
LMP	Low melting point
LPO	Lipid peroxidation
NMP	Normal melting point
PBS	Phosphate buffered saline
PSA	Pisum sativum agglutinin
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBE	Tris-Borate-EDTA
TRPV	Transient receptor potential cation channel subfamily V member 1
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity

fusion [6].

DNA integrity is essential for normal sperm function as well as the genetic health of individuals and their offspring. DNA integrity depends on a series of processes that must take place to allow the cell to package DNA within the nucleus boundaries while maintaining its ability to transcribe and duplicate the entire DNA sequence [7]. Several factors can affect DNA integrity, including exposure to endogenous (such as reactive oxygen species, ROS) or exogenous (such as pathologies, chemicals, or radiation) stressors [8–10].

The DNA of mammalian spermatozoa is especially susceptible to oxidative damage induced by excessive ROS production because its cytoplasm presents less antioxidant enzymes and its plasmatic membrane is rich in polyunsaturated fatty acids. However, during spermiogenesis and epididymal maturation/storage, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) play an important role in protecting sperm function and its DNA integrity from oxidative stress [11]. Marchetti et al. [12] showed that the post-meiotic stage of spermatogenesis is highly susceptible to detrimental effects on DNA. Recently, a retrospective cross-sectional study in men showed that cannabis consumption by at least one cigarette per week, with a history of at least 1 year before semen collection, exerts deleterious effects on sperm nuclear integrity, probably by impairment of spermatogenesis and sperm maturation during epididymal transit [13].

Cannabidiol (CBD) is a natural product that is widely used to treat a variety of conditions and is a cannabinoid present in the Cannabis plant genus. It is well known that this compound is helpful in the treatment of epilepsy, anxiety, and seizures [14]. Moreover, the analgesic and anti-inflammatory properties of CBD [15], as well as its effect in the treatment of children and adolescents with autism spectrum disorder (ASD) have also been observed [16].

Studies of CBD and others cannabis constituents started an era of research in which two G protein-coupled receptors, type 1 and 2 cannabinoid receptors (CB1R and CB2R) were characterized [17,18]. Subsequently, their major endogenous ligands, arachidonoylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG), were identified, becoming known as endocannabinoids [19,20]. The effects of anandamide and 2-AG, via CB1R and CB2R, depend on their concentration at the binding sites, which is controlled by the balance among synthesis and degrading enzymes [21]. Endocannabinoids, cannabinoid receptors and transporters, and regulatory enzymes are collectively known as the endocannabinoid system. CBD has an inhibitory action on the degrading of the enzyme fatty acid amide hydrolase (FAAH). This event could promote an accumulation of endocannabinoids in the synapses [22].

Cannabinoid receptor dysregulation has been associated with DNA damage in somatic and germ cells. The *in vitro* exposure of fetal oocytes from CD1 mice to 1 μM of JWH133, a selective agonist of CB2R, increased the number of DNA-damaged cells. Moreover, DNA breaks were not correctly repaired during meiosis, leading to oocyte apoptosis as a result of CB2R agonist administration [23]. Additionally, another *in vitro* study showed that CBD, at concentrations equal or greater than 6.0 μM, induced DNA damage in human-derived cells (HepG2 and TR146). The authors also showed that in low concentrations (≥0.22 μM) CBD caused nuclear anomalies, as well as the induction of cell death (necrosis and apoptosis) in HepG2 cells [24]. On the other hand, the knowledge concerning the CBD-mediated genotoxicity after *in vivo* exposure remain limited [13,25].

Thus, given the growing therapeutic use of CBD and its possible genotoxic activity and interactions with the endocannabinoid system, the present study was undertaken. Furthermore, other qualitative sperm parameters such as concentration, motility and acrosomal status were assessed in this study. The hypothesis that the chronic exposure to CBD induces deleterious effects on the nuclear integrity of mammalian sperm (germinative cells) by impairment of spermatogenesis and by susceptibility to excessive ROS production was tested *in vivo* in mice. To the best of our knowledge, this is the first time this subject has been addressed. In addition, to test damage specificity, the *in vivo* effects of CBD on the DNA integrity of somatic (leukocytes) cells were also evaluated.

2. Materials and methods

2.1. Animals and experimental groups

Healthy 21-day-old male Swiss mice (*Mus musculus*) with 13–15 g were housed in standard polypropylene cages (40 × 30 × 16 cm) (11 mice per cage) at 23 °C, with a light/dark cycle of 12 h (lights on at 6 a.m.). The animals were obtained from the mating colony mice of the Central Animal House of the UFG. Food (commercial rodent diet Presence®; Neovia, Paulínia, SP, Brazil) and filtered tap water were available *ad libitum.*

Animals were randomly allocated to three experimental groups (n = 11 each) and treated daily by intragastric administration for 34 consecutive days, as follows: (1) CBD 15 group, received 15 mg/kg body weight (bw) of CBD dissolved in sunflower oil; (2) CBD 30 group, received 30 mg/kg (bw) of CBD dissolved in sunflower oil; and (3) control group, received sunflower oil. The volume administered was adjusted daily based on animal bw. Since there is a wide CBD dose range for the treatment of children and adolescents with comorbidities, including epilepsy and ASD [16,26] and due to the complex relationship with the human doses and pharmacokinetic differences in mice, the doses used in this study correspond to CBD concentrations previously used in studies with this animal model [27–30]. The treatment regimen

was established to include four spermatogenic cycles. In mice, each cycle lasts 8.6 days [31]. Thus, the treatment time used is adequate to evaluate reproductive toxicity.

All experimental protocols were approved by the Ethics Committee on the Use of Animals of the Universidade Federal de Goiás - UFG (protocol no. 088–2019 CEUA/UFG) following the guidance for the care and use of laboratory animals (National Institutes of Health) and the ethical principles established by the National Council for the Control of Animal Experimentation (Concea).

2.2. Reagents and test compound

A modified human tubal fluid (HTF) medium was purchased from Irvine Scientific (California, USA). Low melting point (LMP) and normal melting point (NMP) agarose were obtained from Thermo Fisher Scientific (Karlsruhe, Germany). Dithiothreitol (DTT) and Proteinase K were purchased from Promega Corporation (Wisconsin, USA). SYBR Green I was obtained from Thermo Fisher Scientific (Invitrogen, Paisley, UK). Triton X-100, malondialdehyde (MDA), epinephrine bitartrate, 2,4-dinitrophenylhydrazine (DNPH), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), calcium ionophore A23187 and *Pisum sativum* agglutinin (PSA) labeled with fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich Corporation (Missouri, USA). Hydrogen peroxide (50% H_2O_2) and tris(hydroxymethyl) aminomethane (approximately 99.9% purity) were obtained from Gold Analisa Diagnóstica (Minas Gerais, Brazil). All buffer solutions were prepared with Milli-Q water. 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; CAS No. 13956-29-1; Batch No. CBD11-001.

2.3. Sample collection

The animals were decapitated 24 h after the last CBD administration. Blood samples were collected to evaluate sexual hormone levels (progesterone, total testosterone, and estradiol) and to detect DNA damage in leukocytes. To assess sexual hormone levels, an aliquot of the blood was injected into a gel clot activator tube immediately after decapitation and centrifuged (907 g for 20 min) at 4 °C. Another part of the blood sample (30 μl) was injected into ethylenediamine tetra acetic acid (EDTA) vacuum tubes to assess DNA integrity. Testes and epididymis were collected through a mid-abdominal incision and were weighed to determine absolute and relative weight. Both removed cauda epididymides were placed in a cavity microscope slide with a medium (96% v/v HTF; 4% v/v BSA) at 37 °C, and spermatozoa were released by maceration to evaluate motility. An aliquot of the sperm-medium mixture was stored in centrifuge tubes, frozen in liquid nitrogen, and maintained at −80 °C [32] for subsequent assessment of the DNA integrity, acrosome reaction and determination of SOD and CAT activities and MDA levels in sperm cells. Subsequently, another aliquot was maintained at 4 °C and sperm were counted later.

2.4. Hormonal assays

The serum concentrations of progesterone, total testosterone, and estradiol were measured by using a chemiluminescence assay (ARCHITECT; Abbott Park, IL, USA). The sensitivity of hormone detection per assay tube for progesterone, total testosterone, and estradiol was ≤ 0.1 ng/ml, 2.30 ng/dl, and 10 pg/ml, respectively.

2.5. Sperm count

For the sperm count, a solution containing sperm was placed in the chamber of a Neubauer hemocytometer (depth: 0.100 mm; area: 0.0025 mm^2) and the cells were counted under a light microscope (Olympus CX41RF, Philippines) at 400 × magnification. Two hemocytometers per animal were used, and twenty squares from a total of one hundred squares were measured. Data were expressed as the number of sperm per milliliter.

2.6. Spermatogenesis and histomorphometry

The right testes were fixed in Bouin's solution by immersion for 6 h at 4 °C. After fixation, the tissues were washed in water, embedded in paraffin (Histosec®; Merck KGaA, Darmstadt, Germany), sectioned at 5 μm and stained with hematoxylin and eosin. The histological sections were used to evaluate the dynamics of spermatogenesis and morphometry of seminiferous tubules [28]. Spermatogenesis was evaluated under a light microscope (Olympus CX41RF, Philippines) at 400 × magnification 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 [33].

Histomorphometric measurements were performed by evaluating the seminiferous tubules at stages VII–VIII, IX and XII in 45 random cross-sections of tubules (15 tubules for each stage) per animal ($n = 6$ for each experimental group). Analyses were carried out using the Image Pro-Plus 3.0 software. The images ($n = 270$ per group) were taken using a Zeiss Axioscope A1 light microscope (Zeiss, Germany) with a 200 × magnification. The area of the tubule (S_0), lumen (S_1), epithelium (S_2), and the height (h) of the seminiferous epithelium were determined.

2.7. Comet assay

2.7.1. Sperm cells

Sperm comet assay was performed using the neutral method described by Haines et al. [34]. All procedures were carried out in the darkness to avoid additional DNA damage. Sperm were defrosted and an aliquot was placed in LMP agarose (1%) made in phosphate buffered saline (PBS 1X) at pH 7.2 to yield 1000 cells/μl. Then, 100 μl of the agarose-sperm mixture was applied to NMP agarose-coated glass slides (1.5%), cover-slipped and allowed to solidify at 4 °C for 10 min. Slides were incubated for 1 h in lysis buffer (40 mM DTT; 1% v/v Triton X-100 at pH 10) at 22 °C. Proteinase K was added to a final concentration of 100 μg/ml and the slides incubated for a further 3 h at 37 °C. Following lysis, all slides were washed with Milli-Q water to remove all traces of salt and detergent from the gels that could affect DNA migration. The slides were placed in an electrophoresis chamber with neutral buffer (Tris-Borate-EDTA (TBE 1X), pH 8.4) at 4 °C and allowed to equilibrate for 20 min before electrophoresis. After 20 min electrophoresis at 25 V, 0.01 A, and a temperature of 4 °C, slides were immersed for 5 min in absolute ethanol for fixation and air-dried at room temperature (22 °C).

2.7.2. Leukocyte cells

The alkaline comet assay was performed using the method described by Singh et al. [35] with some modifications. The slides were precoated with 1.5% NMP agarose. Plates were prepared with 5 μl of whole blood from each mouse diluted in 120 μl of 0.5% LMP agarose at 37 °C. The slides were immersed in lysis buffer solution (1 ml of Triton X-100; 10 ml of dimethyl sulfoxide; 89 ml of stock lysis solution, pH 10) at 6 °C for 24 h. The slides were then placed in an alkaline buffer (300 mM NaOH; 1 mM EDTA, pH $>$ 13) for 30 min, and the electrophoretic run was conducted for 25 min at 25 V and 300 mA. After electrophoresis, neutralization with 0.4 M Tris buffer solution (pH 7.5) was performed three times for 5 min. The slides were then washed twice with Milli-Q water and, after fixation with absolute ethanol (5 min), allowed to dry overnight at room temperature.

2.7.3. DNA damage analysis

For analysis of both cells (sperm and leukocytes), the slides were stained with 100 μl of SYBR Green I solution (10 ng/ml). Nucleoids were visualized under an Axio Imager epifluorescence microscope (Carl Zeiss®, Jena, Germany), using a magnification of 200 × . Fluorescence images were obtained using Comet Imager 2.2. (MetaSystems®, Altlussheim, Germany). Fifty cells per slide on two slides (n = 900 cells per experimental group) were analyzed, with Comet Imager version 2.2. The evaluated comet assay parameter was the percent of DNA in the comet tail (% tail DNA).

2.8. Antioxidant enzymes and lipid peroxidation in sperm cells

Superoxide dismutase (SOD) activity was spectrophotometrically determined according to the method described by Misra and Fridovich [36] with some modifications. This method is based on the principle that the SOD inhibits the autoxidation of epinephrine. The sperm samples (5 μl) were incubated with epinephrine bitartrate 60 mmol/l, and the sample color intensity was measured at 480 nm. The enzymatic activity is expressed in units (U) of SOD/mg of protein.

Catalase (CAT) activity was spectrophotometrically determined by the H_2O_2 decomposition at 240 nm as described previously by Aebi [37] with some modifications. The sperm samples (6 μl) were incubated with 86 mmol/l H_2O_2 and sodium phosphate buffer (pH 7.0). The enzymatic activity is expressed in units (U) of CAT/mg of protein. One U of enzyme thus decomposed one μmol of H_2O_2 in 1 min at pH 7.0 at 25 °C.

Lipid peroxidation (LPO) in sperm was assessed by a specific spectrophotometry method that measures the thiobarbituric acid reactive substances (TBARS) as malondialdehyde according to the method described by Ohkawa et al. [38] with some modifications. The samples (50 μl) were incubated with thiobarbituric acid, acetic acid (pH 3.4) and SDS at 95 °C for 60 min. The MDA intensity was determined at 532 nm and expressed as equivalents of MDA in nmol/mg protein.

The total protein concentration in 5 μl of sperm samples was measured by the method described by Bradford [39] using BSA as the standard and expressed as mg of protein per ml of sample.

2.9. Sperm motility

For the motility analysis, 10 μl of sperm solution at 37 °C were pipetted on to a glass slide and covered with a coverslip. Video sequences of sperm motility were recorded using imaging software (Motic Images Plus 2.0 ML), and a light microscope (Leica DMLB) at 100 × magnification, connected to a digital camera (Moticam 2300, 3.0 megapixels). The motility analysis from video sequences was performed using computer-assisted sperm analysis (CASA) and Image J software [40]. The first 5 s of the videos recorded were analyzed. The variables measured were sperm motility (%), curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL).

2.10. Acrosome reaction

Acrosome reaction assay was performed using the method described by De Jonge and Barratt [41] with some adaptations. All procedures were performed in darkness. Sperm samples were defrosted, and the capacitation was performed for 30 min at 37 °C. Then, the samples were centrifuged at 500 g for 10 min, before adding 10 μl of calcium ionophore (10 μM) to the supernatant at 37 °C for 15 min, to induce the acrosome reaction in capacitated spermatozoa. An aliquot of 20 μl was removed from the control and the experimental tubes and placed separately on slides. Slides were air-dried at room temperature (22 °C) and immersed for 30 min in 95% ethanol. The slides were then placed into a vertical staining jar containing PSA/FITC stain for 2 h at 4 °C. Finally, the slides were washed with deionized water, air-dried, and mounted with Entellan mounting medium. Two hundred sperm cells were examined and scored under a fluorescence microscope (Zeiss Axiocam MRc-Scope A1, Oberkochen, Germany), using a filter set at 450–490 nm at 1000 × magnification in duplicate. The sperm cells were categorized as acrosome-intact (more than half of the sperm head is brightly and uniformly fluorescing), acrosome-reacted (a band of fluorescence is localized to the equatorial segment or no fluorescence in the acrosomal region), and abnormal acrosome (all other patterns).

2.11. Statistical analysis

Statistical analyses were performed using STATISTICA (Version 7.1) data analysis software system and the graphs were obtained by GraphPad Prism (Version 6.0). Homogeneity was assessed using the Levene test. For multiple comparisons, one-way analysis of variance (ANOVA) was used, and the significant differences were tested by Tukey's *post-hoc* test. For testing the significance of the effects of CBD on progesterone levels, the Wald chi-square statistic (Wald $\chi 2$), computed using Generalized Linear/Nonlinear (GLZ) Models, was used. Poisson and Identity were applied for the distribution and link function of predicted values. The level of significance considered was $p < 0.05$.

3. Results

3.1. Somatic parameters

Neither dose of CBD significantly affected the final body weight of the treated mice and there were no changes in the absolute and relative weights of the testes and epididymides ($p > 0.05$; Table 1).

3.2. Hormone levels

In the present study, we evaluated the serum progesterone, total testosterone, and estradiol concentrations to assess the effects of CBD on the hormone levels of the mice after the last day of treatment. As shown in Fig. 1, no significant differences in the progesterone, total testosterone, and estradiol levels were observed in the CBD 15 and CBD 30 groups when compared to the control group ($p > 0.05$).

3.3. Sperm count

Sperm concentrations in the cauda epididymis from the control, CBD 15 and CBD 30 groups are shown in Fig. 2. CBD did not affect sperm concentrations after five weeks of exposure ($p > 0.05$).

3.4. Spermatogenesis

Significant differences were found among the germinal epithelium stages of the control, CBD 15 and CBD 30 groups (Table 2). Mice exposed

Table 1
Absolute and relative weights of reproductive organs of adult male Swiss mice orally treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw and control group.

Parameters	Weight		
	Control	CBD 15	CBD 30
Absolute			
Final Body (g)	43.4 ± 4.2	42.6 ± 4.1	42.4 ± 5.0
Testis (mg)	104.3 ± 33.1	106.2 ± 27.2	103.1 ± 31.0
Caput/corpus epididymis (mg)	20.4 ± 5.3	18.5 ± 3.4	20.2 ± 7.6
Cauda epididymis (mg)	9.4 ± 1.7	10.2 ± 2.0	9.1 ± 1.0
Relative			
Testis (g/100 g)	0.237 ± 0.052	0.247 ± 0.055	0.240 ± 0.058
Caput/corpus epididymis (g/100 g)	0.047 ± 0.009	0.043 ± 0.008	0.047 ± 0.014
Cauda epididymis (g/100 g)	0.021 ± 0.003	0.024 ± 0.005	0.021 ± 0.002

Values expressed as mean ± standard deviation to ANOVA ($p > 0.05$).

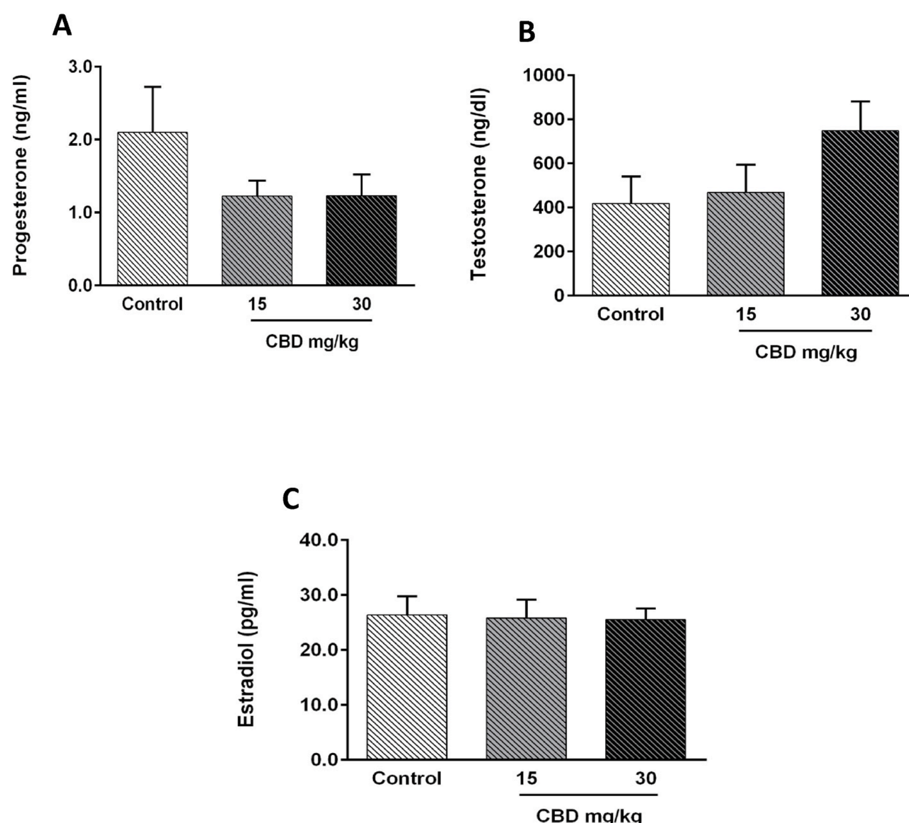

Fig. 1. Serum hormone levels of adult male Swiss mice from the control group and groups treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw. The control group received sunflower oil. (A) progesterone; (B) total testosterone; (C) estradiol. Values expressed as mean ± SEM, $p > 0.05$ to GLZ (progesterone) and ANOVA (total testosterone and estradiol).

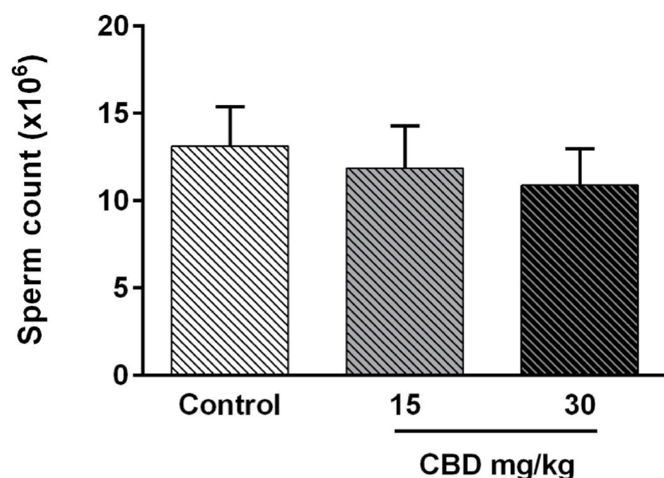

Fig. 2. Sperm count of adult Swiss mice from control group and groups treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw. Control group received sunflower oil. Values expressed as mean ± SEM, $p > 0.05$ to ANOVA.

to both doses of CBD showed a decrease in the number of seminiferous tubules at stages VII-VIII ($F_{(2,19)} = 17.171$; $p < 0.001$) and XII ($F_{(2,19)} = 29.336$; $p < 0.001$), while the number of seminiferous tubules at stage IX was increased ($F_{(2,19)} = 16.688$; $p < 0.001$), when compared to the control group.

Table 2
Frequency of germinal epithelium stages obtained from cross-sections of seminiferous tubules of male Swiss mice orally treated for 34 days with 15 and 30 mg/kg body weight cannabidiol (CBD 15 and CBD 30 groups, respectively) and control group.

Germinal epithelium stage	Frequency		
	Control	CBD 15	CBD 30
I-IV	29.0 ± 3.0	32.0 ± 6.0	31.0 ± 5.0
V-VI	19.0 ± 5.0	21.0 ± 3.0	20.0 ± 3.0
VII-VIII	23.0 ± 3.0	15.0 ± 2.0*	16.0 ± 3.0*
IX	11.0 ± 4.0	19.0 ± 3.0*	20.0 ± 4.0*
X-XI	16.0 ± 5.0	15.0 ± 3.0	13.0 ± 5.0
XII	8.0 ± 1.0	4.0 ± 1.0*	5.0 ± 1.0*

Values expressed as mean ± standard deviation. ANOVA followed by Tukey's post-hoc test (*$p < 0.001$).

3.5. Histomorphometric analysis

Based on histomorphometric analyses, no significant differences were found in seminiferous tubules at stages VII-VIII between the control and CBD-treated groups (Fig. 3). In terms of area, a significant decrease in the tubular lumen area at stage XII ($F_{(2,15)} = 3.802$; $p < 0.05$; Fig. 3B) was observed in the CBD 15 group (Fig. 4E). There were no significant differences in the tubule and epithelium area ($p > 0.05$; Fig. 3A and C) among groups. Regarding the seminiferous epithelium height, both CBD groups showed a decrease at stage IX ($F_{(2,15)} = 12.390$; $p < 0.001$; Figs. 3D and 4B-C) and an increase at stage XII ($F_{(2,15)} = 17.298$; $p < 0.0001$; Figs. 3D and 4E-F).

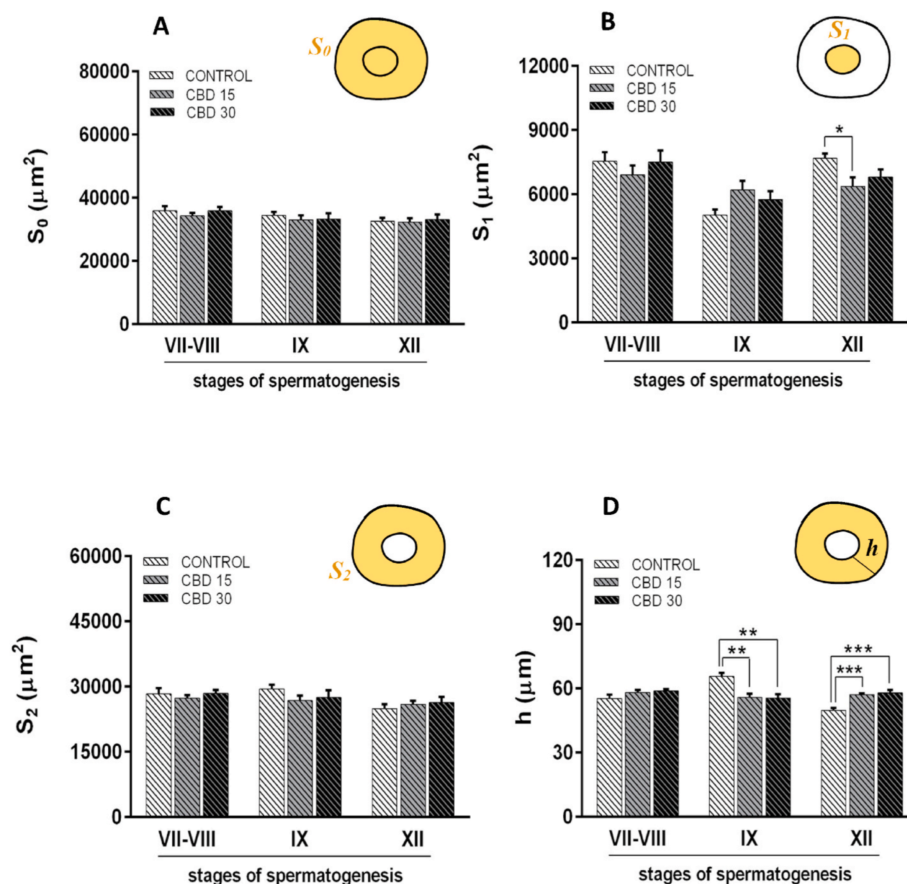

Fig. 3. Histomorphometry of seminiferous tubules at stages VII–VIII, IX and XII of Swiss mice from control group and group treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw. The control group received sunflower oil. (A) tubular area (S_0); (B) lumen area (S_1); (C) epithelium area (S_2); (D) epithelium height (h). Values expressed as mean ± standard error of mean. ANOVA followed by Tukey's post-hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.6. DNA damage

Chronic CBD treatment resulted in increased sperm DNA damage (Fig. 5). The sperm samples from the CBD-treated mice showed an increase of 46.5% in the percentage of tail DNA at doses of 15 (43.4 ± 2.0) and 30 (43.7 ± 1.9) mg/kg when compared with the control group (29.8 ± 1.9) ($F_{(2,23)} = 16.984$; $p < 0.001$). On the other hand, no significant alterations (DNA damage) were observed in the leukocytes between the treated groups (CBD 15 and CBD 30) and the control group ($p > 0.05$; Fig. 6), indicating that the genotoxic effects induced by CBD is cell type dependent.

3.7. Antioxidant enzyme activities

Regarding the control and CBD 15 groups, mice that received CBD at 30 mg/kg bw for 34 consecutive days showed a significant decrease in sperm SOD activity ($F_{(2,20)} = 6.113$; $p < 0.01$; Fig. 7A). Likewise, CAT activity was decreased in the CBD 30 group ($F_{(2,23)} = 11.318$; $p < 0.001$; Fig. 7B) when compared to the control and CBD 15 groups.

3.8. Lipid peroxidation

LPO levels in spermatozoa obtained from the caudal region of the epididymis was found to be significantly increased ($F_{(2,25)} = 4.151$; $p < 0.05$; Fig. 8) in animals in the CBD 30 group, while there were no changes in the control and the CBD 15 groups ($p > 0.05$).

3.9. Sperm motility

Sperm motility and the kinematic variables (curvilinear velocity, average path velocity and straight-line velocity) were affected by CBD treatment. CBD 30 group significantly decreased the percentage of mobile spermatozoa ($F_{(2, 25)} = 5.112$, $p < 0.05$; Fig. 9A) and VCL ($F_{(2, 25)} = 3.9200$, $p < 0.05$; Fig. 9B). VAP ($F_{(2, 25)} = 5.8096$, $p < 0.01$; Fig. 9B) and VSL ($F_{(2, 25)} = 6.5719$, $p < 0.01$; Fig. 9B) were reduced in both CBD groups.

3.10. Acrosome reaction

The results of acrosome reaction are shown in Fig. 10. There was no difference in the number of acrosome-reacted spermatozoa in all groups ($p > 0.05$; Fig. 10B). The frequency of acrosome-intact spermatozoa decreased in the CBD 30 group ($F_{(2, 21)} = 4.985$, $p < 0.05$; Fig. 10B). The CBD 15 and CBD 30 groups had a higher number of abnormal acrosome spermatozoa than the control group ($F_{(2, 21)} = 6.9844$, $p < 0.01$; Fig. 10B).

4. Discussion

The current present study showed the effects of CBD on the reproductive system of male mice after chronic exposure (34 days). The findings complement our previous results [28] and provide more pieces of the puzzle in respect of the regulatory role of the endocannabinoid system on male reproduction. Results suggested that CBD-mediated reproductive toxicity is largely the result of an interaction among CBD and the endocannabinoid system in male reproduction. In fact,

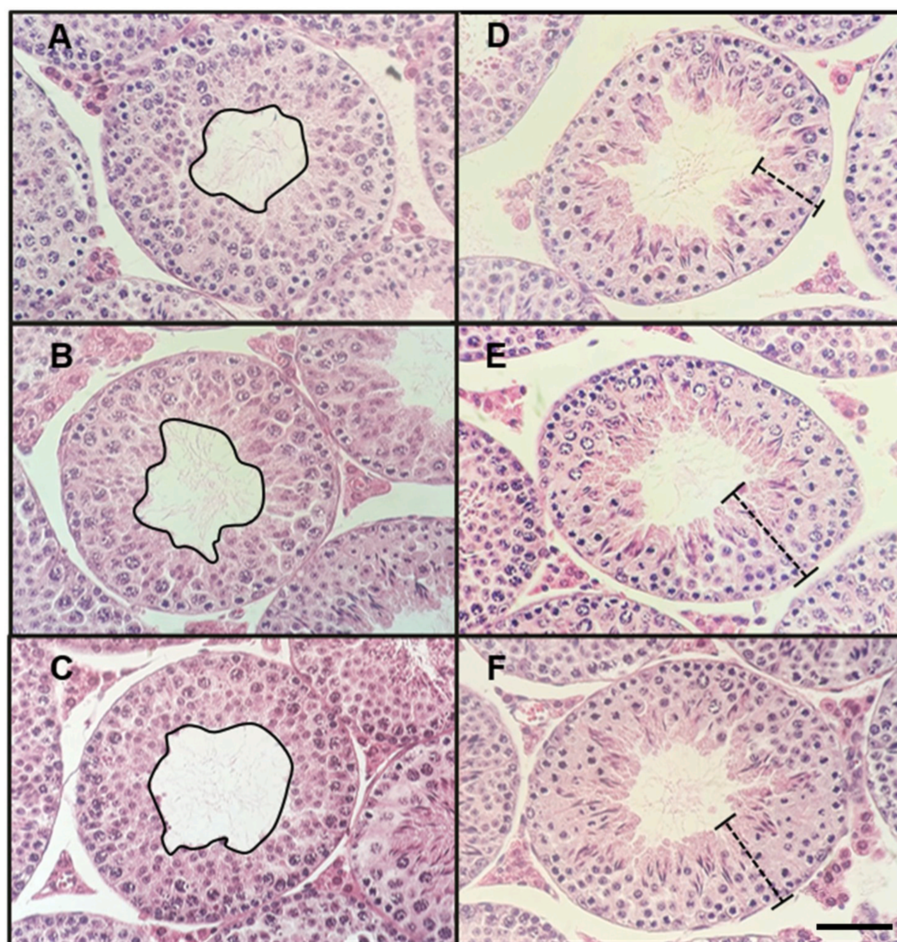

Fig. 4. Photomicrographs of seminiferous tubules (400 × magnification) at stages IX (A–C) and XII (D–F) of Swiss mice from control group (A and D) and group treated for 34 days with cannabidiol (CBD) at a dose of 15 (B and E) and 30 (C and F) mg/kg bw. The control group received sunflower oil. The solid black lines (A–C) represent the lumen area (S1) and dashed black lines (D–F) the epithelium height (h). Hematoxylin and eosin.

researchers have shown wide participation of this system in the control of reproduction, as reviewed by Grimaldi et al. [42].

Our results showed no significant changes in the relative or absolute weights of the testes and epididymis. Likewise, the serum hormonal levels and sperm count were not affected by CBD exposition. Previous data in rodent models has demonstrated that sexual hormones at regular physiological levels is important to sperm production [43,44] and their dysregulation may cause a reduction in epididymal sperm numbers [45]. Thus, the absence of changes in the epididymal sperm count in this study is consistent with the normal serum progesterone, total testosterone, and estradiol levels.

Testicular changes, particularly in the stages of spermatogenesis and tubular morphometry, were promoted by both doses of CBD. The reduction of the stages VII-VIII and XII, as well as the increase of stage IX, show disorders in two important events of spermatogenesis, proliferation, represented by the modification of mitotic stage (IX), as well as in the formation and development of haploid germ cells, represented by the difference in the meiotic (XII) and post-meiotic (VII-VIII) stages [46–48]. However, it is possible to infer from histomorphometric analyses that the increase of stage IX could be adjusted by the reduction of the seminiferous epithelium height, while the reduction of stage XII was compensated by the increase in the epithelium height of mice from both groups exposed to CBD. On the other hand, this compensatory pattern was not observed in stages VII-VIII, showing that the post-meiotic stages were the most affected by chronic exposure to CBD.

Grimaldi et al. [49] demonstrated that mouse spermatogenesis is regulated by an autocrine endocannabinoid signal in the mitotic, meiotic and post-meiotic phases, with high expression of CB1R, CB2R and the transient receptor potential cation channel subfamily V member 1 (TRPV1) in differentiating germ cells. CB1R was observed during meiotic and post-meiotic cells, CB2R showed elevated levels in all stages of spermatogenesis, while TRPV1 showed a strong increase in meiotic cells. Endocannabinoids might promote different effects on spermatogenesis, depending on the receptor target that is activated [42]. CB2R activation by 2-AG showed that this endocannabinoid exerts a differentiative effect on isolated spermatogonial cells, stimulating the entry of germ cells into meiosis [49]. The expression and signalling of anandamide, CB1R and FAAH have also been shown in rodent spermatids [49, 50], highlighting the involvement of the endocannabinoid system in spermiogenesis and maturation.

CBD is associated with the inhibition of the reuptake and degradation of endocannabinoids, increasing the concentration of these substances at their binding sites [22]. Thus, the changes observed in spermatogenesis following CBD exposure are indicative of disturbances in the regulatory function of the endocannabinoid system in the germinal epithelium, particularly during spermiogenesis.

Regarding sperm DNA integrity, the damage was measured using the neutral comet assay, which enables detection of double-stranded DNA breaks (dsDNA) and single-stranded DNA breaks (ssDNA) [51]. The literature has shown that dsDNA breaks are naturally generated during the chromatin remodelling process that occurs in spermiogenesis [52], while ssDNA breaks are produced mainly due to ROS production [53]. The percentage of tail DNA increased by about 50% with chronic CBD treatment at both doses (15 and 30 mg/kg), and is a significant result

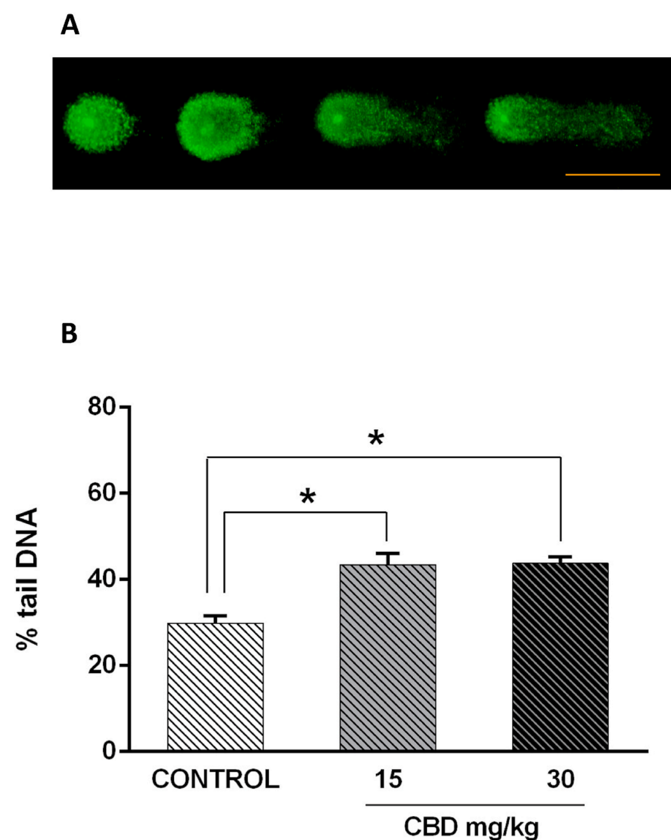

Fig. 5. Representative comet assay image under a fluorescence microscope at 200 × magnification showing sperm cells (stained using SYBR Green I stain) and the percent of DNA in the comet tail (A). DNA damage expressed as % tail DNA in sperm cells of male Swiss mice from control group and group treated for 34 days with cannabidiol (CBD) at 15 and 30 mg/kg bw (B). Control group received sunflower oil. Values expressed as mean ± standard error of mean. ANOVA followed by Tukey's post-hoc test (*$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

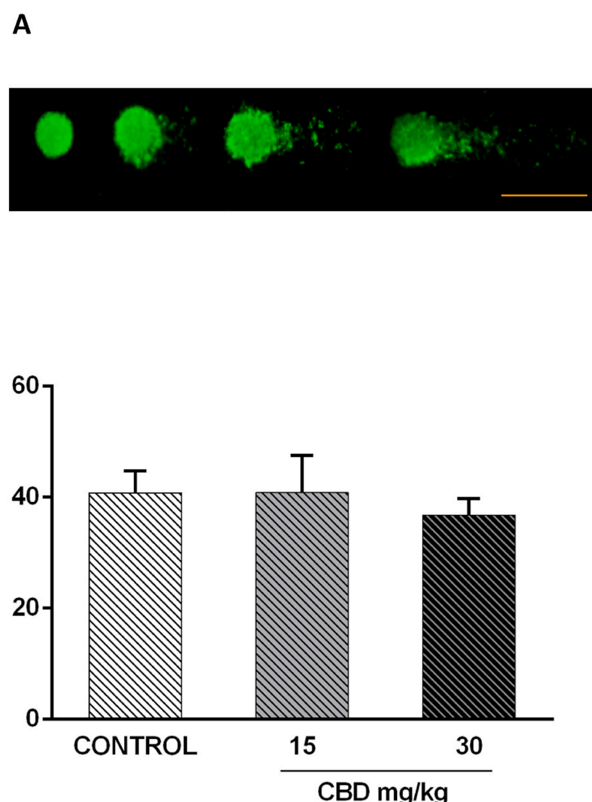

Fig. 6. Representative comet assay image under a fluorescence microscope at 200 × magnification showing leukocytes cells (stained using SYBR Green I stain) and the percent of DNA in the comet tail (A). DNA damage expressed as % tail DNA in leukocytes cells of male Swiss mice from control group and group treated for 34 days with cannabidiol (CBD) at 15 and 30 mg/kg bw (B). The control group received sunflower oil. Values expressed as mean ± standard error of mean, $p > 0.05$ to ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

when compared to the effects of cyclophosphamide (CP), an antineoplastic agent with known genotoxicity (about 100% DNA damage) to the male reproductive system [54]. The reproductive system is vulnerable to this antineoplastic drug due to the presence of rapidly dividing cells [55]. Additionally, CP alters the interaction between protamine and DNA in round spermatids [56].

During spermiogenesis (the post-meiotic phase of spermatogenesis) the chromatin of round spermatids undergoes a remodelling process that continues during epididymal transit [57]. Chromatin-bound histones, such as those in somatic cells, are almost completely replaced by small protamine molecules, which are more efficient for packaging sperm DNA into a limited cytosolic space [7]. This provides a much more condensed and tightly bound nuclear structure, indirectly preserving DNA integrity, and preventing stressors reaching the genome [58]. Clinical studies have reported protamine deficiency in the sperm cells of infertile patients and a positive correlation with DNA fragmentation [59]. In addition, poor protamination makes spermatic DNA sensitive to free radicals [60], resulting in genetic injury.

Spermatogenetic events such as packaging of chromatin and DNA repair are also modulated by endocannabinoid signalling [61]. By excluding genes encoding CB1R in mice, Chioccarelli et al. [62], demonstrated that this receptor plays an important role in regulating spermatid histone displacement/retention. The authors also reported that CB1R signalling disruption interfered with spermiogenesis and was associated with changes in sperm chromatin remodelling and subsequent DNA fragmentation. Thus, failures in the protamination, induced by an endocannabinoid signalling disorder, can explain the genetic damage found after exposition to CBD at doses of 15 and 30 mg/kg. It will be interesting in the future to conduct studies using methods to quantify the protamination and packaging of DNA in sperm cells after CBD exposure in order to better understand the mechanisms by which this cannabinoid induces fragmentation of germline genetic material.

On the other hand, our results showed that *in vivo* exposure to CBD at 30 mg/kg decreased antioxidant enzyme activity while increasing the MDA levels in mice sperm from the cauda epididymis. Normally, the balance between ROS generation and scavenging activities is maintained by adequate levels of antioxidant enzymes such as SOD and CAT. A reduction in these enzymes may result in a high ROS concentration, which is reflected in declining sperm quality, including DNA fragmentation [11]. Studies have shown that in human and mice cells exposed *in vitro* to CBD, there was an increase in ROS production [63,64]. Thus, the harmful effects of CBD on DNA sperm from the CBD 30 group can also be explained by an increase in oxidative damage due to insufficient enzymatic ROS scavengers. Further research to address the ROS-producing enzymes in semen from mice chronically exposed to CBD is required to confirm the data found in this study.

Contrary to the findings in the sperm comet assay, our results showed no changes in the DNA fragmentation of leukocytes in the group exposed to CBD compared to the unexposed group. It was previously seen that there was no correlation between sperm DNA damage and leukocyte cells, thus a specific genotoxic effect of CBD on the male germline is observed in this study.

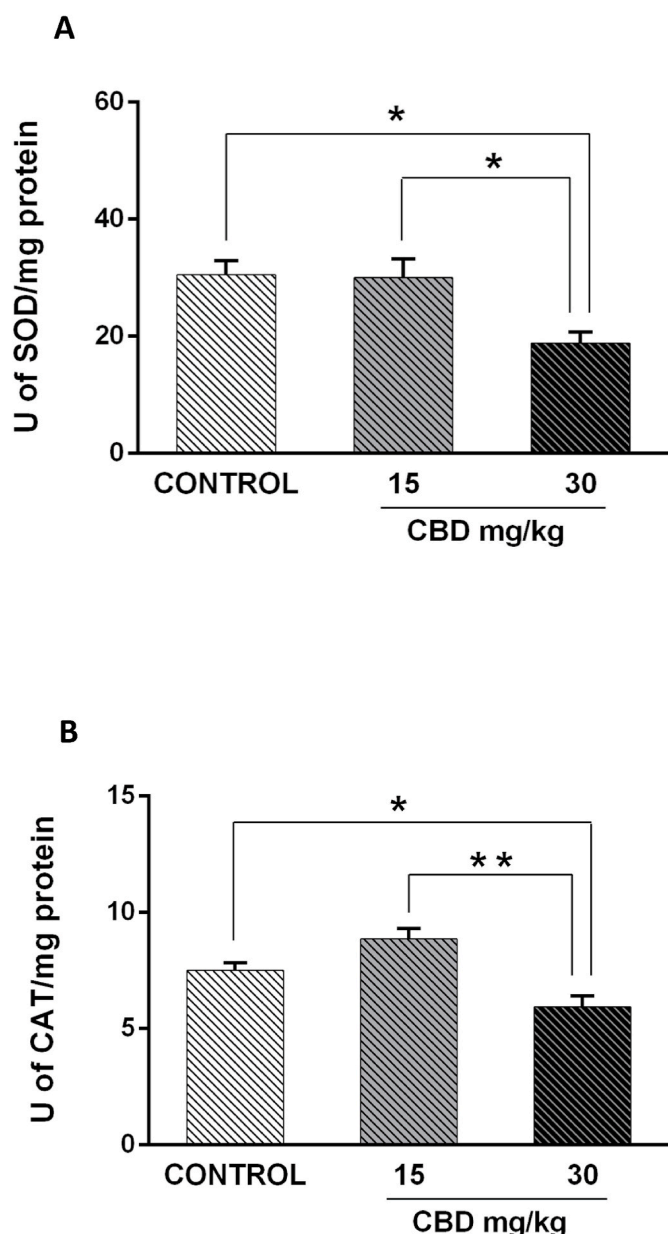

Fig. 7. *In vivo* effect of cannabidiol (CBD) on antioxidant enzymes activities of sperm from cauda epididymis of adult Swiss mice from control group and groups treated for 34 days with CBD at doses of 15 and 30 mg/kg bw. The control group received sunflower oil. (A) SOD activity; (B) CAT activity. Values expressed as mean ± standard error of mean. ANOVA followed by Tukey's post-hoc test (*$p < 0.05$; **$p < 0.001$).

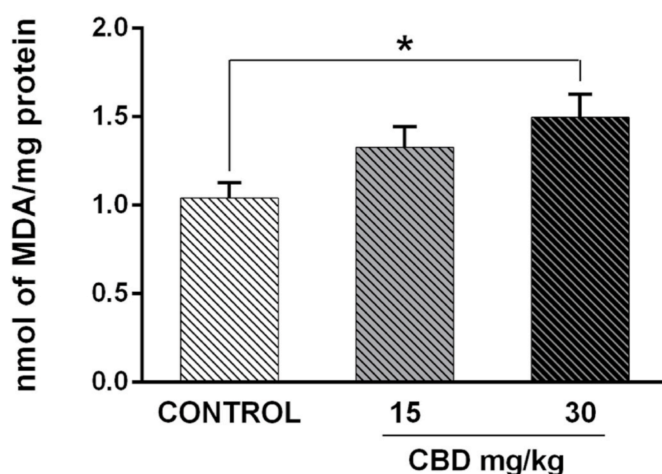

Fig. 8. *In vivo* effect of cannabidiol (CBD) on malondialdehyde (MDA) levels in sperm from cauda epididymis of adult Swiss mice from control group and groups treated for 34 days with CBD at doses of 15 and 30 mg/kg bw. The control group received sunflower oil. Values expressed as mean ± standard error of mean. ANOVA followed by Tukey's post-hoc test (*$p < 0.05$).

To complement our sperm quality investigation, we also evaluated the effects of CBD on sperm motility. We showed that the swimming velocity was decreased in the CBD 15 (VAP and VSL) and CBD 30 (VCL, VAP and VSL) groups, and in the higher dose the percentage of mobile sperm was also reduced. Endocannabinoids play a central role in the control of sperm motility in the male reproductive tract [65]. Mice with diminished FAAH activity show high concentrations of anandamide in the testis and epididymis, thus impairing sperm motility [66]. In humans, it has been shown that CBR1 activation decreases the mobility of spermatozoa and mitochondrial function [67]. Indeed, cannabis constituents have been linked to changes in sperm motion. Morgan et al. [68] exposed mouse spermatozoa to tetrahydrocannabinol (THC) and clearly showed that it reduced the percentage of motile spermatozoa and the kinematic parameters (VSL, VAP and VCL), while ATP levels were also decreased by inhibition of mitochondrial activity.

ATP provides energy essential for flagellar movements of spermatozoa, and a decrease in the energy supply due to mitochondrial injuries can impair sperm motility [67]. The highly lipophilic feature of cannabinoids grants them access to the intracellular compartment, with mitochondria being reported as targets for cannabinoids [69,70]. Rimmerman et al. [71] showed that CBD directly modulates the outer mitochondrial membrane channel in BV-2 microglial cells, as an agonist of the voltage-dependent anion channel 1 (VDAC1), related to the regulation of cell energy.

Regarding endocrine support, there is a positive relationship between serum testosterone levels and sperm motility [72]. However, in the current study, CBD chronic treatment impaired sperm motility even in the presence of normal hormonal status. Thus, we speculate that the motility disorders found in this study may have been caused by a reduction in ATP supply through the direct action of CBD on mitochondrial function, or indirectly through the endocannabinoid system. To evaluate these hypotheses, a biochemical analysis would be necessary.

Finally, in addition to the sperm parameters discussed above, another sperm property that is important for fertilization is acrosomal integrity. The acrosome is a perinuclear vesicle derived from the Golgi apparatus that covers the anterior portion of the sperm head. Its major role is in the acrosome reaction, a calcium-dependent process that occurs during the gamete interaction, allowing the spermatozoa to cross the oocyte membrane [73]. The assembly of the acrosome starts during the meiotic stage of spermatogenesis, in which acrosomal granules are distributed and coalesce to form one vesicle attached to the round spermatid nucleus. Thus, this vesicle is reorganized during spermiogenesis, simultaneously with nuclear remodelling, and further maturation occurs during the sperm epididymal transit and storage [4,73].

To start the acrosomal reaction, the acrosome must have normal morphology. Structural or functional defects in acrosomal status can impair sperm fusion and lead to infertility [4,74]. The effect of CBD on the number of abnormal acrosomes observed in this study may be explained by an alteration in the assembly of acrosome during the spermiogenesis stage. This abnormality can be associated with linkage disorders of the acrosomal vesicle to the nuclear membrane during spermiogenesis, or with abnormal maturation during epididymal transit, resulting in fragmentation of the acrosome.

Studies on sea urchin spermatozoa have shown that cannabinoids (THC, CBD and AEA) negatively affect the acrosome reaction and impair

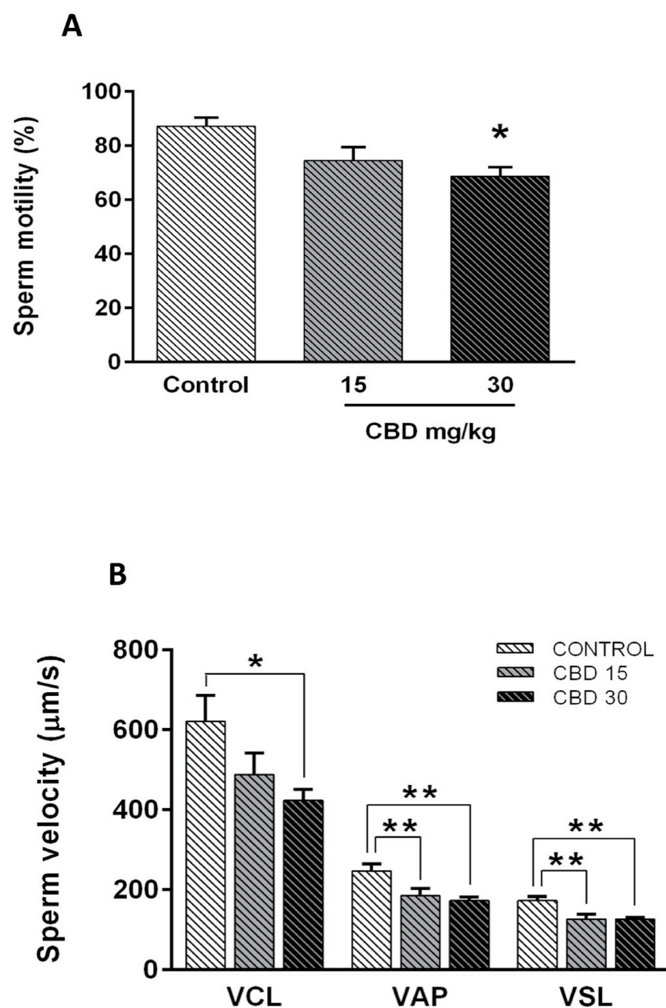

Fig. 9. *In vivo* effect of CBD on motility of sperm from cauda epididymis of adult Swiss mice from control group and groups treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw. Control group received sunflower oil. (A) percentage of motile spermatozoa; (B) kinematic parameters: curvilinear velocity (VCL), average pathway velocity (VAP), straight-line velocity (VSL). Values expressed as mean ± SEM. ANOVA followed by Tukey's post-hoc test (*$p < 0.05$; **$p < 0.01$).

sperm fertilizing ability [75,76]. We have previously shown that in male mice, 30 mg/kg of CBD reduced fertility and the number of pups [29]. Overall, poor sperm quality, as evidenced by a reduction in DNA integrity and motility, in addition to the major effects on acrosomal status, by decreasing the number of acrosome-intact and increasing acrosome abnormalities of sperm, may be linked to the action of the higher dose of CBD on mice fertility.

In summation, chronic exposure (34 days) to CBD at doses of 15 and 30 mg/kg is able to impairs the sperm quality and induce DNA damage and LPO in sperm cells, while no genotoxic effects were observed in leukocyte cells. The impairment of spermatogenesis dynamics from meiotic to spermiogenesis stages by an endocannabinoid system dysregulation, as well as the induction of oxidative damage on the nuclei of male gametes prompts us to suggest that these harmful effects may be involved in the reduced sperm quality from CBD-treated mice. In addition, the way that CBD impairs sperm motility can be explained partially by the fact that it inhibits mitochondrial activity, thereby, starving the exposed spermatozoa of energy. Therefore, further biochemical, and cytogenetic studies are necessary to determine the mechanism of CBD action and the cell-specific responses, since damage to germ cells can affect future generations.

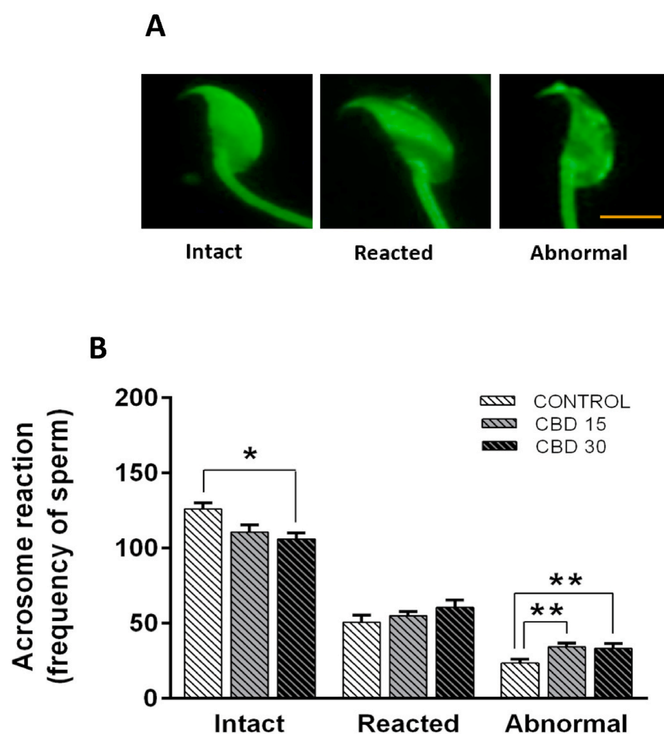

Fig. 10. Representative images of cauda epididymis sperm stained using PSA/FITC under a fluorescence microscope at 1000 × magnification showing the acrosomal status on mice sperm head that were addressed in this study (A). Frequency of sperm with acrosome-intact, reacted and abnormal of adult Swiss mice from control group and groups treated for 34 days with cannabidiol (CBD) at a dose of 15 and 30 mg/kg bw (B). Control group received sunflower oil. Values expressed as mean ± SEM. ANOVA followed by Tukey's post-hoc test (*$p < 0.05$; **$p < 0.01$).

CRediT authorship contribution statement

Renata K. Carvalho: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft. **Thiago L. Rocha:** Resources, Writing – review & editing. **Fábio H. Fernandes:** Validation, Writing – review & editing. **Bruno B. Gonçalves:** Validation, Investigation, Writing – review & editing. **Maingredy R. Souza:** Validation, Investigation. **Amanda A. Araújo:** Validation, Investigation, Writing – review & editing. **Caio C. Barbosa:** Validation, Investigation. **Daniela M. Silva:** Resources, Investigation, Writing – review & editing. **Hericles M. Campos:** Validation, Investigation. **Mariana V. Tomazett:** Resources, Investigation. **Paulo C. Ghedini:** Resources, Validation. **Francisco S. Guimarães:** Resources. **Monica L. Andersen:** Resources, Investigation. **Fernanda C.A. Santos:** Resources, Validation. **Renata Mazaro-Costa:** Conceptualization, Supervision, Resources, Validation, Investigation, Writing – review & editing.

Declaration of competing interest

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

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