JANAINA DA SILVA

THE EFFECTS OF ENVIRONMENTAL (CADMIUM) AND PHARMACOLOGICAL (CBD AND THC) CHEMICALS IN THE SPERMATOGENIC PROCESS

Thesis submitted to the Universidade Federal de Viçosa, in partial fulfillment of the requirements in the Graduate Program in Cellular and Structural Biology, for the degree of *Doctor Scientiae*.

Adviser: Sérgio Luis Pinto da Matta

Co-advisers: Aurore Gely-Pernot
Fabiana Cristina Silveira Alves de Melo

VIÇOSA – MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

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S586e
2021

Silva, Janaina da, 1994-
The effects of environmental (cadmium) and pharmacological (CBD and THC) chemicals in the spermatogenic process / Janaina da Silva. - Viçosa, MG, 2021.
113 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Sérgio Luis Pinto da Matta.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Toxicologia reprodutiva. 2. Toxicologia ambiental. 3. Rotas de resultados adversos. 4. Tetra-hidrocanabinol. 5. Canabidiol.
I. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Biologia Celular e Estrutural.
II. Título.

CDD 22. ed. 615.902

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APPROVED: June 15, 2021

Assent:

Janaina da Silva
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Adviser

To Pedro Casagrande, my perfect friend, I dedicate.

ACKNOWLEDGEMENTS

To God for everything;

To my mother Nilza, my sister Jaqueline, and my nieces Sofia and Heloísa for the support, motivation, and inspiration. You are the women of my life!

To my boyfriend Paul for the love and affection, for making me smile every time;

To my stepfather Osmar and my brother-in-law Wesley, for the support and incentive;

To my supervisor professor Sérgio da Matta for orientation, teaching, trust, encouragement, and care. A person who I have always admired for his wisdom;

To the co-supervisor of my studies abroad, Dr Nathalie Dejucq-Rainsford, for the great opportunity to research in her team and for the teachings;

To my co-supervisors the professors Aurore Gely-Pernot and Fabiana Cristina Silveira Alves de Melo for the teaching, trust, encouragement, and affection.

To the thesis committee, professors Mariella de Freitas, Reggiani Vilela, Clovis Neves, and Marcos de Lucca, for all the contributions;

To the Universidade Federal de Viçosa (UFV) and the Graduate Program in Cellular and Structural Biology for all the teaching, research, and extension opportunities;

To the Institut de recherche en santé, environnement et travail (Irset), specially the Equipe #8 – Physiologie et physiopathologie du tractus uro-génital, for one year of a lot of great learning and for hosting me as a researcher;

To the friends that UFV gave me, in special: Luiz, Talita, Amanda, Fran, Diane, and Vivi for productive happy years. My eternal “amiguinhos” and “manas”;

For my interns, Patricia, Francielle, João Vitor, and Pablo. Thanks for the help, you were amazing!

To the coworkers and friends that Irset gave me, in special: Juliette, Nadège, Annabelle, Indusha, Pauline, Salomé, Maryne, and Edith, for teaching me a lot and being good friends;

To the Brazilian friends that Europe gave me, in special: Tamiris, Luciana, Diana, Diego, and Rodrigo;

To the French friends that Europe gave me, in special: Glannon, Al, and Leïla & JEC;

To all my aunts, uncles, and cousins, in special to my godparents Débora and Fernando;

To my good longtime friends, in special: Pedro, Luis Guilherme, Lêda, and Bárbara;

To Hugo, for helping me to speak English, study math, and go abroad;

To Elizabeth Pena, our dear Beth, the secretary of the Graduate Program in Cellular and Structural Biology, for the help, attention, affection, and care;

To Alexandra Elbakyan for opening the doors to free and universal access to specialized literature;

To all the laboratories involved in carrying out this study;

To the Université de Rennes 1 for hosting me in Rennes as a PhD student;

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the PhD scholarships, in special the CAPES/PrInT (Institutional Internationalization Program);

To all the people who somehow contributed and cheered for me to achieve this dream.

“É justo que muito custe o que muito vale”. (Teresa de Ávila)

ABSTRACT

DA SILVA, Janaina, D.Sc., Universidade Federal de Viçosa, June, 2021. **The effects of environmental (cadmium) and pharmacological (CBD and THC) chemicals in the spermatogenic process.** Adivisor: Sérgio Luis Pinto da Matta. Co-advisors: Aurore Gely-Pernot and Fabiana Cristina Silveira Alves de Melo.

A significant trend towards a worldwide decline in human male fertility has been reported over the last years. In the meantime, exposure to chemicals has been proposed to potentially contribute to male reproductive disorders, in which environmental and pharmacological/recreational chemicals might induce detrimental effects on male reproductive health. Among a complex variety of environmental chemicals, cadmium (Cd) is one of the heavy metals that rank among the priority metals that are of public health significance, since Cd is considered as an endocrine disruptor. Human beings are daily exposure to Cd through occupational (e.g. production of batteries) and non-occupational (e.g. contaminated food/water, cigarette smoke) sources. Furthermore, cannabis consumption for medical and recreational purposes is on the rise. In this context of chemicals and male fertility, exposure to cannabis compounds, called phytocannabinoids (cannabinoids), could result in adverse outcomes to male reproductive health. So, this present thesis aimed to assess the impact of these two global trends, cadmium and cannabinoids, in the spermatogenic process. It is already known that cadmium induces damage to the testis. However, the mechanisms that cadmium leads to testicular histopathologies and the relationship between dose, route, and time of exposure and injuries are poorly understood. In this sense, we conducted a systematic review (**Chapter 1**) to answer these questions. After retrieving original studies on databases, we organized the results into an Adverse Outcome Pathway (AOP) framework. Also, a bias analysis was performed. In the 37 studies selected, it was clear that cadmium induces significant histopathologies in the murine model's testis regarding routes, in a dose- and time-dependent manner. The damages can be observed in the first hours of exposure, mainly vascular damages suggesting that vasculature failure is the primary mechanism involved. The AOP showed that potential molecular initiating events may mimic and interfere with essential elements disrupting proteins (structural and antioxidants), change in the oxidative phosphorylation enzyme activities, and gene expression alteration, which lead to reproductive failure. However, analysis of bias showed that the current evidence presents poor methodological quality. Regarding the cannabinoid impact on male reproductive health, we

performed two biological essays. In one manuscript (**Chapter 2**), we aimed to answer whether the two main phytocannabinoids present in cannabis could represent a hazard for adult human testis. For that, we exposed adult human testis to Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), or CBD/THC (ratio 1:1) at the concentrations from 10^{-9}M to 10^{-5}M for 48 hours, by using our unique *ex vivo* model of organotypic culture. Our findings show no alteration in testis histology, testosterone production, number of apoptotic and proliferative cells, and expression of some genes encoding important testicular proteins. Since some male reproductive problems have been hypothesized to have a fetal origin, in the last study (**Chapter 3**), we assessed whether prenatal exposure to CBD and THC could lead to male reproductive disorders in adult life. For that, we exposed pregnant mice from embryogenic day 6.5 (E6.5) to E15.5 to 5mg/kg/day of CBD/THC (ratio 1:1), by gavage. Then, we observed decreased testicular weight and testicular morphological alterations identified by morphometric analyses in adult offspring. However, these alterations were not followed by changes in the undifferentiated spermatogonia and Sertoli cell numbers. The gene expression analysis revealed an increase in the expression of pro-apoptotic (*Bax*), germ cell differentiation (*Sohlh1*), and regulating meiotic (*Stra8*) genes. Alterations in the serum testosterone levels were not observed. In general, our findings demonstrate that cadmium, an environmental pollutant, can affect the male reproductive health of murine models and lead to male infertility. On the other hand, the two main cannabinoids present in cannabis, THC and CBD, which are also used as pharmacological chemicals, do not cause acute direct effects in the testicular histophysiology using our well-characterized organotypic culture of adult human testis. However, prenatal exposure to a mixture of these cannabis-derived cannabinoids can lead to decreased testis weight of mice in adult life. Also, prenatal exposure can accelerate meiosis and cellular differentiation and induce apoptosis, based on the molecular findings, probably, contributing compensatory to the decreased testis weight. So, in mice, prenatal exposure to a mixture of THC/CBD might affect the testis biology of adult offspring. Overall, the results presented by this thesis provide new insights into male repro-toxicology and reproductive health, which might be considered to preserve fertility across the population.

Keywords: Repro-toxicology. Environmental contaminant. Adverse Outcome Pathway. Δ^9-tetrahydrocannabinol. Cannabidiol. Organotypic culture.

RESUMO

DA SILVA, Janaina, D.Sc., Universidade Federal de Viçosa, junho de 2021. **Os efeitos de químicos ambientais (cádmio) e farmacológicos (CBD e THC) no processo espermatogênico.** Orientador: Sérgio Luis Pinto da Matta. Coorientadores: Aurore Gely-Pernot e Fabiana Cristina Silveira Alves de Melo.

Uma tendência significativa para um declínio mundial na fertilidade masculina humana tem sido relatada nos últimos anos. Enquanto isso, a exposição a químicos tem sido proposta para potencialmente contribuir para os distúrbios reprodutivos masculinos, em que químicos ambientais e farmacológicos/recreacionais podem induzir efeitos prejudiciais à saúde reprodutiva masculina. Entre uma variedade complexa de produtos químicos ambientais, o cádmio (Cd) é um dos metais pesados que se classifica entre os metais prioritários com importância para a saúde pública, uma vez que o Cd é considerado como um desregulador endócrino. Os seres humanos estão expostos diariamente ao Cd por meio de fontes ocupacionais (e.g. produção de baterias) e não ocupacionais (e.g. alimentos/água contaminados e fumaça de cigarro). Além disso, o consumo de cannabis para fins terapêuticos e recreativos está em ascensão. Neste contexto de produtos químicos e fertilidade masculina, a exposição aos compostos da cannabis, chamados fitocanabinoides (canabinoides), pode resultar em resultados adversos para a saúde reprodutiva masculina. Portanto, a presente tese teve como objetivo avaliar o impacto dessas duas tendências globais, cádmio e canabinoides presentes na cannabis, no processo espermatogênico. Já se sabe que o cádmio induz danos ao testículo. No entanto, os mecanismos pelos quais o cádmio promove o aparecimento de patologias testiculares e a relação entre dose, via e tempo de exposição e lesões são pouco conhecidos. Nesse sentido, realizamos uma revisão sistemática (**Capítulo 1**) para responder a essas questões. Depois de recuperar estudos originais em bancos de dados, organizamos os resultados em uma estrutura de Adverse Outcome Pathway (AOP). Além disso, uma análise de viés foi realizada. Nos 37 estudos selecionados, ficou claro que o cádmio induz histopatologias significativas em testículos de modelos murinos independente de via, de maneira dose e tempo-dependente. Os danos podem ser observados nas primeiras horas de exposição, principalmente danos vasculares, sugerindo que a falha na vascularização é o mecanismo primário envolvido. A AOP mostrou que potenciais eventos moleculares iniciais podem ser o mimetismo e a interferência com elementos essenciais desregulando proteínas (estruturais e antioxidantes), alteração na atividade da enzima de

fosforilação oxidativa e alteração da expressão gênica, que levam à falha reprodutiva. No entanto, a análise de viés mostrou que as evidências atuais apresentam qualidade metodológica pobre. Em relação ao impacto dos canabinoides na saúde reprodutiva masculina, realizamos dois ensaios biológicos. Em um manuscrito (**Capítulo 2**), buscamos responder se os dois principais fitocanabinoides presentes na cannabis poderiam representar um perigo para o testículo humano adulto. Para isso, expusemos testículos humanos adultos ao Δ^{9}-tetrahidrocanabinol (THC), canabidiol (CBD), ou CBD/THC (proporção 1:1) nas concentrações de 10^{-9}M a 10^{-5}M por 48 horas, utilizando nosso modelo *ex vivo* único de cultura organotípica. Nossos achados não mostraram alteração na histologia testicular, produção de testosterona, número de células apoptóticas e proliferativas e expressão de genes que codificam importantes proteínas testiculares. Uma vez que alguns problemas reprodutivos masculinos foram hipotetizados como sendo de origem fetal, no último estudo (**Capítulo 3**) avaliamos se a exposição pré-natal ao CBD/THC poderia levar a distúrbios reprodutivos masculinos na vida adulta. Para isso, expomos camundongos fêmeas prenhas do dia de gestação 6,5 (E6,5) ao E15,5 a 5mg/kg/dia de CBD/THC (proporção 1:1), por gavagem. Observamos diminuição do peso testicular e alterações morfológicas testiculares, estas identificadas por análises morfométricas, na prole adulta. No entanto, essas alterações não foram acompanhadas por mudanças nos números de espermatogônias indiferenciadas e células de Sertoli. A análise da expressão gênica revelou um aumento na expressão de genes pró-apoptótico (*Bax*), de diferenciação de células germinativas (*Sohlh1*) e de regulação meiótica (*Stra8*). Não foram observadas alterações nos níveis séricos de testosterona. Em geral, nossos resultados demonstram que o cádmio, um poluente ambiental, pode afetar a saúde reprodutiva masculina de modelos murinos e conduzir à infertilidade masculina. Entretanto, os dois fitocanabinoides presentes na cannabis, THC e CBD, os quais são também utilizados como químicos farmacológicos, não causam efeitos agudos diretos na histofisiologia testicular usando nossa cultura organotípica bem caracterizada de testículo humano adulto. Porém, exposição pré-natal a uma mistura desses dois canabinoides derivados da canabis pode conduzir ao peso testicular diminuído de camundongos na vida adulta. Ainda, a exposição pré-natal pode acelerar a meiose e a diferenciação celular e induzir apoptose, baseado nos achados moleculares, provavelmente, contribuindo de forma compensatória para a diminuição do peso do testículo. Assim, em camundongos, exposição pré-natal a uma mistura de THC/CBD pode afetar a biologia testicular de proles adultas. No geral, os resultados apresentados por esta tese fornecem novos conhecimentos sobre a toxicologia reprodutiva e a saúde

reprodutiva masculina, que podem ser considerados para preservar a fertilidade em toda a população.

Palavras-chave: Toxicologia reprodutiva. Contaminante ambiental. Adverse Outcome Pathway. Δ^9-tetrahidrocanabinol. Canabidiol. Cultura organotípica.

SUMMARY

1. INTRODUCTION

Male infertility is a global and significant health concern (BARRATT et al., 2017). Although it is hard to determine an unbiased prevalence of male infertility in the general population, the current literature indicates that global rates of male infertility range from 2.5% to 12% (AGARWAL et al., 2015). Specifically, 7.5% of men are infertile in Europe, 9.4% in North America, and this percentage is unknown for Latin America. However, it is certain that male reproductive disorders affect the health and overall quality of life of a large number of men over the world.

Male infertility can result from defects in both sperm production and sperm delivery. More than 90% of cases are due to low sperm counts, poor sperm quality, or both. Other causes include anatomical problems, hormonal imbalances, and genetic defects (LEAVER, 2016). Male reproductive disorders may develop during various life phases. Alterations in proper reproductive functioning may be the result of several occurrences throughout fetal development, childhood, adolescence, or adulthood (WORLD HEALTH ORGANIZATION, 2011a). While much is known about the male reproductive system and some disorders, the mechanisms of action for certain pathologies are still unknown. However, the rapid pace of the increase of reproductive disorders suggests that environmental or life-style factors, rather than an accumulation of genomic structural defects, are the most likely causes (SKAKKEBAEK et al., 2001). So, exposure to chemicals has been proposed to potentially contribute to male reproductive disorders (WORLD HEALTH ORGANIZATION, 2011b). Specifically, environmental and pharmacological chemicals may potentially induce detrimental effects on male reproductive health (AJAYI; AKHIGBE, 2020; SHARMA et al., 2020).

Cadmium, an environmental chemical

Cadmium (Cd) is reported in the top 10 priority chemicals on the ATSDR's Substance Priority List based on a combination of its frequency, toxicity, and potential for human exposure (AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, 2020). Human beings are daily exposed to Cd through occupational or non-occupational sources. The primary non-occupational sources of Cd exposure are from the food supply, in which leafy vegetables, potatoes,

grains, seeds, and organ meats such as liver contain high levels of Cd, and contaminated water. Smoking is another primary non-occupational source of Cd exposure since tobacco leaves accumulate high levels of Cd from the soil. Occupational sources include Cd exposure at the workplace from processes mainly involving heating Cd-containing materials such as smelting and electroplating (AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, 2012).

Among a complex variety of heavy metals, cadmium (Cd) is one of the environmental chemicals that rank among the priority metals that are of public health significance because of its high degree of toxicity (TCHOUNWOU et al., 2012). Cadmium is an environmental pollutant known as an endocrine disruptor (KABIR et al., 2015). To date, it is already known that Cd male reproductive toxicity is mediated by several mechanisms, including structural damage to testis vasculature and blood-testis barrier; inflammation; cytotoxicity on Sertoli and Leydig cells; oxidative stress; apoptosis; interference with selected signaling pathways and epigenetic regulation of genes involved in the regulation of reproductive function; and disturbance of the hypothalamus-pituitary-gonadal axis (*reviewed in* DE ANGELIS et al., 2017). However, it remains poorly understood the association between dose, route, and time of exposure and the injury, and the mechanisms that lead to histopathological alterations.

Cannabinoids, pharmacological and recreational chemicals

Drugs, prescribed and non-prescribed, have also been implicated in the etiopathogenesis of reproductive dysfunction (BONDE et al., 1996). Among the illicit drugs, cannabis has the highest consumption rate worldwide (WORLD HEALTH ORGANIZATION, 2021). Furthermore, the use of cannabis compounds, called phytocannabinoids (cannabinoids), has been placed under the spotlight for therapeutic use. To date, more than 100 cannabinoids (terpenophenolic secondary metabolites) have been identified in *Cannabis sativa* plants (GOULD, 2015). The Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant phytocannabinoids, best-studied, and most used in healing treatment because of their promising therapeutic applications (COHEN; WEIZMAN; WEINSTEIN, 2019).

Cannabis-derived chemicals have increased across the population as a potential therapeutic strategy in numerous pathological conditions such as epilepsy, Alzheimer's and Parkinson's diseases, multiple sclerosis, and cancer (FRAGUAS-SÁNCHEZ; TORRES-SUÁREZ, 2018).

Dronabinol, a synthetic THC, has been indicated in the treatment of nausea secondary to cancer chemotherapy and anorexia associated with weight loss in patients with acquired immune deficiency syndrome and is approved by the US Food and Drug Administration for those indications (FOOD AND DRUG ADMINISTRATION, 2017). CBD-based medicines are indicated for the treatment of seizures associated with Lennox-Gastaut syndrome or Dravet syndrome in patients 2 years of age and older (FOOD AND DRUG ADMINISTRATION, 2018). A mixture of THC and CBD can be found at the same medicine (Sativex), which is useful to adjunctive treatment for symptomatic relief of spasticity of neuropathic pain in adult patients with multiple sclerosis (GIACOPPO; BRAMANTI; MAZZON, 2017) or as an analgesic in adult cancer patients (LICHTMAN et al., 2018). Also, in several instances, THC and CBD have been associated with a reduction in cancer size or in the number of circulating cancer cells (HINZ; RAMER, 2019).

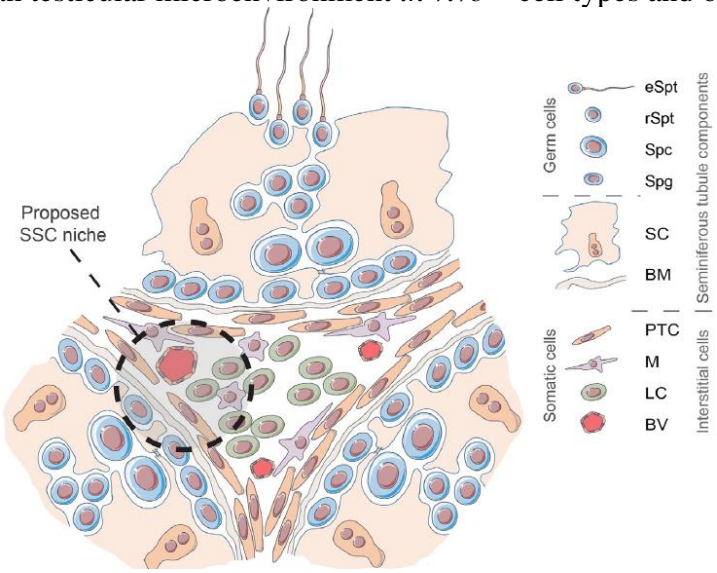
Cannabis's popularity as both recreational and medical drug is increasing especially among men of reproductive age. Since male factor infertility is increasing, exposure to cannabinoid compounds, mainly the two main phytocannabinoids: Δ^9-tetrahydrocannabinol (THC) and/or cannabidiol (CBD), both released by cannabis, could be a contributing cause (DU PLESSIS et al., 2015). However, the current evidence, which is limited and heterogenous, does not suggest clinically significant associations between cannabis use and testicular function (BELLADELLI et al., 2021). Pacey et al. (2014) and Gundersen et al., (2015) highlight that the use of cannabis in men is a risk factor for poor sperm quality. On the other hand, Nassan et al. (2019) reported that cannabis users had higher sperm concentrations. The divergence in outcomes is also observed at the hormonal level, in which increased (GUNDERSEN et al., 2015), decreased (KOLODNY et al., 1974), and non-effected (THISTLE et al., 2017) testosterone levels can be observed in cannabis users. However, for ethical reasons, these studies are limited in the scope to sperm and hormonal analyses. This way, considering the increase of cannabis use for both medical and recreational purposes, well-designed studies are needed to investigate the association between cannabis use and the male reproductive system.

The testicular microenvironment

Male fertility requires a testicular microenvironment where hormone production and spermatogenesis occur. Spermatogenesis is a complex biological process by which spermatogonial

stem cells self-renew and differentiate into haploid spermatids within the highly specialized architecture of the testis microenvironment (HESS; FRANÇA, 2008). The testes are organized into two distinct compartments: the seminiferous tubules and the surrounding interstitial space. The seminiferous tubules are further separated into basal and adluminal compartments by various cellular contacts (e.g. tight, adherence, gap, and desmosome) linking together adjacent Sertoli cells forming the blood-testis barrier. Spermatogenesis takes place in the seminiferous tubule. Spermatogonia reside within the basal compartment, while the spermatocytes and round and elongating spermatids are contained in the adluminal region. The basement membrane, comprised of extracellular matrix proteins such as fibronectins, laminins, and collagens, encloses the seminiferous tubule. Peritubular myoid cells line the outside of the basement membrane providing structural support. The basement membrane together with myoid cells form the tunica propria of the seminiferous tubules. Interstitial tissue is located between seminiferous tubules and hosts testosterone-producing Leydig cells, the vascular network, and various immune cell populations including macrophages, of which there are distinct peritubular and interstitial populations (RUSSELL; FRANÇA, 1995; OLIVER; STUKENBORG, 2020). The testicular cell types and organization are shown in Figure 1.

Figure 1 – The human testicular microenvironment *in vivo* – cell types and organization



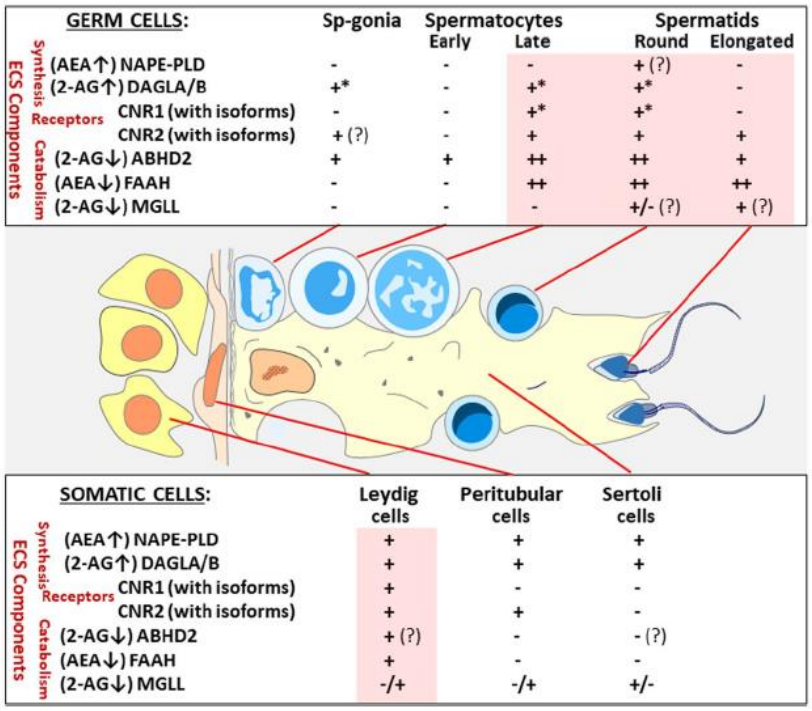
Sertoli cells (SC); Spermatogonia (Spg); Spermatocytes (Spc); Round (rSpt) and elongating (eSpt) spermatids; Basement membrane (BM); Peritubular myoid cells (PTC); Leydig cells (LC); Vascular network (BV); Macrophages (M). The dashed line represents the proposed spermatogonial stem cell (SSC) niche. Source: Oliver and Stukenborg, 2020.

The endocannabinoid system (ECS)

The research on cannabinoids led to the discovery of the cannabinoid receptors (CB) (DEVANE; HOWLETT; MELVIN, 1988) and, consequently, the identification of endogenous ligands of these receptors, which became known as endocannabinoids (eCB), such as Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (DEVANE et al., 1992; SUGIURA et al., 1995). This system of endogenous signals and receptors together with a complex system of synthesizing and hydrolyzing enzymes of eCB constitute the endocannabinoid system (ECS) (KILARU; CHAPMAN, 2020). It has become evident that most components of the mammalian ECS are highly conversed in evolution, pointing to a fundamental modulatory role in basic cellular and organismic functions (ELPHICK; EGERTOVÁ, 2005; ELPHICK, 2012). Accordingly, the ECS is widely expressed in vertebrates, central and peripheral organs, and regulates a large array of physiological functions and behaviors (BOVOLIN et al., 2014). It became clear that cannabinoid receptors and endocannabinoids are involved in re-establishing homeostasis after pathological insults. So, the ECS signaling has been studied as a focus of pharmacological manipulation and potential therapeutic exploitation for multiple pathologies, which justify the therapeutic use of cannabinoids (DI MARZO, 2009).

Recently, the ECS was described in the adult human testis (NIELSEN et al., 2019) (Figure 2). The findings of Nielsen et al. (2019) suggest a direct involvement of the ECS in the physiology of the human testis. Within the seminiferous epithelium, the specific and differential expression patterns of the ECS components were linked to be involved in the regulation of spermatogenesis. The transition from spermatogonia to early primary spermatocytes was associated with the relative silencing of the ECS receptors and metabolizing enzymes. The oppositive was observed in post-meiotic germ cells, with high expression levels of the catabolizing enzymes. Furthermore, their findings suggest the involvement of the ECS with the Leydig cell function, in which the AEA might have participation in steroidogenesis.

Figure 2 – Graphical summary of the main components of the endocannabinoid system (ECS) in different cell types of the adult human testis.



The ECS components are grouped into the synthesizing enzymes (with the main products shown in parentheses), the receptors, and the catabolic enzymes (main substrates in parentheses). Uncertain expression patterns, with some discrepancy between the transcript and protein levels, are denoted by question marks. The asterisks refer to the uncertain subcellular location of the protein (nuclear). The testicular cell types with the greatest activity of the ECS are shaded in pink. Anandamide (AEA); 2-arachidonoylglycerol (2-AG); N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD); diacylglycerol lipase (DAGLA/B); cannabinoid receptor isoforms (CNR1, CNR2); α/β-hydrolase domain-containing protein 2 (ABHD2); fatty acid amide hydrolase (FAAH); monoacylglycerol lipase (MGLL). Source: Nielsen et al. 2019.

Considering that cannabinoids can bind to CB, cannabis use might disrupt the ECS signaling in the male reproductive tract (or at the central level). To date, there is conflicting evidence regarding the phytocannabinoid effects on male reproductive health. Some studies suggest detrimental effects on fertility (RAJANAHALLY et al., 2019; CARVALHO et al., 2020) while others showed inconclusive evidence to support this notion (BELLADELLI et al., 2021; MACCARRONE et al., 2021) or even improvement of sperm quality (NASSAN et al., 2019). Then, we cannot exclude any cannabis' effect because of the limitation and heterogeneity of the studies. Therefore, further studies are needed to understand the association between cannabis use

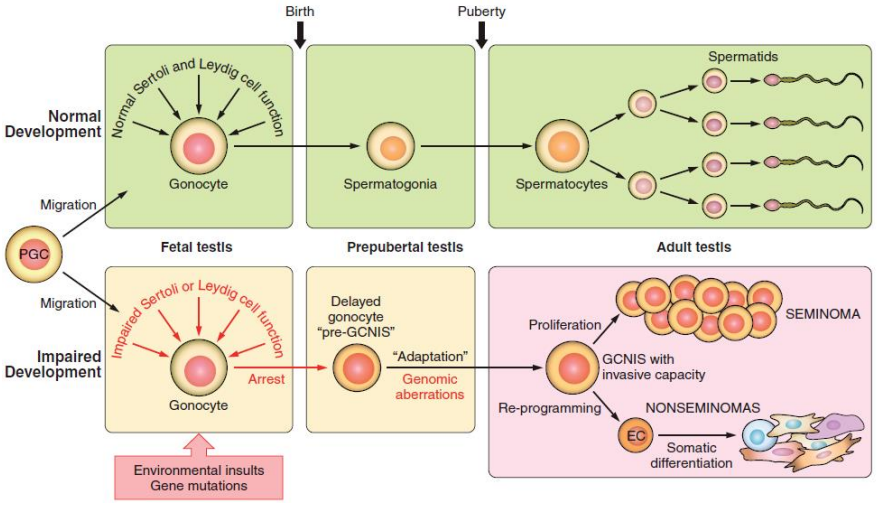
and male reproductive health and even to better describe the involvement of the ECS in male reproductive function.

Fetal origin hypothesis for male reproductive disorders

Interestingly, there is evidence that a large fraction of human male reproductive disorders is of fetal origin (JUUL et al., 2014). Male reproductive problems, specifically poor sperm quality, testis cancer, undescended testis, and hypospadias may be disorders associated with each other, as risk factors for each other, and have been hypothesized to be symptoms of one underlying entity, the testicular dysgenesis syndrome (TDS) (SKAKKEBAEK et al., 2001). The TDS is a resulting phenotype of the association of these male reproductive disorders, in which its histological signs have been identified in many infertile men. This syndrome is thought to be from the disruption of embryonal programming and gonadal development during fetal life (WOHLFAHRT-VEJE et al., 2009), which can be caused by several factors such as adverse environmental exposure, genetic aberrations and epigenetic factors, and lifestyle factors, such as smoking (SKAKKEBAEK et al., 2016).

Testicular germ cell tumors (TGCT) are one of the male reproductive problems hypothesized to be of fetal origin (SKAKKEBAEK et al., 2016). Environmental insults and genetic factors cause failure of normal fetal programming of the differentiation of primordial germ cells through a gonocyte stage into spermatogonia (Figure 3). At the same time, cannabis consumption has been positively associated with TGCT development (GHASEMIESFE et al., 2019; SONG et al., 2020). So, it is plausible to think that phytocannabinoid use during the gestational period might lead to TGCT or even to other male reproductive disorders in adult life.

Figure 3 - Model for the pathogenesis of testicular germ cell tumors (TGCT) of young adults



TGCT are derived from germ cell neoplasia in situ (GCNIS). Primordial germ cells (PGC). Source: Skakkebaek et al., 2016.

Thus, this study aimed to analyze two global trends in male reproductive health problems: cadmium exposure and phytocannabinoids use. This present thesis is divided into three chapters which were written in scientific article format. The first paper is a systematic review regarding Cd exposure and testis susceptibility, which was published in 2020 in the *Biological Trace Element Research Journal*. Systematic review is an invaluable tool for identifying, appraising, and synthesizing, through its standardized methodology and impartially, research-based evidence related to formulated questions (COCHRANE COLLABORATION, 2020). Jointly, systematic reviews have received increasing attention for being a potential tool for answering issues in the field of toxicology (HOFFMANN et al., 2017). So, the first chapter aimed to assess whether Cd exposure (in any dose, route, and time of exposure) causes significant testicular tissue alterations, including any outcome of testicular histomorphology, as well as molecular, biochemical, and hormonal evaluations performed to understand the mechanisms involved in the histomorphological changes, in murine models. The last two chapters are experimental studies regarding the impact of phytocannabinoids on male reproductive biology. The second chapter assessed whether THC and/or CBD represent a hazard for adult human testis, by using a unique *ex vivo* model. The third chapter evaluated the effect of prenatal exposure to a mixture of these phytocannabinoids on the male reproductive health of mice in adult life. Therefore, this thesis brings great insights into male repro-toxicology and reproductive health.

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2. CHAPTER 1

*Article published in the *Biological Trace Element Research Journal*

Biological Trace Element Research
https://doi.org/10.1007/s12011-020-02389-0

Cadmium Exposure and Testis Susceptibility: a Systematic Review in Murine Models

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Received: 25 May 2020 / Accepted: 10 September 2020

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Abstract

It is known that cadmium induces damage to the testis. However, the significant cadmium impact on the testicular architecture and the mechanisms involved in this process are not clear. Besides, the relationship between dose, route, and time of exposure and injuries remains poorly understood. Thus, we aimed to assess whether cadmium exposure in any dose, route, and time of exposure causes significant alteration in the testicular tissue of murine models, as well as the main mechanisms involved. We performed a structured search on the Medline/PubMed and Scopus databases to retrieve studies published until September 2018. The results were organized into an Adverse Outcome Pathway (AOP) framework. Also, a bias analysis of the included studies was performed. We included 37 studies, and most of them identified significant histopathologies in both tubule and intertubule regarding routes, in a dose- and time-dependent manner. The damages were observed after the first hours of exposure, mainly vascular damages suggesting that vasculature failure is the primary mechanism. The AOP showed that potential molecular initiating events may mimic and interfere with essential elements disrupting proteins (structural and antioxidants), change in the oxidative phosphorylation enzyme activities, and gene expression alteration, which lead to reproductive failure (adverse outcome). Analysis of methodological quality showed that the current evidence is at high risk of bias. Despite the high risk of bias, cadmium triggers significant lesions in the testis of murine models, regarding routes, in a dose- and time-dependent manner, mainly due to vascular changes. Therefore, cadmium is a risk factor for male reproductive health.

Keywords: reproductive toxicology, environmental contaminant, adverse outcome pathway (AOP), vascular damage, male reproductive health

2.1 Introduction

Cadmium (Cd) is of great concern due to its ubiquitous and non-biodegradable characteristics, toxicity, and tendency of bioaccumulation [1, 2]. Human beings are daily exposed to this ubiquitous environmental toxicant through non-occupational sources, including food and water ingestion, and occupational sources, mainly in processes involving heating cadmium-containing materials such as the production of alloys and batteries [3, 4]. Cadmium has been reported to cause several health disorders [5, 6], and at the same time, male infertility has been described as a global and significant health problem [7]. Among the causes of male infertility, some substances have shown negative effects on spermatogenesis and sperm quality [8]. In this context, Cd is one of the most investigated heavy metals that play a key role in male infertility due to the association between its high concentration in seminal plasma and decreased sperm quality in infertile men [9, 10].

Due to the exceptionally long half-life, 10 to 35 years, Cd accumulates in the body and can cause injuries [11, 12]. The Cd-induced reproductive toxicity has been reported, even at low doses and brief exposure [13, 14]. Testes are exceedingly susceptible to this persistent heavy metal, and the harmful effect is usually related to multiple mechanisms, such as inflammation, cytotoxicity, oxidative stress, interference with selected signaling pathways, epigenetic regulation of genes, and disruption of the hypothalamus-pituitary-gonadal axis. The testicular injuries include structural damage to the seminiferous epithelium, germinative and somatic cells, blood-testis barrier, and testis vasculature [15, 16]. In this context, understanding the histopathological changes of the testis after Cd exposure and the molecular and biochemical mechanisms involved in these alterations provide useful information about this toxic agent's impact on male reproductive health [17].

Currently, it is known that Cd induces testicular damages [18]. However, the significant impact of Cd on the testicular architecture and the mechanisms involved in this damaging process are not clear. Besides, it remains poorly understood if there is a relationship between dose, route, and time of exposure and the injury intensity. Thus, this information is extremely important to provide a direction for future research in this field and the development of decision making for therapeutic alternatives on the treatment of testicular injuries. Thereby, based on the published data associating Cd and reproductive toxicology and our knowledge that the testis is susceptible to

heavy metal-induced toxicity, we hypothesize that Cd can promote significant injuries in the testicular tissue by several routes, doses, and time of exposure in murine models.

Therefore, considering the fragmented knowledge, we performed this systematic review to assess whether Cd exposure (in any dose, route, and time of exposure) causes significant testicular tissue alterations, including any outcome of testicular histomorphology, as well as molecular, biochemical, and hormonal evaluations performed in order to understand the mechanisms involved in the histomorphological changes, in murine models. We also aimed at reporting what types of histomorphological analyses have been used to understand these effects. Given the uncertainties and controversies surrounding the action of Cd exposure in the testicular damage, the results of this review were organized into an adverse outcome pathway (AOP) framework to provide a systematic and transparent assembly of the evidence. In addition, the AOP's framework constructed in this review allowed the identification of the potential molecular initiating events (MIE) involved in the testicular damage process and the consequences to male reproduction health. The results of this study may help to understand the main mechanisms involved in the alterations triggered by other heavy metals, even other environmental contaminants, and, consequently, the translation to the human health risk assessment. Based on a detailed analysis of methodological bias, we also evaluated the force of the evidence by analyzing the advances and limitations of the studies carried out in this field.

2.2 Materials and Methods

This systematic review was conducted based on PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) [19]. Details of the protocol for this systematic review were registered on PROSPERO - International prospective register of systematic reviews (CRD42019158315). Details of the Population, Exposure, Comparators, and Outcome (PECO) are given in Online Resource 1 - Table S1.

Focus Question

What are the significant effects of Cd exposure considering several doses, routes, and time of exposure in the testicular tissue of adult murine models? What are the main testicular tissue

alterations including any outcome of the testicular histomorphology and consequently, other molecular, biochemistry, and hormonal evaluations involved in these processes?

Search Strategy

An extensive bibliography search was performed using the electronic databases Medline/PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and Scopus (https://www.scopus.com/home.uri), completed on September 21, 2018, at 2:13 p.m. For all databases, the search filters were based on three complementary levels: (i) animals, (ii) testis, and (iii) cadmium, which were combined by Boolean connectors [AND] (Online Resource 1 - Table S2). Search filters were initially developed for PubMed. The search algorisms [MeSH Terms] and [TIAB] were applied to identify indexed records and those recently published in an indexing process, respectively. To detect all *in vivo* animal model studies in PubMed, a standardized and optimized animal filter was used [20]. The terms used to search on PubMed were adapted to Scopus for the recovery of studies, and the "animals" filter was provided by the website itself.

As search strategies within both databases, the keywords "English" and "male" were selected because it is the official scientific language and to increase the search specificity, respectively. In addition, a backward search (hand-search) was performed, in which the reference list of each included study was manually screened for additional eligible studies that were not retrieved by our search.

Selection Criteria

After record identification through both databases, the duplicate studies were removed. Then, an initial selection based on title and abstract was performed. In this initial selection, we included pre-clinical studies in murine models that assessed the Cd effect on testicular architecture that did or did not perform molecular, biochemical, and/or hormonal analyses. All timings, frequencies, routes, and dosages of Cd (and compounds) exposure were eligible for inclusion. We excluded studies that did not evaluate the Cd exposure in the testicular histomorphology of murine models. Secondary (literature reviews, letters to the editor, case studies, comments, and editorials) and *in vitro* studies were also excluded. After the initial screening, all relevant studies were recovered in

full text and evaluated by the eligibility criteria. We excluded studies that either the full text was not available or did not meet the criteria described above.

Evidence Synthesis

Considering our objectives, data extraction was based on descriptive levels as follows: (i) characteristics of publication: authors, publication year, and country; (ii) characteristics of the experimental animals: animal model, age, weight, number of animals, number of animals per group, and number of groups; (iii) exposure: compounds, doses, periodicity of administration, route, duration, and existence of a control group; (iv) main histomorphological outcomes and analyses as well as the main molecular, biochemical, and hormonal results related with the histomorphological alterations; and (v) secondary outcomes. We classified the main histomorphological analyses in two levels: (i) qualitative analyses, in which the study reports the Cd effect in a descriptive way using words such as presence/absence, yes/no, and (ii) quantitative analyses, in which the study reports the Cd effect in an objective way related with numbers that are absolute value/percentage usually being the mean of the group. After the extraction of outcomes, which were identified as "key events," an AOP analysis was performed considering the main outcomes reported by included studies as well as how these key events are related to each other and how they can interact [21].

Bias Analysis

The quality of the studies was assessed by the criteria described on the SYRCLE's Risk of Bias (RoB) tool (Systematic Review Centre for Laboratory Animal Experimentation) designed specifically for animal studies [22]. The following methodological domains based on RoB were evaluated considering the following: selection bias - "Was the allocation sequence adequately generated and applied?", "Were the groups similar at baseline or were they adjusted for confounders in the analysis?", "Was the allocation to the different groups adequately concealed?"; performance bias - "Were the animals randomly housed during the experiment?", "Were the caregivers and/or researchers blinded regarding which intervention each animal received during the experiment?"; detection bias - "Were animals selected at random for outcome assessment?", "Was the outcome assessor blinded?"; attrition bias - "Were incomplete outcome data adequately

addressed?"; reporting bias - "Are reports of the study free of selective outcome reporting?"; other biases - "Was the study apparently free of other problems that could result in high risk of bias?". The items in the RoB tool were scored with "yes" (low risk of bias); "no" (high risk of bias); or "unclear" (indicating that the item was not adequately reported, and therefore, the risk of bias was unknown). Based on these items, we constructed a figure in the Review Manager 5.3 program, based on Cochrane Collaboration (RoB 2.0), to demonstrate the risk of bias across all studies included.

2.3 Results

Included Studies

Our search strategy allowed recovering 2112 records (MEDLINE—1277 and Scopus—835). After the removal of 653 duplicates, 1459 records were screened by reading the title and abstract. Then, 1410 studies were excluded due to inappropriate topics. Forty-nine studies were assessed through the eligibility criteria, of which 30 were included. The reference list screening resulted in 7 other records that met the eligibility criteria. Therefore, 37 records [23–59] were included in this systematic review (Fig. 1).

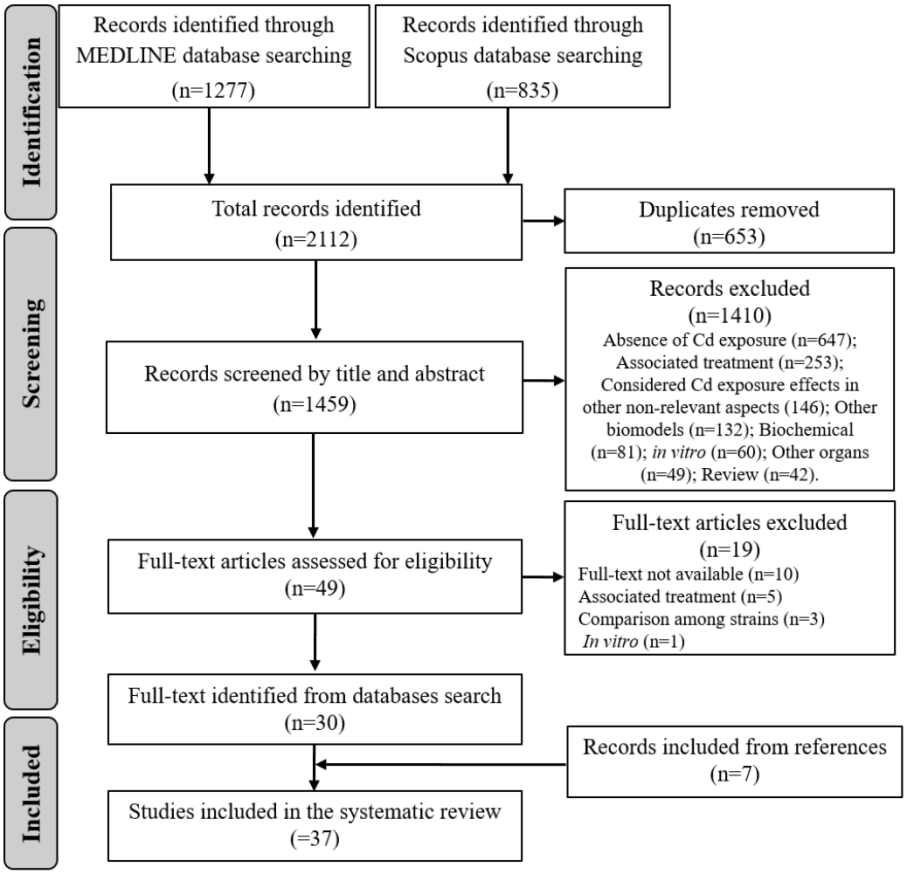

Fig. 1 Flow diagram of the results of the systematic review literature search. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (www.prisma-statement.org)

Characteristics of Publication, Experimental Animals, and Cd Exposure

The studies were published between 1959 and 2017, and they were conducted in several countries, including USA (24.32%), Brazil (10.81%), India, and Spain (8.11% each). Considering animal models, rats were the most used (64.87%), followed by mice (29.73%) and wild rodents (5.41%). Among the studies with rats, Wistar rats were the animal strain most used (54.17%). Among mice, Swiss mice were prevalent (54.55%), and two gerbil species were used in the wild rodents (Online Resource 1 - Table S3).

Concerning the metal exposure, cadmium chloride ($CdCl_2$) was the widely used compound (91.89%). The lowest compound doses used were 0.00005 mg/kg BW (body weight) $CdCl_2$ (0.00003 mg/kg Cd) (oral (*Or*), daily dose for 6 months) [37], and 5 μM/kg BW $CdCl_2$ (0.56 mg/L Cd) (intraperitoneal (*Ip*), single dose) [43]. The highest compound doses used were 326.2 mg/kg

BW $CdCl_2$ (200 mg/kg Cd) (*Or*, single dose) [29] and 1121.9 mg/L $CdCl_2$ (688 mg/L Cd) (*Or*, ad libitum for 70–80 days) [55]. Most studies assessed the Cd effect administrated in a single dose (54.05%), followed by studies that provided Cd *ad libitum* in drink water or food (18.92%). The subcutaneous route (*Sc*) was the most used route of administration (32.43%), followed by *Ip* and *Or* (24.32% each). The experiments lasted from 2 h to 30 months. About the control group, almost 22% of the records did not specify if the animals received something as placebo, and in 8.11%, the control group did not receive any treatment (Online Resource 1 - Table S4).

Main Alterations in the Testicular Tissue (Histopathological Outcomes)

The Cd effects on testicular histomorphology are shown in Fig. 2. Almost 83% of the studies that evaluated both interstitial tissue (intertubule) and seminiferous tubules identified damage in both compartments. Approximately 19% of studies evaluated exclusively the seminiferous tubules and all verified Cd-induced damage in this compartment, and 5% evaluated solely the interstitial tissue and all found injuries. Almost 67% of the studies that evaluated the tunica albuginea found injuries such as thickening and calcification. Only one included study did not observe any testicular histological alteration after Cd exposure.

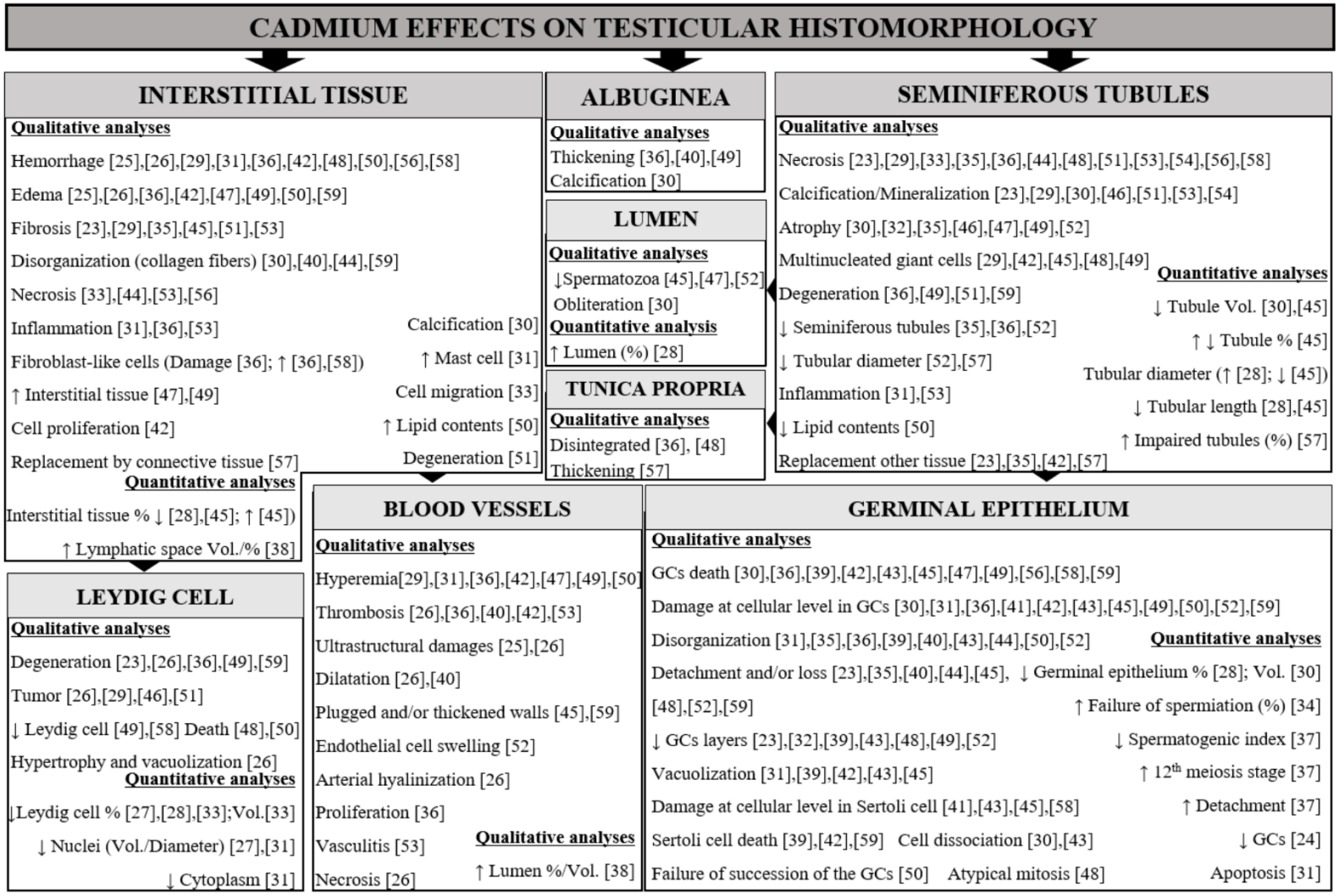

Fig. 2 Main outcomes of Cd exposure on testicular histomorphology in murine models. Vol. = volume; GCs = germ cells

Interstitial Tissue

A total of 31 studies evaluated the interstitial tissue, and almost 87% found damage in this compartment. The main interstitial injuries seen by the studies were hemorrhage, edema, fibrosis, disorganization (collagen fibers), necrosis, and inflammation. In addition to qualitative outcomes, changes in the percentage of this tissue were confirmed by quantitative analyses performed by studies that used this method.

Among the 23 studies that evaluated Leydig cells, 65% reported alterations in these interstitial cells such as degeneration and the presence of tumors considered adenomas. In addition, death in the Leydig cells was also reported in which necrosis was confirmed. The quantitative analyses showed a decrease in nuclei, cytoplasm, volume, and Leydig cell percentage.

Blood vessels were also investigated by 18 studies, and almost 83% confirmed that they are also targets for Cd-induced toxicity. Hyperemia was the most prevalent disorder, followed by thrombosis. Besides, dilatation of blood vessels was also verified by some studies. Ultrastructural changes in the blood vessels, such as tumefaction of endothelial cells, increase of pinocytotic vesicles, and loss of integrity of the desmosome complex were also reported. The quantitative analysis confirmed increase of lumen vessels.

Seminiferous Tubules

Similarly to that observed in the interstitial tissue, it was reported that Cd caused several alterations to the seminiferous tubules. A total of 35 studies evaluated this compartment, and almost 91% found damages. The main injuries reported were necrosis, calcification/mineralization, atrophy, and the presence of multinucleated giant cells. Degeneration of the seminiferous tubules was also observed. Some studies also verified that the damaged tubular areas were replaced by another tissue (fibrous connective tissue or amorphous mass). The quantitative analyses reported changes in the length, diameter, volume, and percentage of seminiferous tubules, and increase of impaired tubule number.

The germinal epithelium was greatly impaired by Cd administration, mainly the germ cells. Almost a third of the studies that evaluated the seminiferous tubules verified Cd induced death in this cellular type. Other damages were also observed at the cellular level in the germ cells such as vacuolization and heterochromatic and disordered nuclei. The injuries possibly led to the decrease of these cells and their layers, which was also reported. After Cd exposure, the germinal epithelium

was reported disorganized, with detachment and/or loss, and vacuolization. The Sertoli cells were also reported as a target of Cd-induced toxicity. Damage at the cellular level, such as vacuolization and disruption of blood-testis barrier, and Sertoli cell death were confirmed. The decrease of the germinal epithelium and spermatogenic index and the increased failure of spermiation and detachment were also confirmed by quantitative analyses.

Considering the lumen changes observed by studies that analyzed the seminiferous tubules, they are described as spermatozoa reduction, lumen obliteration, and a significant increase in its percentage. In addition to seminiferous tubule injuries, some records observed that the tunica propria was disintegrated and thickened.

Main Histomorphological Analyses

Most studies (70.27%) assessed the Cd effects on testicular tissue by qualitative analyses (i.e., descriptive analysis related to words - presence/absence, yes/no). However, quantitative analyses (i.e., objective analysis related to numbers - absolute value/percentage) were also used (13.51%) and some studies (16.22%) used both methodologies.

Regarding seminiferous tubules, approximately 90% of the studies that evaluated this compartment by qualitative analyses reported alterations, and almost 78% of the studies that used quantitative analyses found some tubular changes. Concerning interstitial tissue, 86% of the studies that evaluated this tissue by qualitative methods verified some injuries, and all studies that used quantitative analyses found some alterations in this tissue (Fig. 2).

Correlation Between Study Characteristics and Testicular Tissue Alterations

The characteristics of the studies, mainly the experimental animal model, route, dose, and time of exposure, play a crucial role in the Cd-induced testicular toxicity. Considering the animal model, all studies that used rats looked for seminiferous tubular alteration, in which almost 92% found some injuries in this compartment, and 20 studies looked for intertubular alteration in which 85% found injuries. About mice, among the studies that used this animal model, 81.82% observed the seminiferous tubules and all reported injuries, and 9 studies looked for alteration in the interstitial tissue, which was confirmed by 88.89%. It is important to point out that several alterations observed in both seminiferous tubules and interstitial tissue (Fig. 2) were not specific to any animal model.

Regarding doses, specifically, the lowest Cd compound doses in which histomorphological alteration was reported were 0.005 mg/kg BW $CdCl_2$ (0.003 mg/kg Cd) (*Or*, daily for 6 months) [37] and 15 μM/kg BW $CdCl_2$ (1.68 mg/L Cd) (*Ip*, single dose, 48 h) [43]. Damages at germ cells (pyknosis) and detachment of the germinal epithelium were reported by these studies, respectively. In addition, 45.95% of the studies compared low and high dose-response effects and most of them confirmed that the histopathological outcomes were dose-dependent such as epithelial damages, mainly death of germ cells, and interstitial damages, for example, Leydig cell tumors. Furthermore, when some studies compared several dose frequencies, they observed that a high single dose administration, at the same amount of Cd or even less, is worse than several low doses. They concluded it by the increase of calcification and necrosis of the seminiferous tubules observed by high single Cd doses.

Regarding routes, all studies that used the *Sc* route evaluated the seminiferous tubules and approximately 92% observed some alterations, and ten records analyzed the interstitial tissue, which was reported with changes by 90% of them. All the studies that used the *Ip* route evaluated the seminiferous tubules and found some alterations, while eight of them evaluated the interstitial tissue and damage was confirmed by almost 88%. Six studies that used the *Or* route observed the seminiferous tubules, and damages were reported by 67% of them. Damages in the interstitial tissue were reported by 75% of studies that evaluated this compartment and used the *Or* route.

Regarding the exposure time, although there is not a consensus between chronic and acute exposures, we considered acute exposure until 4 weeks and after this chronic exposure. Fourteen studies evaluated acute Cd exposure, in which all found injuries, and 13 studies evaluated chronic Cd exposure, in which 92% found changes. In addition, ten studies assessed both acute and chronic Cd effects, in which only 20% of them did not report alteration after both exposures.

In acute exposure, seven records evaluated Cd-induced toxicity in the first 2 to 24 h and almost 71% found early morphological changes mainly in the blood vessels, such as loss of integrity of the desmosome complexes (after 2 h) and thrombosis (after 14 h). Edema and hemorrhage (after 4 h) were also seen in the interstitial tissue. Fifteen studies observed the testis between 2 and 7 days of Cd exposure. The injuries were similar to those described initially, with some of them being in a time-dependent manner, as the failure of spermiation. In addition, at this stage, the studies also reported more tubular alterations, such as necrosis, calcification, and even damage to Sertoli cells. Lyses and decrease of Leydig cells were also confirmed. All of 12 studies

that evaluated the testis between 10 days and 4 weeks of Cd exposure also reported damages. Interstitial injuries such as hemorrhage and necrosis were observed, as well as tubular damage such as degeneration of germinal epithelium, germ cell necroses, and damage at Sertoli cells.

Lastly, almost 92% of the studies that assessed the chronic Cd effects (between 35 days and 33 months) reported several histopathological changes in both testicular compartments. Extensive tubular necroses, calcification, decrease of germ cells, and vacuolization of Sertoli cells were the main tubular alterations, while in the interstitial tissue hemorrhage, fibrosis, and damage in the Leydig cells as tumors were confirmed.

Main Mechanisms Involved in the Histopathologies: an AOP Framework

Some records performed molecular, chemical, and/or hormonal analyses to answer whether the Cd-induced toxicity affects other parameters in order to understand the histomorphological alterations. Approximately 16% of the studies [31, 32, 44, 48, 52, 56] evaluated the testis at the molecular level and observed some modifications, such as increase of DNA oxidation with increase of apoptotic index [31] and increase of expression of autophagy-related proteins (e.g., Beclin 1) that are associated with testicular injuries [52]. In addition, another study reported increased expression of *Cmyc* and *Egr1* genes suggesting stress response, and repressed expression of pro-apoptotic (e.g., *Casp3*) and DNA repair genes (e.g., *Msh2*), which possibly also contributes to Cd induced carcinogenesis [56].

The oxidative stress markers were investigated [30, 38], which detected increased antioxidant enzyme activities, such as catalase, superoxide dismutase, and/or total glutathione. This increase suggests that the upregulation of antioxidant enzymes was due to increased generation of reactive oxygen species (ROS), which could justify some alterations such as in the vascular tonus. An increase of lipid and protein peroxidation markers was also observed by these studies, which also contributes to injuries in the tissue. One study assessed the essential mineral content in which microminerals such as selenium (Se), copper (Cu), Iron (Fe), and magnesium (Mg) were significantly reduced, while the calcium (Ca) concentration increased, which justified the calcification seen by this study [30].

In addition, changes in the adiponectin levels were verified, which can affect the testosterone levels or can be affected by it [31]. A decrease of vimentin in fibroblasts and endothelial cells was also reported [40]. Change in the activity of some oxidative phosphorylation enzymes (adenosine

triphosphatase and succinic dehydrogenase) in seminiferous tubules was also confirmed and can lead to increase of ROS [50]. Regarding the hormonal evaluation, the results showed disturbances in luteinizing hormone (LH), follicular stimulating hormone (FSH), and/or testosterone levels, observing that the downregulation of steroidogenesis can affect or be affected by morphological changes, mainly in Leydig cells [31, 39, 57].

The main histomorphological outcomes as well as the molecular, chemical, and hormonal results were organized in an AOP network to provide a systematic and transparent assembly of the evidence regarding Cd effects in the testicular tissue. It was possible to identify by molecular and chemical outcomes three potential molecular initiating events (MIE): (i) mimicry and interference of Cd with essential elements, which disrupt some essential element-dependent proteins (structural or antioxidant enzymes); (ii) change in the activity of oxidative phosphorylation enzymes; and (iii) gene expression alteration. The first cited MIE can disrupt directly the endothelial cells, causing loss of junctional complex integrity (e.g., desmosomes), in which the first histopathologies are related to circulatory failure. These MIE trigger mainly increase in ROS levels and, consequently, oxidative stress, which leads to several histomorphological alterations and even death in endothelial, Leydig, germ, and Sertoli cells. Changes in the testis weight and appearance proceed these alterations as observed by some included studies. All these key events lead to a significant decrease of spermatogenesis and steroidogenesis resulting in reproductive failure (Fig. 3).

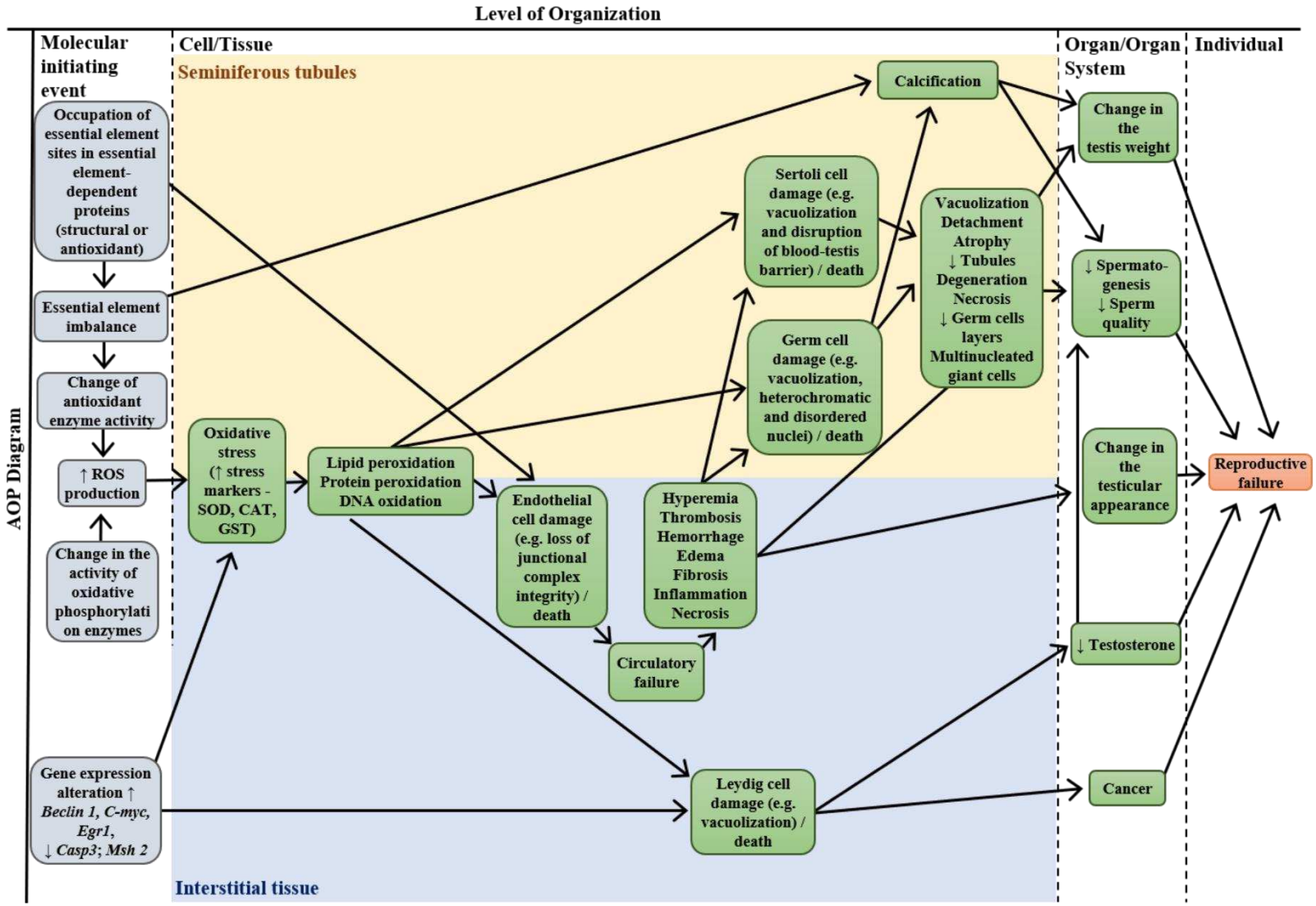
Level of Organization
AOP Diagram
Molecular initiating event
Cell/Tissue
Organ/Organ System
Individual
Seminiferous tubules
Occupation of essential element sites in essential element-dependent proteins (structural or antioxidant)
Essential element imbalance
Change of antioxidant enzyme activity
↑ ROS production
Change in the activity of oxidative phosphorylation enzymes
Gene expression alteration ↑ Beclin 1, C-myc, Egr1, ↓ Casp3; Msh 2
Oxidative stress (↑ stress markers - SOD, CAT, GST)
Lipid peroxidation Protein peroxidation DNA oxidation
Endothelial cell damage (e.g. loss of junctional complex integrity) / death
Circulatory failure
Hyperemia Thrombosis Hemorrhage Edema Fibrosis Inflammation Necrosis
Calcification
Sertoli cell damage (e.g. vacuolization and disruption of blood-testis barrier) / death
Germ cell damage (e.g. vacuolization, heterochromatic and disordered nuclei) / death
Vacuolization Detachment Atrophy ↓ Tubules Degeneration Necrosis ↓ Germ cells layers Multinucleated giant cells
Leydig cell damage (e.g. vacuolization) / death
Change in the testis weight
↓ Spermato-genesis ↓ Sperm quality
Change in the testicular appearance
↓ Testosterone
Cancer
Reproductive failure
Interstitial tissue

Fig. 3 Adverse Outcome Pathway (AOP) analysis based on the main and prevalent results of the included studies. Cadmium (Cd)-induced testicular histopathologies are associated with three potential molecular initiating events (MIE): (i) mimicry and interference of Cd with essential elements, which disrupt some essential element-dependent proteins (structural or antioxidant enzymes); (ii) change in the activity of oxidative phosphorylation enzymes; and (iii) gene expression alteration. The first cited MIE can disrupt directly the endothelial cells, in which the first histopathologies are related to circulatory failure. These MIE trigger mainly increase in ROS levels and, consequently, oxidative stress, which leads to several histomorphological alterations and even death in endothelial, Leydig, germ, and Sertoli cells. Also, the essential element disbalance is directly related to calcification, so this severe histopathology is also observed in acute exposure. All these key events lead to a significant decrease of spermatogenesis and steroidogenesis resulting in reproductive failure. Gray boxes = molecular initiating event; green boxes = key events; orange box = adverse outcome; arrows = key event relationship. ↑ inside the box = increase; ↓ inside the box = decrease; ROS = reactive oxygen species; SOD = superoxide dismutase; CAT = catalase; GST= total glutathione.

Blood and Testicular Cadmium Concentrations

In a complementary manner, 15 studies verified the Cd concentration in the testis and/or blood after exposure. Almost 87% of them measured the amount of Cd in the testis and 20% in blood. Of these that measured the Cd amount, only one did not detect Cd in the testis. However, 60% of the studies did not measure the tissue concentration of Cd after exposure (Fig. 4).

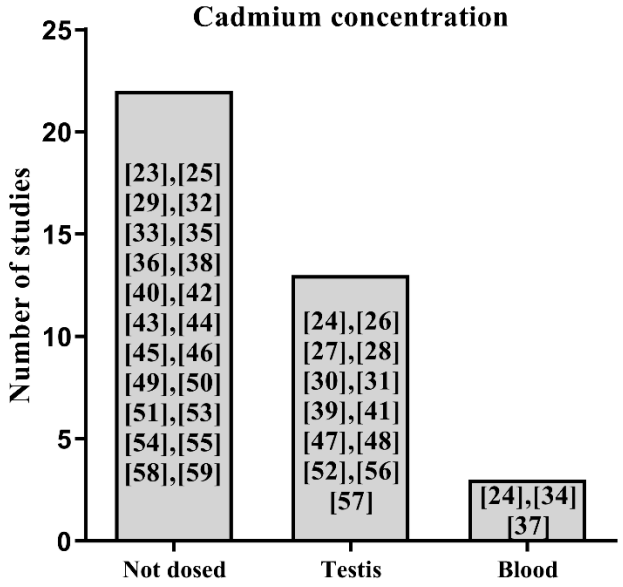

Fig. 4 Studies that did or did not dose the blood and/or testicular cadmium concentration

Risk of Bias

Figure 5 shows the percentage of each risk of bias item across all included studies. The result for the risk of bias assessment of individual studies is available in Online Resource 1 - Fig. S1. No studies fulfilled all methodological criteria analyzed. Ten studies described the baseline characteristics (strain, weight, and age) among the animals, but most studies (n = 27) have some baseline characteristics missing or did not mention whether the experimental groups were similar at baseline characteristics. Twenty-three studies did not mention allocation concealment, and 12 studies reported the randomization of animals among the groups but did not describe the used methods. Two studies did not have more than one experimental group, which presented low risk of bias for this item. More than half of the studies reported that the animals were exposed to standardized room conditions and specified these conditions, whereas in the other studies this information is unclear or not provided. None of the studies reported about random sequence generation, blinding of participants and personnel (caregivers and investigators), random outcome assessment, and blinding of outcome assessment (outcome assessor). Regarding incomplete outcome data, almost 32% of the studies reported whether there was animal exclusion and the reason for the measure, while in the other records this information is not clear, or they did not mention anything about it. Most studies were free of selective outcome reporting. However, some studies did not mention all protocols or did not include all expected outcomes (compared methods and results), which poses a high risk of bias or omitted some important information, which made it unclear. More than half of the studies were seemingly free from other problems that could increase the risk of bias. Conversely, some studies presented unclear risk because either the control animals received nothing as placebo or they did not mention about it. Moreover, some studies presented high risk due to the absence of control or different periods of intervention between control and treatment groups, or they did not report whether wild animals were pathogen-free.

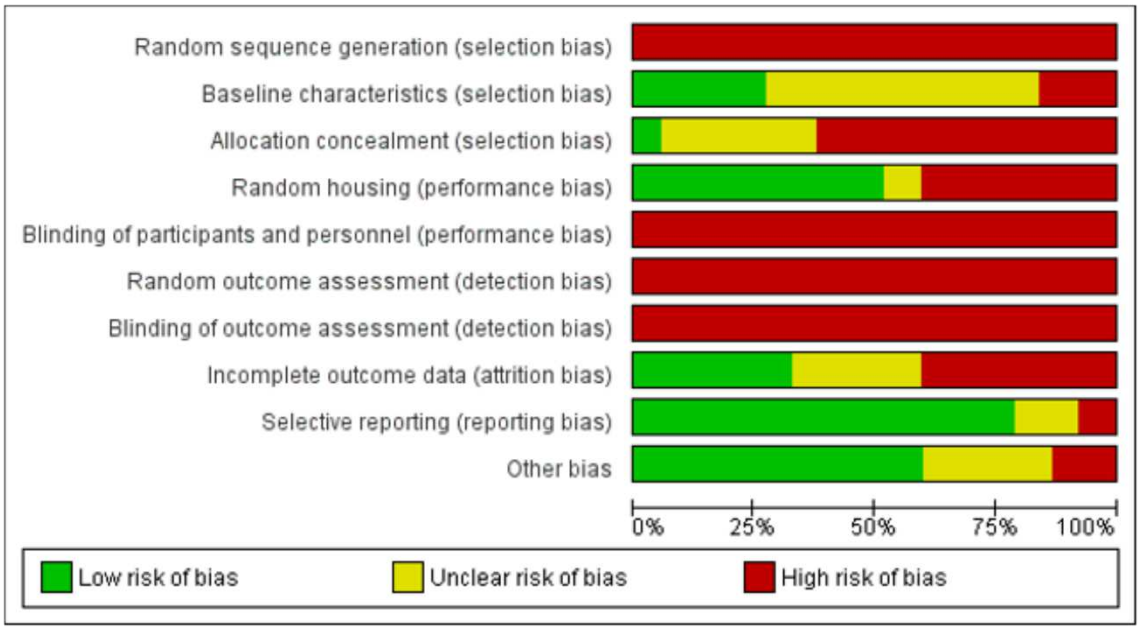

Fig. 5 Results of the risk of bias and methodological quality indicators for all included studies that evaluated the Cd impact on testicular histomorphology of murine models. The items of SYRCLE's RoB tool were scored with "yes" (low risk of bias); "no" (high risk of bias); or "unclear" (indicating that the item was not adequately reported, and therefore, the risk of bias was unknown)

2.4 Discussion

In our study, a systematic review was conducted to assess the significant Cd impact on the testicular tissue of adult murine models, in any dose, route, and time of exposure, and the main mechanisms involved in the damage process. Our results revealed strong evidence that Cd exposure induces severe histopathologies in both seminiferous tubules and interstitial tissue of murine models regarding routes, in a dose- and time-dependent manner. The main mechanisms involved in these processes may be related to three potential molecular initiating events (MIE) identified by the adverse outcome pathway (AOP) framework: mimicry and interference of Cd with essential elements, which disrupt some essential element dependent proteins (structural or antioxidant enzymes); change in the activity of oxidative phosphorylation enzymes; and gene expression alteration. The first reported MIE disrupts directly the endothelial structural proteins, in which the primary mechanism involved in the lesion may be vascular damage. All the MIE contribute to the increase of free radicals and consequently increase of reactive oxygen species (ROS) production, which leads to oxidative stress in the tissue with alterations in the antioxidant enzyme activities

and, therefore, several injuries in the testis. Furthermore, the testis proved to be an accumulation target of this environmental toxicant, which contributes to the development of testicular damages.

Our results showed that the concern about Cd-induced toxicity on testicular histomorphology of murine models has been reported in the literature since 1959 [42]. For this purpose, a wide variety of strains were used by the included records, in which rats were the most commonly used followed by mice. Rats have become a species of choice in toxicological research because of its size and relatively docile and physiological similarities, and mice are generally more economical [60]. However, it has already been reported that sensitive and resistant murine models may exist in toxicological bioassays [61]. Our findings indicate that there is not a species-sensitivity distribution between rat and mouse in Cd-induced histopathologies, in which the Cd is able to induce injuries regarding the murine model and these histopathologies were not species-specific (Fig. 6 (a)). It is probably because rats tend to be more sensitive for determining toxicological outcomes [61] and mice are more susceptible to stress-induced testicular changes [62], so injuries can be found in both species. This result that is not strain-specific is supported by our AOP analysis, in which Cd can trigger the MIE regarding the murine model leading to histopathologies. The sensitivity to Cd-induced testicular toxicity may be related to Cd accumulation [63, 64].

The Cd effect on tissue is directly related to doses of exposure, in which in some pathologies, its action appears to be bidirectional and determined by Cd concentration. For example, in tumor angiogenesis, Cd shows to be either stimulatory or inhibitory depending on the concentration [65]. However, our results show that there is strong evidence about testicular histopathological manifestations that are dose-dependent and the damages are intensified following the increase in Cd concentration. In addition, a high single dose is worse than several low doses, even at the same amount of Cd or less (Fig. 6 (c)).

Regarding routes of exposure, the most common route of environmental Cd exposure in animals and humans is oral (*Or*), and in toxicological studies, it is the intraperitoneal (*Ip*) route [66]. However, our findings indicate that the subcutaneous (*Sc*) route is mostly used to evaluate histomorphological alterations, followed by *Ip* and *Or* routes. We believe that the *Sc* route was preferred because it is less aggressive than *Ip*, and it is possible to know the amount of Cd available on the body, differently from the Or route in which Cd is absorbed by the duodenum and the amount of Cd that reaches the organism is unknown [67]. Also, our findings indicate that there is strong evidence that Cd exposure can cause testicular histopathologies by all these routes in both tubule

and intertubule compartments, even though damages in the seminiferous tubules were more frequently observed by studies that chose direct routes (i.e., *Sc* and *Ip*) other than indirect routes (i.e., *Or*) (Fig. 6 (d)).

Recently, it has already been reported that Cd could affect male reproductive health during both acute and chronic exposure [68], and the severity of Cd-induced reproductive toxicity is also time-dependent [65]. So, our results indicate robust evidence that the Cd-induced testicular toxicity is fast, showing severe testicular damages during the first 24 h or 7 days of exposure. Furthermore, modifications that were expected only in chronic phases such as degeneration, necrosis, and calcification were also observed in the acute phase in the first days of exposure, which support our findings. Some histopathologies were intensified in a time-dependent manner, sustaining that the mechanisms of histopathological injuries start in the acute phase and are increased and enhanced during the chronic phase (Fig. 6 (e)).

The AOP has been a multifaceted framework that supports the twenty-first-century toxicology [69]. AOP is conceptually synonymous of mode of action developed to analyze the relevance of toxicological effects of chemicals and even nonchemical substances observed in animals to human health risk assessment [21]. In addition, AOP has received substantial attention as an organizing framework for reproductive toxicity [70]. Based on this, we constructed an AOP network to better explain the histopathologies and the main mechanisms involved in these damage processes. So, we scored three potential MIE that may be responsible for causing the main testicular histopathologies that lead to reproductive failure: mimicry and interference of Cd with essential elements, which disrupt some essential element dependent proteins (structural or antioxidant enzymes); change in the activity of oxidative phosphorylation enzymes; and gene expression alteration (Fig. 6 (f)). Although the evidence is slightly considerable due to few studies that performed molecular and chemical analyses and the high level of bias presented by some of them, this conclusion is in accordance and consistent with other studies. These MIE have already been reported as the cause of pathologies caused by Cd in other organs [71, 72, 73].

Jointly, it has already been reported that heavy metals can affect the testes disrupting spermatogenesis and steroidogenesis via mechanisms that involve the increase in ROS production [75, 76]. Cadmium is able to occupy the sites of microminerals, such as zinc (Zn), copper (Cu), and selenium (Se), in the antioxidant enzymes, disrupting their activities and decreasing the micromineral concentration [77, 78].

Our findings indicate that the primary mechanism of Cd-induced disorders on testicular architecture is related to circulatory failure due to endothelial injuries, even in low (single dose of 7.5 mg/kg BW $CdCl_2$ [25]) or high (single dose of 20.25 mg/kg BW $CdCl_2$ [42]) doses, independently of exposure route and animal model. This conclusion is supported by the appearance of interstitial injuries related to vasculature changes after brief Cd exposure (3 h [25]; 12 h [50]; 1 day [49]; 5 days [40]; 7 days [31]), such as hemorrhage, edema, thrombosis, and hyperemia (Fig. 6 (g)). In hyperemia, inflammatory mediators are released and cause dilatation and increase in the permeability of vessels which leads to the development of an inflammatory process, edema, hemorrhage, ischemia, and, consequently, degeneration and necrosis in the testis [15, 75] (Fig. 6 (h)). Cadmium affects vessel homeostasis by causing structural [25, 40], metabolic, and functional [16, 26, 80] damages at endothelial cells. Since the endothelial junctional complexes are composed of calcium (Ca^{+2})-dependent proteins (e.g., cadherins), the first cited MIE disrupts directly the integrity of junctional complexes (e.g., desmosomes and adherens junctions) [25, 40, 80]. Then, the primary mechanism involved in Cd-induced toxicity may be vascular damage (Fig. 6 (f)). Interestingly, injuries related to circulatory failure are not restricted to short time exposure, indicating that these injuries do not repair if the exposure continues [26, 47]. Therefore, there is strong evidence that blood vessel alterations are the first manifestation of the histopathologies triggered by Cd exposure, in which this mechanism is activated in the acute phase and continued on the chronic phase.

In addition to injuries in the blood vessels, the main histopathologies in the interstitial tissue were fibrosis, disorganization, and proliferation of fibroblast-like cells (Fig. 6 (h)). All these interstitial changes may affect other cellular populations from the interstitium, mainly the Leydig cells, and seminiferous tubules as a secondary consequence. Leydig cells are important targets of high-dose Cd [56]. Our findings indicate that the morphological changes in the Leydig cells are correlated to high doses or increase in the time of Cd exposure, such as degenerative changes and appearance of tumors (Fig. 6 (i)). In addition, the interstitial injuries can affect the fluid movement inside the testis resulting in injuries at the seminiferous tubules since they are dependent on interstitial tissue homeostasis [45, 65]. As a result, the seminiferous tubules can be affected by the direct Cd impact and interstitial alterations.

Usually associated with interstitial injuries, our findings indicate that Cd exposure causes atrophy, degeneration, necrosis, and calcification of the seminiferous tubules. Necrosis and

calcification/mineralization were the most common histopathological changes in the seminiferous tubules, besides germ cell death. Indeed, cell death and calcification are processes closely related, once increases in Ca^{+2} and inorganic phosphate in blebs (matrix vesicles) formed by apoptotic and/or necrotic cells are seemingly the primary mechanism of calcification. Additionally, membranous cellular degradation products resulting from cellular disintegration are often used as the nidus of this disorder [81]. The similarities between Cd^{+2} and Ca^{+2} allow Cd^{+2} to displace Ca^{+2} in some Ca^{+2}-binding proteins, and to disrupt Ca-mediated signaling pathways that lead to tissue damages [52]. Xie et al. [82] reported that Cd-induced cell death was mediated by the release of Ca^{+2} from intracellular storage, which confirmed that the increased Ca^{+2} intracellular concentration is related to cell death (Fig. 6 (j)).

Although the main histomorphological tool used by studies for understanding the Cd impact on testicular tissue was qualitative analysis, some records, mainly of this century, confirmed the injuries by quantitative tools. The qualitative evaluation of the testicular parenchyma allows the visualization of histomorphological injuries in the tissue caused by Cd, showing cell and tissue modifications that are probably responsible for the organ malfunction [30]. On the other hand, the use of quantitative tools and morphometric and stereological methods is very desirable because they are very sensitive instruments to detect non-evident alterations, which could not be confirmed merely by the observation of the testicular morphology [45, 83]. Indeed, all methods are fundamental for the searches, so relying on only one type of data (i.e., number or words) is extremely limiting. By using qualitative and quantitative techniques within the same framework, researchers can incorporate the strengths of both methodologies, adopting the principle of complementarity [84, 85].

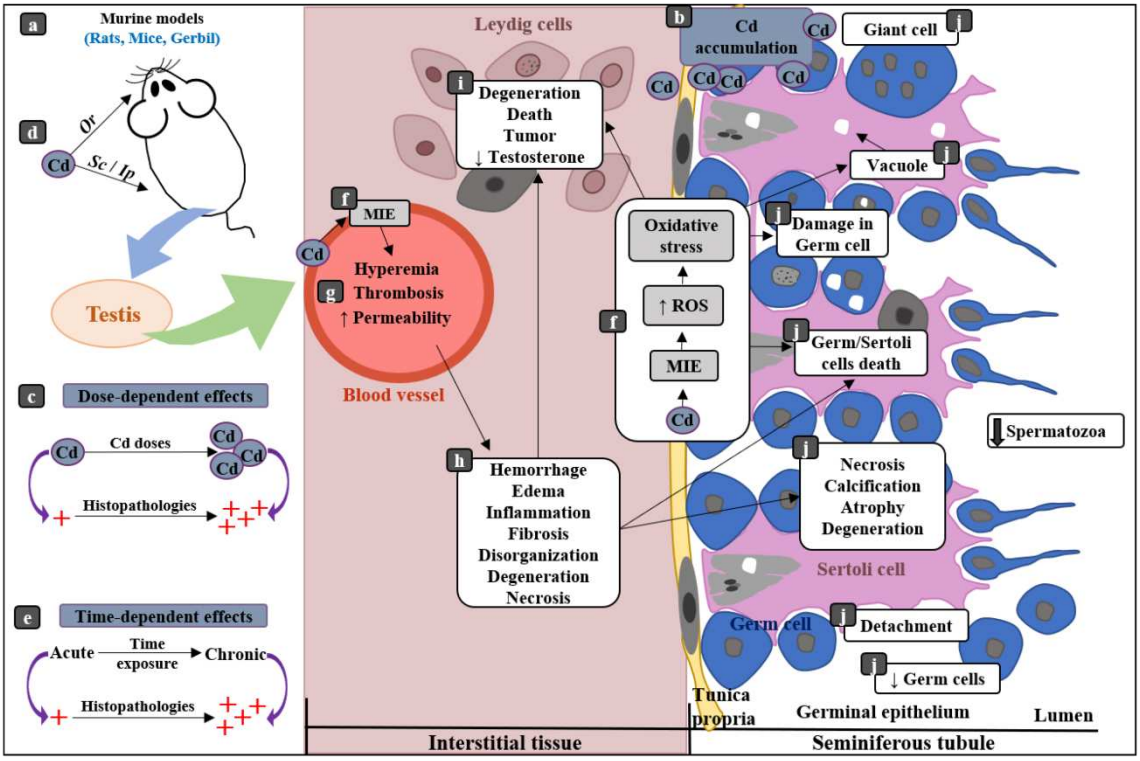

Fig. 6 Current understanding of the Cd effects on the testicular tissue of murine models. (a) All murine models are susceptible to Cd-induced testicular toxicity. (b) Cd accumulation in the testis. (c) The effects of Cd on testicular tissue are dose-dependent. (d) Main routes of Cd administration: Or = oral; Sc = subcutaneous; Ip = intraperitoneal. (e) The effects of Cd on testicular tissue are time-dependent. (f) Molecular initiating events (MIE) identified by the adverse outcome pathway (AOP) framework. (g) Histopathologies related to circulatory failure. (h) Main histopathological secondary consequences of vascular damages in the interstitial tissue. (i) Main histopathological damages in the Leydig cells. (j)Main histopathological damages in the seminiferous tubules including the germ and Sertoli cells. ↑ inside boxes = increase; ↓ inside boxes = decrease

Strength and Limitations of the Current Review

Recently, studies about exposure to environmental contaminants and male reproductive health are on the rise. However, no systematic review has been reported to investigate cadmium exposure and testicular tissue outcomes. The main strength of this study is its novelty and the applied findings that can be useful to provide a direction for future researches in this field and the development of decision making for therapeutic alternatives. Also, the AOP network of this study may help to understand the main mechanisms involved in the alterations triggered by other environmental contaminants, and, consequently, the translation to the human health risk assessment.

This review also has some limitations. The bias analysis demonstrated that fundamental characteristics, such as random sequence generation or random outcome assessment and blinding of participants (caregivers and outcome assessor), were not reported in the studies. In addition, some records provided incomplete outcome data and insufficient information about the control group, which affect the accuracy of the results. Overall, the evidence of the individual studies showed wide heterogeneity and so it was not possible to compare the data statistically. This kind of comparison should be avoided because it generates evidence that presupposes an apparent external validity (generalizability), which is in fact not supported by the available data set. In this sense, we identified that each study presented marked differences regarding the experimental model and methods of data collection, analysis, and interpretation, as well as the accuracy of the scientific report. In individual studies, each element of methodological bias is associated with some degree of variability in the research outcomes, with a direct impact on the quality of evidence. However, it is important to emphasize that all types of review have limitations and these limitations are more evident in systematic review studies once flaws methodological and incomplete reports can produce inaccurate and unreliable conclusions. In our case, the major limitation was the heterogeneity of the studies, which makes it an arduous task to compare them. Therefore, considering these analytical limitations, we developed a systematic review admitting its intrinsic qualitative nature by describing important points of bias. We hope to contribute to future studies on avoiding those elements of bias that impair the quality of evidence.

2.5 Conclusions

Our results support that the Cd exposure induces significant histopathologies in the testis of all murine models regarding routes, in a dose- and time-dependent manner, in which damages, even some expected only in the chronic phase (e.g., degeneration and necrosis), can be observed during the first hours of exposure. These results allowed us to conclude that the mechanisms involved in the histopathological process start in the acute phase and are increased and enhanced during the chronic phase.

The AOP shows that the main mechanisms involved in Cd-induced histomorphologies may be related to three potential MIE: mimicry and interference of Cd with essential elements; change in the activity of oxidative phosphorylation enzymes; and gene expression alteration. The first cited

MIE disrupts directly the endothelial structural proteins, in which the primary mechanism involved in the lesion may be vascular damage. All the MIE contribute to the increase in free radicals and consequently increase in ROS production, which leads to oxidative stress in the tissue with alterations in the antioxidant enzyme activities and, consequently, several injuries in the testis. Overall, our findings provide new insights into mechanisms of Cd-induced testicular toxicity.

2.6 Acknowledgments

The authors would like to thank Amanda Alves de Melo for English assistance, the support provided by Fundação do Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, processes PPM-00687-17 and PPM-00077-18), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processes 303972/2017-3, 423594/2018-4, 305093/2017-7, and MCTIC 408503/2018-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, finance code 001). Also, we thank CAPES for the PhD scholarship provided to Silva J.

2.7 Conflict of interest

The authors declare that they have no conflict of interest.

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2.9 Supplementary material

Table S1. PECO (Population, Exposure, Comparator, Outcomes) statement.

Parameter	Definition
Population	Any species of adult male murine models (mice, rats, gerbil).
Exposure	Exposure to cadmium as singular or in any cadmium compound. Any dose, route, and time of exposure.
Comparator	Control group consisting of animals undergoing treatment (i.e. placebo-only treatment) or not at all.
Outcomes	Any examination of the testicular tissue, as well as other evaluations performed together with histomorphology to understand the main mechanism involved in this process (e.g. molecular, biochemical, hormonal).

Table S2. Search strategy in PubMed and Scopus databases.

DATABASE	DESCRIPTORS/FILTERS	ITEMS FOUND	TIME	DATE
PUBMED	#1. LABORATORY ANIMALS PART 1 ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms])	6,320,352	14h04	09/21/2018
	#2. LABORATORY ANIMALS PART 2 (animals[Tiab] OR animal[Tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[Tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[Tiab] OR cottonrats[Tiab] OR hamster[Tiab] OR hamsters[Tiab] OR cricetinae[Tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[Tiab] OR swines[Tiab] OR piglets[Tiab] OR piglet[Tiab] OR boar[Tiab] OR boars[Tiab] OR "sus scrofa"[Tiab] OR ferrets[Tiab] OR ferret[Tiab] OR polecat[Tiab] OR polecats[Tiab] OR "mustela putorius"[Tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosphila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR pan[Tiab] OR paniscus[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR troglodytes[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR	4,549,355	14h04	09/21/2018

Table S2. (*Continuation*) Search strategy in PubMed and Scopus databases.

DATABASE	DESCRIPTORS/FILTERS	ITEMS FOUND	TIME	DATE
PUBMED	#2. LABORATORY ANIMALS PART 2 (*continuation*) prosimians[Tiab] OR "bush baby"[Tiab] OR prosimian[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR pongo[Tiab] OR pygmaeus[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutans[Tiab] OR pygmaeus[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR pongo[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR "fathead minnow"[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR "sea lamprey"[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR minks[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR			

Table S2. (*Continuation*) Search strategy in PubMed and Scopus databases.

DATABASE	DESCRIPTORS/FILTERS	ITEMS FOUND	TIME	DATE
PUBMED	#2. LABORATORY ANIMALS PART 2 (*continuation*) fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab] NOT medline[subset])			
	#3. TESTIS ["testis"[MeSH Terms] OR "spermatogenesis"[MeSH Terms] OR "testosterone"[MeSH Terms] OR "spermatogonia"[MeSH Terms] OR "spermatocytes"[MeSH Terms] OR "spermatids"[MeSH Terms] OR testis[Tiab] OR testicle[Tiab] OR testicles[Tiab] OR testes[Tiab] OR spermatogenesis[Tiab] OR testosterone[Tiab] OR spermatogonia[Tiab] OR spermatogonias[Tiab] OR spermatocyte[Tiab] OR spermatocytes[Tiab] OR spermatid[Tiab] OR spermatids[Tiab] OR leydig cells[Tiab] OR seminiferous tubules[Tiab] OR blood-testis barrier[Tiab] OR seminiferous epithelium[Tiab] OR sertoli cells[Tiab]]	202,821	14h05	09/21/2018
	#4. CADMIUM ("cadmium"[MeSH Terms] OR "cadmium poisoning"[MeSH Terms] OR "cadmium compounds"[MeSH Terms] OR "cadmium radioisotopes"[MeSH Terms] OR cadmium[Tiab] OR cadmium poisoning[Tiab] OR cadmium compounds[Tiab] OR cadmium radioisotopes[Tiab] OR cadmium radioisotope[Tiab] OR cadmium chloride[Tiab]] OR Cd[Tiab])	141,315	14h06	09/21/2018
	TOTAL: #1 OR #2 AND #3 AND #4	1,557	14h07	09/21/2018
	TOTAL: #1 OR #2 AND #3 AND #4 Short by: English	1,466	14h07	09/21/2018
	TOTAL: #1 OR #2 AND #3 AND #4 Short By: English; Male	1,277	14h08	09/21/2018
SCOPUS	#1. TESTIS (TITLE-ABS-KEY("testis") OR TITLE-ABS-KEY("spermatogenesis") OR TITLE-ABS-KEY("testosterone") OR TITLE-ABS-KEY("spermatogonia") OR TITLE-ABS-KEY("spermatocytes") OR TITLE-ABS-KEY("spermatids") OR TITLE-ABS-KEY("testicle") OR TITLE-ABS-KEY("testicles") OR TITLE-ABS-KEY("testes") OR TITLE-ABS-KEY("spermatogonias") OR TITLE-ABS-KEY("spermatocyte") OR TITLE-ABS-KEY("spermatid") OR TITLE-ABS-KEY("leydig cells") OR TITLE-ABS-KEY("seminiferous tubules") OR TITLE-ABS-KEY("blood-testis barrier") OR TITLE-ABS-KEY("seminiferous epithelium") OR TITLE-ABS-KEY("sertoli cells"))	296,559	14h10	09/21/2018
	#2. CADMIUM (TITLE-ABS-KEY ("cadmium") OR TITLE-ABS-KEY ("cadmium poisoning") OR TITLE-ABS-KEY ("cadmium compounds") OR TITLE-ABS-KEY("cadmium radioisotopes") OR TITLE-ABS-KEY ("cadmium radioisotope") OR TITLE-ABS-KEY ("cadmium chloride") OR TITLE-ABS-KEY ("Cd"))	458,944	14h10	09/21/2018
	TOTAL: #1 AND #2	2,891	14h11	09/21/2018
	TOTAL: #1 AND #2 LIMIT-TO "Animal"	996	14h11	09/21/2018
	TOTAL: #1 AND #2 LIMIT-TO "Male"	905	14h12	09/21/2018
	TOTAL: #1 AND #2 LIMIT-TO "Male"; "English"	835	14h12	09/21/2018

Standardized descriptors were defined by MeSH algorithm and non-MeSH descriptors were characterized by the [TIAB] algorithm, which was also used to recover recently published but not indexed studies (*in process*) on the PubMed database. A previously published and optimized animal filter was applied in the PubMed search interface [21]. The same search filters used were adapted for Scopus. The own Scopus animal filter (Keyword – animal [limit to]) was used in this database.

Table S3. Characteristics of publication and experimental animals of the studies that assessed the Cd effect on testicular histomorphology in murine models.

CHARACTERISTICS OF PUBLICATION		EXPERIMENTAL ANIMALS					
Reference	**Country**	**Animal model**	**Age (days)**	**Weight (g)**	**Number of animals**	**Animals per group**	**Groups**
Allanson M. and Deanesly R., 1962 [23]	England	Hooded rats	?	?	?	?	?
Aoyagi T., et al., 2002 [24]*	Japan	Sprague-Dawley rats	?	?	?	C=1 T=3	?
Berliner A. and Jones-Witters P., 1975 [25]	EUA	Gerbil *Meriones unguiculatus*	84-140	?	?	4	4
Blanco A., et al., 2010 [26]	Spain	Swiss mice	84	?	40	C=4 T=6	4
Blanco A., et al., 2009 [27]	Spain	Swiss mice	84	?	48	C=4 T=6 Wd=4	4
Blanco A., et al., 2007 [28]*	Spain	Swiss mice	84	?	48	C=4 T=6 Wd=4	4
Bomhard E., et al., 1987 [29]	German	Wistar rats	91-112	FE: 248 SE: 325	FE: 160 SE: 85	FE: C=10 T=30 SE: T=25 or 35	FE: 8 SE: 3
Cupertino M., et al., 2017 [30]	Brazil	Wistar rats	70	236	30	6	5
Cupertino M., et al., 2017 [31]	Brazil	Wistar rats	63	288	30	6	5
Davis J. and Coniglio J., 1967 [32]	EUA	Sprague-Dawley rats	105	?	5	5	1
Favino A., et al., 1966 [33]	Italy	Wistar rats	?	150-200	60	C=3 T=4	?
Hew k., et al., 1993 [34]	EUA	Sprague-Dawley rats	70	352	?	15	?
Ito T. and Sawauchi K., 1966 [35]	Japan	Japanese mice	PreT: 26 T: 28	?	40	?	6
Kar A. and Das R., 1962 [36]*	India	Albino rats	?	100-135	?	?	?

Table S3 (*Continuation*). Characteristics of publication and experimental animals of the studies that assessed the Cd effect on testicular histomorphology in murine models.

CHARACTERISTICS OF PUBLICATION		EXPERIMENTAL ANIMALS					
Reference	**Country**	**Animal model**	**Age (days)**	**Weight (g)**	**Number of animals**	**Animals per group**	**Groups**
Krasovskii G., et al., 1976 [37]	Russia	White random-bred rats	?	FE: ? SE: 300	?	?	?
Leite R., et al., 2015 [38]	Brazil	Wistar rats	70	?	20	5	4
Li X., et al., 2016 [39]	China	BALB/c mice	?	18-22	189	?	3
Marettová M., et al., 2010 [40]	Slovakia	Wistar rats	?	?	15	C=5 T=10	2
Medina M., et al., 2017 [41]	Argentina	Wistar rats	42-56	167	36	18	2
Meek E., 1959 [42]*	England	Albino mice	84	?	?	C=? T=30	?
Niknafs B., et al., 2015 [43]*	Iran	BALB/c mice	56-70	?	?	5	8
Oliveira H., et al., 2012 [44]	Portugal	Swiss mice	56	?	20	5	4
Predes F., et al., 2010 [45]	Brazil	Wistar rats	?	?	36	12	3
Reddy J., et al., 1973 [46]	EUA	Fisher-344 rats	28-35	80-100	30	C=10 T=20	2
Saygi Ş., et al., 1991 [47]	Turkey	Wistar rats	35	100	28	C=8 T=20	2
Selypes A., et al., 1992 [48]	Hungary	CFLP mice	70-84	?	70	10 or 25	4
Sharma, S. and Kaur S., 2012 [49]*	India	Swiss mice	?	20-25	?	?	3
Singh K. and Mathur R., 1968 [50]	India	Gerbil *Meriones hurrianae*	?	?	?	?	?

Table S3 (*Continuation*). Characteristics of publication and experimental animals of the studies that assessed the Cd effect on testicular histomorphology in murine models.

CHARACTERISTICS OF PUBLICATION		EXPERIMENTAL ANIMALS					
Reference	**Country**	**Animal model**	**Age (days)**	**Weight (g)**	**Number of animals**	**Animals per group**	**Groups**
Waalkes M., et al., 1988 [51]	EUA	Wistar [Crl:(WI)BR] rats	42	225	315	C=15 T=30	12
Wang Y., et al., 2016 [52]	China	Wistar rats	?	220-260	40	10	4
Wong K. and Klaassen C., 1980 [53]	EUA	Sprague-Dawley rats	70	?	?	?	?
Wong K., et al., 1980 [54]	EUA	Sprague-Dawley rats	49	?	?	5, 10 or 11	3
Zenick H., et al., 2009 [55]	EUA	Sprague-Dawley rats	100	?	20	5	4
Zhou T., et al., 2004 [56]	EUA	Swiss mice	49	?	?	6	?
Zielinska-Psuja B., et al., 1979 [57]*	Poland	Wistar rats	84	180	96	8	12
Zschauer A. and Hodel C., 1980 [58]	Switzerland	Albino rats	56	250	?	?	?
Öner H., et al., 2004 [59]	Turkey	Wistar rats	?	200-250	15	5	3

? = Not informed; FE = First Experiment; SE = Second Experiment; PreT = Pretreatment; C = Control; T = Treatment; Wd = Withdrawn. * Studies included by hand-search.

Table S4. Characteristics of exposure to Cd of the studies that assessed its effect on testicular histomorphology in murine models.

REFERENCE	EXPOSURE						
	Compound	Compound dose	Cd dose	Administration	Route	Duration	Control
Allanson M. and Deanesly R., 1962 [23]	$CdCl_2$	0.456, 0.912, 1.277, or 1.824mg/rat	0.280, 0.559, 0.783, 1.118mg/rat	SD	Injection	2 to 266 D	Yes (?)
Aoyagi T., et al., 2002 [24]	$CdCl_2$	0.979mg/kg	0.6mg/kg	DD, 6x/W	Sc	1, 2, 3, 4, 5, or 6 W	Yes (Sal)
Berliner A. and Jones-Witters P., 1975 [25]	$CdCl_2$	7.5mg/kg	4.6mg/kg	SD	Sc	C=1h T=2, 3, or 4h	Yes (Sal)
Blanco A., et al., 2010 [26]	$CdCl_2$	15mg/L	9.2mg/L	*Ad libitum*	Or (DW)	3, 6, 12, or 18 M	Yes (?)
Blanco A., et al., 2009 [27]	$CdCl_2$	15mg/L	9.2mg/L	*Ad libitum*	Or (DW)	1, 3, 6, or 12 M	Yes (?)
Blanco A., et al., 2007 [28]	$CdCl_2$	15mg/L	9.2mg/L	*Ad libitum*	Or (DW)	1, 3, 6, or 12 M	Yes (?)
Bomhard E., et al., 1987 [29]	$CdCl_2$	FE: 0.41 (WD-Sc), 4.08 (SD-Sc), 8.15 (WD-Or), or 81.54mg/kg (SD-Or) SE: 3.3 (SD-Sc), 163.1, or 326.2mg/kg (SD-Or)	FE: 0.25 (WD-Sc), 2.5 (SD-Sc), 5 (WD-Or), or 50mg/kg (SD-Or) SE: 2 (SD-Sc), 100, or 200mg/kg (SD-Or)	SD or WD (10W)	Or (gavage) or Sc	FE: 12, 18, or 30 M SE: 6 M	FE: Yes (Dist.) SE: ?
Cupertino M., et al., 2017 [30]	$CdCl_2$	1.1, 1.2, 1.4, or 1.8mg/kg	0.67, 0.74, 0.86, or 1.1mg/kg	SD	Ip	7 D	Yes (Sal)
Cupertino M., et al., 2017 [31]	$CdCl_2$	1.1, 1.2, 1.4, or 1.8mg/kg	0.67, 0.74, 0.86, or 1.1mg/kg	SD	Ip	7 D	Yes (Sal)
Davis J. and Coniglio J., 1967 [32]	$CdCl_2$	0.2mg/kg	0.123mg/kg	SD	*In situ*	28 D	Yes (Sal)
Favino A., et al., 1966 [33]	$CdCl_2$	10mg/kg	6.132mg/kg	SD	Sc	10, 50, 100, or 150 D	Yes (?)
Hew k., et al., 1993 [34]	$CdCl_2$	0.5 or 1mg/kg	0.307 or 0.613mg/kg	SD	Ip	4, 24, 48, or 72h	Yes (Deion.)
Ito T. and Sawauchi K., 1966 [35]	$CdCl_2$	PreT: 1/2, 1/4, 1/8, or 1/16 of T T: 0.405mg/rat	PreT: 1/2, 1/4, 1/8, or 1/16 of T T: 0.248mg/rat	SD or 2 doses (0 and 48h)	Sc	5 W	Yes (Unt.)

Table S4 (*Continuation*). Characteristics of exposure to Cd of the studies that assessed its effect on testicular histomorphology in murine models.

REFERENCE	EXPOSURE						
	Compound	Compound dose	Cd dose	Administration	Route	Duration	Control
Kar A. and Das R., 1962 [36]	$CdCl_2$	0.05, 0.10, 0.15, or 0.20mg/kg	0.031, 0.061, 0.092, or 0.123mg/kg	SD	*In situ*	2, 7, 15, 30, or 45 D	Yes (Dist.)
Krasovskii G., et al., 1976 [37]	FE: ? SE: $CdCl_2$	FE: 4.9, 14.7, 44mg/kg SE: 0.00005, 0.0005, or 0.005mg/kg	FE: 3, 9, or 27mg/kg SE: 0.00003, 0.0003, 0.003mg/kg	FE: ? SE: DD	Or	FE: ? SE: 6 M	Yes (?)
Leite R., et al., 2015 [38]	$CdCl_2$	40.8, 81.5 or 122.3mg/L	25, 50 or 75mg/L	*Ad libitum*	Or (DW)	30 D	Yes (DW)
Li X., et al., 2016 [39]	CdTe quantum dots	0.2 or 2nmol	NL	SD	Iv	3h or 1, 3, 7, 15, 30 60, or 90 D	Yes (Sal)
Marettová M., et al., 2010 [40]	$CdCl_2$	3mg/kg	1.84mg/kg	SD	Ip	5 D	Yes (?)
Medina M., et al., 2017 [41]	$CdCl_2$	10mg/kg	1.23mg/rat/D	DD, 5x/W	Or	1, 2, or 3 M	Yes (Dist.)
Meek E., 1959 [42]	$CdCl_2$	20.25mg/kg	12.42mg/kg	SD	Sc	2 to 96h, or 5, 21, or 49 D	Yes (Unt.)
Niknafs B., et al., 2015 [43]	$CdCl_2$	5, 10, 15, 20, 25, 30, or 40µM/kg	0.56, 1.12, 1.67, 2.25, 2.81, 3.37, 4.49mg/L	SD	Ip	48h	Yes (?)
Oliveira H., et al., 2012 [44]	$CdCl_2$	1, 2, or 3mg/kg	0.613, 1.226, 1.839mg/kg	SD	Sc	35 D	Yes (Sal)
Predes F., et al., 2010 [45]	$CdCl_2$	1 or 1.2mg/kg	0.613, 0.736mg/kg	SD	Ip	7 or 56 D	Yes (Sal)
Reddy J., et al., 1973 [46]	$CdCl_2$	5.499mg/kg	3.372mg/kg	SD	Sc	1 Y	Yes (Sal)
Saygi Ş., et al., 1991 [47]	NL	NL	10mg/L	*Ad libitum*	Or (DW)	28, 40, 52, or 56 W	Yes (Tap)
Selypes A., et al., 1992 [48]	$CdCl_2$	1.631mg/kg	1mg/kg	SD	Ip	3 D or 6 M	Yes (Sal)

Table S4 (*Continuation*). Characteristics of exposure to Cd of the studies that assessed its effect on testicular histomorphology in murine models.

REFERENCE	EXPOSURE						
	Compound	Compound dose	Cd dose	Administration	Route	Duration	Control
Sharma, S. and Kaur S., 2012 [49]	$CdCl_2$	0.1 (DD) or 2mg/kg (SD)	0.061 (DD) or 1.226mg/kg (SD)	SD or DD	Ip	1 or 7 D (SD) 15 or 30 D (SD and DD)	Yes (Dist.)
Singh K. and Mathur R., 1968 [50]	$CdCl_2$	5.6mg/kg	3.434mg/kg	SD	Sc	12 or 24h	Yes (Dist.)
Waalkes M., et al., 1988 [51]	$CdCl_2$	PreT=0.917mg/kg T=1.833 or 3.666mg/kg. Others: 1, 1/2, 1/4, 1/8, 1/16, or 1/40 of 7.333 (SD); or 0.917mg/kg (4 doses)	PreT=0.562mg/kg T=1.124 or 2.247mg/kg. Others: 1, 1/2, 1/4, 1/8, 1/16, or 1/40 of 4.497 (SD); or 0.562mg/kg (4 doses)	SD, 2 (0 and 48h) or 4 doses (0, 48, 96, and 168h)	Sc	104 W	Yes (Sal)
Wang Y., et al., 2016 [52]	$CdCl_2$	0.2, 0.4, or 0.8mg/kg	0.123, 0.245, 0.491mg/kg	DD, 5x/W	Ip	5 W	Yes (Sal)
Wong K. and Klaassen C., 1980 [53]	$CdCl_2$	1.631mg/kg	1mg/kg	SD	Iv	3 D or weekly intervals after injection to 3 M	Yes (Sal)
Wong K., et al., 1980 [54]	$CdCl_2$	3.262, 4.892mg/kg	2 or 3mg/kg	DD	Sc	7 D	Yes (Sal)
Zenick H., et al., 2009 [55]	$CdCl_2$	280.5, 560.9, 1,121.9mg/L	172, 344, or 688mg/L	*Ad libitum*	Or (DW)	70-80 D	Yes (Dist.)
Zhou T., et al., 2004 [56]	$CdCl_2$	1.495, 2.989mg/kg	0.917 or 1.833mg/kg	SD	Sc	12, 24, or 72h	Yes (Sal)
Zielinska-Psuja B., et al., 1979 [57]	$CdCl_2$	8.8 or 88mg/kg/D	5.396, 53.96mg/kg/D	*Ad libitum*	Or (Pellet)	3, 6, 12, or 15 M	Yes (Unt.)
Zschauer A. and Hodel C., 1980 [58]	$CdCl_2$	1 or 2mg/kg	0.613, 1.226mg/kg	SD	Or (gavage) or Sc	2, 4, or 6 W	?

Table S4 (*Continuation*). Characteristics of exposure to Cd of the studies that assessed its effect on testicular histomorphology in murine models.

REFERENCE	EXPOSURE						
	Compound	Compound dose	Cd dose	Administration	Route	Duration	Control
Öner H., et al., 2004 [59]	Cd (II) complex	150mg/kg	20.94mg/kg	3-day intervals	Sc	15 D	Vehicle (Corn oil to 10% DMSO)

$CdCl_2$ = Cadmium chloride; CdTe = Cadmium telluride; ? = Not informed; NL = Not listed; FE = First Experiment; SE = Second Experiment; PreT = Pretreatment; C = Control; T = Treatment; SD = Single dose; WD = Weekly dose; DD = Daily dose; Ip = Intraperitoneal; Sc = Subcutaneous; Iv = Intravenous; Or = Oral; DW = Drink water; D = Day; W = Week; Y = Year; Sal = Saline; Dist. = Distilled water; Deion. = Deionized water; Tap = Tap water; Unt. = Untreated. When the Cd dose was not listed by a recovered record, it was calculated from $CdCl_2$ dose, as well as the $CdCl_2$ dose was calculated from Cd dose when it was not provided (100mg $CdCl_2$ contains 61.32mg Cd; 100µM $CdCl_2$ contains 11.24mg/L Cd; 100mg Cd (II) complex contains 13.96mg Cd; Cd quantity in CdTe quantum dots was not informed).

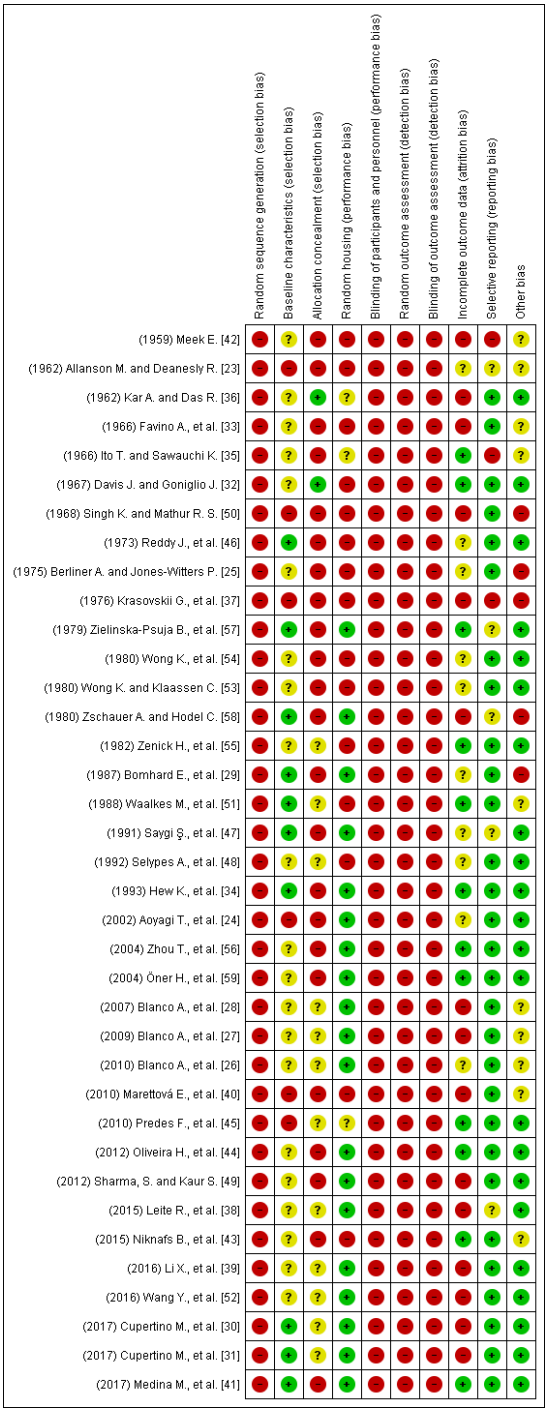

Fig. S1 Results of the risk of bias assessment for each included study that evaluated the Cd impact on the testicular histomorphology of murine models in chronological order. Green = Low risk of bias; Yellow = Unclear risk of bias; Red = High risk of bias. Note that the risk of bias of the studies did not change along the time

3. CHAPTER 2

Short-term exposures of the adult human testis to cannabinoids THC and CBD have no major effects *ex vivo*

Abstract

Study question: Do Δ^9-tetrahydrocannabinol (THC) and/or cannabidiol (CBD) represent a hazard for human testis *ex vivo*?

Summary answer: THC and/or CBD did not affect testis biology at gonadal level.

What is known already: Conflicting evidence exists about an association between cannabis use and testicular functions in men. However, available data accord to the positive relation between incident testicular germ cell tumors (TGCT) and cannabis exposure.

Study design, size, duration: Adult human testis explants from 8 different donors were placed in culture and exposed to CBD, THC, or CBD/THC [ratio 1:1] from 10^{-9} to 10^{-5}M for 48 h. The controls were exposed to DMSO, ethanol (EtOH), or DMSO/EtOH, respectively.

Participants/materials, setting, methods: Human adult testes were obtained from multiorgan donors who had no antiandrogen treatment (mean age: 55.5±19.9 y). After *ex vivo* exposure to the investigated cannabinoids, we evaluated the testis explants histology, the testosterone production in the medium after 24 h and 48 h of exposure, the number of positive cells for apoptosis and proliferation, and RNA expression of genes encoding proteins involved in germ cell differentiation, meiosis, Sertoli cell functions, steroidogenesis, and endocrine signaling.

Main results and the role of chance: *Ex vivo* exposure of adult human testis to a range of concentrations of CBD, THC, or CBD/THC mixture did not affect the testis histology, testosterone production in both exposure times, number of apoptotic and proliferative cells, nor expression of transcripts encoding important testicular proteins.

Limitations, reasons for caution: These findings reflect a relatively short-term exposure *ex vivo* of the human testis to the main components of cannabis and cannot account for potential effects of regular or chronic uses.

Wider implications of the findings: these findings suggest that there are no acute effects on the testis of exposure to cannabis-derived cannabinoids.

Study funding/competing interest(s): This work was financed by University Rennes 1 and the Institut national de la santé et de la recherche médicale (Inserm). Also, this study was supported by the Institutional Internationalization Program (PrInt) from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Brazil) for the scholarship provided to J Da Silva. All authors declare they have no current or potential competing financial interests.
Trial registration number: N/A

Keywords: phytocannabinoids, Spermatogenesis, Steroidogenesis, organotypic culture, adult human testis.

3.1 Introduction

Cannabis is the most widely cultivated, produced, trafficked, and consumed drug worldwide (World Health Organization, 2021). In the last decade, several countries in Europe and outside including Canada (Government of Canada, 2018) and states from the USA (Chandra *et al.*, 2019) legalized cannabis for recreational and/or medical use, while other countries such as Greece and Ireland continue with severe penalties for its supply (European Monitoring Centre for Drugs and Drug Addictions, 2018). In 2018, the global number of cannabis users was 192 million (United Nations Office on Drugs and Crime, 2020). The use of cannabis has increased in young people with most consumers starting smoking cannabis in their mid to late teens (Schneider, 2009). However, this is a sensitive window for reproductive health, which its integrity is required for lifelong fertility (Geyer, 2017).

To date, more than 500 compounds have been identified in *Cannabis sativa* plants including more than 100 terpenophenolic secondary metabolites, which are named phytocannabinoids (Gould, 2015). The most relevant cannabinoids (CA) of cannabis are the Δ^9-tetrahydrocannabinol (THC) and the cannabidiol (CBD). These two compounds are the best-studied and most used for therapeutic applications such as epilepsy, Alzheimer's and Parkinson's diseases, multiple sclerosis, and cancer (Fraguas-Sánchez and Torres-Suárez, 2018). THC and CBD act on specific G protein-coupled receptors called cannabinoid receptors, CB1 and CB2 (Morales *et al.*, 2017). Both receptors also respond to naturally synthesized cannabinoids, so-called endocannabinoids (eCA), whose levels are tightly regulated. The eCA and their receptors, together with their synthesis and degradation enzymes and the transporters comprise the endocannabinoid system (ECS) (Kilaru and Chapman, 2020).

Gonads synthesize eCA, which were found in rodents to control Sertoli and Leydig cells activity, germ cell progression, as well as the acquisition of sperm functions (Bovolin *et al.*, 2014). Our laboratory recently evidenced the expression of the main components of the ECS in adult human testis (Nielsen *et al.*, 2019). Notably, both CB1 and CB2 were expressed in Leydig cells and post-meiotic germ cells, along with ECS enzymes. eCA has been also found in human reproductive fluids (Schuel *et al.*, 2002). In men, the semen eCA is involved in sperm quality, such as morphology, and the acquisition of sperm functions related to fertilization, such as mobility (Zufferey *et al.*, 2020).

Scientific evidence based on observational findings from cannabis smokers has produced conflicting data about the effect of cannabinoids on male reproductive health. Some studies highlight the negative effect of cannabis consumption on sperm parameters and testosterone secretion, whereas others failed to observe any impact (Maccarrone *et al.*, 2021). A recent meta-analysis concluded that the current evidence does not support a clinically significant association between cannabis use and testicular function in men (Belladelli *et al.*, 2021). Nevertheless, frequent cannabis use has been associated with the development of testicular germ cell tumors (TGCT), suggesting that recreational and therapeutic uses of it or its compounds, may represent a risk factor for the development of testis cancer in humans (Gurney *et al.*, 2015; Ghasemiesfe *et al.*, 2019; Song *et al.*, 2020).

In this conflicting data context, our study aimed to identify the impact of THC and CBD alone or in a mixture, on germ cell lineage as well as on the steroidogenic function, using our well-characterized organotypic culture of adult human testis (Roulet *et al.*, 2006). This study will also help to discriminate the gonadal from the central effect of phytocannabinoids.

3.2 Materials and methods

Chemicals

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

Ethical approval

Testes were obtained from multiorgan donors (n = 8; mean age: 55.5±19.9 y). The protocol was approved by the local ethics committee (Agence de la Biomédine; authorization no. PFS09-015), and informed consent was obtained from all donors or their next of kin.

Ex Vivo Organ Model: Testis Explant Assay

To determine the direct effect of CBD and/or THC on adult human testis histophysiology, we used a validated organ model assay (Testis Explant Assay) (Roulet *et al.*, 2006). After collection

of each testis, the organ was placed at 4°C and rapidly processed for experimentation. Observation by transillumination allowed us to discard the rare testes not displaying spermatogenesis. Four 3-mm^3 testis explants were placed onto a polyethylene terephthalate insert (Falcon Labware, USA) at the interface of air in 1 mL of Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, 31053028) supplemented with antibiotics, 4 mM glutamine, 1 mM sodium pyruvate, 10µg/ml insulin, 5µg/ml transferrin, 200ng/ml vitamin E, 100 ng/ml vitamin A, 50ng/ml vitamin C, and 1 IU/ml hCG for culture, in a well of a 12-well plate. The medium contained 0.1% DMSO, EtOH, CBD or THC, or 0.2% of DMSO/EtOH [ratio 1:1] or CBD/THC. Drugs were used from 10^{-5}M to 10^{-9}M. For each condition, randomized testis explants were cultivated in three different wells corresponding to technical replicates for 48 h with a change of medium at 24 h. Media from 24 and 48 h were stored at -80ºC for testosterone measurements. At the end of the exposure, three explants, one of each well of each condition, were collected at random and stored at -80ºC for reverse transcription quantitative real time PCR (RT-qPCR) or fixed in Bouin's solution and embedded in paraffin. The fixed explants were cut into 5.0µm-thick sections and stored at 4ºC until staining.

To avoid risk of bias and increase our methodological quality, in all the analyses described below, the samples were selected at random for outcome assessment. Furthermore, they had blind outcome assessors from knowing which intervention each sample received.

Hormone measurement

Testosterone levels were assayed in duplicate in the culture medium with a specific radioimmunoassay (RIA, Beckman Coulter, Czech Republic). The intra- and inter-assay coefficients of variation were 11.6 and 13.5%, respectively. Control testis explants produced an average of 15.13 ± 2.90 ng testosterone per ml of medium after 24 h of culture and 17.41 ± 3.98 ng testosterone per ml of medium after 48 h of culture.

Histology and Immunohistochemistry

Histological sections of adult human testis explants from each treatment condition were stained with hematoxylin-eosin. The testicular histology was checked under a light microscope (Zeiss microscope).

Immunohistochemistry (IHC) was performed in histological sections of adult human testis explants to observe Leydig cells and estimate the number of testicular cells undergoing apoptosis

and proliferation as already described (Desdoits-Lethimonier *et al.*, 2012; Desdoits-Lethimonier *et al.*, 2017). Briefly, Leydig cells were labeled with a rabbit primary antibody (1:500; Sigma-Aldrich, Germany) directed against the cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) followed by biotinylated polyclonal goat anti-rabbit secondary antibody (1:200; Dako, Denmark). For apoptosis assessment, histological sections were labeled with a polyclonal rabbit primary antibody directed against cleaved caspase-3 (1:100; Asp175, Cell Signaling Technology, Ozyme, France) and then with the secondary antibody as already described. For proliferation assessment, histological sections were labeled with a monoclonal mouse primary antibody directed against Ki-67 (1:100; Dako, Denmark) and then a biotinylated polyclonal goat anti-mouse secondary antibody (1:200, Dako, Denmark). To quantify the number of cells in apoptosis (Caspase-3 positive cells) or in proliferation (Ki-67 positive cells), we counted positive cells in the seminiferous tubules and interstitial tissue in 10 histological sections of each condition using a light microscope (Bh2 Olympus Microscope) coupled to Mercator Expert Software (Explorer NOVA). The surface area of each section was measured to indicate the cell counts per surface area.

RNA analysis

RNA was extracted from adult human testis explants, which were frozen, using RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of RNAs was estimated by nanodrop D-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed in 500ng of RNA using QuantiTect Reverse Transcription (Qiagen) according to the protocol provided by the manufacturer. RT-qPCR was performed according to the manufacturer's instructions with iTaq Universal SYBR Green Supermix (Bio-Rad) and 2µL cDNA template in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The amplification program was as follows: an initial denaturation of 3 min at 95 °C; 40 cycles of 10-s denaturation at 95 °C; and 30 s at 62 °C for annealing and extension. Dissociation curves were produced with the thermal melting profile performed after the last PCR cycle. To avoid amplification of contaminating genomic DNA, primer pairs were selected on two different exons (For genes studied, see Table 1). Rplp0 and Rps20 mRNA were used as internal controls for normalization. Results were calculated by the ΔΔCT method as *n*-fold differences in target gene expression with respect to the reference gene and the calibration sample.

Table 1. Primers used in RT-qPCR in adult human testis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
AMHR2	GGGCATTACTTCCCACAGCT	GCAGCTCTCCCAGTGTCTTT	105
CYP11A1	AGACCTGGAAGGACCATGTG	TCCTCGAAGGACATCTTGCT	435
CYP17A1	GTGGAGACCACCACCTCTGT	GCTGAAACCCACATTCTGGT	108
CYP19A1	CCAGGTCCTGGCTACTGCAT	GATCCCCATCCACAGGAATCT	69
LHR	GCCGGTCTCACTCGACTATC	TCATTAAGTCCTCTGAAAGCTTGA	77
STAR	GGCTGGCATGGCCACAGACT	TTGGGCAGCCACCCCTTGA	162
AMH	CGCCTGGTGGTCCTACAC	GAACCTCAGCGAGGGTGTT	162
CLDN1	GAATGCAACCACGGCATCTG	AGGAAGAGGAAGGCGCTCGT	176
FSHR	TGCCATTGAACTGAGGTTTG	GGTCCCCAAATCCTGAAAAT	74
INHBB	GCGTTTCCGAAATCATCAG	GCCTTCGTTGGAGATGAAGA	86
SOX9	AACGCCTTCATGGTGTGG	TCTCGCTCTCGTTCAGAAGTC	124
TJP1	CGGGCTACGCTATTGAATGTCC	ACCGCTGGTCAGGAGATCGT	166
ALPP	TCTGGGTACTCAGGGTCTGG	ATCGCTACGCAGCTCATCTC	101
DAZ1	GAAGGCAAAATCATGCCAAACAC	CTTCTGCACATCCACGTCATTA	186
DMC1	TACCCTCTGTGTGACAGCTCA	GAAGATGCCAGCTTCTTCATG	152
KIT	TTCTTACCAGGTGGCAAAGG	AAATGCTTTCAGGTGCCATC	209
LIN28A	AAGCTGCACATGGAAGGGTT	CCGCCTCTCACTCCCAATAC	138
POU5F1	TACTCCTCGGTCCCTTTCC	CAAAAACCCTGGCACAAACT	131
RAD51	GGTGAAGGAAAGGCCATGTA	GGGTCTGGTGGTCTGTGTT	148
REC8	TTTGGGATGATGTCTGTGGA	CTGGGATTGCAGCCTCTAAG	85
SPO11	TCGAGGAAGATGGCACCAAA	TTGTCATCTAGGAGCCGCTGAA	154
SYCP3	TGCTGGAAGGAGTTGGAGTTGA	TCCCACTGCTGAAACAAAGTCA	193
STRA8	GAGGGCAGCGTGAAGGACAG	GCAGCTGGCAAGCACTGAAC	165
TFAP2C	CCGGTCCTTGCGGGAGAAGTT	CTGGTTTACTAGGAAATTCGGCTTCACA	164
INSL3	CCCAGAGATGCGTGAGAAGT	CCAGCCACTGTAGCAACTCA	229
Reference genes			
RPLP0	TCTACAACCCTGAAGTGCTTGAT	CAATCTGCAGACAGACACTGG	167
RPS20	AACAAGCCGCAACGTAAAA	ACGATCCCACGTCTTAGAA	96

AMHR2 – anti-Mullerian hormone receptor type 2; *CYP11A1* - cytochrome P450 family 11 subfamily a member 1; *CYP17A1* - cytochrome P450 family 17 subfamily a member 1; *CYP19A1* - cytochrome P450 family 19 subfamily a member 1; *LHR* - luteinizing hormone receptor; *STAR* -

steroidogenic acute regulatory protein; *AMH* - anti-Mullerian hormone; *CLDN1* - claudin 1; *FSHR* - follicle-stimulating hormone receptor; *INHBB* - inhibin subunit beta; *SOX9* - SRY-box transcription factor 9; *TJP1* - tight junction protein 1; *ALPP* - alkaline phosphatase, placental; *DAZ1* - deleted in azoospermia 1; *DMC1* - DNA meiotic recombinase 1 ; *KIT* - KIT proto-oncogene, receptor tyrosine kinase; *LIN28A* - lin-28 homolog A; *POU5F1* - POU class 5 homeobox 1; *RAD51* - RAD51 recombinase; *REC8* - REC8 meiotic recombination protein; *SPO11* - SPO11 initiator of meiotic double stranded breaks; *SCP3* - synaptonemal complex protein 3; *STRA8* - stimulated by retinoic acid 8; *TFAP2C* - transcription factor AP-2 gamma; *INSL3* - insulin like 3; *RPLP0* - ribosomal protein lateral stalk subunit P0; *RPS20* - ribosomal protein S20.

Statistical analyses

The data normality was checked by the Shapiro-Wilk test. Quantitative variables were compared using one-way ANOVA followed by Tukey's post hoc comparison test (parametric data) or only Kruskal-Wallis' test (nonparametric data). Data were expressed divided by their respective controls. The statistical significance threshold was set at 0.05. The data are expressed as mean ± SEM.

3.3 Results

Justification of the molecules and their doses used in the study

The two main cannabinoids present in cannabis are THC and CBD and both of them have an interesting range of potential therapeutic indications. Pharmacokinetic data show that peak plasma concentrations (Cmax) are different depending on the mode of administration of cannabis. THC Cmax after smoking a cannabis cigarette are higher (2.5 10^{-7}M and 7.3x10^{-7}M for cigarettes containing 19 mg (Ohlsson *et al.*, 1980) and 69.4 mg (Hunault *et al.*, 2008) of THC, respectively) than after oral intake (1.9x10^{-7}M for 20 mg ingested) (Ohlsson *et al.*, 1980), while plasma concentrations 5 min after intravenous injection of THC (0.053 mg/kg body weight) range from 2.6x10^{-7}M to 2x10^{-6}M (Naef *et al.*, 2004). The same happens for CBD, in which CBD Cmax after smoking a cannabis cigarette are higher (ranges from 1.8x10^{-5}M to 2.9x10^{-5}M for 1.5 mg CBD ingested) than after oral intake (ranges from 1.9x10^{-6}M to 2.6x10^{-6}M for 1.5 mg CBD ingested) (Swortwood *et al.,* 2017). Furthermore, the oral intake of the medicament Sativex® results in THC Cmax of 1.6x10^{-8}M and 1.5x10^{-7}M for 5.4 and 16.2mg THC ingested, respectively, and CBD Cmax of 5.1x10^{-8}M and 2.1x10^{-8}M for 5 and 15mg CBD ingested, respectively (Karschner et al., 2011). Based on these data, we chose to test THC or CBD from 10^{-9} to 10^{-5}M in order to cover various

recreational and medical modes of exposure. Also, the half-life of the THC is around 30 h (Huestis *et al.*, 1996) and the CBD is around 24 h (Millar *et al.,* 2018) for an infrequent user depending on the mode of administration.

Given the bibliographic data which show very diverse exposures depending on the mode of administration and the type of intake (recreational or medical), we have chosen to achieve dose-effects of THC and CBD, surrounding the maximum concentrations observed in the plasma of the study subjects, from 10^{-9} to 10^{-5} M. Taking into account the additive effects of molecules acting in cocktails (Gaudriault *et al.*, 2017) and the use in therapy of a mixture of CBD and THC, we evaluate the dose-response effects for each compound alone or in a combination based on equimolarity.

THC and/or CBD did not affect the morphology of adult human testis explants

To analyze the effect of CBD and/or THC on male human germ cells, we observed the effects of these compounds on the testis morphology. Analysis of the sections stained with hematoxylin/eosin did not reveal any alteration of human testis explants even at the dose of 10^{-5}M of compounds when compared to the control conditions (Figure 1).

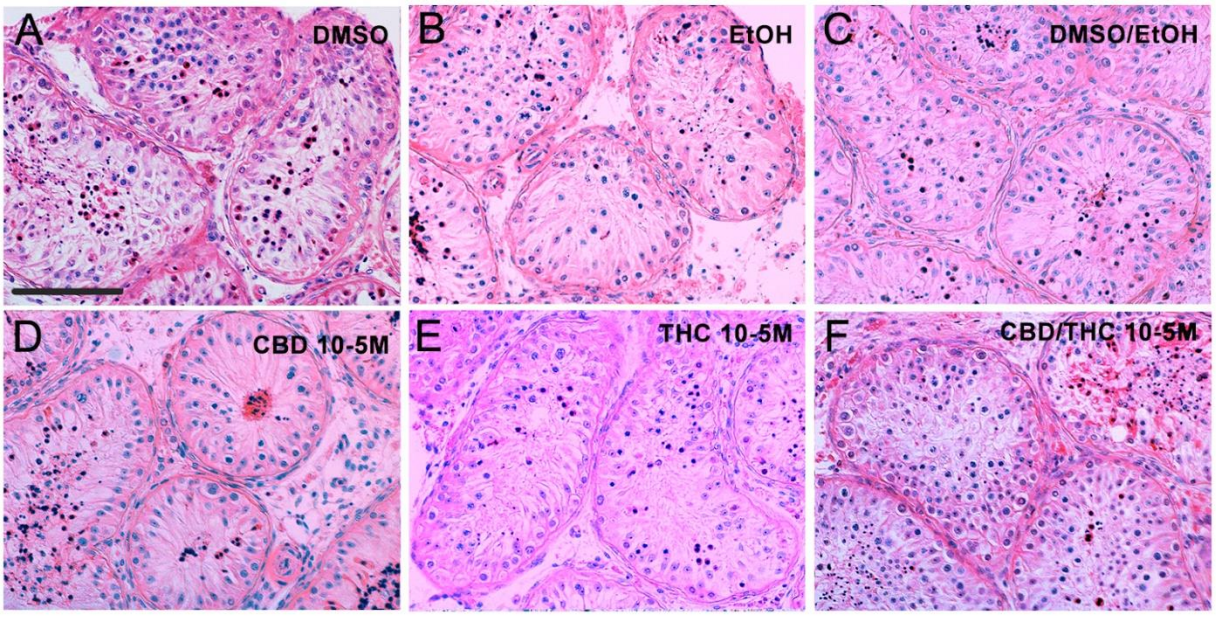

Figure 1. **CBD (cannabidiol) and THC (Δ^9-tetrahydrocannabinol), alone or in a mixture, did not affect the morphology of adult human testis explants**. H&E staining of the histological sections of adult human testis explants cultured for 48h in the presence of (A) DMSO (Control CBD), (B) EtOH (Control THC), (C) DMSO/EtOH (Control CBD/THC), (D) CBD, (E) THC, or (F) CBD/THC (1:1 ratio). Bar = 100µm.

THC and/or CBD did not affect the secretion of testosterone by adult human testis explants

Since exposure to cannabis was described to possibly modulate the level of circulating testosterone in men, we assessed the endocrine function of explants cultured following treatment. Our data showed that neither THC nor CBD, alone or in a mixture, were able to modify the secretion of testosterone in the culture media after 24 or 48h of culture (Figure 2). Immunohistochemistry of the testis sections using CYP11A1 as a specific marker of Leydig cells did not highlight any modification of this cell population in treated condition compared to control (data not shown).

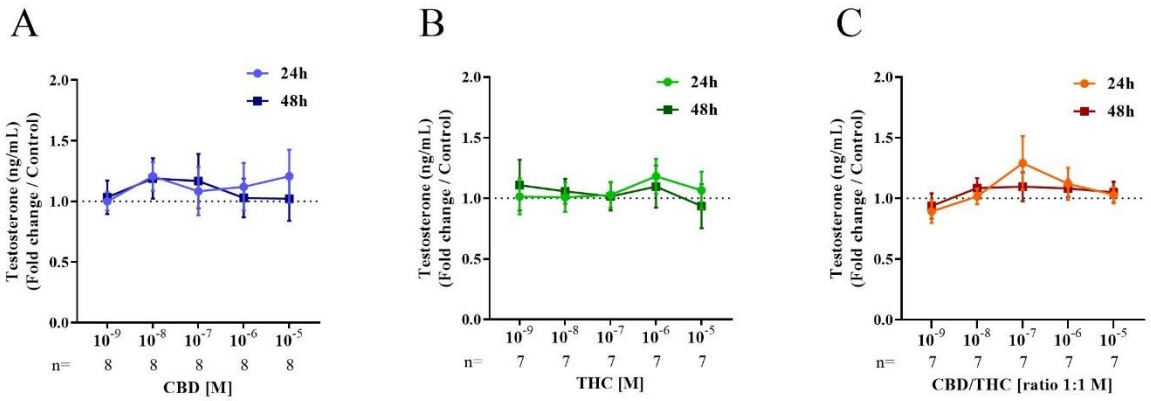

Figure 2. CBD (cannabidiol) and THC (Δ^9-tetrahydrocannabinol), alone or in a mixture, did not affect the secretion of testosterone by human testis in organotypic culture. Amount of testosterone in the supernatants of adult human testis explants cultured for 24h or 48h in presence of (A) CBD, (B) THC, and (C) CBD/THC at 10^{-9} to 10^{-5} M. No significant difference ($p<0.05$) among the groups by ANOVA followed by Tukey test (parametric data) or Kruskal-Wallis test (nonparametric data). Data presented as mean ± SEM divided by the control mean.

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

As our result did not highlight morphological effects of tested drugs, we determined whether exposure to CBD and THC alone or in a mixture was able to affect an early sign of cell death in the organotypic culture of adult human testis. To this aim, we decided to check by IHC the cleaved-caspase 3, a specific marker of apoptosis. After quantification of the number of caspase-3 positive cells per surface area in both seminiferous tubules, containing germ cells and Sertoli cells, and interstitial compartments, containing among other Leydig cells, our results showed that neither CBD nor THC impacted cell viability (Figure 3).

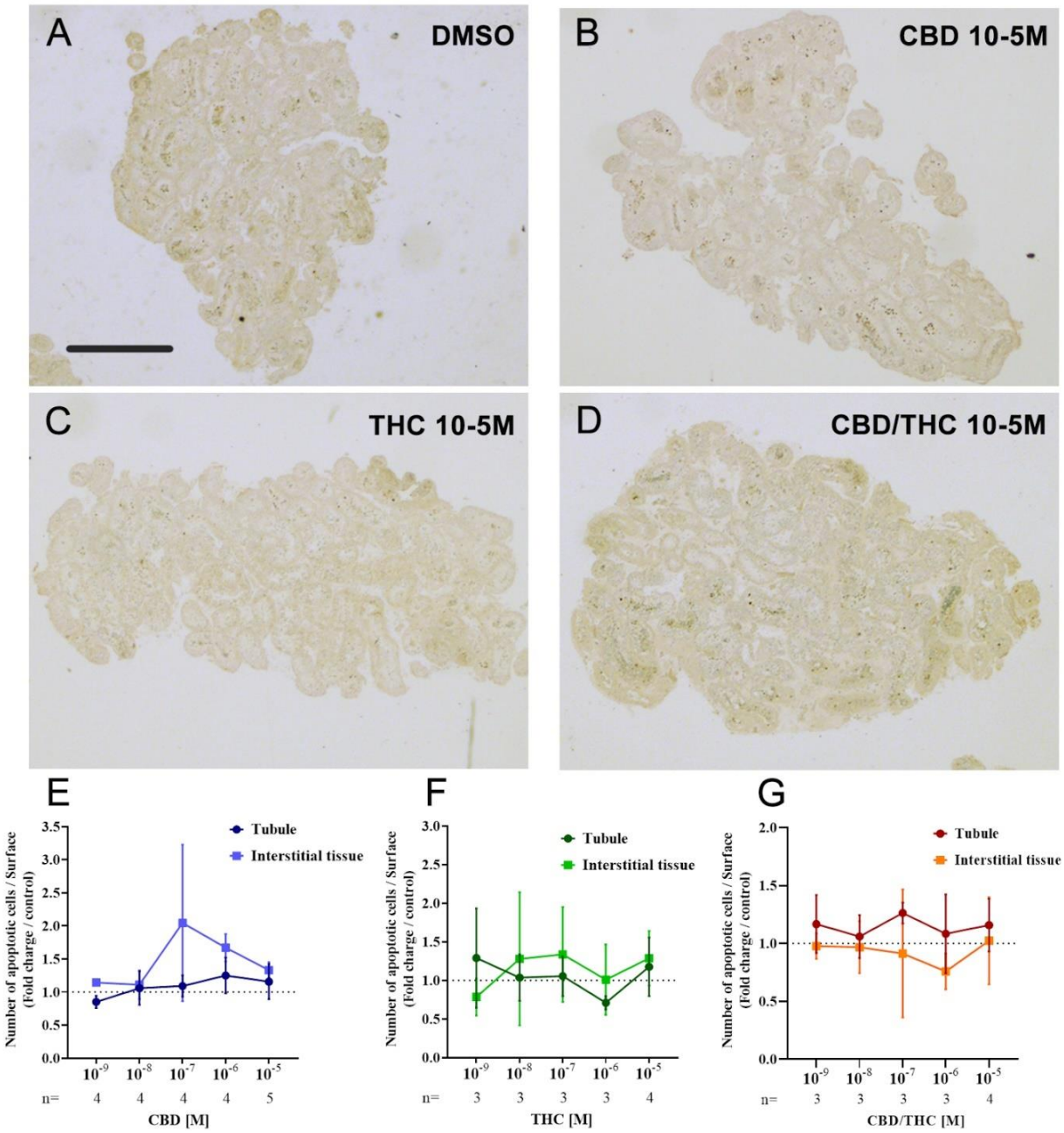

Figure 3. CBD (cannabidiol) and THC (Δ^9-tetrahydrocannabinol), alone or in a mixture, did not affect the number of apoptotic cells in adult human testis explants. Immunostaining using an anti-caspase 3 antibody on histological sections of testis explants cultured for 48h in the presence of CBD, THC, or CBD/THC (1:1 ratio). (A-D) Panels show whole explants stained with caspase 3 (apoptotic cells in brown). Bars = 1000µm. (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 ($p<0.05$) among the groups by ANOVA followed by Tukey test. Data presented as mean ± SEM divided by the control mean.

To evaluate the effect of CBD and/or THC on the proliferating rate in adult human testis explants, we performed an IHC using a specific marker of proliferating cells, Ki-67. After quantification of the number of Ki-67 positive cells per surface area in both seminiferous tubules and interstitial compartments, our results showed that CBD or THC, alone or in combination, did not affect the capacity of cells to proliferate (Figure 4).

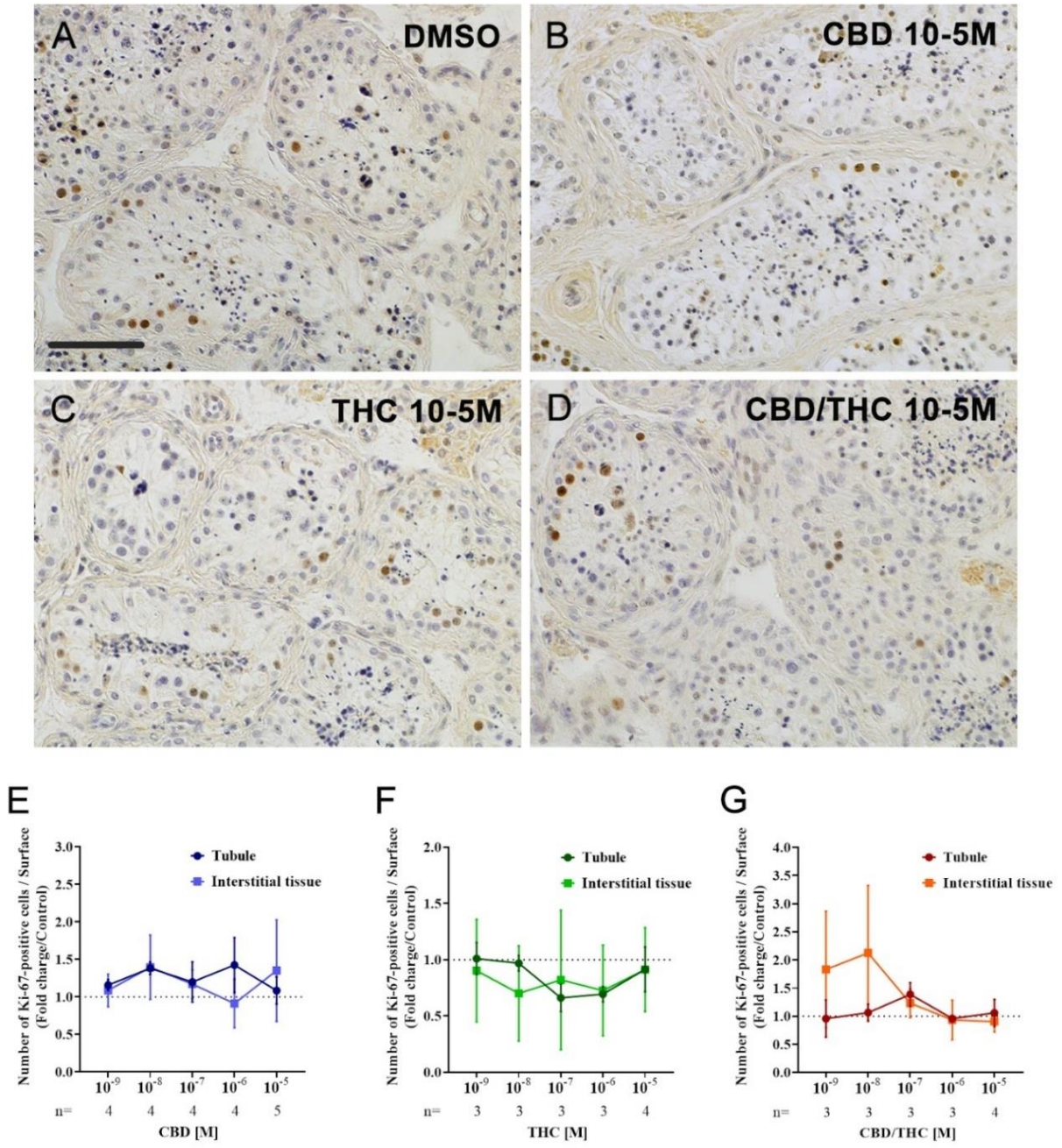

Figure 4. CBD (cannabidiol) or THC (Δ^9-tetrahydrocannabinol), alone or in a mixture, did not affect the number of proliferating cells in adult human testis explants. Immunostaining using an anti-Ki-67 antibody on histological sections of testis explants cultured for 48h in the presence of CBD, THC, or CBD/THC (1:1 ratio). **(A-D)** Ki-67-positive cells appear in brown and in most of cases are on the peripheral site of the seminiferous tubules. Bars = 100µm. (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 ($p<0.05$) among the groups by ANOVA followed by Tukey test. Data presented as mean ± SEM divided by the control mean.

Expression of genes involved in spermatogenesis and steroidogenesis was not affected by CBD and/or THC exposure

To reveal the effects of CBD and/or THC exposure on gene expression in organotypic culture of adult human testis, we performed RT-qPCR. We performed analysis on six different replicates coming from the organotypic culture of testis treated with the highest dose of CBD and/or THC and their related controls. For the analysis, we chose genes encoding proteins involved in germ cell differentiation (*POU5F1*, *LIN28A*, *KIT, ALPP, TFAP2C*), meiosis (*DMC1*, *DAZ1*, *REC8*, *SPO11*, *STRA8*, *SYCP3*, *RAD51*), Sertoli cell function (*AMH, AMHR2*, *INHBB*, *SOX9, TJP1, CLDN1,*), steroidogenesis (*CYP11A1*, *CYP17A1, CYP19A, STAR, INSL3*) and endocrine signaling (*FSHR*, *LHR*) (Table 1). Our results showed that exposure to CBD and/or THC at the dose of 10^{-5}M did not affect the expression of genes tested in our experiment (data not shown).

3.4 Discussion

This study is the first to investigate the impact of cannabis-extract cannabinoids CBD and THC, alone or in a combination, in adult human testis. Using our validated model of adult human testis in organotypic culture (Kristensen *et al.*, 2018; Matusali *et al.*, 2018), we aimed to reveal the potential direct effects of cannabinoids used for recreational and therapeutic purposes on male germ cell differentiation. We provide evidence that 48 h of exposure of testis explants to CBD and THC, alone or in a mixture, has no major effects on the germ cell lineage as well as the steroidogenic function based on our unique *ex vivo* culture assay. Our results, from the range of cannabinoid concentrations used, show no alteration in the testis histology, testosterone production, number of apoptotic and proliferative cells, and expression of some genes encoding important testicular proteins involved in spermatogenic and steroidogenic functions.

The ECS has already been described as a critical regulator of male vertebrate reproduction, in which its action modulates mainly the secretion/release of male hormones (Bovolin *et al.*, 2014). At the central level, the ECS regulates the release of the gonadotropin-releasing hormone (GnRH), a key neuropeptide for reproductive physiology, and influences 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 (Corradi *et al.*, 2016). However, the clinical data in the literature regarding the relationship between cannabis and male reproductive hormone production are inconsistent and discrepancies. Kolodny *et al.*, (1974) observed a decrease in plasma testosterone levels while other studies did not observe any alteration (Mendelson *et al.*, 1974; Thistle *et al.*, 2017; Lisano *et al.*, 2019). The plausible explanation for heterogeneous results may be related to the temporal and reversible effects of cannabis on hormone levels (Rajanahally *et al.*, 2019). The dosage of testosterone production by the adult human testis explants after 24 h and 48 h of exposure to CBD, THC, or CBD/THC did not show any time-related effect. As a result, we did not observe any significant alteration in both exposure times. We suggest that short-term exposure to CBD and THC does not modify testosterone production by Leydig cells, in support to a recent meta-analysis (Belladelli *et al.*, 2021). However, we cannot rule out an indirect effect through a modification of gonadotropins released by the pituitary and in turn a disruption of the hypothalamic-pituitary-gonadal axis (HPG axis), or a direct effect on testis after following longer/repeated exposures.

The histological analysis of the adult human testis explants by using hematoxylin and eosin staining did not reveal any perceptive change in testis morphology and the number of germ cell layers. A normal testosterone level is required for spermatogenesis (Neto *et al.*, 2016). So, the maintenance of testosterone production contributed to keeping the cell morphology and the number of germ cell layers. However, storage of phytocannabinoids, such as THC, in tissues due to insufficient pharmacokinetic mechanisms can dysregulate cellular processes, including apoptosis of spermatogenesis cells in rats (Huestis, 2007). After staining histological slides using an antibody against Caspase-3, we observed no effect of the phytocannabinoids on the number of apoptotic cells. This result suggests that short-term exposure to CBD and THC, alone or in combination, does not impact testicular cell survival.

More than 90% of testicular cancers are testicular germ cell tumors (TGCT) (American Cancer Society, 2018). TGCT are due to a deregulation of gonocyte differentiation during development which results in the formation of germ cell neoplasia in situ (GCNIS) in adult testis (Skakkebæk, 1972; Skakkebæk *et al.*, 2016; Baroni *et al.*, 2019). GCNIS corresponds to a precursor lesion that could develop into TGCT after exposure to a risk factor (Skakkebæk *et al.*, 1987; Baroni *et al.*, 2019). As TGCT incidence has been associated with cannabis consumption (Gurney *et al.*, 2015; Ghasemiesfe *et al.*, 2019; Song *et al.*, 2020), we can speculate that cannabis is able to affect the state of differentiation and proliferation of the germ cell. Ki-67, a proliferation marker, has

been used for the evaluation and prognosis of TGCT (Datta *et al.*, 2000). In order to observe the CBD and THC impact on the testicular cell proliferation, we stained histological sections using an antibody against Ki-67, and we found that the CBD and/or THC exposure did not affect the number of Ki-67 positive cells. This result could be due to a short time of exposure in our experiments. In fact, TGTC cases are observed in frequent cannabis users and the risk of developing this cancer increase with years of use (Daling *et al.*, 2009; Trabert *et al.*, 2011). However, Lacson *et al.*, (2012) did not observe this lifetime pattern because the lower risk was among men who reported more than 10 years of cannabis use.

Currently, the mechanisms that may explain the cannabis-TGCT association are not known, but some possibilities can be speculated based on available information. Cannabinoids could bind to CB receptors expressed in germ cells or testicular somatic cells altering germ cell development and triggering the cancerous transformation (Barchi *et al.*, 2019). Also, the biological plausibility of the link between cannabis exposure during adult life and TGCT is thought to be related to disruption to the HPG axis (Gurney *et al.*, 2015; Ghasemiesfe *et al.*, 2019). Indeed, the transformation of delayed gonocytes (pre-GCNIS) into invasive tumors is dependent on factors such as gonadotropins and/or testicular steroids (Skakkebæk *et al.*, 1987). It is thus likely that phytocannabinoids, by binding to cannabinoid receptors at a central level, could target the hypothalamic–testis axis, thus disrupting normal hormone regulation of spermatogenesis and leading to carcinogenesis (Barchi *et al.*, 2019). Since we aimed to evaluate the phytocannabinoids effects directly in germ cell lineage and testicular steroidogenic function discriminating the gonadal from the central effect, the indirect effects caused by them on the testis as a consequence of effects in other organs were excluded due to our culture system.

Since we did not observe any impact at cellular and histophysiological levels, we performed RT-qPCR analyses for checking if phytocannabinoid exposure could have any effect at molecular level. Our results from the RNA expressions of 26 genes involved in germ cell differentiation, Sertoli cell function, and steroidogenesis showed that testicular functions were not affected. This outcome confirms that there is no association between cannabis exposure and impaired testicular functions. A recent meta-analysis identified driver genes for initiation of GCNIS, including KIT, POU5F1, and LIN28A (Von Eyben and Parraga-Alava, 2020). Our findings show no alteration in the RNA expressions of these important genes of the pathogenesis of TGCT in adult human testis. To further understand efficiently the biology of induction of testis cancer by cannabis, studies on

the capacity of GCNIS to respond to cannabinoids would be crucial to investigate, as the expression of ECS in these lesions.

The role of the ECS in the physiopathology of cancer is not totally clear yet, as well as the phytocannabinoid action in cancer biology. There are intriguing current data about cannabinoids in cancer biology. CBD and THC exert antiproliferative and proapoptotic effects in several tumor cell lines, including human breast and prostate carcinomas (Ligresti *et al.*, 2016). Although the activation of CB receptors has been associated with anti-cancer effect, high concentration of cannabinoids and high expression of their receptors might be protumorigenic and plays an essential role in the development of cancer (Śledziński *et al.*, 2018). It seems that phytocannabinoids may have bidirectional roles in carcinogenesis depending on the cells (noncancerous or cancerous) and tumor type.

The existing literature about the association between cannabis exposure and male fertility in humans is scarce and inconsistent while this subject is of contemporary significance. Holmes *et al.*, (1983) observed no effect of CBD and THC in human Sertoli cell culture. However, previous heterogeneous studies in humans are focused mainly on the adverse effects of phytocannabinoids on sperm quality and hormone levels. So far, there is no association between cannabis use and a decrease in sperm quality and serum hormone levels (Belladelli *et al.*, 2021). Here, we provided evidence that cannabis-extract cannabinoids CBD and THC do not directly affect germ cell lineage and testosterone production in adult human testis.

3.5 Conclusions

Our findings suggest that there are no acute direct effects of exposure to cannabis-derived cannabinoids THC and CBD on the testicular structure and function *ex vivo*, from the range of concentrations used in phytotherapy. In addition, this study cooperates with the safety of therapies based on plant-derived cannabinoids. A more understanding of the link between cannabis consumption and TGCT development is required.

3.6 Acknowledgments

The authors would like to thank the Institutional Internationalization Program (PrInt) from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Brazil) for the scholarship provided to J Da Silva.

3.7 Funding

This work was supported by University Rennes 1 and Institut national de la santé et de la recherche médicale (Inserm).

3.8 Conflict of interest

The authors declare that they have no competing interests.

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4. CHAPTER 3

Prenatal phytocannabinoid exposure and its long-term outcomes on male reproductive biology in adult mouse offspring

Abstract

Ingestion of cannabis derivatives during pregnancy might result in adverse outcomes to the offspring during later life. This way, we aimed to determine the impact of prenatal exposure to a mixture of the two main cannabinoids present in cannabis, CBD and THC, in the male reproductive biology of adult mice. Also, we aimed to extrapolate our results to provide information on the involvement of the endocannabinoid system (ECS) in male reproductive biology during the prenatal period. We exposed pregnant mice to a mixture of 5mg/kg of each CBD and THC from embryogenic day 6.5 to 15.5, by oral route (gavage). Our results showed decreased testicular weight and morphometric alterations, such as decreased tubular lumen and increased epithelium height, in adult offspring. However, these alterations were not followed by changes in the undifferentiated spermatogonia and Sertoli cell numbers. The RNA RT-qPCR expression analysis using the whole testes revealed an increase in the expression of pro-apoptotic (*Bax*), germ cell differentiation (*Sohlh1*), and regulating meiotic (*Stra8*) genes. We did not observe alterations in the serum testosterone levels. So, CBD/THC prenatal exposure leads to decreased testis weight in the adult life of mice, which is thought to be an organ growth restriction. Also, based on our molecular findings, prenatal exposure to cannabis-derived cannabinoids accelerates meiosis and cellular differentiation, which could be an indicator of spermatogenic activity contributing compensatory to the decreased testis weight. At the same time, prenatal exposure increases the proapoptotic gene expression (*Bax*). This "density regulation" might be necessary in the processes of acceleration of meiosis and cellular differentiation. Furthermore, we can suggest that the ECS is involved in the testis growth in mice.

Keywords: THC, CBD, spermatogenesis, steroidogenesis, repro-toxicology

Running head: Phytocannabinoids on male reproductive biology

4.1 Introduction

Cannabis use is rising globally and its consumption has been cited as the most used illicit drug during pregnancy (Schauberger *et al.*, 2014). Of concern, 22% of pregnant female younger than 18 years and 19% of pregnant females aged 18 to 24 years screened positive for marijuana use in 2016 in U. S. (Young-Wolff *et al.*, 2017). Between 2002-2003 and 2016-2017 at the same cited country, the self-reported medical and nonmedical past-month cannabis use increased from 3.4 to 7.0% among pregnant women overall and from 5.7 to 12.1% only among pregnant women during the first trimester (Volkow *et al.*, 2019). Also, medical-only cannabis use disregards the pregnancy status (pregnant or nonpregnant). However, ingestion of cannabis derivatives during pregnancy might result in adverse outcomes to the offspring, since cannabinoids are able to cross the placental barrier (Hutchings *et al.*, 1989).

The Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant cannabinoids present in cannabis, best-studied, and most used in healing treatment because of their promising therapeutic effects (Cohen *et al.*, 2019). THC and CBD can have biological activities because they can bind mainly to receptors from the endocannabinoid system (ECS) denominated cannabinoid receptors (CB1R, CB2R) (Morales *et al.*, 2017). However, the ECS has its endogenous ligands known as endocannabinoids (eCB) being anandamide (AEA) and 2-arachidonoylglycerol (2-AG) the best described (Zou and Kumar, 2018). In the past decade, there is more and more evidence that suggests eCB involvement from early to late pregnancy (Correa *et al.*, 2016). The presence of cannabinoid receptors (CB1R and CB2R) in fetal murine gonads has already been confirmed (De Domenico *et al.*, 2017). They observed the pro-meiotic role of CB2 in male and female germ cells and suggested that the use of cannabis by pregnants might represent a risk for fertility in the adult life of female offspring.

This way, prenatal cannabinoid exposure might cause long-term consequences (Schneider, 2009). With an emphasis on male reproductive health, little is known about prenatal phytocannabinoid exposure and its consequence on adult life. THC prenatal exposure was reported to decrease the testis weight (Dalterio and Bartke, 1979), as well as decrease the serum levels of luteinizing hormone (LH) and testosterone (Dalterio and deRooij, 1986) in adult rat offspring. CBD prenatal exposure was also reported to decrease testis weight of rats (Dalterio and deRooij, 1986). Cannabinol, another cannabinoid present in cannabis, was able to disrupt the hypothalamic-

pituitary-gonadal axis of adult male rat offspring after prenatal exposure (Dalterio *et al.*, 1984). Furthermore, although these three phytocannabinoid were not able to affect the number of spermatogenic cells in adult offspring, prenatal exposure to them decreased the rate of successful gestation and the number of live pups in rats (Dalterio and deRooij, 1986). However, to the best of our knowledge, nothing is documented about the effect of a mixture of phytocannabinoids and the importance of the ECS during the prenatal period for adult life.

The origin of some diseases in later life has been often referred to as the "Fetal Origin Hypothesis"(Barker *et al.*, 2002; Barker, 2007). Interestingly, testicular germ cell tumors (TGCT) have been hypothesized to be from a fetal origin and should be considered a late-onset disease due to failure of normal fetal programming of the differentiation of primordial cells through a gonocyte stage into spermatogonia (Skakkebaek *et al.*, 2016). At the same time, frequent cannabis use has been associated with the development of testicular germ cell tumors (TGCT) (Gurney *et al.*, 2015; Song *et al.*, 2020; Ghasemiesfe *et al.*, 2019). Once that cannabinoid receptors are involved in germ cell differentiation during the development, it raises the more specific possibility that phytocannabinoids used for recreational or therapeutic purposes might aberrantly sustain cannabinoid signaling of primordial germ cells.

As cannabis consumption gains acceptance and there is a light of evidence that suggests ECS plays essential role during the development period, the concern regarding outcome effects of prenatal cannabis use on male reproductive health during adult life is of total interest. Then, we aimed to determine the impact of prenatal exposure to a mixture of CBD and THC in the male reproductive biology of mice in adult life. Also, we aimed to extrapolate our results to provide information on the importance and involvement of the ECS in male reproductive biology during the prenatal period.

4.2 Materials and methods

Chemicals

The chemicals used in our study were the CBD (Axon Medchem, Netherlands) diluted in dimethylsulfoxide (DMSO) solution and the THC (Sigma Aldrich; CAS number 1972-08-3, USA) diluted in ethanol (EtOH) solution. To treat female mice, a mixture of CBD/THC (ratio 1:1) was prepared. The same amount of a mixture of DMSO/EtOH was used as the control.

Ethical statement

All animal procedures were performed according to the guidelines for animal models in research defined by the Ethics Committee and approved by the Ministry of France (reference project number is APAFIS#20984). All experiments were performed by AGP who is qualified for laboratory animal care and use.

Animal exposure

Four-month-old (m.o.) adult outbred Swiss mice (n = 14) were treated for each experimental condition (n = 7 for each experimental group). The day of vaginal plug detection was considered as embryonic day 0.5 (E0.5) and a mixture of CBD and THC was administered by gavage from E6.5 to E15.5 at the concentration corresponding to 5 mg/kg/day of CBD and 5 mg/kg/day of THC based on average body weight equal to 40 g at the beginning of the experiment. Treatments were provided in PBS Solution in a final volume of 150 µl. The control group received PBS containing the corresponding amount of DMSO and EtOH of the treated group. Control and treated adult mice (10 weeks old) were euthanized, and reproductive organs were dissected. Experiments were done on at least 5 animals derived from at least 5 different litters in each group (Figure 1).

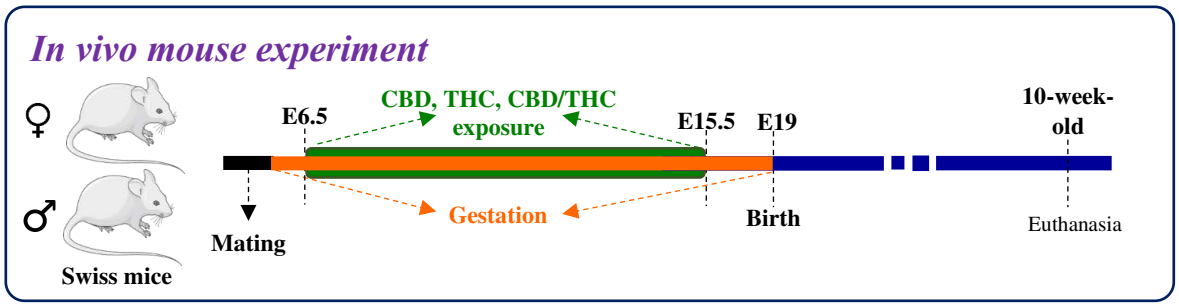

Figure 1. Experimental design.

Early cannabis administration as well as the use of very high cannabinoid doses produce morphological abnormalities that are resulting from neurotoxic effects with little relation to the endocannabinoid system (Fernández-Ruiz et al., 2000; Schneider, 2009). So, we administered the cannabinoids from E6.5 to E15.5 and we used a dose and route reflecting human cannabinoid use for therapeutic purposes. For example, the recommended oral starting dosage for Epidiolex®, a CBD-based medicine for the treatment of seizures associated with Lennox-Gastaut syndrome,

Dravet syndrome, or tuberous sclerosis complex in children and adults, is 5mg/kg/day (Food and Drug Administration, 2018).

To avoid risk of bias and increase our methodological quality, in all the analyses described below, the animals were selected at random for outcome assessment. Furthermore, they had blind outcome assessors from knowing which intervention each animal received.

Testosterone measurement

Serum was collected from ketamine/xylazine-anesthetized adult animals by terminal cardiac exsanguination and aliquots were stored at -80°C. Testosterone levels in the serum were assayed in duplicate using a commercial radioimmunoassay (RIA) based on competitive binding with I125-labeled testosterone (Immunotech, Ref. IM1087, Czech Republic), according to the manufacturer's recommendations. The intra- and inter-assay coefficients of variation were 11.6 and 13.5%, respectively.

Analysis of histology, morphometry, and stereology

For these analyses, testis samples were fixed in Bouin's solution and embedded in paraffin. Histological sections (5 μm-thick) were stained with hematoxylin and eosin (H&E). The testicular histology of each animal from each condition was checked under a light microscope (Zeiss microscope).

For morphometry and stereology of testicular tissue from adult mice, 20 digital images from each animal of each condition were obtained using a light microscope connected to a digital camera (Olympus AxioCam ICc 1). The analyses were performed using ImageJ (National Institute of Health, USA) Software as already described (Mouro *et al.*, 2019). Briefly, the proportion among the components that constitute the testis parenchyma was obtained using square grids placed over the digital images (200x magnification). A total of 2.660 intersection points were counted per animal classifying those on interstitial space and seminiferous tubule and its components (seminiferous epithelium, lumen, and tunica propria). The percentage of each component was determined by the ratio between the number of total counted points and the number of counted points at the studied structure). The volume of the testicular components was obtained by multiplying the percentage of each component by the testis weight, considering the density of the mammalian testis equal to one (Johnson and Neaves, 1981).

The seminiferous tubule diameter was obtained after measuring it at 20 random circular seminiferous tubule cross-sections from each animal. These sections were also used to obtain the seminiferous epithelium height, from the tunica propria to the tubular lumen, as the mean of two diametrically opposed measurements. The tubular lumen diameter was calculated after subtracting the seminiferous epithelium height from the seminiferous tubule diameter. The seminiferous tubule and lumen areas were calculated according to the following equation: area = $\pi.R^2$, in which R is the tubular radius or the lumen radius, respectively. The epithelium area was obtained after subtracting the luminal area from the tubular area. The seminiferous tubule length was assessed by the ratio of seminiferous tubule volume and seminiferous tubule area. Finally, the tubule epithelium ratio was calculated by dividing the tubule area by epithelium area.

Immunohistochemistry analysis

For immunohistochemistry (IHC), the adult offspring were perfused, and the testes were fixed for 24h in 4 % (wt/vol) paraformaldehyde (PFA) and then embedded in paraffin. So, to estimate the number of Sertoli cells (SC) and undifferentiated spermatogonia (SG), sections from the 10 weeks-old testes were stained with monoclonal rat anti-GATA1 (sc-265, 1:50, Santa Cruz Biotechnology) and polyclonal goat anti-PLZF primary antibody (AF2944, 1:500, R&D systems) as already described (Gely-Pernot *et al.*, 2018). To quantify the number of SC (GATA1-positive cells) and SG (ZBTB16 (PLZF)-positive cells), we manually counted the cells on around 30 sections of the seminiferous epithelium at stage VII taking in three different random areas of the testis for each biological replicate. The perimeter of each tubule section was measured using ImageJ to indicate the cell counts per micrometer of tubule circumference. The ratio of the number of SC per SG was also calculated.

RNA extraction and Reverse Transcription quantitative real-time PCR (RT-qPCR)

RNA was extracted from mouse testes samples, which were frozen, using RNeasy® Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. The quantity of RNAs was estimated by nanodrop. Reverse transcription was performed in 1µg of RNA using QuantiTect Reverse Transcription (QIAGEN) according to the protocol provided by the manufacturer. RT-qPCR was performed according to the manufacturer's instructions with iTaq Universal SYBR Green Supermix (Bio-Rad) and 2µL cDNA template in a CFX384 Touch Real-Time PCR Detection

System (Bio-Rad). The amplification program was as follows: an initial denaturation of 3 min at 95 °C; 40 cycles of 10-s denaturation at 95 °C; and 30 s at 62 °C for annealing and extension. Dissociation curves were produced with the thermal melting profile performed after the last PCR cycle. To avoid amplification of contaminating genomic DNA, primer pairs were selected on two different exons (For genes studied, see Table 1). PCR amplification of the coding regions of *Actb* and *Rplp0* was used for normalization. The data from at least 6 samples were analyzed. Results were calculated by the ΔΔCT method as *n*-fold differences in target gene expression with respect to the reference gene and the calibration sample.

Table 1. Primers used for RT-qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Product size (bp)
Bax	TTTGCTACAGGGTTTCATCCAG	CCAGTTCATCTCCAATTCGCC	137
Bcl2	CCTGTGGATGACTGAGTACC	GTATGCACCCAGAGTGATG	184
Dazl	CAGCACTCAGTCTTCATCAG	GACACACCAGTTCGATCAGT	186
Zbtb16	GCAGCTATATTTGCAGTGAG	CAGCCATTACACTCATAGGG	190
Kit	GTACATGGCTGCATTCTGAC	CATCTTTAGCCACATGGAGT	173
Sall4	ACACGGTGTGGAAAGAACT	GTTCTCTATGGCCAGCTTC	195
Sohlh1	ACTTGGTCTTGGCATCTGT	AGTCAGGGAAGATGCTGTC	300
Sohlh2	TCAGTGAGCCGCTGACCTTG	AAAAACGCCCTCCGAGTTCAC	150
Stra8	GATGCTTTTGACGTGGCAAGTTTCC	TCATCATCTGGGGGCTCTGGTTC	197
Star	TCTCTGCTTGGTTCTCAACT	AAACACCTTGCCCACATCTG	192
Cyp26b1	GAATGTCTTCTCTCTGCCAG	CTCAATGAGAATGTCCAGGG	160
Inha	GTTGGGATGGCCGGAATACA	GGCGTCTGCCTCGAAGACAT	189
Lhcgr	TGCCTTTGACAACCTCCTCA	TCGAAACATCTGGGAGGGTC	152
Reference genes			
Actb	GACAGCAGTTGGTTGGAG	GGGTGAGGGACTTCCTGTAA	156
Rplp0	ACCCTGAAGTGCTCGACATC	AGGAAGGCCTTGACCTTTTC	208

Bax - BCL2-associated X protein; *Bcl2* - B cell leukemia/lymphoma 2; *Dazl* - deleted in azoospermia-like; *Zbtb16* - zinc finger and BTB domain containing 16; *Kit* - KIT proto-oncogene receptor tyrosine kinase; *Sall4* - spalt like transcription factor 4; *Sohlh1* - spermatogenesis and oogenesis specific basic helix-loop-helix 1; *Sohlh2* - spermatogenesis and oogenesis specific basic helix-loop-helix 2; *Stra8* - stimulated by retinoic acid gene 8; *Star* - steroidogenic acute regulatory protein; *Cyp26b1* - cytochrome P450, family 26, subfamily b, polypeptide 1; *Inha* - inhibin alpha; *Lhcgr* - luteinizing hormone/choriogonadotropin receptor; *Actb* - actin, beta; *Rplp0* - ribosomal protein, large, P0.

Sperm analyses

Freshly dissected portions of the epididymis cauda from adult mice were placed in a petri dish and cut around five times. After, it was added 50µl of tris-citric acid-sucrose (tris 3.025g, citric acid 1.7g, sucrose 1.25g miliqH_20 100ml q.s.) for 5min at 34ºC to enable the release of spermatozoa. Aliquots of this fluid were collected for sperm analyses. Sperm motility was evaluated immediately post-collection by adding 10 µl of epididymal fluid to 200 µl of tris-citric acid-sucrose. The spermatozoa were classified as either motile or immotile in three random fields in 2 histological slides of each animal and the total motility was expressed as percentage. For sperm morphology, epididymal fluid was fixed in 100 µl 2% buffered formaldehyde. Two hundred spermatozoa were examined under phase-contrast microscopy (400x magnification) and classified as normal, defects in the head, midpiece, principal piece or terminal segment, coiled tail, and cytoplasmic droplet. The results were expressed in percentage.

Statistical analyses

Quantitative variables were compared by t-test using GraphPad Prism software v8.0.1 (GraphPad Software, USA). A p-value < 0.05 was considered statistically significant. The data are expressed as means ± SEM.

4.3 Results

Prenatal exposure to a mixture of CBD and THC affected testicular weight, morphometry, and stereology of adult mouse offspring

To analyze the effect of prenatal CBD/THC exposure on body weight as well as on reproductive organs weight, we weighted the animals at the euthanasia moment and the organs immediately after dissection. As a result, the body weight of adult mouse offspring did not change as well as the epididymis and vesicle weights. However, the absolute and relative testicular weights were lower in adult mice after prenatal exposure to a mixture of the phytocannabinoids CBD and THC, as shown in Table 2.

Table 2. Biometric parameters from adult mice after prenatal exposure to CBD/THC.

Parameters	Control	CBD/THC
Body weight (g)	37.49 ± 0.16	37.36 ± 0.82
Testis weight (g)	0.13 ± 0.00	0.11 ± 0.00*
Relative testis weight (%)	0.34 ± 0.01	0.30 ± 0.01*
Epididymis weight (g)	0.04 ± 0.00	0.04 ± 0.00
Relative epididymis weight (%)	0.11 ± 0.01	0.12 ± 0.00
Seminal vesicle weight (g)	0.25 ± 0.03	0.26 ± 0.01
Relative seminal vesicle weight (%)	0.66 ± 0.07	0.71 ± 0.04

*Significant difference between Control and CBD/THC groups by t-test $p<0.05$ (n=6 control; n=7 CBD/THC). Data presented as mean ± SEM.

In order to understand the decrease in the testicular weight, we checked the histology of adult mouse testis. Analysis of the sections stained with hematoxylin/eosin did not show any noticeable alteration in seminiferous tubules or interstitial tissue morphology caused by prenatal exposure to a mixture of phytocannabinoids CBD and THC when compared to the control (Figure 2).

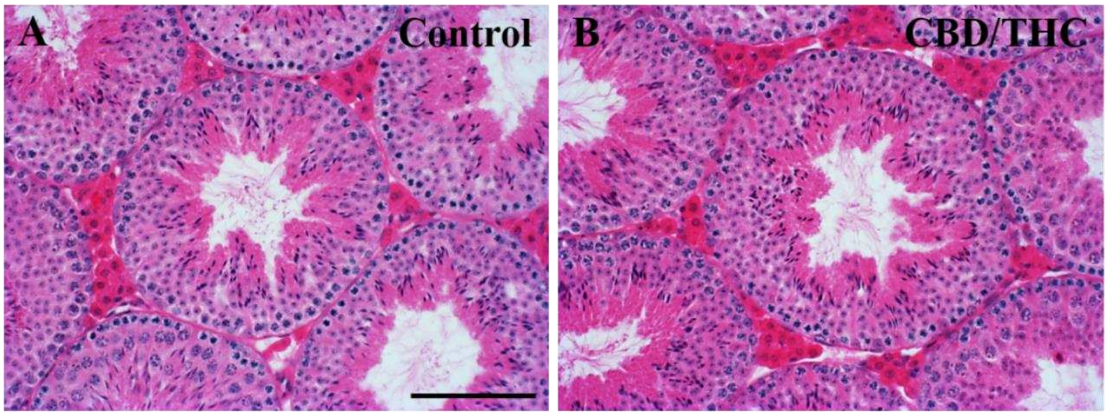

Figure 2. Histology of adult mice testis after prenatal exposure to: a) a vehicle (Control) or b) a mixture of phytocannabinoids (CBD/THC). Bars = 100µm.

Although the prenatal exposure to phytocannabinoids did not affect noticeably the histology of adult testis, we performed the morphometry and stereology of the testicular tissue to check if there is any alteration that was not perceptive to our eyes in the observation of testicular morphology. Our findings showed that the proportion among the testicular components (seminiferous tubule, seminiferous epithelium, tunica propria, lumen, and interstitial tissue) did not change after CBD/THC prenatal exposure. However, when we checked the volume of these

components, we found that the lumen volume was almost 25% lower after CBD/THC prenatal exposure when we compared it to the control group (Table 3).

Table 3. Proportion and volume of testicular components of adult mice after prenatal exposure to CBD/THC.

Parameters	Control	CBD/THC
Tubule (%)	89.86±0.86	88.91±1.71
Epithelium (%)	78.92±1.41	79.17±1.66
Lumen (%)	9.46±1.16	8.37±1.07
Tunica propria (%)	1.48±0.22	1.38±0.22
Interstitial tissue (%)	10.14±0.86	11.09±1.71
Tubule (μL)	115.92±10.92	98.93±14.51
Epithelium (μL)	101.76±9.09	88.14±13.3
Lumen (μL)	12.24±2.21	9.25±1.44*
Tunica propria (μL)	1.92±0.44	1.54±0.39
Interstitial tissue (μL)	13.06±1.06	12.32±2.36

*Significant difference between Control and CBD/THC groups by t-test $p < 0.05$ (n=5 control; n=6 CBD/THC). Data presented as mean ± SEM.

Also, although the tubule diameter was not significantly altered by the prenatal treatment, the epithelium height was higher by 5.6% and the lumen diameter was lower by 16.6% as shown in Table 4. The lumen changes were also verified in its area, which was almost 31% lower. The tubule/epithelium ratio was also lower after prenatal exposure to phytocannabinoids (3.48%). The tubule and epithelium areas as well as the tubule length were not modified by the treatment.

Table 4. Testicular morphometry of adult mice after prenatal exposure to CBD/THC.

Parameters	Control	CBD/THC
Tubule diameter (µm)	222.44±3.36	216.89±2.48
Epithelium height (µm)	70.73±1.02	74.67±1.12*
Lumen diameter (µm)	80.99±4.09	67.56±2.05*
Tubule area (10-² mm²)	3.89±0.12	3.70±0.09
Epithelium area (10-² mm²)	3.37±0.08	3.34±0.08
Lumen area (10-² mm²)	0.52±0.05	0.36±0.02*
Tubule length/testis (m)	2.99±0.13	2.67±0.13
Tubule length/g.testis (m)	23.20±0.73	24.12±0.55
Tubule/epithelium ratio	1.15±0.01	1.11±0.01**

*Significant difference between Control and CBD/THC groups by t-test $p < 0.05$. **Significant difference between Control and CBD/THC groups by t-test $p < 0.01$ (n=5 control; n=6 CBD/THC). Data presented as mean ± SEM.

Prenatal exposure to a mixture of phytocannabinoids CBD/THC did not affect the testicular steroidogenic function of mice in adult life

We assessed the steroidogenic function of the testes by measuring the serum testosterone of adult mice. As result, prenatal exposure to a mixture of phytocannabinoids CBD and THC did not affect significantly the serum testosterone concentration of male mice in adult life (Figure 3).

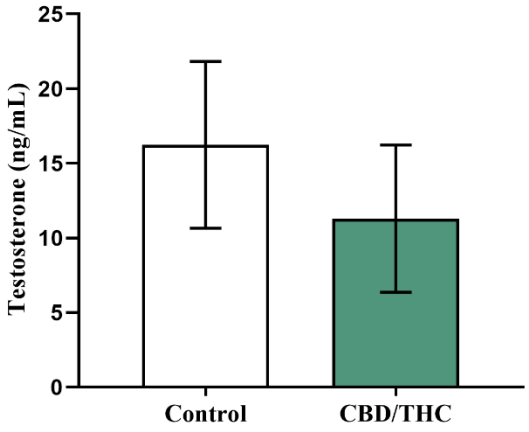

Figure 3. Serum testosterone levels of adult mice after prenatal exposure to CBD/THC. No significant difference between Control and CBD/THC groups by t-test (n=6 control; n=7 CBD/THC). Data presented as mean ± SEM.

Prenatal exposure to CBD/THC did not change the numbers of Sertoli cells and undifferentiated spermatogonias in adult mouse offspring

To check whether prenatal exposure to the two main cannabinoids present in cannabis could affect Sertoli cells and undifferentiated spermatogonia, we quantified positive cells for GATA1 and ZBTB16, respectively. As result, the number of both cell types was not affected by the phytocannabinoids exposure, as well as the ratio of the number of Sertoli cells per undifferentiated spermatogonias (Figure 4).

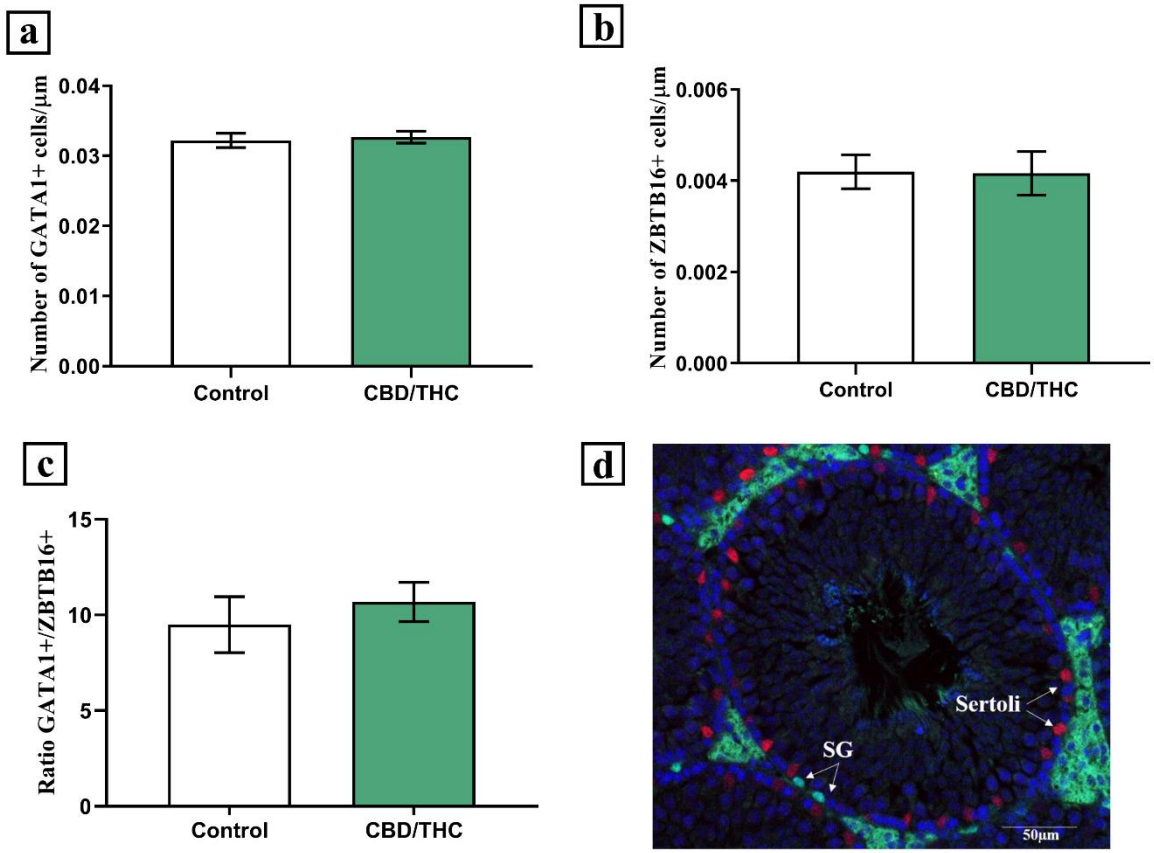

Figure 4. Numbers of Sertoli cells (GATA1) and undifferentiated spermatogonia (ZBTB16) in the testis of adult mice after prenatal exposure to CBD/THC. a) Sertoli cell number; b) undifferentiated spermatogonia number; c) the ratio of the number of Sertoli cells per undifferentiated spermatogonias; d) representative image of the teste section showing Sertoli cells labeled in red and undifferentiated spermatogonia (SG) labeled in green. The cells were counted at stage VII of the seminiferous epithelium. The values shown indicate the cell counts per micrometer of tubule circumference. No significant difference between control and CBD/THC groups by t-test (n=5 control; n=6 CBD/THC). Data presented as mean ± SEM.

Prenatal exposure to CBD/THC altered mRNA expression in the testis of adult mouse offspring

To reveal the effect of prenatal exposure to CBD/THC on testicular gene expression of adult mice, we performed RT-qPCR. We chose important genes involved in apoptosis (*Bax, Blc2*), germ cell differentiation (*Dazl, Zbtb16, Kit, Sall4, Sohlh1, Sohlh2*), meiosis (*Stra8*), steroidogenesis (*Cyp26b1*), Sertoli cell (*Inha*), and endocrine signaling (*Star, Lhcgr*). We found that prenatal exposure to CBD/THC increased the expression of *Bax*, *Sohlh1*, and *Stra8* genes in the testis of mice in adult life (Figure 5).

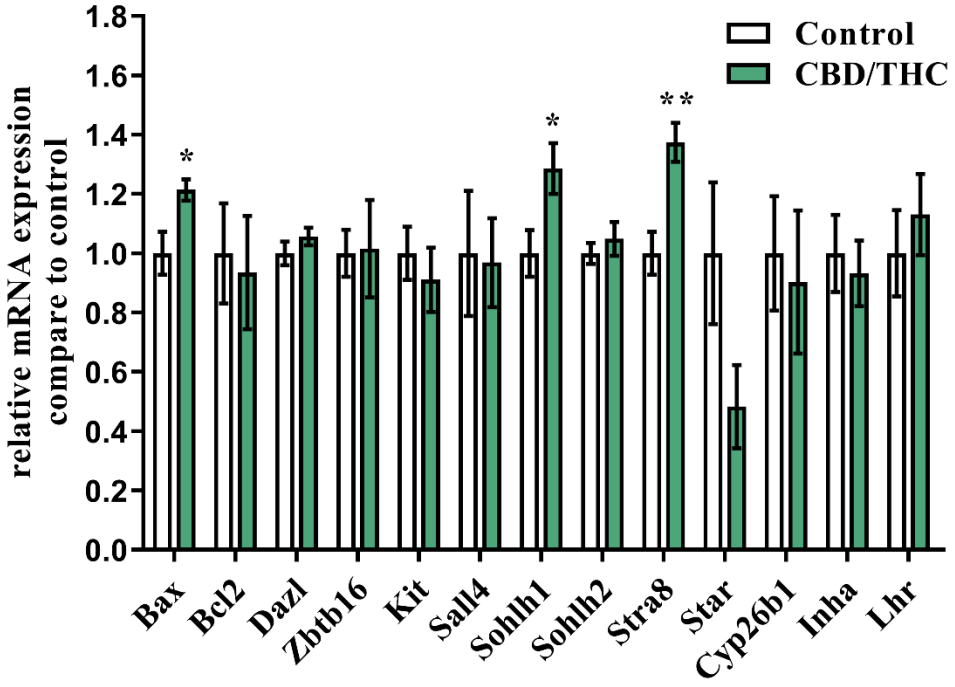

Figure 5. RT-qPCR of some genes expressed in the adult mouse testis after prenatal exposure to CBD/THC. *Significant difference between Control and CBD/THC groups by t-test p< 0.05. **Significant difference between Control and CBD/THC groups by t-test p< 0.01 (n=5 control; n=7 CBD/THC). Data presented as mean ± SEM.

Prenatal exposure to CBD/THC did not change the sperm quality of adult mice

To check the sperm quality of adult mice after prenatal exposure to CBD/THC, we evaluated the sperm motility just after euthanasia and sperm morphology in fixed sperm (Figure 6). We observed that there were motile spermatozoa in both experimental groups and this motility did not change significantly by the CBD/THC prenatal exposure when we compared to the control. Also,

we did not observe a significant alteration in the sperm morphology when we compare the percentage of normal sperm between the groups. Furthermore, we did not see a significant difference even when we quantified the percentage of coiled tail, cytoplasmic droplet, or defects at the specific sperm regions (head, midpiece, principal piece, and terminal segment).

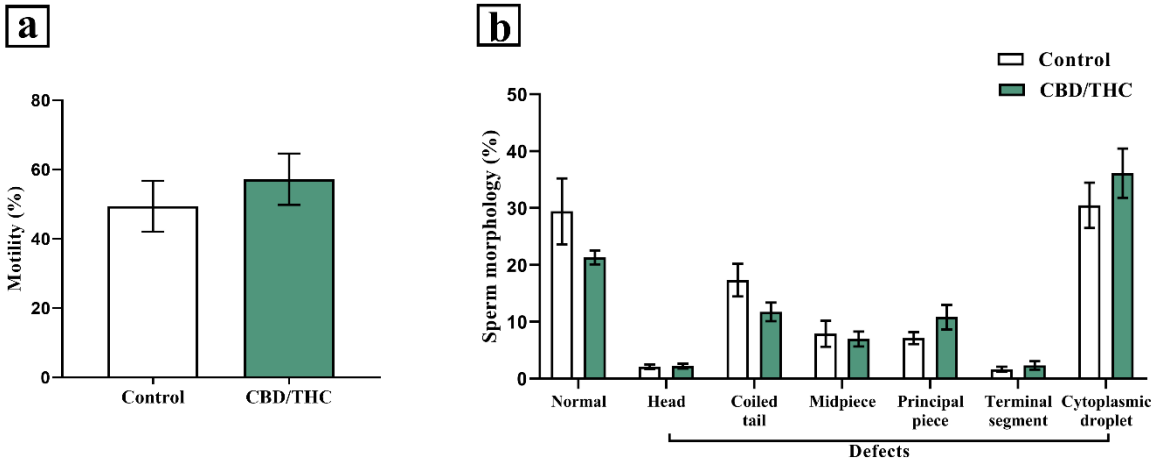

Figure 6. Sperm quality of adult mice after prenatal exposure to CBD/THC. a) Sperm motility; b) Sperm morphology. No significant difference between Control and CBD/THC groups by t-test $p < 0.05$ (n=6 control; n=7 CBD/THC). Data presented as mean ± SEM.

4.4 Discussion

Main findings

Several diseases in adult life have been associated to have a "fetal origin", in which insults during the prenatal period might result in pathological conditions in later life. Then, we aimed to determine the impact of prenatal exposure to a mixture of CBD and THC in the male reproductive biology of adult mice. Also, we aimed to extrapolate our results to provide information on the importance and involvement of the ECS during the prenatal period. Although prenatal exposure to the two main cannabinoids present in cannabis did not affect the serum testosterone, the numbers of Sertoli cells and undifferentiated spermatogonias, and the sperm quality, our results revealed that prenatal exposure to CBD/THC decreases the testicular weight, causes changes in the testicular morphology, and upregulates some important testicular genes. By extrapolation, our findings suggest the role of the ECS in the testis growth

THC prenatal exposure (single dose of 50mg/kg at E20) was already reported to decrease the testis weight of mice (Dalterio and Bartke, 1979), as well as CBD prenatal exposure (single dose of 50mg/kg at E12) (Dalterio and deRooij, 1986). Natale *et al.* (2020) concluded that THC exposure during rat pregnancy leads to symmetrical fetal growth restriction. They observed decreased birth weight, liver, and brain. However, these results were followed by postnatal catch-up growth. So, we hypothesized that the decrease of the testicular weight found by our study was actually an organ growth restriction, which was not reversed after birth.

Some effects of cannabinoids on the endocrine system may not become apparent until the maturational or environmental factors require physiological responses (Dalterio *et al.*, 1984). However, we did not find changes in the serum testosterone levels or the expression of steroidogenesis or endocrine signaling genes in the testes of adult offspring. Our result is in accordance with Dalterio *et al.* (1984) and Dalterio and Bartke (1979), who did not find change in the serum testosterone levels after prenatal exposure to a single dose of 50mg of THC or CBD. In fact, the effect of cannabis consumption on testosterone level was shown to be related to the recency of use, in which the serum testosterone was inversely associated with the time since last use in men (Thistle *et al.*, 2017).

The mixture of CBD/THC promoted alterations in the tubular structure such as decreased lumen and increased epithelium height. Carvalho *et al.* (2018) also observed an increase in epithelium height of mice after CBD exposure in adult life. They associated this result as an indicator of spermatogenic activity, which contributing compensatory to an increase of germ cells since other parameters, such as stage frequency, were affected. In our study, we also found dysregulated expression of some genes. We observed increased expression of the *Bax* gene, a proapoptotic member of the *Bcl-2* family that is required for normal spermatogenesis in mice (Russell *et al.*, 2002). Russell *et al.* (2002) found that the increased cell death in mature mice could be explained by disruption of the normal germ cell death earlier in development. This meets our experimental design (prenatal exposure). However, germ cell apoptosis occurs normally during spermatogenesis which is called "density-dependent regulation", possibly mediated by *Bax*, among others (Hess and França, 2008). We also identified an increased expression of *Sohlh1* gene, an essential germ-cell-specific transcription factor for spermatogonial differentiation (Ballow *et al.*, 2006). *Stra8* was also upregulated, which is a marker of meiosis initiation and it is required for germ cells to undergo meiotic prophase (Ma *et al.*, 2018). Dysregulation of meiosis and

differentiation signaling may be implicated in the pathogenesis of germ cell neoplasia *in situ* (GCNIS), the precursor lesion of testicular germ cell tumors (TGCT) (Jørgensen *et al.*, 2013). However, our histological findings did not reveal any alteration to support a tumor process.

So, based on our molecular findings, prenatal exposure to a mixture of THC and CBD accelerates meiosis and cellular differentiation, which could be an indicator of spermatogenic activity contributing compensatory to the decreased testis weight. Also, one possibility is that, since apoptosis during spermatogenesis is a homeostatic mechanism to limit germ cells to the number that can be supported by available Sertoli cells (Hess and França, 2008), we found increased expression of proapoptotic gene *Bax*, considering that the number of Sertoli cells, staining with GATA-1, did not change. This "density regulation" might be necessary in the processes of acceleration of meiosis and cellular differentiation.

Overall, these findings provide an initial investigation into the effects of CBD/THC prenatal exposure to the testis of adult offspring. By extrapolation, our findings suggest the role of the ECS in testis growth.

Strengths and limitations

Our preliminary study is based on robust, simple, and reproductive techniques. We applied standard techniques on at least 5 non-littermates from each condition to improve the accuracy. In the situations in which we analyzed two or more littermates, the data were considered the mean of the littermates' values.

Important considerations for the validity and significance of the effects of maternal cannabinoid administration obtained from animal studies are the timing of treatment and cannabinoid doses. Early cannabis administration as well as the use of very high cannabinoid doses produce morphological abnormalities that are resulting from neurotoxic effects with little relation to the endocannabinoid system (Fernández-Ruiz *et al.*, 2000; Schneider, 2009). So, we administrated the cannabinoids from E6.5 to E15.5 and we used a dose reflecting human cannabinoid use for therapeutic purposes.

Based on some literature, the testis growth restriction observed after cannabinoid exposure is likely attributable to an indirect effect resulting from impaired placental function or a direct effect resulting from the binding of THC/CBD of cannabinoids to the CB1R and CB2R in the developing testes (Natale *et al.*, 2020). The current study is limited in its scope and independent studies will

be needed to evaluate mainly the placenta. Such assessment, while beyond the scope of this study, would significantly contribute to the global understanding of the mechanisms that phytocannabinoids can lead to testis growth restriction.

Although our results of sperm motility and morphology corroborate with the maintenance of sperm quality, we did not check the sperm production and the fertilizing capacity of these sperms. We believe that decreased testis weight found here might reflect on the testicular function.

Perspectives

In the present study, prenatal CBD/THC exposure occurred from E6.5 to E15.5. Then, we analyzed the serum testosterone, the testis, and the sperm quality of the progeny at 10-week-old. Although our findings provide direct evidence regarding prenatal phytocannabinoid exposure on the male reproductive biology in late life, this study is part of a wider project.

The entire project aims to determine the impact of prenatal exposure (E6.5 to E15.5) to CBD and THC, alone or in combination, on the male reproductive health of fetus and afterbirth offspring at several ages. The project will be performed in three experimental blocks: prenatal exposure to a mixture of CBD/THC, CBD alone, and THC alone. In all these experiments, the male reproductive biology of the treated mouse progenies: fetuses (E15.5), newborns (E19), postnatal day 20 (PND20), 10-week-old (as presented in this study), and 9 months will be assessed. In this way, further experiments and analyses will be performed by the research team #8 from Institut de Recherche en Santé, Environnement et Travail, Rennes, France. Specifically, the embryos results will constitute another doctorate thesis of a Ph.D. student from the same Institution.

4.5 Conclusion

Taking together, our findings provide an initial investigation into the effects of phytocannabinoid prenatal exposure to the testis of adult offspring. CBD/THC prenatal exposure, used as herbal medicines, leads to decreased testis weight in the adult life of mice. This result is thought to be an organ growth restriction, which is not reversed after birth. Also, based on our molecular findings, prenatal exposure to cannabis-derived cannabinoids accelerates meiosis and cellular differentiation, which could be an indicator of spermatogenic activity contributing compensatory to the decreased testis weight. At the same time, prenatal exposure increases the proapoptotic gene expression (*Bax*). This "density regulation" might be necessary in the processes of acceleration of meiosis and cellular differentiation. Furthermore, we can suggest that the ECS is involved in the testis growth in mice.

4.6 Acknowledgments

The authors sincerely thank the Institutional Internationalization Program (PrInt) from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Brazil) for the scholarship provided to J Da Silva.

4.7 Funding

This work was supported by University Rennes 1 and Institut national de la santé et de la recherche médicale (Inserm).

2.8 Conflict of interest

The authors declare that they have no competing interests.

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5. FINAL CONSIDERATIONS

In this study, it has demonstrated the effects of chemicals on male reproductive biology. Cadmium, an environmental pollutant, can affect the male reproductive health of murine models and lead to male infertility. After revising systematically the existent literature, it was possible to conclude that Cd exposure induces testicular histopathologies in murine models regarding routes, in a dose- and time-dependent manner. With the AOP flamework, it was possible to identify the main mechanisms responsible for Cd-induced histomorphologies. Mimicry and interference of Cd with essential elements; change in the activity of oxidative phosphorylation enzymes; and gene expression alteration seem to be the potential MIE. The first MIE disrupts directly the endothelial structural proteins, in which the primary mechanism involved in the lesion may be vascular damage. All the MIE contribute to the increase of free radicals and consequently increase in ROS production, which leads to oxidative stress in the tissue with alterations in the antioxidant enzyme activities and, consequently, several injuries in the testis. Regarding phytocannabinoids exposure, our findings showed that short-term exposure to THC and CBD, pharmacological chemicals, does not impact the testicular physiology using *ex vivo* human testis. So, our findings do not corroborate with the notion that CBD and/or THC, used as herbal medicines, have acute direct effects on the adult human testis. On the other hand, prenatal exposure to a mixture of these cannabis-derived cannabinoids can lead to decreased testis weight in the adult life of mice. So, in mice, prenatal exposure to a mixture of them might affect the testis biology of adult offspring. By extrapolation, our results suggest the involvement of the ECS during prenatal time, which might influence the testis growth in mice. Overall, all the results presented by this thesis provide new insights into male repro-toxicology and reproductive health, which might be considered to preserve fertility across the population.