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Janaina da Silva, Juliette Dochez-Arnault, Chritèle Desdoits-Lethimonier, Nathalie Dejudcq-Rainsford, Aurore Gely-Pernot. The Acute Exposure of Human Adult Testis Tissue to Cannabinoids THC and CBD Does Not Impact Testosterone Production Nor Germ Cell Lineage. *The World Journal of Men's Health*, 2023, 41, pp.e34. 10.5534/wjmh.220210 . hal-04099943

HAL Id: hal-04099943

<https://hal.science/hal-04099943>

Submitted on 31 May 2023

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The Acute Exposure of Human Adult Testis Tissue to Cannabinoids THC and CBD Does Not Impact Testosterone Production Nor Germ Cell Lineage

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Purpose: While an increased risk of developing germ cell tumors has been established in regular cannabis consumers, there is conflicting evidence about an association between cannabis use and testosterone levels in those regular consumers. In this context, we aimed to determine whether Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD), the two major and best-studied cannabinoids present in cannabis, also the most used for therapeutic applications, can directly impact the steroidogenic function and germ cell lineage of the human adult testis.

Materials and Methods: We used our well-characterized organotypic culture of human testis, in which adult testis explants were exposed to CBD, THC, or CBD/THC [ratio 1:1] from 10^{-9} to 10^{-5} M for up to either 48 hours or 9 days of culture. The testes were obtained from multi-organ donors (n=13; mean age: 55.15 ± 5.62 y).

Results: The testosterone production and the spatial distribution of Leydig cells did not change upon CBD and/or THC exposure *versus* controls after 48 hours or 9 days. The overall tissue morphology of the cannabinoids-exposed testis explants was similar to their control upon 24 hours or 9 days of exposure, a finding confirmed by morphometric analyses on short-term cultures. In line, the number of apoptotic cells was not affected by either 48 hours or 9 days of cannabinoids treatment *versus* mock. Cannabinoids had no impact on the number of proliferating cells nor on mRNA expression of genes encoding proteins involved in germ cell differentiation, meiosis, or Sertoli and Leydig functions after 24 hours exposure.

Conclusions: Altogether, these findings show an absence of acute direct effects of exposure to cannabis-derived cannabinoids THC and CBD on testicular testosterone production and germ cells *ex vivo*. Further studies are warranted to explore an indirect impact of cannabinoids on testis functions through the hypothalamic-pituitary-testis axis, as well as the potential effects of long-term exposures.

Keywords: Cannabis; Leydig cells; Male infertility; Spermatogenesis

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Received: Oct 5, 2022 **Revised:** Dec 21, 2022 **Accepted:** Dec 29, 2022 **Published online** Mar 7, 2023

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INTRODUCTION

Cannabis remains the most widely used drug worldwide. In 2020, more than 4 per cent of the global population aged 15–64 years (209 million people) had used cannabis in the past year. The use of cannabis has increased in young people of reproductive age, with a majority of consumers starting smoking cannabis in their mid to late teens. Of concern, puberty represents a sensitive window for reproductive health, which integrity is required for lifelong fertility. Furthermore, there has been a renewed interest in the potential medical use of cannabis, cannabis extracts, and pharmaceutical products (containing cannabinoids) [1].

In animals, a reduction of serum testosterone level is observed after acute or chronic exposure to cannabis [2,3] but scientific evidence based on observational findings from cannabis smokers has produced conflicting data about the effect of cannabinoids on male reproductive health. Some studies highlighted the negative effect of cannabis consumption on sperm parameters [4,5] and testosterone secretion [6,7], whereas others failed to observe any impact [8-11]. In the last few years, a review [12] and a meta-analysis [13] concluded that the current evidence does not support a clinically significant association between cannabis use and testicular function, assessed through published clinical data of semen quality and serum hormone levels. Nevertheless, it is now established that frequent cannabis use is associated with the development of testicular germ cell tumors (TGCTs), suggesting that recreational and therapeutic uses of cannabis or its compounds may impact the testis [14,15]. Furthermore, a recent systematic review indicated that the most consistent consequence of cannabis addition is its negative effects on sperm morphology, while its negative impact on the hormonal profile was still questionable [16]. Also, the evidence that cannabis depresses testosterone levels relies heavily on animal studies [4].

The two major cannabinoids of cannabis are Δ^9 -tetrahydrocannabinol (THC), responsible for the psychotropic effect, and cannabidiol (CBD). These two compounds are the best-studied and most used as an alternative for therapeutic applications such as epilepsy, Alzheimer's and Parkinson's diseases, multiple sclerosis, and cancer [17]. THC and CBD act on specific G protein-coupled receptors called cannabinoid receptors, CB1 and CB2. These receptors, together with their nat-

urally synthesized cannabinoids, so-called endocannabinoids (eCAs), and their synthesis/degradation enzymes and transporters, constitute the endocannabinoid system (ECS) [18]. Gonads synthesize eCAs, which in rodents control Sertoli and Leydig cells activity, germ cell progression, as well as the acquisition of sperm functions [19]. Our laboratory evidenced the expression of the main components of the ECS in the human adult testis [20]. Notably, both CB1 and CB2 were expressed in Leydig cells and post-meiotic germ cells, along with ECS enzymes.

Since ECS is involved in reproductive function and data about the effect of cannabis consumption on reproductive health are conflicting, our study aimed to determine whether THC and CBD, alone or in a mixture, directly impact testis steroidogenic function and germ cell lineage. To this end, we tested the effect of these cannabinoids in our well-characterized organotypic culture of human adult testis, which is routinely used to identify anti-androgenic compounds [21-24].

MATERIALS AND METHODS

1. Chemicals

CBD (Axon Medchem) was diluted in dimethylsulfoxide (DMSO) solution, while THC (CAS number 1972-08-3; Sigma Aldrich) was diluted in ethanol (EtOH) solution. The same amount of DMSO, EtOH, and a mixture of DMSO/EtOH was used as control of CBD, THC, and the mixture of THC/CBD, respectively.

2. Ethical statement

Testes were obtained from 13 multi-organ donors (mean age: 55.15 ± 5.62 y). The protocol was approved by the local ethics committee (Agence de la Biomédecine; authorization no. PFS09-015) and informed consent was obtained from the donors' families.

3. Ex Vivo organ model

We used our previously validated organotypic culture of human adult testis [21]. Testis tissues were examined by transillumination upon receipt. Only tissues displaying spermatogenesis were further processed. Four 3 mm^3 testis explants were placed onto a polyethylene terephthalate insert (Falcon Labware) at the interface of air in 1ml of Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific). This medium was supplemented with antibiotics, 4 mM glutamine, 1 mM

sodium pyruvate, 10 µg/mL insulin, 5 µg/mL transferin, 200 ng/mL vitamin E, 100 ng/mL vitamin A, 50 ng/mL vitamin C, and 1 IU/mL hCG for culture, in a well of a 12-well plate. For 9 day-culture, 10% of fetal calf serum without steroids were added to supplemented medium. The explants were exposed to a medium containing either CBD or 0.1% of DMSO, THC or 0.1% EtOH, CBD/THC or 0.2% of DMSO/EtOH [ratio 1:1]. Drugs were used at 10^{-9} M to 10^{-5} M. For each condition, testis explants were cultured in three different wells corresponding to technical replicates. For 48 hours cultures (aiming to mimic a single exposure, taking into account the half-life of the cannabinoids), medium was replenished at 24 hours. Media from 24 and 48 hours were stored at -80°C for testosterone measurements. For 9 day-culture (aiming to mimic a repeated exposure), explants were cultured during the first 24 hours of culture (D0) without any drug to define a baseline for hormone production in each well. Media were changed and collected at 3, 6, and 9 days. At the end of the exposure, three explants, one of each well of each condition, were randomly collected and stored at -80°C

for RT-qPCR or fixed in Bouin's or paraformaldehyde (PFA) 4% (w/v) and embedded in paraffin (Supplement Fig. 1).

4. Histology and morphometry

The Bouin's-fixed explants were cut into 5.0 µm-thick sections and stained with hematoxylin-eosin. The testicular histology was checked under a light microscope (Zeiss microscope). Morphometric analysis was performed as previously described [25], using the ImageJ software (US National Institutes of Health). Briefly, the components that constitute the testicular tissue were visualized in square grids placed over digital images. It was counted 2,260 Intersection points per condition, including those in the seminiferous tubules (STs; distinguishing seminiferous epithelium and tunica propria) and in the interstitial tissue (IT). The proportion of each component/point in the distinct compartments was then determined and expressed as a % of all components. The ST diameter was obtained by measuring 20 random circular ST cross sections from each condition. The ST area was determined using the following

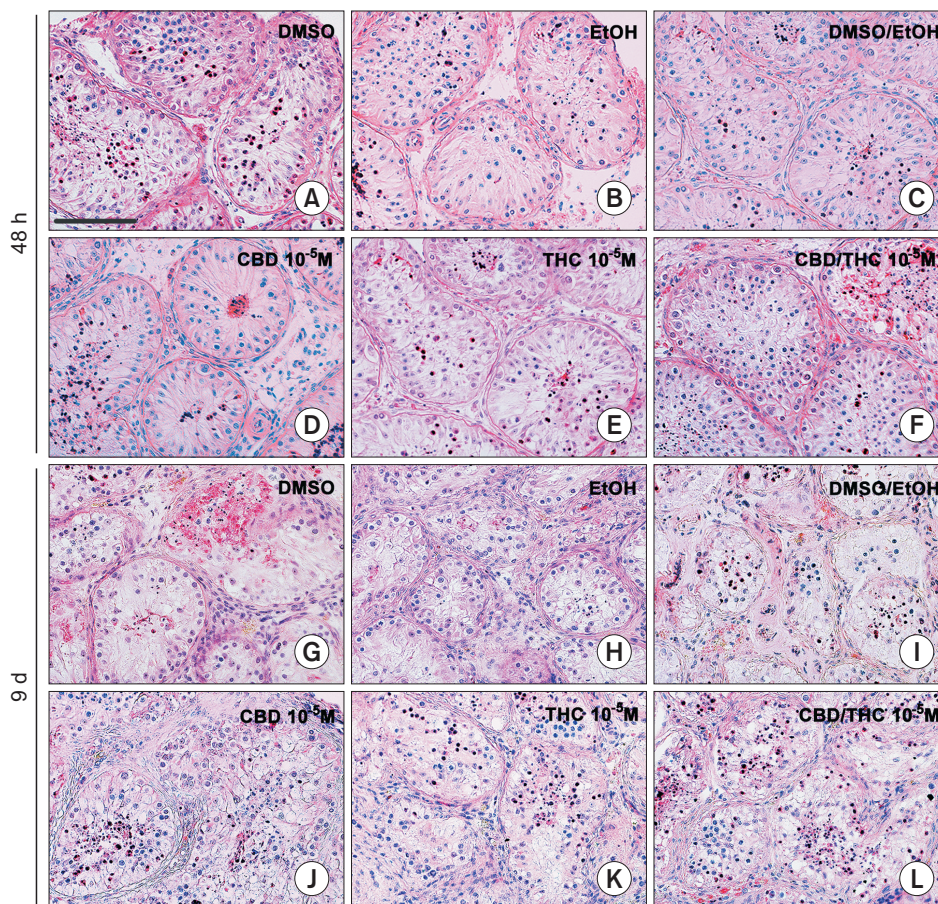


Fig. 1. CBD and THC, alone or in a mixture, do not affect the morphology of human adult testis explants. H&E staining of the histological sections of human adult testis explants cultured for 48 hours (A-F) or 9 days (G-L) in the presence of (A, G) DMSO (control CBD), (B, H) EtOH (control THC), (C, I) DMSO/EtOH (control CBD/THC), (D, J) CBD, (E, K) THC, or (F, L) CBD/THC (1:1 ratio). Pictures showed are representative of all tested cannabinoid concentrations performed on 6 donors for the short-term culture and 5 donors for the longer-term culture. Bar=100 µm. CBD: cannabidiol, DMSO: dimethylsulfoxide, EtOH: ethanol, THC: Δ^9 -tetrahydrocannabinol.

equation: $area = \pi R^2$, in which R is the tubule radius.

5. Hormone measurement

Testosterone levels were assayed in duplicate in the culture medium using a specific radioimmunoassay (Beckman Coulter). Control testis explants from the short-term culture produced an average of 15.08 ± 2.96 ng/mL testosterone after 24 hours of culture and 17.37 ± 4.03 ng/mL testosterone after 48 hours of culture. For the longer-term culture, the testosterone levels in the controls were 9.53 ± 1.79 , 29.95 ± 5.96 , and 166.31 ± 11.42 ng/mL after 24 hours (D0), D3, and D9, respectively.

6. Immunohistochemistry

Immunohistochemistry was performed on PFA 4%-fixed histological sections of explants to observe Leydig cells and estimate the number of testicular cells undergoing apoptosis and proliferation, as previously described [22-24]. Briefly, Leydig cells were labeled with a rabbit primary antibody (1:500; Sigma-Aldrich) against the cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) followed by biotinylated goat anti-rabbit secondary antibody (1:200; Dako) [23]. For apoptosis assessment, histological sections were labeled with a rabbit primary antibody against cleaved caspase-3 (1:100; Asp175; Cell Signaling Technology) and then with the secondary antibody already described [24]. For proliferation assessment, histological sections were labeled with a mouse primary antibody against Ki-67 (1:100; Dako) and then a biotinylated goat anti-mouse secondary antibody (1:200, Dako). The relative Leydig cells area was estimated by determining the ratio between the surface occupied by the CYP11A1 positive Leydig cells and that of the total tissue surface on a whole section, on 8-10 randomly selected histological explant sections using ImageJ software. To quantify the number of cells in apoptosis or proliferation, for each condition, positive cells were counted in the STs and IT in 10 histological sections using a light microscope (Bh2 Olympus Microscope; Olympus) coupled to Mercator Expert Software (Explorer Nova).

7. RT-qPCR

RNA was extracted from the frozen explants using RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using QuantiTect Reverse Transcription (Qiagen) according to the protocol provided. RT-qPCR

was performed as described previously [26] using iTaq Universal SYBR Green Supermix (Bio-Rad) and 5.7 ng cDNA template in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Used primers are listed in the Supplement Table 1. Results were calculated by the $\Delta\Delta CT$ method as *n*-fold differences in target gene expression with respect to the reference gene and the calibration sample.

8. Statistical analyses

The data normality was checked by Shapiro–Wilk test. More than two groups were compared using one-way ANOVA followed by Holm–Sidak comparison test (parametric data) or Kruskal–Wallis test followed by Dunn multiple comparisons test (nonparametric data). For comparing two groups only, the unpaired t-test (parametric data) or Mann–Whitney test (nonparametric data) was used. The statistical significance threshold was set at 0.05. All statistical analyzes were performed using GraphPad Prism 6 software (Graph-Pad).

RESULTS

1. THC and/or CBD do not affect the morphology of human adult testis explants

We first determined the effects of a broad range of concentrations of CBD, THC, and CBD/THC mixture on the testis morphology following either a 48 hours or 9-day exposure, in an attempt to reflect various can-

Table 1. Morphometry of the testicular explant parenchyma of CBD/THC exposure after 48 hours

Parameter	48 h	
	EtOH/DMSO	CBD/THC 10 ⁻⁵ M
Seminiferous tubules (%)	73.94±1.77	74.53±1.68
Seminiferous epithelium (%)	65.89±2.80	66.03±2.27
Tunica propria (%)	8.05±1.36	8.50±0.89
Interstitial tissue (%)	26.06±1.77	25.47±1.68
Tubule diameter (µm)	188.43±7.98	191.22±6.12
Tubule area (µm ²)	0.0288±0.002	0.0296±0.002

Values are presented as mean±standard error of the mean. CBD: cannabidiol, DMSO: dimethylsulfoxide, EtOH: ethanol, THC: Δ^9 -tetrahydrocannabinol. Morphometric analysis of H&E-stained histological slides from testis explants cultured in the presence of EtOH/DMSO (control) or CBD/THC 10⁻⁵ M during 24 hours (n=6). No significant difference (*i.e.*, $p > 0.05$) among the groups by Mann–Whitney test of unpaired t-test.

nabis usages, *i.e.*, single or more regular exposure to different administration modes. The analysis of the sections stained with hematoxylin/eosin showed that the overall structure of the testis explants exposed to different concentrations of cannabinoids was not impacted by either a 48 hours (Fig. 1A-1F) or 9 day treatment (Fig. 1G-1L). This result was confirmed by a morphometric analyses (Table 1) performed in the CBD/THC 10^{-5} M and control groups of short-term culture. After 48 hours, the proportion between the two testicular compartments, *i.e.*, STs and IT, remained unchanged ($p>0.99$) in the CBD/THC group (ST: 74.53%; IT: 25.47%) compared to the control (ST: 73.94%; IT: 26.06%). The proportion of seminiferous epithelium ($p>0.97$) and tunica propria ($p>0.78$), as well as the tubule diameter

($p>0.78$) and area ($p>0.81$) were also unaffected. In the long-term culture, the STs appeared smaller and containing less cells in both control and exposed conditions, in line with our previous observations of a progressive decline of the most differentiated germ cells number in cultured testis explants [21]. This impact of the culture at 9 day prevented histomorphometric analyses.

2. THC and/or CBD have no effect on the secretion of testosterone, spatial distribution, relative surface or gene expression of Leydig cells

Our assessment of the endocrine function of cultured testis explants exposed to cannabinoids demonstrated that neither THC, CBD, nor the mixture significantly

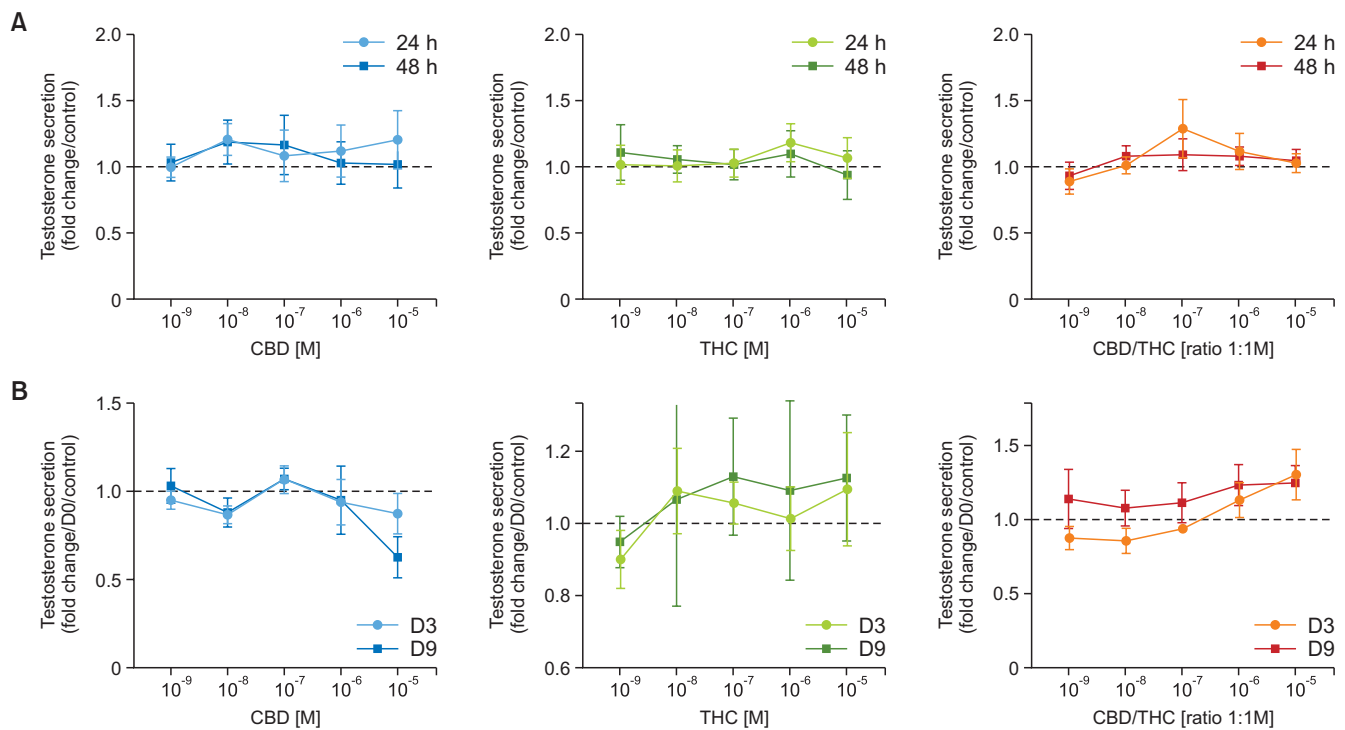


Fig. 2. CBD and THC, alone or in a mixture, do not affect the secretion of testosterone by human testis in organotypic culture. Amount of testosterone in the supernatants of human adult testis explants cultured for 24 or 48 hours (A) and 3 or 9 days (B) in presence of CBD (in blue), THC (in green), and CBD/THC (in orange) at 10^{-9} to 10^{-5} M. There was no significant difference (*i.e.*, $p>0.05$) among the groups by ANOVA followed by Holm–Sidak test (parametric data) or Kruskal–Wallis test followed by Dunn multiple comparisons test (nonparametric data). Data presented as mean \pm SEM divided by their respective controls. Testosterone released by testis explants from 8 donors for the short-term culture and 5 donors for the longer-term culture. (C) Immunostaining using an anti-CYP11A1 antibody on histological sections of whole explants cultured for 48 hours or 9 days in the presence of CBD, THC, or CBD/THC (1:1 ratio) at 10^{-5} M. Pictures showed are representative of minimum 3 independent experiments performed on 6 donors for the short-term and 5 longer-term culture. Bar=500 μ m. (D) Relative Leydig cell area estimated by relating the surface occupied by the CYP11A1 positive Leydig cells on the total surface of 6–8 randomly selected histological explant sections from CBD/THC at 10^{-5} (C/T) and DMSO/EtOH (D/E) after 48 hours ($n=6$) or 9 days ($n=5$) of culture. Data presented as percentage of section occupied by CYP11A1 positive Leydig cells \pm SEM. No significant difference (*i.e.*, $p>0.05$) among the groups by Mann–Whitney test. (E) RT-qPCR analysis using testis explants cultured for 48 hours in the presence of CBD, THC, or CBD/THC (1:1 ratio) at 10^{-5} M. The effect of the treatments was investigated on *INSL3* and *CYP11A1* gene expression. No significant difference (*i.e.*, $p>0.05$) among the groups by ANOVA followed by Holm–Sidak test. Presented data are normalized on 2 reference genes (*RPLP0* and *RSP20*) and control condition (as mean \pm SEM). CBD: cannabidiol, *CYP19A1*: cytochrome P450 family 19 subfamily a member 1, DMSO: dimethylsulfoxide, EtOH: ethanol, *INSL3*: insulin like 3, THC: Δ^9 -tetrahydrocannabinol.

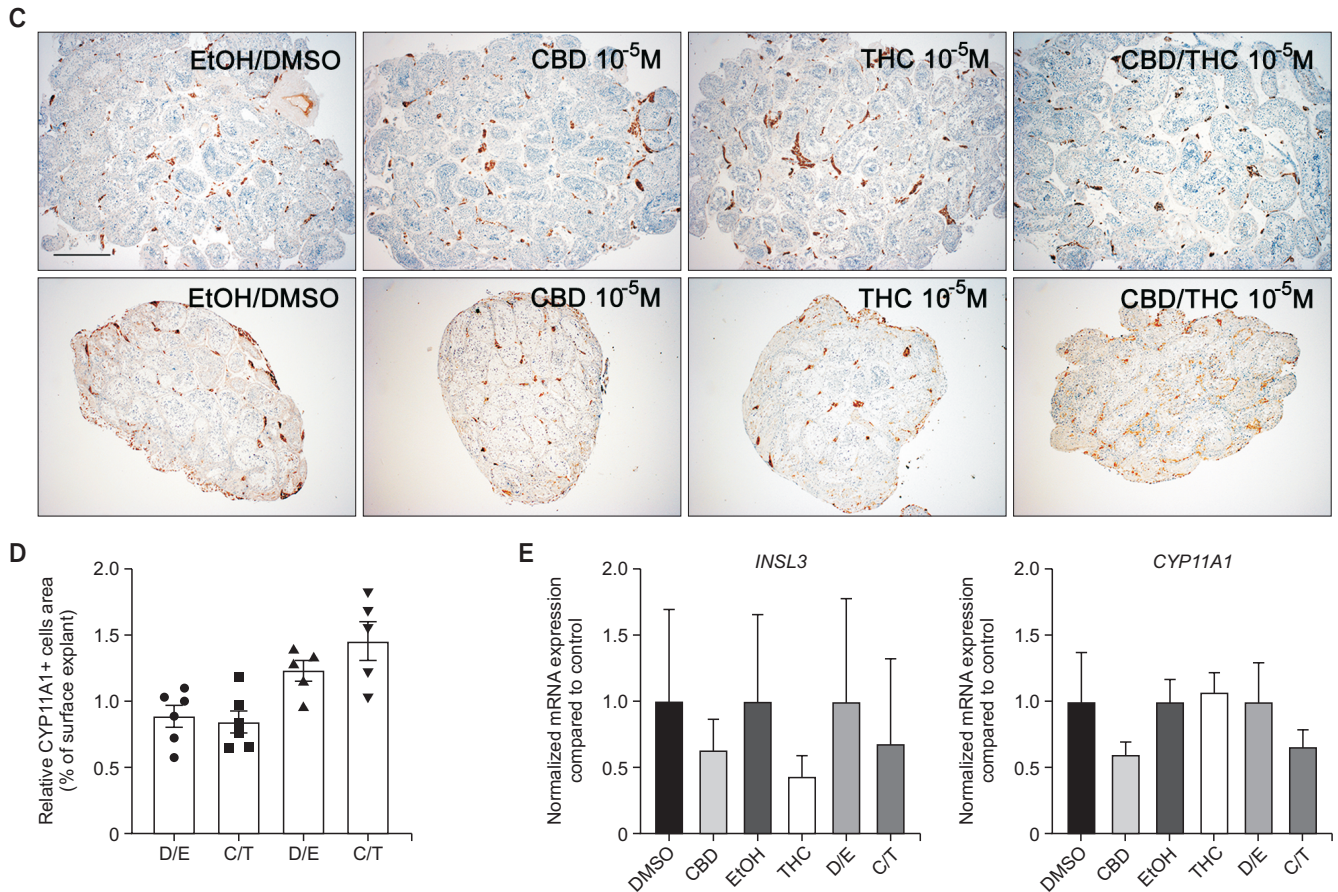


Fig. 2. Continued.

modified ($p \geq 0.05$) the secretion of testosterone in the culture media after 24, 48 hours, 3, or 9 days of exposure (Fig. 2A, 2B). In line, immunohistochemistry of the testis sections using CYP11A1, a specific marker of Leydig cells, did not highlight any change in Leydig cell spatial distribution in treated conditions compared to controls, whether at 48 hours or after 9 day of treatment (Fig. 2C). The quantitative measurement of the relative surface occupied by Leydig cells after 24 hours of CBD/THC 10⁻⁵ M exposure (0.85%) versus control condition (0.89%) showed no significant impact of the treatment ($p=0.69$). Similarly, 9 days of CBD/THC 10⁻⁵ M exposure (1.23%) versus DMSO/EtOH (1.46%) had no significant effect ($p=0.31$) (Fig. 2D). Furthermore, the quantitative analysis of genes specifically expressed by Leydig cell (*INSL3*, *CYP19A1*) did not reveal any significant changes ($p \geq 0.05$) upon 48 hours drug exposures (Fig. 2E).

3. THC and/or CBD do not affect the number of apoptotic and proliferating cells in human adult testis explants

Since we did not detect any morphological changes after CBD and/or THC exposure, we focused on early signs of cell death in the exposed testis explants. After quantification of the number of positive cells for cleaved caspase-3 per surface area in both STs and interstitial space, our results showed that neither CBD, THC, nor CBD/THC impacted germ cells, Sertoli cells, Leydig cells or other interstitial cells' viability after 48 hours in the range of concentrations used ($p \geq 0.05$) (Fig. 3). In the longer-term culture, we also verified that CBD, THC, or CBD/THC at 10⁻⁵ M did not impact the number of apoptotic cells (Supplement Fig. 2).

As proliferation is more active at the beginning of the culture, we next evaluated the short-term effect of CBD and/or THC on the proliferating rate in human adult testis explants, using the specific marker Ki-67. After quantification of the number of Ki-67 positive cells per surface area in both STs and interstitial space,

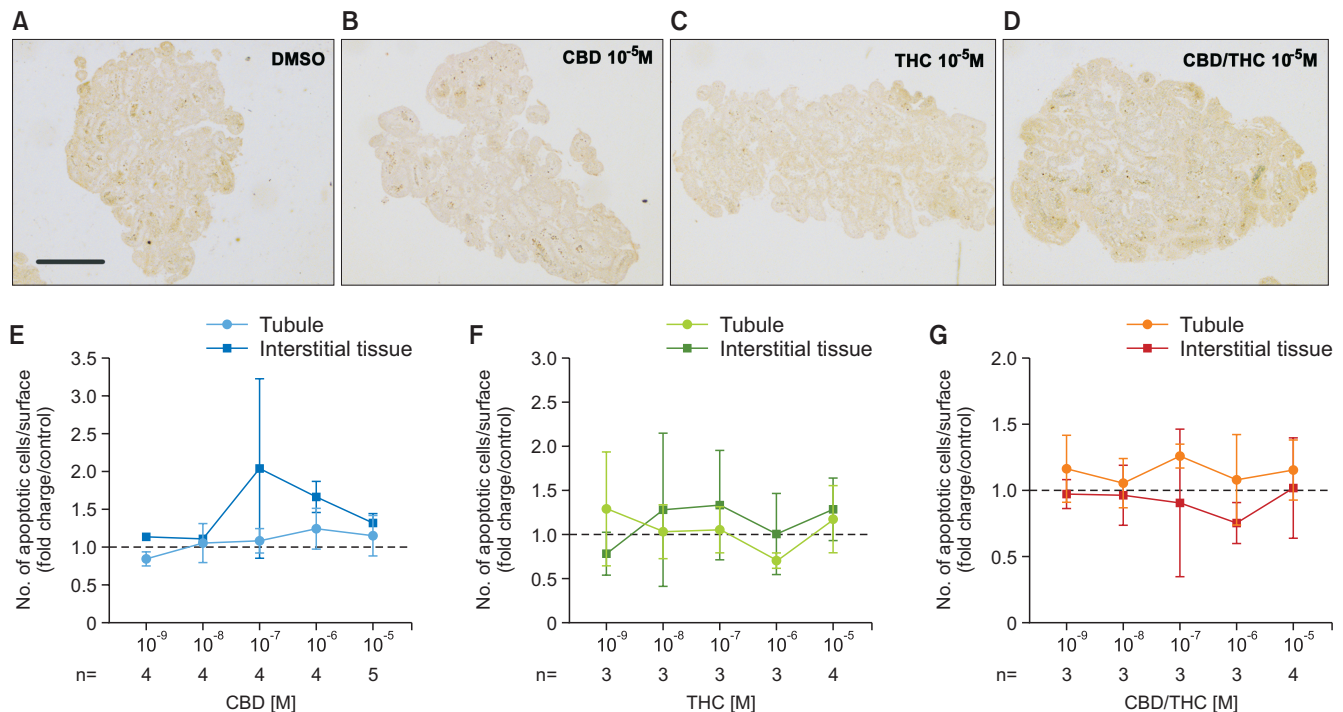


Fig. 3. CBD and THC, alone or in a mixture, do not affect the number of apoptotic cells in human adult testis explants after 48 hours of culture. Immunostaining using an anti-cleaved caspase-3 antibody on histological sections of testis explants cultured for 48 hours in the presence of CBD, THC, or CBD/THC (1:1 ratio). (A-D) Panels show whole explants stained with cleaved-caspase 3 (apoptotic cells in brown). Pictures showed are representative of minimum 3 independent experiments performed on 5 donors. Bar=500 μ m. (E-G) Quantification of positive cells per surface area in both tubule and interstitial compartments after CBD (E), THC (F), and CBD/THC (G) exposure from 10⁻⁹ to 10⁻⁵ M. No significant difference (i.e., $p > 0.05$) among the groups by ANOVA followed by Holm–Sidak test. Data presented as mean \pm SEM divided by their respective controls. Quantification performed in at least 3 donors (the exact n for each dose is indicated at the bottom of the graph). CBD: cannabidiol, THC: Δ^9 -tetrahydrocannabinol.

our results show that CBD and/or THC did not affect ($p \geq 0.05$) the cell's capacity to proliferate after 48 hours of exposure (Fig. 4).

4. Expression of genes involved in spermatogenesis and Sertoli cell function are not affected by 48 hours exposure of testis explants to CBD and/or THC

To investigate the potential effects of CBD and/or THC exposure on gene expression of human adult testis, we performed RT-qPCR on a range of genes markers of germ cell populations and Sertoli cells. Testis explants treated during 48 hours with the highest dose of CBD and/or THC and their related controls were used. For the analysis, we chose genes encoding proteins involved in germ cell differentiation (*DAZZ*, *LIN28A*, *POU5F1*), meiosis (*SYCP3*, *STRA8*, *REC8*), and Sertoli cell function (*INHBB*, *SOX9*, *FSHR*). Our results show that exposure to CBD and/or THC at the highest dose (10⁻⁵ M) for 48 hours did not significantly affect ($p \geq 0.05$) the expression of any of the genes (Fig. 5).

DISCUSSION

This study is the first to investigate the direct impact of the plant-extract cannabinoids CBD and THC, alone or in a combination, on the human adult testis. Using our validated model of human adult testis in organotypic culture [21-24,26], we investigated whether exposure to cannabinoids used for recreational and therapeutic purposes may directly impact testis functions. We provide evidence that 48 hours or 9 day exposures of testis explants to CBD and THC alone or in a mixture - which duration spans the 1.3 days half-life of cannabis measured in infrequent users [27] for the range of cannabinoid concentrations used - has no major effect on steroidogenic function and germ cell lineage.

The ECS has been previously described as a critical regulator of male vertebrate reproduction, notably through the modulation of the secretion of male hormones [19]. At the central level, the ECS regulates the release of the gonadotropin-releasing hormone (GnRH),

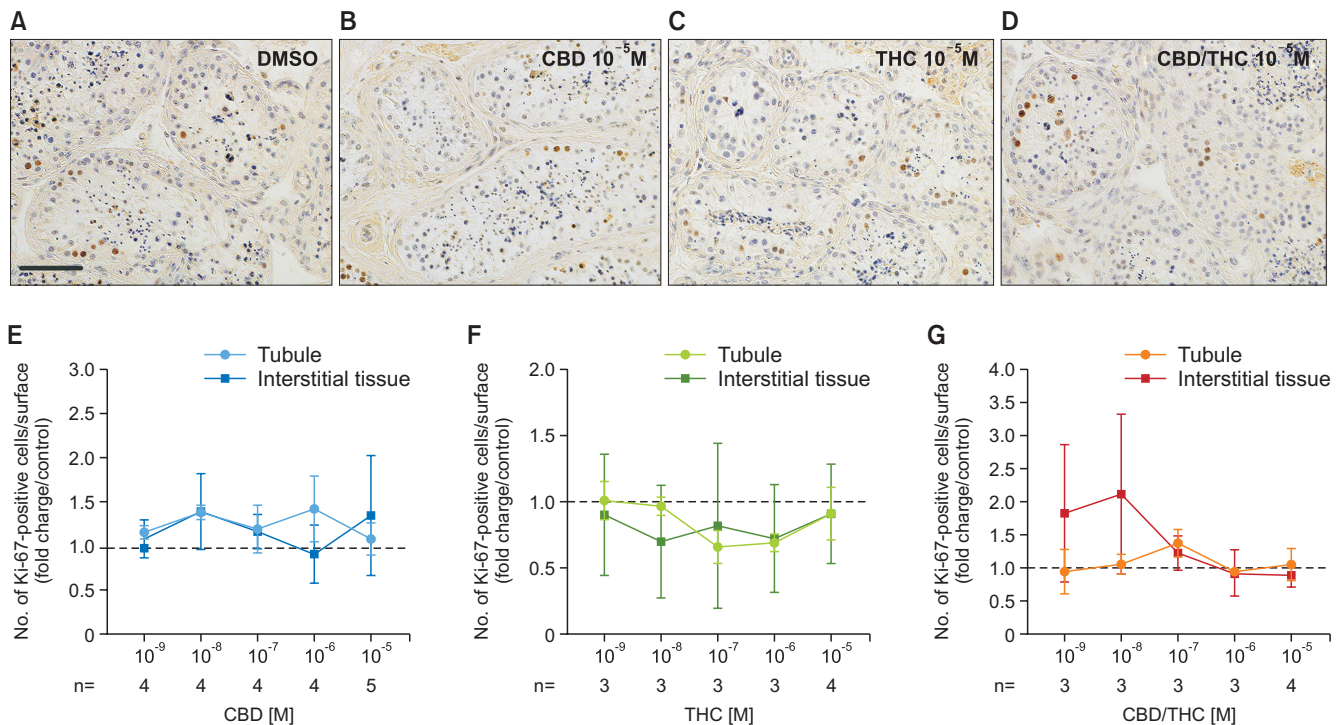


Fig. 4. CBD and THC, alone or in a mixture, do not affect the number of proliferating cells in human adult testis explants. Immunostaining using an anti-Ki-67 antibody on histological sections of testis explants cultured for 48 hours in the presence of CBD, THC, or CBD/THC (1:1 ratio). (A-D) Ki-67 positive cells appear in brown and in most of the cases are on the peripheral site of the seminiferous tubules. Pictures showed are representative of minimum 3 independent experiments performed on 5 donors. Bar=100 μ m. (E-G) Quantification of positive cells per surface area in both tubule and interstitial compartments after CBD (E), THC (F), and CBD/THC (G) exposure at 10^{-9} to 10^{-5} M. No significant difference (*i.e.*, $p>0.05$) among the groups by ANOVA followed by Holm–Sidak test. Data presented as mean \pm SEM divided by their respective controls. Quantification performed in at least 3 donors (the exact n for each dose is indicated at the bottom of the graph). CBD: cannabidiol, THC: Δ^9 -tetrahydrocannabinol.

a key neuropeptide for reproductive physiology, and lowers the levels of the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) released by the anterior pituitary. FSH and LH are respectively involved in the function of Sertoli cells, the somatic testicular cells that nurse the germ cells and are essential for their differentiation, and in the production of testosterone by Leydig cells [28]. However, the available clinical data on the relationship between cannabis and male reproductive hormone production are inconsistent. Thus, while some studies showed a decrease in the levels of LH [29] and FSH [5] in cannabis users *versus* non-users, others did not find any differences [6,11]. Kolodny et al [6] observed a modification of plasma testosterone level associated to a chronic and intensive use of cannabis (at least four days a week for a minimum of six months), whereas other studies did not observe any alteration [9,10]. An explanation for these heterogeneous results could be related to the temporal and reversible effects of cannabis on hormone levels. Indeed, the effect of cannabis consumption on testos-

terone level is related to the recency of use [10]. Nevertheless, the dosage of testosterone production by the human adult testis explants after CBD, THC, or CBD/THC exposure did not show any time-related effect as no alteration were observed for either exposure times.

Our human testis explants model has been extensively validated for its capacity to reproduce the deleterious effects of a wide range of chemicals onto the testis upon short term exposure, and even allowed to unveil the subtle effects of widely used molecules such as paracetamol onto the testis endocrine functions [22,24,26]. It is therefore unlikely that the lack of effect of cannabinoids on testosterone secretion during the testis culture time frame reflects a limitation of this model.

Furthermore, we did not observe any histological nor molecular alterations of Leydig cells in histological analysis and RT-qPCR. Our results are consistent with that of a recent meta-analysis [13], and suggest that exposure to CBD and THC does not modify testosterone production by Leydig cells. However, we cannot rule

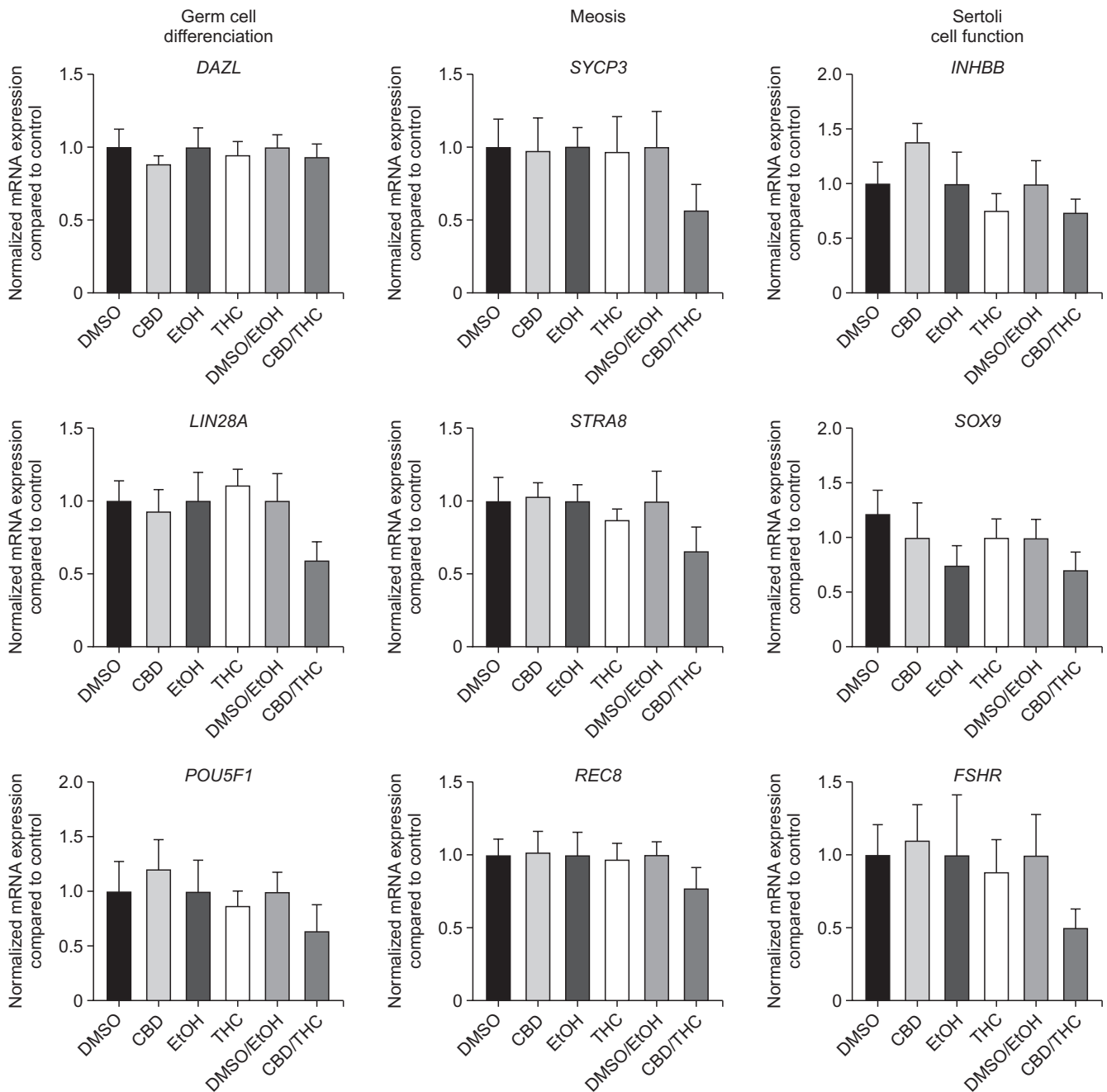


Fig. 5. CBD and THC, alone or in a mixture, do not affect the expression of genes specific to germ cells and Sertoli cells in human adult testis explants. RT-qPCR analysis using testis explants from 5–6 donors cultured for 48 hours in the presence of CBD, THC, or CBD/THC (1:1 ratio) at 10^{-5} M. From the left to the right column, the effect of the treatments was investigated on genes involved in germ cell differentiation (*DAZL*, *LIN28A*, *POU5F1*), meiosis (*SYCP3*, *STRA8*, *REC8*) and Sertoli cell function (*INHBB*, *SOX9*, *FSHR*). No significant difference (*i.e.*, $p > 0.05$) among the groups by ANOVA followed by Holm–Sidak test. Presented data are normalized on 2 reference genes (*RPLP0* and *RSP20*) and control condition (as mean \pm SEM). CBD: cannabidiol, *DAZL*: deleted in azoospermia 1, DMSO: dimethylsulfoxide, EtOH: ethanol, *FSHR*: follicle-stimulating hormone receptor, *INHBB*: inhibin subunit beta, *LIN28A*: lin-28 homolog A, *POU5F1*: POU class 5 homeobox 1, *REC8*: REC8 meiotic recombination protein, *RPLP0*: ribosomal protein lateral stalk subunit P0, *RSP20*: ribosomal protein S20, *SOX9*: SRY-box transcription factor 9, *STRA8*: stimulated by retinoic acid 8, *SYCP3*: synaptonemal complex protein 3, THC: Δ^9 -tetrahydrocannabinol.

out an indirect effect through a modification of the gonadotropins released by the pituitary, and in turn a disruption of the hypothalamic-pituitary-gonadal axis

(HPG axis), nor a direct effect on testis after longer/repeated exposures.

Over 90% of testicular cancers are TGCT [30]. TGCT

are believed to be primarily caused by a deregulation of gonocyte differentiation during development, resulting in the formation of germ cell neoplasia *in situ* (GCNIS) in adult testis. GCNIS corresponds to a precursor lesion that may develop into TGCT after exposure to risk factors [30]. TGCT incidence has been associated with cannabis consumption [14,15]. Thus, TGCT cases have been observed in frequent cannabis users, and the risk of developing this cancer was reported to increase with years of use, speculating that cannabis is able to affect the state of differentiation and proliferation of testicular germ cells. Ki-67, a proliferation marker, has been used for the evaluation and prognosis of TGCT [31]. After quantifying the Ki-67 positive cells in the explants, we did not observe a change after 48 hours of cannabinoids' exposure. To date, the mechanisms that may explain cannabis-TGCT association are unknown and further studies are required. RT-qPCR performed after 48 hours of exposure did not reveal any change in the expression of genes involved in steroidogenesis, Sertoli cell function, and germ cell differentiation, including those recently identified as driver genes for initiation of GCNIS, such as POU5F1 and LIN28A [32]. This outcome confirms the lack of acute effect of cannabis on testis function in our *ex vivo* model.

However our model has several limitations. For instance, we cannot rule out a discrete effect of the cannabinoid compounds on a small number of testicular cells, which may have been missed in our bulk molecular analyses. In that respect, an analysis at single cell level would be useful. The gradual loss of the more differentiated germ cells during culture could also impair the detection of an impact of the drugs on a restricted germ cell population after 9 days. Moreover, as mentioned above, our model can only mimic a direct exposure of the testis outside its body environment, i.e. without external hormonal regulations.

Our results, obtained in a validated organotypic culture of human adult testis [21] that has been extensively used to uncover the direct anti-androgenic activity of a wide range of chemicals [22,24,26], provide important complementary evidence to the heterogeneous epidemiological studies performed on cannabis users. They strongly suggest that cannabis has no direct acute impact on Leydig cells. However, further studies are warranted to explore: (1) an indirect impact of cannabinoids on testis functions through the hypothalamic-pituitary-testis axis; (2) the potential effects of longer-

term exposures.

CONCLUSIONS

Our study indicates that there are no direct effects of a 48 hours or 9-day exposure to cannabis-derived cannabinoids CBD and THC on steroidogenesis and testicular cells, including germ cell lineage in the human testis *ex vivo*. While these findings do not support the notion that a direct acute exposure to the phyto-cannabinoids CBD and THC have harmful effects on testosterone production and human male reproductive health, a better understanding of the link between cannabis consumption and TGCT development is required, as well as that of the effect of regular/continuous exposures of teenagers or adult men.

Conflict of Interest

The authors have nothing to disclose.

Funding

This work was supported by University Rennes 1 and Institut national de la santé et de la recherche médicale (Inserm). This study was also supported by the Institutional Internationalization Program (PrInt) from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Brazil) through a scholarship for J Da Silva.

Acknowledgements

We would like to thank Professors Sérgio da Matta and Fabiana Alves de Melo for the help in the achievement of the collaborative period abroad of J Da Silva. This paper is dedicated to the memory of Bernard Jégou, who prompted this research and passed away prematurely.

Author Contribution

Conceptualization: AGP, NDR. Data curation: JDS, JDA, AGP, CDL. Formal analysis: JDS, AGP. Funding acquisition: AGP, NDR. Investigation: JDS, AGP, NDR. Methodology: AGP, JDS, CDL, NDR. Project administration: AGP. Resources: AGP, NDR. Software: AGP, CDL. Supervision: AGP, NDR. Validation: AGP, JDA, NDR. Visualization: AGP, JDS. Writing – original draft: JDS, AGP, NDR. Writing – review & editing: JDS, AGP, NDR, CDL, JDA.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Materials

Supplementary materials can be found *via* <https://doi.org/10.5534/wjmh.220210>.

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