EFFECTS OF CANNABIS ON OVARIAN HEALTH AND FUNCTION

EFFECTS OF CANNABIS CONSTITUENTS ON OVARIAN HEALTH AND FUNCTION: A FOCUS ON PRENATAL EXPOSURE

By ANNIA A. MARTÍNEZ-PEÑA, M.Sc.

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree Doctor of Philosophy

DOCTORATE OF PHILOSOPHY (2022)

Graduate Program in Medical Sciences

McMaster University, Hamilton, Ontario, Canada

TITLE: Effects of cannabis constituents on ovarian health and function: a focus on prenatal exposure

AUTHOR: Annia A. Martínez-Peña, M.Sc.

SUPERVISOR: Dr. Alison Holloway

SUPERVISORY COMMITTEE: Dr. Lauren Foster

Dr. Deborah Sloboda

NUMBER OF PAGES: xx, 176

LAY ABSTRACT

The use of cannabis by pregnant women and women of reproductive age has increased considerably during the past decades, particularly in developed countries. This thesis evaluated the impact of delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, on ovarian health and function, with a focus on gestational exposure. Our results suggest that cannabis use during pregnancy may have detrimental effects on the offspring's reproductive health, as prenatal THC exposure resulted in altered ovarian follicle dynamics, decreased vascularization and increased follicular apoptosis that could lead to subfertility or premature reproductive senescence. These alterations were associated with changes in epigenetic mechanisms that regulate important growth factors in the ovary. In addition, cannabis use during adulthood may be involved in the progression of gynaecological disorders such as ovarian hyperstimulation syndrome, polycystic ovary syndrome and ovarian cancer. More studies are necessary in order to fully understand the effects of cannabis on female reproductive health.

ABSTRACT

As cannabis use increases, it is important to understand its impact on human health. Particularly, the effect of cannabis constituents on female reproductive health and the long-term effects on the offspring, when used during pregnancy, are not fully understood. The goal of this thesis was to address this knowledge gap by evaluating the impact of delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, on ovarian health and function, focusing on the long-term effects of prenatal exposure and the underlying mechanisms. Gestational THC exposure resulted in altered follicle dynamics, decreased vascularization and increased follicular apoptosis in the adult rat ovary. These alterations were associated with changes in ovarian miRNA and mRNA expression, as well as altered protein levels of important regulating factors. Specifically, prenatal THC exposure increased the expression of miR-122-5p and decreased the expression of its target gene insulin-like growth factor 1 receptor (IGF-1R), involved in the regulation of folliculogenesis, angiogenesis, and granulosa cell proliferation and apoptosis. Reduced vascularization in THC-exposed ovaries was also associated with decreased expression of the angiogenic factor vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2), as well as increased expression of the antiangiogenic factor thrombospondin 1 (TSP-1). The differential expression of these important factors, along with the decreased vascularization and increased follicular apoptosis may partially explain the follicle dynamics observed in prenatally THC-exposed rats at 6 months of age, as they had accelerated folliculogenesis with follicular development arrest, which could lead to premature reproductive senescence. The direct impact of THC on rat ovarian explants and granulosa cells was also evaluated, revealing similarities and differences between

the *in utero*, *ex vivo* and *in vitro* models. Taken together, our results indicate that cannabis constituents have the ability to alter key pathways in the developing ovary that may lead to detrimental effects on female reproductive health and fertility.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Alison Holloway; for your endless guidance and support throughout the years, especially when things got tough. You will always be an important part of who I am as a researcher and person. Thank you for always helping me find my way, for taking my thoughts and ideas into consideration while still pushing me to grow. I can't thank you enough for taking a chance on me and giving me the opportunity to do what I truly love.

To my amazing committee, Dr. Lauren Foster and Dr. Deborah Sloboda; thank you both for your invaluable time and input throughout the way. It is always a pleasure to discuss science with you.

To Dan Hardy and Jim Petrik, without whom this wouldn't have been possible. Thank you for all your help and hard work. I look forward to continue working with you both.

To my labmates, past and present, from whom I learned so much, in and outside of lab. I will always cherish the lunch table talks.

To my parents, Juan Martínez and Gisela Peña, for your unconditional love and support. I am the person I am today because of you, and I will be forever grateful and proud to be your daughter.

To my incredible family, who has taught me what it means to always be there for one another. I am truly lucky to have you all in my life.

To my chosen family, Metzli Aldrete and Eugenia Ávila. Thank you both for all the amazing moments throughout the years. No one will ever be able to make me laugh like you do.

To Renata Otero, for making me feel loved, understood and taken care of. I wouldn't have gotten here if it wasn't for you, and I will always be thankful for that.

To my cousin, friend and confidant, Adriana Martínez; I hope you know how much of an impact you had on my life. You will always be with me.

To my dearest friends, Guadalupe Argüero, Alejandra Hernández, Elisa Gutiérrez and Andrea Peña. You have all made my life so much better.

Last, but certainly not least, to the amazing friends I made along the way. Xiao, Melih, Shawna, Kumru, Tinajero, Lety, Mark, Camile, Carlos and all the Fairweather and soccer gang. Thank you for all the fun times and good talks; you made me feel at home again.

PREFACE

This thesis is prepared in the “sandwich” format as outlined in the “Guide for the preparation of Master’s and Doctoral Theses” available through the School of Graduate Studies at McMaster University. Chapter 1 consists of a general introduction for the entire body of work. Sections of this chapter have been published in a review article (doi.org/10.3390/ijms22168576). Chapters 2, 3 and 4 consist of reprints of original research articles, all published at the time of thesis submission. Chapter 5 includes a general discussion, limitations and future directions of this research project. The author of this thesis is also the first author of all four published articles included.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 2

Chapter 3

Chapter 4

Supplementary figures

LIST OF ALL ABBREVIATIONS AND SYMBOLS

2-AG – 2-arachidonoyl glycerol

5-Aza-Cdr – 5-aza-2'-deoxycytidine

ACC1/ACC2 – Acetyl-CoA carboxylase 1/2

ACEA – Arachidonyl-2-chloroethylamide

ACLY – ATP citrate lyase

AEA – Anandamide

AKT – AKT serine/threonine kinase 1

ANOVA – Analysis of variance

ARRIVE – Animal Research: Reporting of in vivo experiments

AUP – Animal use protocol

B2m – Beta-2-microglobulin

BaP – Benzo(a)pyrene

Bcl9 – B-cell lymphoma 9 protein

bFGF – Basic fibroblast growth factor

BK – Bradykinin

BMP4/BMP15 – Bone morphogenetic protein 4/15

BORN – Better Outcomes Registry & Network

BPA – Bisphenol A

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

Casp3 – Caspase 3

CB1/CB2 – Cannabinoid receptor 1/2

CBD – Cannabidiol

CD – Chlordecone

Cd28/CD31 – Cluster of differentiation 28/31

COX1/COX2 – Cyclooxygenase 1/2

CV – Coefficient of variation

Cyp1b1/Cyp19/Cyp11a1 - Cytochrome P450 family 1 subfamily B member 1/family 19 subfamily A member 1/family 11 subfamily A member 1

DAB – 3',3'-Diaminobenzidine

DAGL – Diacylglycerol lipase

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA/cDNA – Deoxyribonucleic acid/Complementary DNA

DNMT3b – DNA methyltransferase 3 beta

D-PBS – Dulbecco's phosphate-buffered saline

DRD2 – Dopamine receptor D2

DTT - Dithiothreitol

dUTP – Deoxyuridine triphosphate

eCG – Equine chorionic gonadotropin

ECS – Endocannabinoid system

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

ERK – Extracellular signal-regulated kinase

Esr2 – Estrogen receptor 2

FAAH – Fatty acid amide hydrolase

FASN – Fatty acid synthase

FBS – Fetal bovine serum

FOXO3/FOXL2 – Forkhead box O3/L2

FSH – Follicle stimulating hormone

GABA – Gamma-aminobutyric acid

GCT – Granulosa cell tumour

GD – Gestation day

GDF9 – Growth differentiation factor 9

GLUT1 – Glucose transporter 1

GnRH – Gonadotropin releasing hormone

GPR55 – G protein-coupled receptor 55

Gsk3b – Glycogen synthase kinase 3 beta

H2AX – H2A.X variant histone

H3K4me3/H4ac – Histone H3 trimethylation/Histone H4 acetylation

HASM – Human airway smooth muscle

HCC – Human hepatocellular cancer cells

HEPES – N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

HPG – Hypothalamic-pituitary-gonadal

Hprt1 – Hypoxanthine phosphoribosyltransferase 1

Hras – HRas proto-oncogene, GTPase

HRP – Horseradish peroxidase

HSD – Hydroxysteroid dehydrogenase

IGF-1 – Insulin-like growth factor 1

Igf1r – Insulin-like growth factor 1 receptor

IHC – Immunohistochemistry

Inha/Inhba – Inhibin subunit alpha/subunit beta A

IP – Intraperitoneal

IQ – Intelligence quotient

IVF – In vitro fertilization

KIT/KITL – KIT proto-oncogene, receptor tyrosine kinase/KIT ligand

LBW – Low birth weight

LH – Luteinizing hormone

Lhx8 – LIM homeobox 8

LNA – Locked nucleic acid

LPI – Lysophosphatidylinositol

LRBP – Luteinizing hormone receptor mRNA binding protein

LRP-1 – Low density lipoprotein receptor-related protein 1

MAGL – Monoacylglycerol lipase

Mapk3 – Mitogen-activated protein kinase 3

Max – MYC associated factor X

MRI – Magnetic resonance imaging

mTORC1 – Mammalian target of rapamycin kinase complex 1

MXC – Methoxychlor

NAc – Nucleus accumbens

NAPE-PLD – N-acyl phosphatidylethanolamine phospholipase D

NOBOX – NOBOX oogenesis homeobox

Nr1h3 – Nuclear receptor subfamily 1 group H member 3

OHSS – Ovarian hyperstimulation syndrome

PCOS – Polycystic ovary syndrome

PGE_2 – Prostaglandin E2

PHH3 – Phospho-histone H3

PI3K/PI3kr1 – Phosphoinositide 3-kinase/Phosphoinositide-3-kinase regulatory subunit 1

PKA – Protein kinase A

PLA2 – Phospholipase A2

PNA – Prenatally androgenized

PND – Postnatal day

POF – Premature ovarian failure

PTEN – Phosphatase and tensin homolog

PTGS1/PTGS2 – Prostaglandin-endoperoxide synthase 1/2

PVDF – Polyvinylidene fluoride

RNA/mRNA/miRNA – Ribonucleic acid/Messenger RNA/Micro RNA

RNU5G – RNA, U5G small nuclear

RT-qPCR – Real time quantitative polymerase chain reaction

Runx2 – RUNX family transcription factor 2

SCD1 – Stearoyl-CoA desaturase 1

SCOPE – Screening for Pregnancy Endpoints

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SIGC – Spontaneously immortalized granulosa cell

Smad4 – SMAD family member 4

Sohlh1 – Spermatogenesis and oogenesis specific basic helix-loop-helix 1

Srf – Serum response factor

Stat3 – Signal transducer and activator of transcription 3

TBS-T – Tris-buffered saline with 0.1% Tween 20

THC/Δ^9-THC – delta-9-tetrahydrocannabinol

THC-COOH – 11-Nor-9-carboxy-tetrahydrocannabinol

TNFα – Tumor necrosis factor alpha

TRPV1 – Transient receptor potential cation channel subfamily V member 1

TSC1 – Tuberous sclerosis complex subunit 1

TSP-1/Thbs1 – Thrombospondin 1

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick-end labeling

UK – United Kingdom

USA – United States of America

UTR – Untranslated region

VEGF/Vegfa – Vascular endothelial growth factor A

VEGFR-2 – Vascular endothelial growth factor receptor 2

DECLARATION OF ACADEMIC ACHIEVEMENT

Annia A. Martínez Peña contributed to the writing, experimental design, conducting of experiments, literature research, data analysis, interpretation of results and figure generation for all chapters of this thesis.

Alison C. Holloway, Daniel B. Hardy and James J. Petrik contributed to the experimental design and interpretation of results for chapters 2, 3 and 4.

Kendrick Lee contributed to the animal experiments included in chapters 2 and 3.

Madison Pereira contributed to the immunohistochemical staining included in chapter 3.

CHAPTER 1: INTRODUCTION

Cannabis

Prevalence of cannabis use

Cannabis, which is the generic term that denotes several preparations of the plant *Cannabis sativa,* is the most widely used illicit drug in the world, with around 4% of the global population (aged 15 to 64) consuming it [1]. Cannabis use has increased considerably during the last decades, particularly in developed countries, in which there is a growing tendency towards the legalization of its use for both medicinal and recreational purposes [1]. In fact, in 2018 Canada became the first developed nation to legalize cannabis for recreational use.

Legalization of recreational cannabis use has potential risks such as increased availability and social acceptance, decreased perception of harm of use, as well as diversification of cannabis products that may increase interest in use [2]. Since legalization of recreational cannabis use in Canada is still relatively recent, there is limited research examining the outcomes and implications of this policy change. However, a study that evaluated data from four different Canadian population-based sources (The National Cannabis Survey, The Canadian Cannabis Survey, The Canadian Student Tobacco, Alcohol and Drugs Survey and The Centre for Addiction and Mental Health Monitor) reported a slight increase in the prevalence of cannabis use following legalization (2017 to 2020) [3]. Likewise, a study that assessed the prevalence of cannabis use among college students (19.6±2.4 years old) in Colorado (United States), where recreational cannabis has been available for people over 21

since 2014, revealed a significant increase in the number of people who have consumed cannabis after legalization, regardless of their age [2].

Recent estimates suggest that 15 to 25% of Canadians over the age of 15 use cannabis [4,5]. The use of cannabis is estimated to be 2-fold higher in Canadians aged 16 to 24 when compared to those aged 25 or older [4]. Similarly, around 7.8% of the population aged 15 to 64 in Western and Central Europe reported past-year cannabis use in 2019, with a similar difference in usage in younger (aged 15 to 34) people. In the United States, in addition to the number of cannabis users, the frequency of use and quantity of cannabis consumed have also increased dramatically during the past decade [1].

Cannabis use during pregnancy

The legalization of cannabis may have a negative impact on the general public's perception of the risks and benefits of this drug and its constituents. This is especially concerning considering that the use of cannabis during pregnancy has also increased significantly in developed countries, particularly among younger women [6–8], whose perception of cannabis as a harmful drug has decreased over the past decade [1,9]. A study by Ko and colleagues revealed that 70% of pregnant and non-pregnant women in the United States believe that there is little to no harm in using cannabis once or twice per week [9]. Moreover, a longitudinal prospective study in the United Kingdom discovered that 48% of women who used cannabis in the year prior to their pregnancy continued to smoke throughout gestation [10].

Studies that have investigated the prevalence of cannabis use in developed countries revealed that approximately 4-7% of women reported using cannabis during pregnancy [7,8,11,12]. A retrospective cohort study assembled from the Better Outcomes Registry & Network (BORN) (Ontario, Canada) database reported a 61% increase in the overall prevalence of cannabis use during pregnancy between 2012 and 2017. Prevalence was highest among women aged 15–24 years old, who reported an increase in usage from 4.9% in 2012 to 6.5% in 2017 [6]. Another study that evaluated cannabis use during pregnancy in Ontario after legalization (2019) reported that 11% of women (aged 19 to 41 years) consumed cannabis at some point during pregnancy, including the time before they were aware they were pregnant, and 4% continued to consume cannabis after knowing they were pregnant [13]. In addition, 65% of the women who continued to use cannabis during pregnancy did so at least once a week [13]. In the United States, the 2019 National Survey on Drug Use and Health revealed that approximately 5.4% of pregnant women reported using cannabis during the past month [8]. Data from the Screening for Pregnancy Endpoints (SCOPE) study, which includes nulliparous women with singleton pregnancies between 2004 and 2011 from Australia, New Zealand, Ireland, and the United Kingdom, revealed that self-reported cannabis use during pregnancy was approximately 4% [7]. Additionally, given that most of these studies rely solely on self-report, it is likely that these data underestimate actual prevalence of cannabis use during pregnancy [14,15].

Cannabis is typically used for its antiemetic properties in mitigating pregnancy-related nausea and vomiting, more commonly during the first trimester [16–18]. In some cases, cannabis is also used to relieve pain and to aid with disorders such as anxiety and depression throughout pregnancy [19]. In spite of this, there are currently limited studies that evaluate the long-term effects of prenatal cannabis exposure on the offspring's health.

Furthermore, there is conflicting information available to the public regarding the risks and benefits of cannabis use during pregnancy. While most obstetricians and gynaecologists do not recommend the use of cannabis during pregnancy [20,21], if pregnant women do not feel comfortable discussing health risks of cannabis use with their healthcare providers or if they are dissatisfied with the quality of the information they receive, it is likely that they resort to alternative sources of information [22]. Indeed, a recent study that analyzed posts containing the words "cannabis" and "pregnancy" on the social media platform Twitter discovered that 36% of these posts addressed the safety of cannabis use during pregnancy, while 2.7% discussed the use of cannabis to alleviate pregnancy-related symptoms [23]. In addition, although most Canadian dispensaries (93%) recommend against the use of cannabis during pregnancy [24], a study in Colorado revealed that almost 70% recommended treatment of morning sickness with cannabis products, mostly based on personal opinion [25]. Thus, health practitioners and communication planners should be encouraged to discuss the risks of cannabis use, with patients and the general public

respectively, in order to prevent the spread of misinformation from the media and non-healthcare-related sources [23].

Cannabis composition and potency

The cannabis plant contains more than 500 compounds from several chemical classes including cannabinoids (phytocannabinoids), mono- and sesquiterpenes, sugars, hydrocarbons, flavonoids, steroids, nitrogenous compounds, amino acids, and simple fatty acids [26,27]. Among these, the phytocannabinoid delta-9-tetrahydrocannabinol (THC) is one of the most studied constituents, as it is the major cannabinoid present in most cannabis products and it is known for its psychoactive properties [28,29].

Although THC is normally hydroxylated and oxidized to its non psychoactive metabolite (THC-COOH) in the liver, with prolonged exposure THC can accumulate in lipid storage compartments and slowly be released back into the blood stream [30]. This redistribution, along with a significant enterohepatic circulation, contributes to a long terminal half-life for THC in plasma, reported to be greater than 4.1 days in chronic cannabis users [31]. Furthermore, it is known that THC efficiently crosses the placental barrier and can be detected in cord blood and fetal tissue when cannabis is used during pregnancy [32].

In addition to the growing prevalence of cannabis use, the concentration of THC has also increased considerably in cannabis and cannabis products during the last few decades [1,33–35]. In Canada, the percentage of THC in dried cannabis has

increased from an average of 3% in the 1980s to an average of 15% in 2018, with some strains containing as much as 30% [36]. The percentage of THC in cannabis that was seized as part of law enforcement activities has increased fourfold in the United States between 1995 and 2018, and almost doubled in Europe between 2002 and 2018 [1]. Despite this trend, the percentage of adolescents who consider regular use of cannabis to be harmful decreased significantly over the same period of time [1].

The endocannabinoid system

The most studied mechanism of action of THC and other cannabinoids in the human body is through their interaction with the endocannabinoid system (ECS) [26,37]. The ECS is comprised of cannabinoid receptors, such as the G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2, respectively), endogenous cannabinoids that include 2-arachidonoyl glycerol and anandamide (2-AG and AEA, respectively), as well as the enzymes involved in their synthesis and degradation (i.e., NAPE-PLD, FAAH, MAGL, DAGL) [38,39]. It is known that THC and other cannabinoids can bind to and activate the cannabinoid receptors, which modulate several different signaling pathways including the inhibition of adenylyl cyclase activity and therefore intracellular cyclic adenosine monophosphate (cAMP) levels and PKA phosphorylation [40]. While ECS components were first identified in the nervous and immune systems, there is increasing evidence that demonstrates their peripheral role in endocrine and reproductive health [38,41,42].

The ECS and female reproduction

Components of the ECS have been located throughout the female reproductive tract, including the ovary [43,44], fallopian tubes [45], myometrium [46] and endometrium [47]. Studies have shown that the ECS is involved in the regulation of reproductive processes such as fertilization, implantation, embryonic development and placental growth [42,48–50]. Although the physiological implications of the ECS ligand-receptor interactions are not completely understood, it has been suggested that they are also linked to the hypothalamic-pituitary-gonadal (HPG) axis, the opioid, GABAergic, dopaminergic, noradrenergic, serotonergic, cholinergic, prostaglandin and glucocorticoid systems [51,52]. While it is clear that disruption of the HPG axis can lead to negative reproductive outcomes, the dysregulation of other systems, such as the prostaglandin and glucocorticoid systems, that have a profound interaction with components of the reproductive system, may also have an indirect impact on reproductive health [53–56].

The ECS and the developing fetus

The ECS also plays a crucial role in fetal development, from embryo implantation to neurodevelopment and peripheral organogenesis [50,57–60]. In mice, components of the ECS, such as CB1, CB2, NAPE-PLD and FAAH, have been detected as early as the one-and two-cell stages of embryonic development [61,62]. AEA-CB1 signaling is involved in preimplantation embryo development, blastocyst activation and implantation [63,64]. Endocannabinoids have also been detected in fetal tissue, with levels of 2-AG being much higher than those of AEA [65]. While concentrations

of AEA gradually increase throughout development until reaching maximal concentrations in adulthood [65], fetal levels of 2-AG are similar to those observed in young and in adult brains. Collectively, the evidence suggests that ECS component expression and activity must be tightly regulated from very early stages of development and throughout pregnancy [66]. Therefore, dysregulation of the ECS due to exogenous compounds such as THC may lead to negative impacts on the offspring's health.

Cannabis and female reproductive health

To date there are limited studies on the effects of cannabis on female reproductive health [67]. However, it is generally believed that cannabis may have a negative impact on human fertility and pregnancy [68], as its use has been associated with altered luteinizing hormone levels [69], menstrual cycle dysregulation and ovulatory issues [70,71], fewer and poorer quality oocytes, as well as lower pregnancy rates by *in vitro* fertilization (IVF)[72].

Animal studies have shown that THC alone can disrupt the HPG axis. In both male and female rats, exposure to THC lowered gonadotropin releasing hormone (GnRH) concentrations in the hypothalamus [73,74]. Studies with primates and rodents have reported an inhibition of luteinizing hormone (LH) production, as well as a significant decrease in ovulation as a result of THC exposure [75–77]. THC has also been shown to affect prenatal development, increasing the number of resorptions and decreasing fetal weight [78–81].

In addition, it has been proposed that THC may also have a direct effect on the gonads. Indeed, THC has been shown to inhibit progesterone and estradiol production in rat isolated Graafian follicles [82], as well as progesterone production in rat luteal cells *in vitro* [83]. In a study using rat granulosa cells, THC inhibited the follicle stimulating hormone (FSH)-enhanced accumulation of estrogen and progesterone, as well as the increase in LH receptors. The authors proposed that at least part of this inhibition was due to a post-cAMP mechanism of action, since they determined that THC inhibits pregnenolone biosynthesis and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity [84]. In another study with rat granulosa cells, however, THC exhibited a concentration dependent inhibition of the FSH-mediated cAMP accumulation necessary for ovulation [85], pointing out the possible existence of diverse mechanisms of action for this compound.

Effects of cannabis use during pregnancy

Overall, reports about the effects of cannabis use during pregnancy in humans are conflicting [12]. While many researchers have demonstrated that prenatal cannabis use is associated with an increased risk of stillbirth [86,87], preterm birth [7,86,88], small for gestational age [88–90], low birth weight [91–93], and increased admission to neonatal intensive care units [88,90], others have reported no association between prenatal cannabis use and adverse pregnancy or neonatal outcomes [94–96]. As with all drugs of abuse, clinical research on cannabis use during pregnancy has been limited to epidemiologic and retrospective studies, since there are no controlled human experiments due to ethical considerations. Inconsistent

conclusions from maternal cannabis studies in humans could therefore be a result of confounding variables related to socio-demographics, sample size, maternal nutrition, poly-substance use, cannabis potency as well as frequency and duration of use.

A recent review by Singh and colleagues concluded that studies on the effects of prenatal cannabis use in humans are very heterogeneous with respect to study design, study population, data sources and other covariates, which precluded the meaningful pooling of data [12]. The authors pointed out the need for quantification of cannabis exposure by gestational age or frequency, toxicological data rather than self-reported cannabis use, as well as harmonisation in the reporting of pregnancy outcomes in order to have a better understanding of the effects of prenatal cannabis use [12]. On the other hand, the use of animal and *in vitro* models offers an alternative to evaluate the effects of gestational cannabis exposure with greater control over experimental conditions, allowing a deeper comprehension of the underlying mechanisms.

Long-term effects of prenatal cannabis exposure

In addition to the effects observed during pregnancy and immediately after birth, prenatal exposure to cannabis may also result in long-term alterations in the offspring's health, as the developing organs are particularly sensitive to exogenous compounds [97]. While investigations into the long-term effects of cannabis exposure are limited, most of the research effort has focused on the long-term

neurobehavioural effects of prenatal cannabis exposure. In humans, major prospective longitudinal studies have found that cannabis-exposed offspring had diminished verbal and memory skills at 3 to 4 years of age [98,99], increased impulsivity and hyperactivity, as well as decreased concentration, IQ score, and verbal reasoning at 6 or 10 years of age [100,101]. As young adults (18 to 22 years of age), cannabis-exposed offspring presented with alterations in response inhibition and altered neural functioning during visuospatial working memory processing, as assessed by functional magnetic resonance imaging (fMRI) [102,103]. Interestingly, rodent studies have also shown impaired learning, attention and memory skills as a result of prenatal exposure to both THC and a synthetic CB1 agonist, pointing out the role of the ECS in these long-term effects [104].

Although the effects of cannabis on other physiological aspects of the developing fetus have received less attention, animal studies have suggested that prenatal cannabinoid exposure may result in long-lasting metabolic, cardiovascular and reproductive abnormalities in the offspring [102,105–109].

The developing ovary

The ovary is one of the major organs involved in female reproductive and endocrine health, as it is responsible for the development and release of mature oocytes (folliculogenesis and ovulation), as well as the regulation of the menstrual/estrus cycle and other sexual characteristics through the synthesis and secretion of endocrine factors, such as steroid hormones [38,110]. Since the establishment of

the ovarian primordial follicle pool, which is critical for the long-term reproductive capacity of female mammals, takes place during *in utero* development, adverse prenatal conditions may have negative impacts on the offspring's reproductive health later in life [111,112].

Impact of adverse prenatal conditions on reproductive health

It is known that adverse prenatal conditions, such as maternal undernutrition, hormonal imbalance or exposure to exogenous chemicals, have the potential to alter ovarian development, with major implications for reproductive health and fertility [113]. Alterations in ovarian development that affect the establishment of the ovarian reserve (primordial follicle pool), for example, have been implicated in premature ovarian failure [114].

In humans, low birth weight (LBW), commonly associated with maternal undernutrition and smoking [115], has been linked to early pubertal onset [116,117], lower ovulation rates [118], poor pregnancy outcomes [119] and early menopause [120]. Studies with rodents confirmed that fetal nutrient restriction leads to reduced birth weight, and that LBW offspring enter puberty early and have increased ovarian oxidative stress as well as a premature loss of follicles as adults [121].

Altered hormone levels *in utero* also have an impact on the developing fetus. Animal studies have shown that prenatal exposure to an excess of androgens results in ovarian alterations similar to those observed in polycystic ovary syndrome later in life (PCOS) [122]. Furthermore, rats that were prenatally exposed to the synthetic

glucocorticoid dexamethasone had reduced ovarian volumes and a lower number of primordial and primary follicles at postnatal days 16 and 38 [123].

Prenatal exposure to exogenous compounds has also been linked to detrimental effects on reproductive health. Female mice exposed *in utero* to the endocrine disruptor benzo(a)pyrene (BaP) had a significant reduction in the number of developing follicles at 10 weeks of age [124]. In a similar study, adult rats that were perinatally exposed to bisphenol-a (BPA), another known endocrine disruptor, had a significant decrease in the number of growing follicles, largely at the expense of primary follicles, suggesting an inhibition of primordial follicle recruitment [125]. Gestational exposure of mice to an environmentally relevant phthalate mixture resulted in decreased hormone levels, as well as increased primordial and decreased pre-antral follicles, suggesting an effect on follicle development, which is critical for ovulation [126].

Cannabinoids and the developing ovary

It has previously been shown that THC exposure during pregnancy leads to placental insufficiency and symmetrical fetal growth restriction in rats [78]. It is known that placental insufficiency can cause severe malnutrition of the fetus, and that the preferential blood flow to vital organs may impact the development of other organs. Indeed, studies have suggested that fetal growth restriction due to placental insufficiency negatively affects ovarian development and reproductive aging both in humans and rats [127,128].

In addition, it is possible that THC may have a direct impact on the developing ovary. The expression of CB1 and CB2 has been detected in both male and female gonads, with detection as early as embryonic day 11.5 (E11.5) in mice [107]. Exposure to a CB2 agonist (JWH133) has been shown to induce activation of the meiotic program in both male and female gonads *in vitro*. While gonocytes became arrested at early stages of prophase I, oocytes showed accelerated meiosis along with an increase in Ser-139-phosphorylated histone variant H2AX (γ-H2AX)-positive pachytene and diplotene cells and terminal deoxynucleotidyl transferase-mediated dUTP fluorescein nick-end labeling (TUNEL)-positive cells, suggesting that DNA double-strand breaks were not correctly repaired, leading to oocyte apoptosis [107]. Administration of the same agonist to pregnant females resulted in a significant reduction of primordial and primary follicles in ovaries from newborn mice, as well as a diminished reproductive capacity as adults [107]. In a similar study, prenatal exposure to the synthetic CB1/CB2 agonist WIN55212, resulted in a decrease in ovarian reserve at 90 days of age. The same decrease was not observed following co-administration with a CB1 inverse agonist (SR141716), suggesting that the effects of WIN55212 may be CB1-mediated. Interestingly, prenatal exposure to SR141716 alone resulted in an increase in the ovarian reserve compared to the vehicle group [109]. These studies suggest that perturbances of the fetal endocannabinoid system can result in negative reproductive outcomes later in life.

Fetal programming

The analysis of epidemiological data has led to the conclusion that adverse conditions during prenatal development may be associated with an increased risk of the development of diseases later in life [129,130]. One of the theories that attempts to explain this association states that while the fetus adapts to adverse intrauterine conditions by favouring the development of organs that ensure survival, this reprogramming has harmful long-term effects on the other organs, particularly as the offspring ages [131]. Although it was originally thought that prenatal stressors might lead to changes in the genetic sequence or single nucleotide polymorphisms, there is accumulating evidence that indicates that alterations in epigenetic mechanisms may be the primary risk factor in these disorders [132,133].

Epigenetic mechanisms

The term “epigenetics” defines all meiotically and mitotically heritable changes in regulatory components of gene expression that are not coded in the DNA sequence itself [134]. The three major epigenetic mechanisms are DNA methylation, histone modifications and regulation by non-coding RNA [135]. From these, the most extensively studied mechanism is the methylation and demethylation of cytosine nucleotides, commonly those adjacent to guanosine, in the promoter region of genes [135]. In general, hypomethylation of the cytosine bases in CpG islands in the promoter sequence increases gene expression, and hypermethylation results in decreased gene expression [135].

Histones are a family of basic proteins that associate with DNA in the nucleus and help condense it into chromatin. Post-transcriptional modifications of histone terminal tails, such as methylation, acetylation, phosphorylation and ubiquitination, alter chromatin structure and status, and subsequently regulate gene expression [136]. Generally, the acetylation of histones marks transcriptionally active regions, whereas hypoacetylated histones are found in transcriptionally inactive regions. Histone methylation, on the other hand, can be a marker for both active and inactive chromatin regions [134].

Among many different non-coding RNA molecules, microRNAs (miRNAs) have emerged as essential players in post-transcriptional gene regulation. They are small, single-stranded RNA molecules with a length of approximately 21 to 25 nucleotides [137]. Mature miRNAs regulate gene expression by binding to the 3'-UTR region and promoting mRNA cleavage or translational inactivation [138]. A single miRNA may have multiple mRNA targets, and a single mRNA transcript may be regulated by several different miRNAs [138].

Epigenetic alterations in the ovary

As several studies have discovered alterations in epigenetic regulation as a result of prenatal stressors, both in humans and animals, these changes are thought to be responsible for the long-term observable effects [139]. In particular, diverse animal studies have reported alterations in different epigenetic mechanisms in the ovary as a result of adverse prenatal conditions including maternal undernutrition, altered

hormone levels as well as exposure to endocrine disruptors and pollutants such as pesticides and heavy metals [140–144].

Gestational exposure of female rats to BPA, a known endocrine disruptor, resulted in an increased expression of miRNA-224 and aromatase in ovarian granulosa cells in adulthood, along with elevated estradiol and decreased FSH levels in serum [141]. Perinatal exposure of rats to methoxychlor (MXC), another known endocrine disruptor, led to significant changes in the DNA methylation patterns in the ovaries [145]. Several loci involved in the regulation of ovarian processes were hypermethylated both at postnatal day (PND) 7 and PND60, and the expression of a portion (25%) of those genes was reduced [145]. The hypermethylated and downregulated genes at both time points included *Cyp1b1*, *Hras*, *Hsd17b7*, *Igf1r*, *Max*, and *PI3kr1*, which are involved in the regulation of estrogen metabolism, folliculogenesis, and ovulation [145].

On the other hand, gestational exposure of mice to the insecticide chlordecone (CD) resulted in delayed puberty, a decreased number of primordial follicles and an increased number of atretic follicles. These changes were associated with altered gene expression (*Esr2, Inhba, Inha* and *Smad4*), as well as reduced H3K4me3 (histone H3 trimethylation) and H4ac (histone H4 acetylation) in fully grown oocytes of CD-exposed ovaries [140].

The exposure of rats to dexamethasone from gestation day (GD) 9 to GD20 resulted in a decrease in ovarian aromatase expression, serum estradiol and altered follicle

dynamics in 8-week-old offspring (F1 generation). The downregulation of miRNA320a-3p in F1 ovaries was associated with the upregulation of its target *Runx2*, as well as with increased binding of *Runx2* to the aromatase gene promoter region. Interestingly, these changes were also present in ovaries from F2 and F3 generations, when F1 females were mated with vehicle-treated males [146].

A study that used dihydrotestosterone-induced prenatally androgenized (PNA) mice as a model for PCOS reported a total of 3432 mRNAs and 16 miRNAs that were differentially expressed in the ovaries of these animals when compared with controls. After identifying miRNA-mRNA interactions, the authors confirmed the downregulation of miR-106a-5p and miR-155-5p, as well as the upregulation of target genes (*Stat3*, *Gsk3b*, *Nr1h3* and *Cd28*) involved in the regulation of insulin resistance, inflammation and immune response, all related to PCOS. Interestingly, miR-106a-5p and miR-155-5p were also downregulated in granulosa cells from patients with PCOS [144].

Taken together, these studies demonstrate that adverse prenatal conditions, such as exposure to chemical insults, have the potential to disrupt epigenetic mechanisms including miRNA expression in the ovary, which in turn is associated with altered ovarian function later in life.

Cannabis exposure and epigenetic changes

Several studies have shown that cannabis and its constituents (i.e., THC and cannabidiol) have the ability to induce epigenetic changes such as altered DNA

methylation, microRNA (miRNA) expression and histone modifications in multiple tissues, resulting in long-lasting effects [147–156].

A study that evaluated follicles from patients undergoing IVF treatment revealed that cannabis use, determined by the presence of phytocannabinoids in follicular fluid, was associated with a significant decrease in the expression of the de novo methylating enzyme DNMT3b [157]. This effect was confirmed by exposing control granulosa cells to THC *in vitro* [157]. Along with the decrease in DNMT3b, THC-exposed granulosa cells had a significant decrease in global DNA methylation [157]. On the other hand, treatment of the human granulosa cell line KGN with a specific CB1 agonist (ACEA) after stimulation with FSH, resulted in a decreased expression of miR-23a, miR-24, miR-181a and miR-320a, which are involved in the regulation of ovarian processes such as CYP19 (aromatase) expression and estradiol release [158].

In addition, it has been shown that prenatal exposure to cannabis results in epigenetic alterations associated with adverse health outcomes in the offspring. Gestational cannabis exposure has previously been shown to reduce dopamine receptor D2 (DRD2) expression in the ventral striatum (nucleus accumbens (NAc)), a key reward region, in the human fetal brain. To explore the underlying mechanisms, the authors established a prenatally THC-exposed rat model and evaluated NAc from neonatal and adult offspring. Interestingly, the expression of *Drd2* was significantly decreased in NAc of both neonatal and adult offspring as a result of prenatal exposure to THC. Chromatin immunoprecipitation revealed an

increase in the repressive 2meH3K9 (dimethylated lysine 9 on histone 3) mark and a decrease in 3meH3K4 (trimethylated lysine 4 on histone 3), a mark of transcriptional activity, at the *Drd2* gene locus in NAc from prenatally THC-exposed adults. Prenatally THC-exposed adults also exhibited increased sensitivity to opiate reward, consistent with the reduced *Drd2* expression in their NAc [147].

Taking these studies into account, it has been proposed that gestational cannabis exposure may cause epigenetic changes in the developing fetus which could lead to adverse effects on the offspring's health later in life [97]. However, little is known about the possible long-term effects of prenatal cannabis exposure on the epigenetic regulation of the reproductive system.

HYPOTHESIS

Considering that the rodent embryo expresses the cannabinoid receptors to which THC is known to bind to, and that gestational exposure to THC has previously been shown to result in intrauterine growth restriction, the central hypothesis of this thesis was that prenatal exposure to delta-9-tetrahydrocannabinol would result in significant alterations in ovarian health and function during adulthood, and that changes in epigenetic regulation would underlie these alterations.

OBJECTIVES

The specific objectives of this thesis were:

1. To assess the effect of prenatal exposure to THC on ovarian follicle dynamics and vascularization in adult rat offspring.
2. To assess the effect of prenatal exposure to THC on ovarian miRNA profile in adult rat offspring.
3. To evaluate the effect and mechanism of action of THC on VEGF and the prostanoid pathway in rat granulosa cells.

CHAPTER 2: GESTATIONAL EXPOSURE TO Δ^9-THC IMPACTS OVARIAN FOLLICULAR DYNAMICS AND ANGIOGENESIS IN ADULTHOOD IN WISTAR RATS

This chapter is a reprint of the original article with the same title, published in *Journal of Developmental Origins of Health and Disease*, 2020 December;12(6):865-869 (doi: 10.1017/S2040174420001282). Author list: Martínez-Peña AA, Lee K, Petrik JJ, Hardy DB, and Holloway AC. Copyright and licensing can be found in Appendix A.

While it has been suggested that cannabis affects adult female fertility, little is known regarding the long-term impact of gestational cannabis exposure on ovarian development. Since ovarian primordial follicle assembly takes place during this time and largely determines the long-term reproductive capacity of female mammals, exposure to exogenous chemicals during this sensitive window may have persistent effects throughout the lifespan. Given that THC efficiently crosses the placental barrier and that the human and rodent embryo express the cannabinoid receptors to which it binds to from very early stages of development, this compound could have direct effects on the developing ovary. In addition, prenatal THC-exposure has been shown to result in placental insufficiency and symmetrical fetal growth restriction, two conditions that are known to negatively affect ovarian development in humans and rats. Taking all of this into account, we hypothesized that prenatal exposure to THC would have direct and/or indirect effects on fetal ovarian development that would manifest during adulthood.

Abstract

With the legalization of marijuana (*Cannabis sativa*) and increasing use during pregnancy, it is important to understand its impact on exposed offspring. Specifically, the effects of Δ-9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of cannabis, on fetal ovarian development and long-term reproductive health are not fully understood. The aim of this study was to assess the effect of prenatal exposure to Δ^9-THC on ovarian health in adult rat offspring. At 6 months of age, Δ^9-THC-exposed offspring had accelerated folliculogenesis with apparent follicular development arrest, but no persistent effects on circulating steroid levels. Ovaries from Δ^9-THC-exposed offspring had reduced blood vessel density in association with decreased expression of the pro-angiogenic factor VEGF and its receptor VEGFR-2, as well as an increase in the anti-angiogenic factor thrombospondin 1 (TSP-1). Collectively, these data suggest that exposure to Δ^9-THC during pregnancy alters follicular dynamics during postnatal life, which may have long-lasting detrimental effects on female reproductive health.

Introduction

Marijuana (*Cannabis sativa*) is the most widely cultivated, trafficked, and abused illicit drug in the world.[1] In recent years, the use of cannabis during pregnancy in North America has increased considerably, particularly among younger women.[2] Indeed, recent estimates suggest that approximately 3%–7% of pregnant women in North America use cannabis.[2] An integrative review concluded that women who used cannabis during pregnancy believed that there were no significant risks to the

mother or fetus.[3] In fact, it is not uncommon for women to use cannabis to treat pregnancy-related nausea and vomiting.[3]

While it has been shown that cannabis negatively affects female fertility in adults,[4] little is known regarding the long-term influence of maternal exposure to cannabis on ovarian development in the offspring. Given that Δ-9-tetrahydrocannabinol (Δ^9-THC) efficiently crosses the placental barrier and can be detected in cord blood and fetal tissue,[3] this compound could have direct effects on ovarian development. Since ovarian primordial follicle assembly takes place during this time and largely determines the long-term reproductive capacity of female mammals, exposure to exogenous chemicals during this sensitive window may have persistent effects throughout the lifespan.[5] In addition, cannabinoid receptors 1 and 2 (CB1 and CB2), which bind to cannabinoids such as Δ^9-THC, are expressed in the human and rodent embryo from very early stages of development.[6,7] The preimplantation mouse embryo has been shown to express both receptors from the two-cell stage, and CB1 and CB2 have been detected in the ectoplacental cone from gestational day (GD) 8 in the rat.[6] A recent study reported that *in utero* treatment with a selective CB2 agonist influenced oocyte reserve and reduced the reproductive capacity of mice.[8]

We have previously shown that Δ^9-THC exposure during pregnancy leads to placental insufficiency and symmetrical fetal growth restriction.[9] It is known that placental insufficiency can cause severe malnutrition of the fetus, and that the preferential blood flow to vital organs may impact the development of other organs. Indeed, studies have suggested that fetal growth restriction due to placental

insufficiency negatively affects ovarian development both in humans and rats.[10,11] In one of these studies, a significantly lower number of primordial follicles were observed in intrauterine growth restricted animals at postnatal day (PND) 20. Interestingly, no differences were observed in follicle density at PND40, suggesting that compensatory mechanisms occurred during the prepubertal window. In addition, the expression of genes involved in cellular processes such as proliferation, apoptosis, and metabolism was affected at all ages evaluated, potentially implying long-term genetic alterations.[10] Taking all of these studies into account, we hypothesized that prenatal exposure to Δ^9-THC would adversely affect ovarian function in adult rats.

Methods

All procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care. Pregnant Wistar rats were purchased from Charles River (La Salle, St. Constant, QC, Canada) and throughout the experimental procedure were maintained at 22 °C on a 12:12-h light–dark cycle with access to food and water *ad libitum*. Dams were randomly assigned to receive a daily intraperitoneal (IP) injection of either vehicle (1:18 cremophor:saline; $n = 5$) or 3 mg/kg Δ^9-THC (Sigma-Aldrich, St Louis, MO, USA; $n = 6$) from GD6 to GD22 as previously described.[9] This dose results in maternal blood concentrations (8.6–12.4 ng/ml) comparable to those detected after moderate marijuana smoking in adults (13–63 ng/ml), as well as in aborted fetal tissue (4–287 ng/ml) after maternal cannabis use.[12–14] Moreover, it has been previously demonstrated that 3 mg/kg Δ^9-THC per day does not cause fetal

demise, alterations in litter size, gestational length, or maternal weight gain.[9] Dams were allowed to deliver normally and litters were randomly culled to four female pups and four male pups each. Offspring were then euthanized at 6 months of age by IP pentobarbital overdose. Blood was drawn by cardiac puncture for quantification of estrogen (Biovision, Milpitas, CA, USA), progesterone (Cusabio, Wuhan Huamei Biotech Co., Wuhan, China), and free testosterone (R&D Systems, Minneapolis, MN, USA) by ELISA. The ovaries were collected, fixed in 10% formalin, and embedded in paraffin for histological analysis.

Paraffin-embedded ovaries were serially sectioned (8 μm) and stained with hematoxylin–eosin to carry out follicle quantitation and morphometric analysis. Every 10th section was analyzed and follicles were counted and classified as primordial, transitioning, primary, secondary, antral, and atretic as previously described.[15] The area of each section was measured using an Olympus IX81 microscope and Infinity Analyze software, and the total number of each type of follicle was normalized to the total area analyzed from each ovary.

Immunohistochemistry (IHC) was performed to assess the expression of the pro-angiogenic factor vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2), as well as the anti-angiogenic factor thrombospondin 1 (TSP-1) in ovarian sections. Immunofluorescence was also performed to determine the levels of the endothelial marker CD31. For IHC, tissues were deparaffinized using reagent-grade xylene and subjected to a series of decreasing ethanol concentrations for rehydration. Endogenous peroxidase activity was quenched through a 10-minute

incubation period in 3% hydrogen peroxide followed by antigen retrieval using citrate buffer with Tween 20. To reduce nonspecific binding of antibodies, samples were blocked in 5% bovine serum albumin (with 0.02% sodium azide) for 10 min at room temperature. Sections were exposed to the following primary antibodies overnight at 4 °C: anti-VEGF (Abcam; 1:600); anti-VEGFR-2 (Cell Signaling; 1:400); anti-TSP-1 (Abcam; 1:100). Slides were then incubated in anti-mouse or anti-rabbit (Sigma; 1:100) biotinylated secondary antibody for 2 h at room temperature followed by Extravidin (Sigma, 1:50) for 1 h at room temperature. Antigens were visualized using 3,30 -Diaminobenzidine Tetrahydrochloride (DAB; Sigma) and tissues were counterstained with hematoxylin. Slides were imaged using bright-field microscopy, and the percentage of immunopositive tissue was quantified using morphometry software (ImagePro Plus, Media Cybernetics, Rockville, MD, USA). For immunofluorescence, tissues were deparaffinized and rehydrated as above. Slides were exposed to 0.2% sodium borohydride in PBS for 12 min and 5% bovine serum albumin for 15 min to reduce nonspecific binding. Sections were then stained with anti-CD31 (Abcam; 1:25) overnight at 4 °C. The following day, tissue samples were stained with secondary antibody (Alexa Fluor594nm, red). Slides were counterstained and cured using Prolong Gold anti-fade mounting medium. Representative images (n = 4/ovary) were obtained and image analysis was conducted using Metamorph imaging software (Molecular Devices, San Jose, CA, USA).

For hormone levels, follicle quantitation, IHC, and immunofluorescence, one female offspring per dam were included in the statistical analysis. A Student's t-test was performed between vehicle (n = 5) and Δ^9-THC-exposed (n = 6) animals for each outcome measure. Data are presented as the mean plus the standard error of the mean and a p-value of less than 0.05 was considered statistically significant.

Results

Since sex steroids are known to play a crucial role in the regulation of ovarian function and follicular development, at 6 months of age, blood was collected from Δ^9-THC-exposed offspring for hormone quantification. No differences were found in the levels of circulating estrogen (vehicle: 57.92 ± 2.53 pg/ml; Δ^9-THC: 61.11 ± 2.86 pg/ml; intra-assay CV: 4.99%), progesterone (vehicle: 94.12 ± 15.48 ng/ml; Δ^9-THC: 87.76 ± 7.52 ng/ml; intra-assay CV: 10.6%), or testosterone (vehicle: 3.26 ± 0.65 ng/ml; Δ^9-THC: 3.46 ± 0.74 ng/ml; intra-assay CV: 7.25%).

When the ovaries from adult offspring were examined, Δ^9-THC-exposed animals had significantly more transitioning follicles/area unit (Fig. 1c). However, this difference did not persist in later stages of follicle development (Fig. 1d–g). Additionally, there was a significant increase in the percentage of TSP-1-positive granulosa cells, as well as a significant decrease in the percentage of VEGF and VEGFR-2-positive granulosa cells in ovaries from Δ^9-THC-exposed offspring. Consistent with this shift to an anti-angiogenic phenotype, a significant decrease in ovarian blood vessel density, assessed by CD31 levels, was also detected (Fig. 2).

Discussion

Given that the rodent embryo expresses the cannabinoid receptors from very early stages of development, and that gestational Δ^9-THC exposure has been shown to result in intrauterine growth restriction, we suspected that prenatal exposure to this compound may have direct and/or indirect effects on fetal ovarian development that could manifest during adulthood.

We have previously reported that fetal exposure to Δ^9-THC resulted in placental insufficiency culminating in symmetrical fetal growth restriction in the animals used in this study;[9] this growth restriction is similar to what has been reported in humans following maternal cannabis use.[3] Interestingly, studies indicate that low birth weight may be a risk factor for earlier age at menopause in women.[16] Since the age at which menopause occurs is thought to be a reflection of both the size of the initial follicle pool, as well as the rate of oocyte loss,[17] factors that affect either or both of these parameters may lead to premature reproductive aging.

With this in mind, we assessed ovarian follicle dynamics of prenatally Δ^9-THC-exposed rats at 6 months of age. Female rats that were exposed to Δ^9-THC during fetal development had a significant increase in the number of transitioning follicles/area unit. This could be a result of an increase in the activation of dormant primordial follicles, similar to what has been reported in other models of *in utero* insults.[15] Since this difference was not observed in later stages of follicle development (i.e., primary, secondary, and antral), it is possible that a significant

portion of the transitioning follicles did not continue to develop. It has previously been suggested that primordial follicles that are prematurely recruited into the growing pool are subsequently lost, resulting in no net loss or gain of developed follicles.[15] In support of this hypothesis, we did not observe any differences in the levels of circulating estradiol, progesterone or testosterone, or in the number of developed follicles, which are largely responsible for the synthesis of steroid hormones. However, the increase in transitioning follicles may lead to a premature loss of the primordial follicle pool and therefore early reproductive senescence,[18] although this remains to be confirmed.

Since ovarian follicle development is largely dependent on angiogenesis,[19] ovarian sections that were not used for follicle dynamics evaluation were used to assess levels of pro and anti-angiogenic proteins. It has previously been reported that cannabis and Δ^9-THC exposure during pregnancy contributed to reduced blood vessel formation in human and rodent placenta, suggesting that this compound has anti-angiogenic effects.[9,20] Moreover, early life insults, which result in reduced birth weight have been reported to reduce ovarian vascularization.[21] Similarly, in the current study, ovaries from Δ^9-THC-exposed animals had reduced blood vessel density in association with decreased expression of the pro-angiogenic factor VEGF and its receptor VEGFR-2, and increased expression of the anti-angiogenic factor TSP-1. TSP-1 is a potent inhibitor of VEGF signaling, and VEGF and TSP-1 are inversely expressed throughout folliculogenesis, with VEGF levels increasing and TSP-1 decreasing as follicle progression occurs.[22] While primordial follicles rely on

the stroma for nutrient delivery, after activation and development of a primary follicle, endothelial cells are recruited from neighboring blood vessels so that follicular development can proceed.[23] Therefore, the increase in TSP-1 and decrease in VEGF and VEGFR-2 could, at least in part, underlie the follicle dynamics observed in Δ^9-THC-exposed animals, since a significant decrease in ovarian blood vessel formation could result in follicular development arrest after stages that rely solely on the stroma.

On the other hand, *in vitro* treatment of immortalized rat granulosa cells with TSP-1 has been demonstrated to increase the expression of pro-apoptotic factors,[22] while VEGF-VEGFR-2 signaling appeared to have opposing effects.[24] The increase in TSP-1 and decrease in VEGF and VEGFR-2 observed in this study could therefore manifest as increased apoptosis (atresia), and ultimately a loss of developing follicles. Although we did not observe a statistically significant increase in the number of atretic follicles in the Δ^9-THC-exposed animals at 6 months of age ($p = 0.197$), this may be a reflection of the small sample size ($n = 6$). Alternatively, it is possible that the deficits in angiogenesis may manifest more clearly as increased follicle atresia and depletion of the follicle reserve as the animal ages. Thus, to further investigate the effects of gestational exposure to Δ^9-THC on adult reproductive health, it would be prudent to monitor the ovarian follicle reserve at a more advanced age, closer to that at which rats typically experience reproductive senescence (9–12 months of age). Additionally, it would be of great interest to assess the fertility of

these animals, since it is possible that not only the number but also the quality of the oocytes could be affected.

In conclusion, at 6 months of age, animals that were exposed to Δ^9-THC *in utero* had accelerated folliculogenesis with apparent follicular development arrest, as well as decreased blood vessel density. These results suggest for the first time that the popular use of cannabis during pregnancy may have long-term effects on ovarian development in the offspring, which may negatively impact postnatal reproductive health.

Acknowledgments. None.

Financial support. This study was supported by a CIHR Project Grant to ACH (PJT-155981) and an HSFC Grant-in-Aid to DBH (G-19-0,026,343). AAMP was sponsored by the Mexican National Council of Science and Technology (CONACYT).

Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Canadian Council of Animal Care) and has been approved by the institutional animal care committee at Western University.

References

1. World Health Organization (WHO). The Health and Social Effects of Nonmedical Cannabis Use. At https://www.who.int/substance_abuse/publications/cannabis_report/en/

2. Singh S, Filion KB, Abenhaim HA, Eisenberg MJ. Prevalence and outcomes of prenatal recreational cannabis use in high-income countries: a scoping review. BJOG. 2020; 127, 8–16.

3. Carlier J, Huestis MA, Zaami S, Pichini S, Busardò FP. Monitoring perinatal exposure to cannabis and synthetic cannabinoids. Ther Drug Monit. 2020; 42, 194–204.

4. Brents LK. Marijuana, the endocannabinoid system and the female reproductive system. Yale J Biol Med. 2016; 89, 175–191.

5. Allais A, Albert O, Lefèvre PLC, et al. In utero and lactational exposure to flame retardants disrupts rat ovarian follicular development and advances puberty. Toxicol Sci. 2020; 175, 197–209.

6. Buckley NE, Hansson S, Harta G, Mezey E. Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. Neuroscience. 1998; 82, 1131–1149.

7. Biegon A, Kerman IA. Autoradiographic study of pre- and postnatal distribution of cannabinoid receptors in human brain. Neuroimage. 2001; 14, 1463–1468.

8. De Domenico E, Todaro F, Rossi G, et al. Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads and impairs ovarian reserve. Cell Death Dis. 2017; 8, e3085.

9. Natale BV, Gustin KN, Lee K et al. Δ9-tetrahydrocannabinol exposure during rat pregnancy leads to symmetrical fetal growth restriction and labyrinth-specific vascular defects in the placenta. Sci Rep. 2020; 10, 544.

10. Pampanini V, Jahnukainen K, Sahlin L, et al. Impact of uteroplacental insufficiency on ovarian follicular pool in the rat. Reprod Biol Endocrinol. 2019; 17, 10.

11. De Bruin JP, Dorland M, Bruinse HW, et al. Fetal growth retardation as a cause of impaired ovarian development. Early Hum Dev. 1998; 51, 39–46.

12. Klein C, Karanges E, Spiro A, et al. Cannabidiol potentiates Delta(9)-tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. Psychopharmacology (Berl). 2011; 218, 443–457.

13. Schwope DM, Karschner EL, Gorelick DA, Huestis MA. Identification of recent cannabis use: Whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. Clin Chem. 2011; 57, 1406–14.

14. Falcon M, Pichini S, Joya J,et al. Maternal hair testing for the assessment of fetal exposure to drug of abuse during early pregnancy: comparison with testing in placental and fetal remains. Forensic Sci Int. 2012; 218, 92–96.

15. Tsoulis MW, Chang PE, Moore CJ, et al. Maternal high-fat diet-induced loss of fetal oocytes is associated with compromised follicle growth in adult rat offspring. Biol Reprod. 2016; 94, 94.

16. Steiner AZ, D'Aloisio AA, DeRoo LA, Sandler DP, Baird DD. Association of intrauterine and early-life exposures with age at menopause in the Sister study. Am J Epidemiol. 2010; 172, 140–148.

17. Gougeon A, Chainy GB. Morphometric studies of small follicles in ovaries of women at different ages. J Reprod Fertil. 1987; 81, 433–442.

18. Nelson LM. Clinical practice. Primary ovarian insufficiency. N Engl J Med. 2009; 360, 606–614.

19. Robinson RS, Woad KJ, Hammond AJ, et al. Angiogenesis and vascular function in the ovary. Reproduction. 2009; 138, 869–881.

20. Chang X, Li H, Li Y, et al. RhoA/MLC signaling pathway is involved in Δ9 - tetrahydrocannabinol-impaired placental angiogenesis. Toxicol Lett. 2018; 285, 148–155.

21. Chan KA, Bernal AB, Vickers MH, et al. Early life exposure to undernutrition induces ER stress, apoptosis, and reduced vascularization in ovaries of adult rat offspring. Biol Reprod. 2015; 92, 110.

22. Greenaway J, Lawler J, Moorehead R, et al. Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). J Cell Physiol. 2007; 210, 807–818.

23. Fraser HM, Wulff C. Angiogenesis in the primate ovary. Reprod Fertil Dev. 2001; 13, 557–566.

24. Greenaway J, Connor K, Pedersen HG, et al. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. Endocrinology. 2004; 145, 2896–2905.

Figures and tables

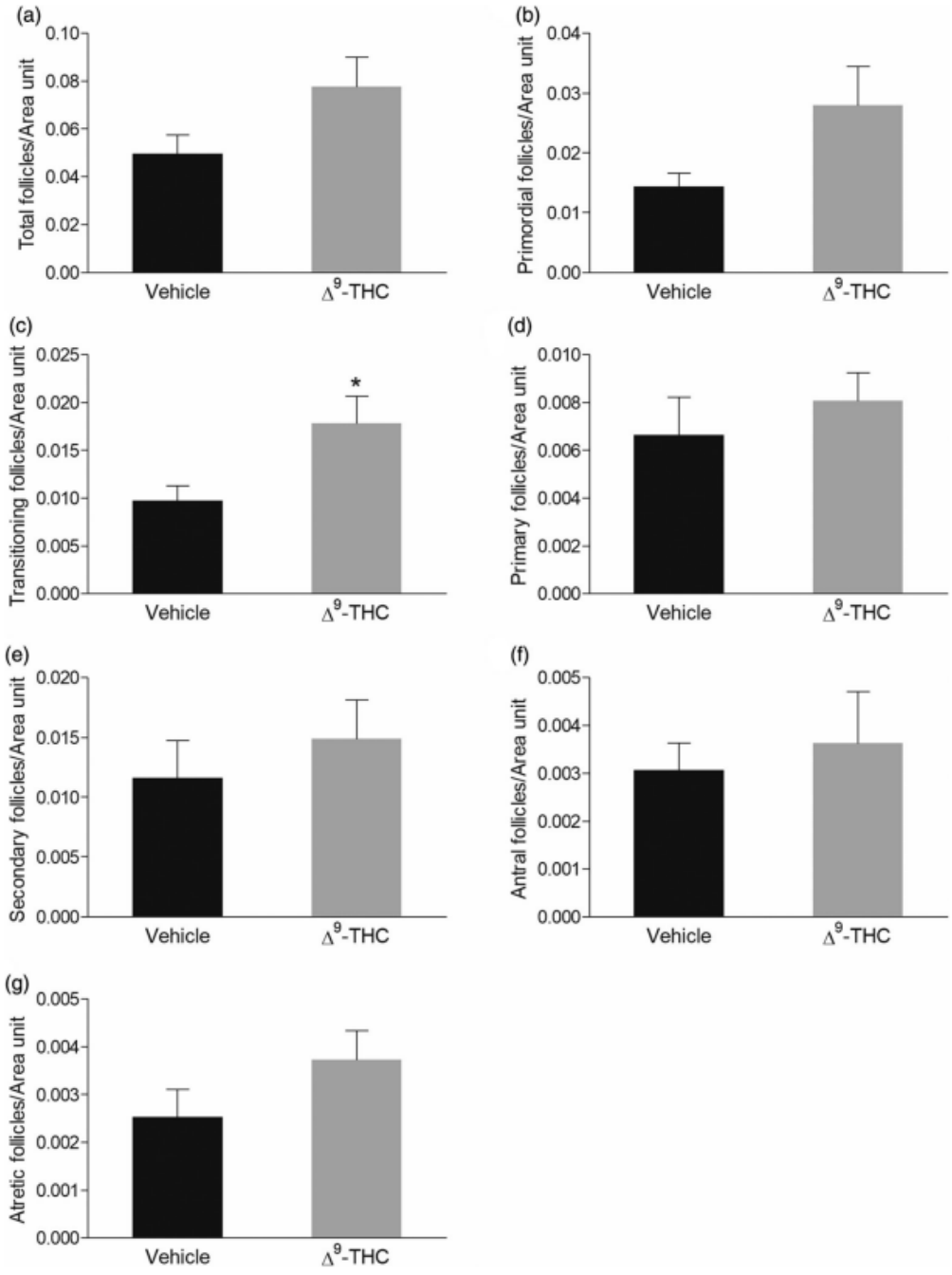

Fig. 1. Number of (a) total, (b) primordial, (c) transitioning, (d) primary, (e) secondary, (f) antral, and (g) atretic follicles per area unit in vehicle (n= 5) and prenatally Δ^9-THC-exposed (n = 6) rat ovaries. Mean + SEM. Student's t-test; *$p < 0.05$.

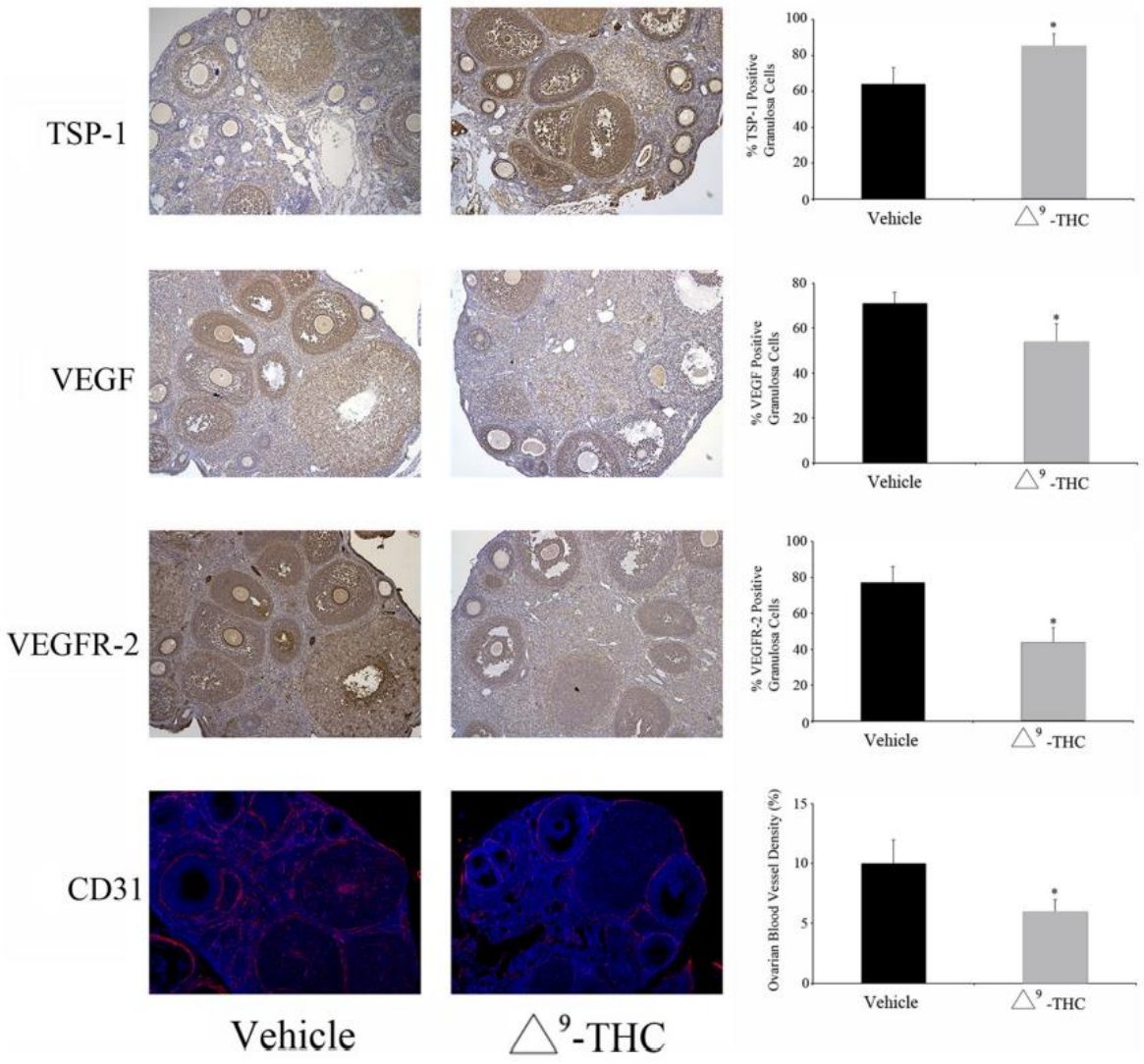

Fig. 2. Expression of thrombospondin 1 (TSP-1), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR-2), and CD31 in vehicle (n = 5) and prenatally Δ^9 -THC-exposed (n = 6) rat ovaries. Mean + SEM. Student's t-test; *$p < 0.05$.

CHAPTER 3: PRENATAL EXPOSURE TO DELTA-9-TETRAHYDROCANNABINOL (THC) ALTERS THE EXPRESSION OF MIR-122-5P AND ITS TARGET IGF1R IN THE ADULT RAT OVARY.

This chapter is a reprint of the original article with the same title, published in *International Journal of Molecular Sciences*, 2022 July; 23(14), 8000 (doi.org/10.3390/ijms23148000). Author list: Martínez-Peña AA, Lee K, Pereira M, Ayyash A, Petrik JJ, Hardy DB, and Holloway AC. Copyright and licensing can be found in Appendix A.

It has been shown that adverse prenatal conditions such as maternal undernutrition, hormonal imbalance and exposure to exogenous chemicals have the potential to alter ovarian development, with major implications for reproductive health. There is accumulating evidence that indicates that alterations in epigenetic mechanisms may be responsible for the increased risk of diseases as a result of prenatal stressors. Furthermore, THC and CB1 activation have been shown to affect DNA methylation and miRNA expression in the ovary, while prenatal THC-exposure resulted in altered histone modifications and gene expression in the adult rodent brain. Considering the long-term observable effects of gestational THC exposure in adult rat ovaries described in chapter 2, and that acute and in utero exposure to THC has been shown to affect epigenetic mechanisms, we hypothesized that prenatal exposure to THC resulted in significant changes in the ovarian miRNA profile, and that these changes were responsible for the observable alterations in the adult offspring.

Abstract

As cannabis use during pregnancy increases, it is important to understand its effects on the developing fetus. Particularly, the long-term effects of its psychoactive component, delta-9-tetrahydrocannabinol (THC), on the offspring's reproductive health are not fully understood. This study examined the impact of gestational THC exposure on the miRNA profile in adult rat ovaries and the possible consequences on ovarian health. Prenatal THC exposure resulted in the differential expression of 12 out of 420 evaluated miRNAs. From the differentially expressed miRNAs, miR-122- 5p, which is highly conserved among species, was the only upregulated target and had the greatest fold change. The upregulation of miR-122-5p and the downregulation of its target insulin-like growth factor 1 receptor (*Igf1r*) were confirmed by RT-qPCR. Prenatally THC-exposed ovaries had decreased IGF-1R-positive follicular cells and increased follicular apoptosis. Furthermore, THC decreased *Igf1r* expression in ovarian explants and granulosa cells after 48 h. As decreased IGF-1R has been associated with diminished ovarian health and fertility, we propose that these THC-induced changes may partially explain the altered ovarian follicle dynamics observed in THC-exposed offspring. Taken together, our data suggests that prenatal THC exposure may impact key pathways in the developing ovary, which could lead to subfertility or premature reproductive senescence.

Introduction

The use of cannabis, which is the generic term that denotes several preparations of the plant *Cannabis sativa*, has increased considerably in recent decades [1]. The increase in cannabis use has been more prominent in developed countries, where there is a growing tendency towards legalization, and specifically among the younger population, whose perception of cannabis as a harmful drug has decreased over the past decade [1]. Regarding cannabis use during pregnancy, several studies have confirmed that consumption during gestation has also increased during the last decades [2–4]. Ko and colleagues reported that 70% of pregnant and nonpregnant women in the US believe that there is little to no harm in using cannabis once or twice per week [5], and a longitudinal prospective study discovered that 48% of women in the UK who used cannabis in the year prior to their pregnancy continued to smoke throughout gestation [6]. Despite advice against its use during pregnancy [7], cannabis is often used to mitigate pregnancy-related symptoms such as pain, nausea and vomiting [8–10]. Studies that have investigated the prevalence of cannabis use in developed countries revealed that approximately 4–7% of women reported using cannabis during pregnancy [3,4,11,12]. However, given that most studies rely solely on self-reporting, it is likely that these data underestimate the actual prevalence of cannabis use during gestation [13,14].

Many researchers have demonstrated that prenatal cannabis use is associated with an increased risk of stillbirth [15,16], preterm birth [4,15,17,18], being small for gestational age [17–20], low birthweight [17,21–23], increased admission to

neonatal intensive care units [18,20] and death within one year of birth [17]. In addition, prenatal exposure to cannabis may also result in long-term alterations in the offspring's health [24]. Considering that delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis [25], efficiently crosses the placental barrier and can be detected in cord blood and fetal tissue [26], this compound could have direct effects on the developing organs, which are particularly sensitive to exogenous chemicals [27,28]. It is known that adverse prenatal conditions are associated with an increased risk of disease later in life, and it has been proposed that while the fetus adapts to these adverse conditions by favoring the development of organs that ensure survival, this reprogramming has harmful long-term effects on other organs, including the ovaries [29]. Indeed, it has been shown that prenatal conditions such as maternal undernutrition, hormonal imbalance or exposure to exogenous chemicals have the potential to alter ovarian development, with major implications for reproductive health and fertility later in life [30].

Studies have suggested that prenatal cannabinoid exposure results in long-lasting neurobehavioural, metabolic, cardiovascular and reproductive abnormalities in offspring [31–38]. Given that these effects are observed long after exposure, one of the proposed mechanisms is that cannabis may cause epigenetic changes in the developing fetus [24]. Alterations in epigenetic regulation can be a consequence of exposomes, including environmental factors (e.g., nutrition and oxygen levels) and/or exogenous compounds (e.g., drugs), which results in heritable phenotypic changes without affecting the DNA sequence [39]. Indeed, several studies have

shown that cannabis constituents (i.e., THC and cannabidiol) have the ability to induce epigenetic changes such as altered DNA methylation, histone modifications and microRNA (miRNA) expression in multiple tissues, resulting in long-lasting effects [40–48].

MiRNAs are small, endogenous, noncoding single-stranded RNA molecules with a length of approximately 22 to 24 nucleotides, which act as posttranscriptional regulators of gene expression [49,50]. Mature miRNAs can regulate gene expression through different mechanisms, including mRNA cleavage and translational inactivation [51]. Given that miRNAs can bind to the 3′-UTR region with partial sequence homology, a single miRNA may have multiple mRNA targets, and a single mRNA transcript may be targeted by several different miRNAs [52]. MiRNAs are involved in the regulation of several cellular processes, including differentiation, proliferation, apoptosis and hormone biosynthesis and secretion [53,54]. In the ovary, miRNAs play an important role in steroidogenesis, oocyte maturation, ovulation, luteinization, follicular development and atresia, and their dysregulation has been associated with disorders such as polycystic ovary syndrome (PCOS), premature ovarian failure (POF) and ovarian cancer [55,56].

A previous study from our research group revealed that prenatal exposure of rats to THC resulted in altered ovarian follicle dynamics and vascularization in the adult offspring [57]. Considering that miRNAs are involved in the regulation of key ovarian processes, and that cannabis constituents have been shown to affect miRNA expression, the aims of this study were to assess the effect of prenatal exposure to

THC on the ovarian miRNA profile and to determine the possible impact of these changes on ovarian health and function.

Materials and Methods

Animals

Pregnant Wistar rats were purchased from Charles River (La Salle, St. Constant, QC, Canada) and maintained at 22 °C at Western University Animal House Facility on a 12:12 h light:dark cycle with access to food and water *ad libitum* throughout the experiment. All animal experiments were done based on the approved animal use protocol by the subcommittee of the Canadian Council of Animal Care, Western University (AUP# 2019-126) in accordance with the ARRIVE guidelines (https://arriveguidelines.org, accessed on 16 July 2022). Dams were randomly assigned to receive a daily intraperitoneal (IP) injection of either vehicle (1:18 cremophor:saline) or 3 mg/kg THC (Sigma-Aldrich, St Louis, MO, USA) from gestation day (GD) 6 to GD22. This dose has been shown to result in maternal blood concentrations (8.6–12.4 ng/mL) comparable to those detected after moderate cannabis smoking in adults (13–63 ng/mL), as well as in aborted fetal tissue (4–287 ng/mL) after maternal cannabis use [82–84]. We have previously demonstrated that 3 mg/kg THC per day does not cause fetal demise, alterations in litter size or gestational length, or maternal weight gain [85]. Dams were allowed to deliver normally, and litters were randomly culled to four female pups and four male pups each. At 6 months of age, prenatally THC-exposed offspring were euthanized by IP pentobarbital overdose. Per female, one ovary was fixed in 10% formalin and

embedded in paraffin for histological analysis, while the other ovary was flash frozen in liquid nitrogen to evaluate miRNA and mRNA expression.

Nanostring Analysis

To determine the effect of gestational THC exposure on the miRNA profile in adult rat ovaries, RNA was extracted from the ovaries of THC-exposed offspring with the use of a mirVana™ miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), obtaining RNA integrity numbers that ranged from 7.4 to 9.3. The Rat v1.5 miRNA Assay (Nanostring Technologies, Seattle, WA, USA) was used to determine the expression levels of 420 biologically relevant rat miRNA targets according to the manufacturer's instructions. Results were analyzed with the use of nSolver® analysis software and ROSALIND®. A statistically significant effect was considered to be a fold change of ≥1.5 or ≤−1.5 and a p value < 0.05.

miRNA Real-Time Quantitative PCR

In order to confirm the results obtained with the Nanostring assay, the expression of the miRNA target with the greatest fold change (miR-122-5p) was evaluated by real-time quantitative PCR (RT-qPCR). For this, the miRCURY™ LNA miRNA PCR Assay system (Qiagen N.V., Hilden, Germany) was used. Complementary DNA (cDNA) was synthesized from the extracted RNA with the use of a miRCURY™ LNA RT kit (Qiagen). The cDNA was then amplified and detected with the use of a miRCURY™ LNA SYBR® Green PCR kit (Qiagen) and a CFX384 Touch™ Real-

Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). RT-qPCR results were analyzed with the $2^{-\Delta\Delta CT}$ method [86] using RNU5G snRNA and miR-191-5p as internal references (Table 2). These reference genes were chosen based on previous studies and showed the lowest variation between vehicle and THC-exposed samples when analyzed using RefFinder [87–89].

Immunohistochemistry

Paraffin-embedded ovaries were sectioned (8 µm) and deparaffinized using reagent-grade xylene, and subjected to a series of decreasing ethanol concentrations for rehydration. Endogenous peroxidase activity was quenched through a 10-min incubation period in 3% hydrogen peroxide, followed by antigen retrieval using 10 mM sodium citrate buffer with Tween 20 (0.05%) for 12 min. To reduce nonspecific binding of antibodies, samples were blocked with 5% bovine serum albumin (with 0.02% sodium azide) for 10 min at room temperature. Sections were exposed to either rabbit polyclonal anti-IGF-1R (Abcam, Cambridge, UK; 1:400) or anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA; 1:300) overnight at 4 °C. Slides were then incubated with biotinylated anti-rabbit secondary antibody (Thermo Fisher Scientific; 1:100) for 2 h at room temperature followed by Extravidin (Sigma-Aldrich; 1:50) for 1 h at room temperature. Antigens were visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), and tissues were counterstained with hematoxylin. Slides were imaged using bright-field microscopy at 200x magnification. The percentage of immunopositive follicular cells was quantified by the same individual, who was blinded to the treatment group until all

the data had been collected, with the use of an integrated morphometry software (MetaMorph Inc., Nashville, TN, USA). The average of 5 fields of view/ovary was used to calculate the percentage of immunopositive cells.

Ovarian Explant Culture

For ex vivo experiments, ovaries were collected from nulliparous Wistar rats (268.09 ± 2.96 g) and transferred to sterile D-PBS (Corning Inc., New York, NY, USA). In a biosafety cabinet, ovaries were trimmed of fat, washed with D-PBS and cut into four equal pieces using a sterile blade. Each piece was transferred to a single well in a 24-well plate containing DMEM/F12 media with L-glutamine (Corning) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (Thermo Fisher Scientific). After 24 h of culture, explants were exposed to vehicle or 3 μM THC for 48 h, changing the media daily. This concentration of THC was based on a pharmacokinetic study that reported similar levels in the serum of cannabis users [90].

Cell Culture

For in vitro experiments, spontaneously immortalized rat granulosa cells (SIGCs) were cultured in DMEM/F12 media with L-glutamine (Corning Inc.) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin. As with ex vivo experiments, cells were cultured with either vehicle or 3 μM THC for 48 h after confirming this concentration had no effect on cell viability (data not shown).

RNA Isolation and RT-qPCR

To evaluate gene expression, total RNA was extracted from THC-exposed ovary explants and SIGCs. Briefly, samples were homogenized in TRIzol™ reagent (Thermo Fisher Scientific) by sonication, and RNA was extracted by precipitation with isopropanol and subsequent ethanol washes. RNA concentration and purity were assessed using a NanoDrop™ One micro-UV/vis spectrophotometer (Thermo Fisher Scientific), and cDNA was synthesized with the use of a High capacity cDNA reverse transcription kit (Thermo Fisher Scientific).

In order to confirm the effects of miRNA dysregulation, the online databases miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php, accessed on 16 July 2022) and TargetScan (https://www.targetscan.org/vert_80/, accessed on 16 July 2022) were used to select validated target genes involved in essential ovarian processes. With this in mind, the expression of the validated miR-122-5p target insulin-like growth factor 1 receptor (*Igf1r*) was determined. In addition, given that both miR-122-5p and IGF-1R are involved in the regulation of apoptosis [58,66], and that alterations in follicle dynamics were previously observed in the prenatally THC-exposed ovaries [57], the expression of the apoptotic marker caspase-3 was evaluated.

For the ovaries from prenatally THC-exposed offspring, the ovary explants and the SIGCs, RT-qPCR was performed using PerfeCTa SYBR® Green FastMix

(Quantabio, Beverly, MA, USA) and the CFX384 Touch™ real-time PCR detection system (Bio-Rad). RT-qPCR results were analyzed with the $2^{-\Delta\Delta CT}$ method using beta-2-microglobulin (*B2m*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) as internal references (Table 3).

Statistical Analysis

For in vivo assessments, one female offspring per dam was included in the statistical analysis. For ex vivo and in vitro assessments, results from 5 independent experiments are presented. The Nanostring assay results were analyzed with the use of nSolver® and ROSALIND® analysis software, considering a fold change ≥ 1.5 and a *p*-value < 0.05 as statistically different. For miRNA and mRNA RT-qPCR, as well as for immunohistochemical evaluation, statistical analyses were performed using SigmaPlot®. A Student's t-test was performed to determine statistically significant differences ($p < 0.05$) between vehicle and THC-exposed samples.

Results

Gestational Exposure to THC Alters miRNA Profile in Adult Rat Ovaries

The differential expression of miRNA targets in adult rat ovaries as a result of gestational exposure to THC is shown in Figure 1. Of the 420 miRNAs evaluated by the Nanostring assay, prenatal exposure to THC altered the expression of 12 miRNA targets (fold change ≥ 1.5, $p < 0.05$).

From the 12 differentially expressed miRNAs, 11 were downregulated (miR-154-5p, miR-214-5p, miR-3552, miR-18a-5p, miR-296-3p, miR-874-3p, miR-211-5p, miR-

20b-3p, miR-598-3p, miR-330-5p and miR-190b-5p) and only miR-122-5p was upregulated (Table 1). For this reason, along with the fact that it exhibited the greatest fold change in expression, miR-122-5p (miR-122) was chosen for further evaluation.

Prenatal THC-Exposure Increases Ovarian miR-122-5p Expression in Adult Offspring

In order to confirm the increase in miR-122 in the prenatally THC-exposed ovaries, the expression of this target was assessed by RT-qPCR. As shown in Figure 2B, RT-qPCR results revealed that miR-122 was significantly upregulated in the ovaries from THC-exposed offspring, similar to the results obtained with the Nanostring assay (Figure 2A).

Gestational Exposure to THC Decreases Igf1r Expression in the Adult Ovary

Changes in miRNA expression may affect the expression of their target genes [51]. Given that gestational THC-exposure resulted in a significant increase in miR-122, the online databases TargetScan and miRTarBase were used to determine validated miR-122 target genes involved in the regulation of ovarian processes. Insulin-like growth factor 1 receptor (*Igf1r*) was identified as a key ovarian miR-122 target based on its role in steroidogenesis, folliculogenesis, angiogenesis, cell proliferation and apoptosis [58,59]. Real-time qPCR revealed that prenatal exposure to THC significantly decreased the relative expression of *Igf1r* in the ovary, as well as the percentage of IGF-1R-positive follicular cells, as determined by immunohistochemistry (IHC) (Figure 3).

Prenatal Exposure to THC Increases Follicular Apoptosis in the Adult Ovary

It has previously been established that IGF-1/IGF-1R signaling plays an essential role in follicular growth and atresia, mostly by regulating granulosa cell proliferation and apoptosis [58]. Considering that gestational exposure to THC resulted in a significant decrease in IGF-1R, the expression of the apoptotic marker caspase-3 was evaluated. As shown in Figure 4, while there were no changes in the steady-state mRNA expression of *Casp3*, gestational exposure to THC significantly increased the percentage of cleaved caspase-3-positive ovarian follicular cells.

Acute Exposure to THC Decreases Expression of Igf1r in the Ovary

In order to determine if THC had a direct effect on the expression of *Igf1r* in the ovaries, ex vivo and in vitro models were used. While it is possible to observe short-term effects of direct THC exposure on gene expression in both models, the use of ovarian explants represents a microenvironment and cell diversity that more closely resemble in vivo conditions. On the other hand, the use of an immortalized cell line provides information on the effect of the compound on that specific cell type. Ovarian explants and spontaneously immortalized rat granulosa cells were cultured and exposed to 3 μM THC for 48 h. As observed in Figure 5, exposure to 3 μM THC significantly decreased the relative expression of *Igf1r* in the ovary explants (Figure 5A) and the granulosa cells (Figure 5B).

Discussion

As the use of cannabis during pregnancy increases, it is important to understand the possible effects of its constituents on the developing fetus. The present study examined the effect of gestational exposure to THC on the miRNA profile in adult rat ovaries, as a proposed mechanism through which THC-induced changes in miRNA expression could influence the altered ovarian follicle dynamics and vascularization previously observed in these offspring [57]. Prenatal exposure to THC resulted in the differential expression of 12 miRNA targets, as determined by a Nanostring assay that evaluated the expression of 420 biologically relevant rat miRNAs. While there are several studies that report in vivo alterations of miRNA expression as a consequence of direct exposure of primates and rodents to THC [60–62], work from our group has also shown that gestational exposure to THC resulted in altered miRNA profiles in the liver of adult rat offspring [37]. Similarly, in this study we report an altered miRNA profile in the ovaries of THC-exposed female offspring.

Of the 420 miRNAs included in the Nanostring assay, miR-122-5p (miR-122) was the only upregulated target, as well as having the largest fold-change compared to the controls. As miR-122 is a highly conserved miRNA among multiple species, including rat and human [63], this target was chosen for further evaluation. RT-qPCR confirmed the upregulation of miR-122 in the prenatally THC-exposed ovaries. There is growing evidence that suggests miR-122 is involved in the regulation of cell proliferation, differentiation, migration and apoptosis [64,65]. A recent study revealed increased expression of miR-122 in atrophic chicken ovaries compared to healthy

ones [63]. Flow cytometry revealed that granulosa cell apoptosis was significantly decreased by a miR-122 inhibitor, while treatment with a miR-122 mimic increased apoptosis and caspase-3 protein levels in vitro [63]. Similarly, Zhang and colleagues recently reported increased granulosa cell apoptosis in a rodent model of primary ovarian insufficiency following treatment with a miR-122 mimic, while a miR-122 inhibitor reduced granulosa cell apoptosis [66]. Furthermore, Menon and colleagues observed that miR-122 plays an important role in the regulation of the luteinizing hormone receptor mRNA binding protein (LRBP) during FSH-induced follicular growth, and therefore may impact folliculogenesis and ovulation [67,68].

To evaluate the effects of the upregulation of miR-122 in the ovaries, the online databases TargetScan and miRTarBase were used to identify validated miR-122 targets involved in the regulation of ovarian processes. The validated miR-122 target insulin-like growth factor 1 receptor (*Igf1r*) was selected for further evaluation. Studies have reported that miR-122 can directly bind to and decrease *Igf1r* transcript in vitro [69–71]. Moreover, IGF-1R has been shown to play a role in the regulation of folliculogenesis, ovulation, angiogenesis and granulosa cell proliferation and apoptosis [58,72]. IGF-1R is a receptor tyrosine kinase that, among other functions, activates the AKT pathway, linked to prevention of apoptosis, and the ERK pathway, which is associated with growth and proliferation [73]. IGF-1R expression has been detected in most structures of the rodent ovary, including the stroma, oocytes, corpus luteum and theca cells, with the strongest staining found in granulosa cells [58]. Gestational exposure to THC significantly decreased mRNA expression of *Igf1r*

and the percentage of IGF-1R-positive follicular cells in the ovaries. These results are in accordance with the observed increase in miR-122 and with studies that have reported the downregulation of *Igf1r* as a result of miR-122 overexpression [69– 71]. Furthermore, the decrease in *Igf1r* expression appears to be directly attributable to the effect of THC on the ovary, as this compound significantly decreased the relative expression of *Igf1r* in ovary explants and spontaneously immortalized rat granulosa cells (SIGCs) exposed to 3 µM for 48 h. Considering the key role of IGF-1R in the ovary [58,59], it is possible that a similar mechanism may partially explain the adverse reproductive outcomes such as an increased risk of infertility due to ovulatory abnormalities [74], fewer and poorer quality oocytes and lower pregnancy rates by in vitro fertilization (IVF) [75] that have been reported in adult cannabis users.

Protein levels of IGF-1R have been shown to be reduced in granulosa cells from patients with PCOS and ovarian tissue of PCOS rat models [76]. This reduction was linked to decreased AKT phosphorylation and increased caspase-3 activity, suggesting that increased granulosa cell apoptosis plays a role in the abnormal folliculogenesis and anovulation in PCOS [76]. It has also been shown that knockdown of *Igf1r* in granulosa cells results in a lack of response to FSH in vitro, and that inhibiting IGF-1R activity in vivo prevents eCG-induced follicular growth [72]. Moreover, Baumgarten and colleagues demonstrated that female mice with a conditional *Igf1r* knockdown in granulosa cells were sterile, with small ovaries lacking antral follicles, even after stimulation with gonadotropin [58]. Accordingly, these

animals failed to ovulate after a superovulation protocol, and their granulosa cells expressed significantly lower levels of preovulatory markers [58]. Similar to what has been observed in PCOS models, ovaries with reduced IGF-1R had impaired AKT activation, as well as increased levels of caspase-3-dependent apoptosis in follicles transitioning from the primary to the large secondary stages [58]. The authors concluded that the lack of IGF-1R signaling in the granulosa cells resulted in increased apoptosis and failure to respond to FSH, which in turn led to the complete arrest of folliculogenesis and the subsequent loss of fertility. In the present study, in addition to reduced *Igf1r* expression, THC-exposed offspring had an increased percentage of cleaved caspase-3-positive follicular cells in the ovaries, suggesting an increase in apoptosis. In addition, IHC revealed that cleaved caspase-3 was predominantly expressed in granulosa cells, which could suggest increased granulosa cell apoptosis, an important factor of follicular atresia [77].

The reduction in *Igf1r* expression may also help explain the altered follicle dynamics in the ovaries of THC exposed offspring previously reported by our group [57]. In the same cohort of animals, we observed that ovaries from THC-exposed offspring had a significant increase in transitioning follicles without any differences in later stages of follicular development, suggesting a portion of the follicles did not continue to develop. While this was partially attributed to the reduced blood vessel formation in these ovaries, it is possible that the increased apoptosis revealed in the present study also contributed to the impaired follicular development. The reduced vascularization of these ovaries was associated with a decrease in VEGF and

VEGFR2 expression and an increase in TSP-1. This is interesting considering it has been shown that activation of IGF-1R results in increased VEGF expression and secretion in bovine luteal cells [78]. Additionally, it has been demonstrated that in vitro treatment of immortalized rat granulosa cells with TSP-1 increases the expression of pro-apoptotic factors [79], while VEGF-VEGFR2 signaling appears to have the opposite effect [80]. It was therefore proposed that the dysregulation of these factors could manifest as increased apoptosis (atresia) and ultimately a loss of developing follicles [57]. Although there were no statistically significant differences in the number of atretic follicles in the THC-exposed ovaries (p = 0.197), this could be a reflection of the small sample size. It is also possible that the increase in apoptosis may manifest more clearly as follicle loss as the animal ages. Indeed, a rodent model with granulosa cell-specific silencing of *Vegf* revealed a decrease in *Igf1r* expression and increased granulosa cell apoptosis [81]. According to the authors, these animals were subfertile, and the effect seemed to be greater as the mice aged [81]. It would therefore be of great interest to continue monitoring the ovarian follicle reserve as the animals approach reproductive senescence and to assess the THC-exposed offspring's fertility, since the observed effects may have an impact on other reproductive aspects such as oocyte quality.

Conclusions

We have previously shown that prenatal exposure to delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, resulted in altered follicle dynamics and vascularization in the adult rat ovary. In this study, we propose that gestational exposure to THC altered miRNA expression in the ovary, which in turn affected ovarian health in adulthood. Particularly, prenatal THC exposure resulted in a significant increase in the expression of miR-122-5p and a significant decrease in the expression of its validated target gene insulin-like growth factor 1 receptor (*Igf1r*). Importantly, decreased IGF-1R expression has been linked to increased apoptosis and abnormal folliculogenesis, both of which have been observed in the THC-exposed offspring. Taken together, these data suggest that prenatal THC-exposure may impact key pathways in the developing ovary that could lead to subfertility or premature reproductive senescence. As the use of cannabis during pregnancy increases, it is important to understand the safety of this drug and its constituents, as well as the possible long-term effects on the offspring's endocrine and reproductive health.

Author Contributions: Conceptualization, A.A.M.-P., J.J.P., D.B.H. and A.C.H; methodology, A.A.M.-P., K.L. and M.P.; formal analysis, A.A.M.-P.; resources, J.J.P., D.B.H. and A.C.H.; writing— original draft preparation, A.A.M.-P.; writing— review and editing, A.A.M.-P., A.A., J.J.P., D.B.H. and A.C.H.; supervision, A.C.H.; funding acquisition, D.B.H. and A.C.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a Canadian Institutes of Health Research (CIHR) (Canada)) Project Grant to ACH (PJT-155981) and an HSFC (Heart and Stroke Foundation of Canada (Canada)) Grant-in-Aid to DBH (G-19-0026343). AAMP was sponsored by CONACYT (National Council of Science and Technology (Mexico)).

Institutional Review Board Statement: All procedures were performed according to guidelines set by the Canadian Council of Animal Care, and Animal Use Protocol (AUP #2019-126) was approved by the Animal Care Committee at The University of Western Ontario. All investigators understood and followed the ethical principles outlined by Grundy, and study design was informed by ARRIVE guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Labor Unooda. World Drug Report 2021 (Set of 5 Booklets); United Nations: 2022. Available online: https://www.unodc.org/unodc/en/data-and-analysis/wdr2021.html (accessed on 16 July 2022).

2. Corsi, D.J.; Hsu, H.; Weiss, D.; Fell, D.B.; Walker, M. Trends and correlates of cannabis use in pregnancy: A population-based study in Ontario, Canada from 2012 to 2017. Can. J. Public Health. 2018, 110, 76–84. https://doi.org/10.17269/s41997-018-0148-0.

3. 2019 National Survey on Drug Use and Health: Women|CBHSQ Data n.d. Available online: https://www.samhsa.gov/data/report/2019-nsduh-women (accessed on 4 June 2022).

4. Leemaqz, S.Y.; Dekker, G.A.; McCowan, L.M.; Kenny, L.C.; Myers, J.E.; Simpson, N.A.B.; Poston, L.; Roberts, C.T., SCOPE Consortium. Maternal marijuana use has independent effects on risk for spontaneous preterm birth but not other common late pregnancy complications. Reprod. Toxicol. 2016, 62, 77–86. https://doi.org/10.1016/j.reprotox.2016.04.021.

5. Ko, J.Y.; Farr, S.L.; Tong, V.T.; Creanga, A.A.; Callaghan, W.M. Prevalence and patterns of marijuana use among pregnant and nonpregnant women of reproductive age. Am. J. Obstet. Gynecol. 2015, 213, 201.e1–201.e10. https://doi.org/10.1016/j.ajog.2015.03.021.

6. Moore, D.G.; Turner, J.D.; Parrott, A.C.; Goodwin, J.E.; Fulton, S.E.; Min, M.O.; Fox, H.C.; Braddick, F.M.B.; Axelsson, E.L.; Lynch, S.; et al. During pregnancy, recreational drug-using women stop taking ecstasy (3,4-methylenedioxy-N-methylamphetamine) and reduce alcohol consumption, but continue to smoke tobacco and cannabis: Initial findings from the Development and Infancy Study. J. Psychopharmacol. 2010, 24, 1403–1410. https://doi.org/10.1177/0269881109348165.

7. PregnancyInfo. Available online: https://www.pregnancyinfo.ca/learn-more/ (accessed on 16 July 2022).

8. Volkow, N.D.; Han, B.; Compton, W.M.; McCance-Katz, E.F. Self-reported Medical and Nonmedical Cannabis Use Among Pregnant Women in the United States. JAMA 2019, 322, 167–169. https://doi.org/10.1001/jama.2019.7982.

9. Westfall, R.E.; Janssen, P.A.; Lucas, P.; Capler, R. Survey of medicinal cannabis use among childbearing women: Patterns of its use in pregnancy and retroactive

self-assessment of its efficacy against ‘morning sickness’. Complement. Ther. Clin. Pract. 2006, 12, 27–33. https://doi.org/10.1016/j.ctcp.2005.09.006.

10. Roberson, E.K.; Patrick, W.K.; Hurwitz, E.L. Marijuana Use and Maternal Experiences of Severe Nausea During Pregnancy in Hawai’i. Hawaii J. Med. Public Health 2014, 73, 283–287.

11. Bayrampour, H.; Asim, A. Cannabis Use During the Pre-Conception Period and Pregnancy After Legalization. J. Obstet. Gynaecol. Can. 2021, 43, 740–745. https://doi.org/10.1016/j.jogc.2021.02.119.

12. Singh, S.; Filion, K.B.; Abenhaim, H.A.; Eisenberg, M.J. Prevalence and outcomes of prenatal recreational cannabis use in high-income countries: A scoping review. BJOG Int. J. Obstet. Gynaecol. 2020, 127, 8–16. https://doi.org/10.1111/1471-0528.15946.

13. Lozano, J.; García-Algar, O.; Marchei, E.; Vall, O.; Monleon, T.; Giovannandrea, R.D.; Pichini, S. Prevalence of gestational exposure to cannabis in a Mediterranean city by meconium analysis. Acta Paediatr. 2007, 96, 1734–1737. https://doi.org/10.1111/j.1651-2227.2007.00535.x.

14. Metz, T.D.; Silver, R.M.; McMillin, G.A.; Allshouse, A.A.; Jensen, T.L.; Mansfield, C.; Heard, K.; Kinney, G.L.; Wymore, E.; Binswanger, I.A. Prenatal Marijuana Use by Self-Report and Umbilical Cord Sampling in a State with Marijuana Legalization. Obstet. Gynecol. 2019, 133, 98–104. https://doi.org/10.1097/AOG.0000000000003028.

15. Luke, S.; Hutcheon, J.; Kendall, T. Cannabis Use in Pregnancy in British Columbia and Selected Birth Outcomes. J. Obstet. Gynaecol. Can. 2019, 41, 1311–1317. https://doi.org/10.1016/j.jogc.2018.11.014.

16. Varner, M.W.; Silver, R.M.; Rowland Hogue, C.J.; Willinger, M.; Parker, C.B.; Thorsten, V.R.; Goldenberg, R.L.; Saade, G.R.; Dudley, D.J.; Coustan, D.; et al. Association between stillbirth and illicit drug use and smoking during pregnancy. Obstet. Gynecol. 2014, 123, 113–125. https://doi.org/10.1097/AOG.0000000000000052.

17. Shi, Y.; Zhu, B.; Liang, D. The associations between prenatal cannabis use disorder and neonatal outcomes. Addict. Abingdon Engl. 2021, 116, 3069–3079. https://doi.org/10.1111/add.15467.

18. Hayatbakhsh, M.R.; Flenady, V.J.; Gibbons, K.S.; Kingsbury, A.M.; Hurrion, E.; Mamun, A.A.; Najmna, J.M. Birth outcomes associated with cannabis use before and during pregnancy. Pediatr. Res. 2012, 71, 215–219. https://doi.org/10.1038/pr.2011.25.

19. Hurd, Y.L.; Wang, X.; Anderson, V.; Beck, O.; Minkoff, H.; Dow-Edwards, D. Marijuana impairs growth in mid-gestation fetuses. Neurotoxicol. Teratol. 2005, 27, 221–229. https://doi.org/10.1016/j.ntt.2004.11.002.

20. Warshak, C.R.; Regan, J.; Moore, B.; Magner, K.; Kritzer, S.; Van Hook, J. Association between marijuana use and adverse obstetrical and neonatal outcomes. J. Perinatol. 2015, 35, 991–995. https://doi.org/10.1038/jp.2015.120.

21. Campbell, E.E.; Gilliland, J.; Dworatzek, P.D.N.; Vrijer, B.D.; Penava, D.; Seabrook, J.A. Socioeconomic Status and Adverse Birth Outcomes: A Population-Based Canadian Sample. J. Biosoc. Sci. 2018, 50, 102–113. https://doi.org/10.1017/S0021932017000062.

22. El Marroun, H.; Tiemeier, H.; Steegers, E.A.P.; Jaddoe, V.W.V.; Hofman, A.; Verhulst, F.C.; van den Brink, W.; Huizink, A.C. Intrauterine Cannabis Exposure Affects Fetal Growth Trajectories: The Generation R Study. J. Am. Acad. Child

Adolesc. Psychiatry 2009, 48, 1173–1181. https://doi.org/10.1097/CHI.0b013e3181bfa8ee.

23. Gabrhelík, R.; Mahic, M.; Lund, I.O.; Bramness, J.; Selmer, R.; Skovlund, E.; Handal, M.; Skurtveit, S. Cannabis Use during Pregnancy and Risk of Adverse Birth Outcomes: A Longitudinal Cohort Study. Eur. Addict. Res. 2021, 27, 131–141. https://doi.org/10.1159/000510821.

24. Smith, A.; Kaufman, F.; Sandy, M.S.; Cardenas, A. Cannabis Exposure during Critical Windows of Development: Epigenetic and Molecular Pathways Implicated in Neuropsychiatric Disease. Curr. Environ. Health Rep. 2020, 7, 325–342. https://doi.org/10.1007/s40572-020-00275-4.

25. Mechoulam, R.; Gaoni, Y. A Total Synthesis of Dl-Delta-1-Tetrahydrocannabinol, the Active Constituent of Hashish. J. Am. Chem. Soc. 1965, 87, 3273–3275. https://doi.org/10.1021/ja01092a065.

26. Carlier, J.; Huestis, M.A.; Zaami, S.; Pichini, S.; Busardò, F.P. Monitoring Perinatal Exposure to Cannabis and Synthetic Cannabinoids. Ther. Drug Monit. 2020, 42, 194–204. https://doi.org/10.1097/FTD.0000000000000667.

27. Banu, S.K.; Stanley, J.A.; Sivakumar, K.K.; Arosh, J.A.; Barhoumi, R.; Burghardt, R.C. Identifying a novel role for X-prolyl aminopeptidase (Xpnpep) 2 in CrVI-induced adverse effects on germ cell nest breakdown and follicle development in rats. Biol. Reprod. 2015, 92, 67. https://doi.org/10.1095/biolreprod.114.125708.

28. Pepe, G.J.; Lynch, T.J.; Albrecht, E.D. Regulation of baboon fetal ovarian development by placental estrogen: Onset of puberty is delayed in offspring deprived of estrogen in utero. Biol. Reprod. 2013, 89, 132. https://doi.org/10.1095/biolreprod.112.107318.

29. Barker, D.J.P.; Eriksson, J.G.; Forsén, T.; Osmond, C. Fetal origins of adult disease: Strength of effects and biological basis. Int. J. Epidemiol. 2002, 31, 1235–1239. https://doi.org/10.1093/ije/31.6.1235.

30. Smith, P.; Wilhelm, D.; Rodgers, R.J. Development of mammalian ovary. J. Endocrinol. 2014, 221, R145–R61. https://doi.org/10.1530/JOE-14-0062.

31. Moore, B.F.; Sauder, K.A.; Shapiro, A.L.B.; Crume, T.; Kinney, G.L.; Dabelea, D. Fetal Exposure to Cannabis and Childhood Metabolic Outcomes: The Healthy Start Study. J. Clin. Endocrinol. Metab. 2022, 20, 1–8. https://doi.org/10.1210/clinem/dgac101.

32. Campolongo, P.; Trezza, V.; Ratano, P.; Palmery, M.; Cuomo, V. Developmental consequences of perinatal cannabis exposure: Behavioral and neuroendocrine effects in adult rodents. Psychopharmacology 2011, 214, 5–15. https://doi.org/10.1007/s00213-010- 1892-x.

33. Smith, A.M.; Fried, P.A.; Hogan, M.J.; Cameron, I. Effects of prenatal marijuana on visuospatial working memory: An fMRI study in young adults. Neurotoxicol. Teratol. 2006, 28, 286–295. https://doi.org/10.1016/j.ntt.2005.12.008.

34. Gillies, R.; Lee, K.; Vanin, S.; Laviolette, S.R.; Holloway, A.C.; Arany, E.; Hardy, D.B. Maternal exposure to Δ9- tetrahydrocannabinol impairs female offspring glucose homeostasis and endocrine pancreatic development in the rat. Reprod. Toxicol. 2020, 94, 84–91. https://doi.org/10.1016/j.reprotox.2020.04.070.

35. De Domenico, E.; Todaro, F.; Rossi, G.; Dolci, S.; Geremia, R.; Rossi, P.; Grimaldi, P. Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads and impairs ovarian reserve. Cell Death Dis. 2017, 8, e3085. https://doi.org/10.1038/cddis.2017.496.

36. Castel, P.; Barbier, M.; Poumerol, E.; Mandon-Pépin, B.; Tassistro, V.; Lepidi, H.; Pelissier-Alicot, A.L.; Manzoni, O.J.; Courbiere, B. Prenatal cannabinoid exposure alters the ovarian reserve in adult offspring of rats. Arch Toxicol. 2020, 94, 4131–4141. https://doi.org/10.1007/s00204-020-02877-1.

37. Oke, S.L.; Lee, K.; Papp, R.; Laviolette, S.R.; Hardy, D.B. In Utero Exposure to Δ9-Tetrahydrocannabinol Leads to Postnatal Catch-Up Growth and Dysmetabolism in the Adult Rat Liver. Int. J. Mol. Sci. 2021, 22, 7502. https://doi.org/10.3390/ijms22147502.

38. Lee, K.; Hardy, D.B. Metabolic Consequences of Gestational Cannabinoid Exposure. Int. J. Mol. Sci. 2021, 22, 9528. https://doi.org/10.3390/ijms22179528.

39. Egger, G.; Liang, G.; Aparicio, A.; Jones, P.A. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004, 429, 457–463. https://doi.org/10.1038/nature02625.

40. DiNieri, J.A.; Wang, X.; Szutorisz, H.; Spano, S.M.; Kaur, J.; Casaccia, P.; Dow-Edwards, D.; Hurd, Y.L. Maternal cannabis use alters ventral striatal dopamine D2 gene regulation in the offspring. Biol. Psychiatry 2011, 70, 763–769. https://doi.org/10.1016/j.biopsych.2011.06.027.

41. Murphy, S.K.; Itchon-Ramos, N.; Visco, Z.; Huang, Z.; Grenier, C.; Schrott, R.; Acharya, K.; Boudreau, M.H.; Price, T.M.; Raburn, D.J; et al. Cannabinoid exposure and altered DNA methylation in rat and human sperm. Epigenetics 2018, 13, 1208–1221. https://doi.org/10.1080/15592294.2018.1554521.

42. Prini, P.; Penna, F.; Sciuccati, E.; Alberio, T.; Rubino, T. Chronic Δ8 -THC Exposure Differently Affects Histone Modifications in the Adolescent and Adult Rat Brain. Int. J. Mol. Sci. 2017, 18, E2094. https://doi.org/10.3390/ijms18102094.

43. Pucci, M.; Rapino, C.; Di Francesco, A.; Dainese, E.; D'Addario, C.; Maccarrone, M. Epigenetic control of skin differentiation genes by phytocannabinoids. Br. J. Pharmacol. 2013, 170, 581–591. https://doi.org/10.1111/bph.12309.

44. Todd, S.M.; Zhou, C.; Clarke, D.J.; Chohan, T.W.; Bahceci, D.; Arnold, J.C. Interactions between cannabidiol and Δ9-THC following acute and repeated dosing: Rebound hyperactivity, sensorimotor gating and epigenetic and neuroadaptive changes in the mesolimbic pathway. Eur. Neuropsychopharmacol. 2017, 27, 132–145. https://doi.org/10.1016/j.euroneuro.2016.12.004.

45. Yang, X.; Hegde, V.L.; Rao, R.; Zhang, J.; Nagarkatti, P.S.; Nagarkatti, M. Histone modifications are associated with Δ9- tetrahydrocannabinol-mediated alterations in antigen-specific T cell responses. J. Biol. Chem. 2014, 289, 18707–18718. https://doi.org/10.1074/jbc.M113.545210.

46. Watson, C.T.; Szutorisz, H.; Garg, P.; Martin, Q.; Landry, J.A.; Sharp, A.J.; Hurd, Y.L. Genome-Wide DNA Methylation Profiling Reveals Epigenetic Changes in the Rat Nucleus Accumbens Associated With Cross-Generational Effects of Adolescent THC Exposure. Neuropsychopharmacology. 2015, 40, 2993–3005. https://doi.org/10.1038/npp.2015.155.

47. Sido, J.M.; Yang, X.; Nagarkatti, P.S.; Nagarkatti, M. Δ9-Tetrahydrocannabinol-mediated epigenetic modifications elicit myeloid-derived suppressor cell activation via STAT3/S100A8. J. Leukoc. Biol. 2015, 97, 677–688. https://doi.org/10.1189/jlb.1A1014-479R.

48. Juknat, A.; Gao, F.; Coppola, G.; Vogel, Z.; Kozela, E. miRNA expression profiles and molecular networks in resting and LPS-activated BV-2 microglia-Effect of cannabinoids. PLoS ONE 2019, 14, e0212039. https://doi.org/10.1371/journal.pone.0212039.

49. Kim, V.N. Small RNAs: Classification, biogenesis, and function. Mol. Cells 2005, 19, 1–15.

50. Kim, J.; You, S. Comprehensive analysis of miRNA-mRNA interactions in ovaries of aged mice. Anim. Sci. J. 2022, 93, e13721. https://doi.org/10.1111/asj.13721.

51. Tufarelli, C.; Stanley, J.A.S.; Garrick, D.; Sharpe, J.A.; Ayyub, H.; Wood, W.G.; Higgs, D.R. Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat. Genet. 2003, 34, 157–165. https://doi.org/10.1038/ng1157.

52. Brennecke, J.; Stark, A.; Russell, R.B.; Cohen, S.M. Principles of microRNA-target recognition. PLoS Biol. 2005, 3, e85. https://doi.org/10.1371/journal.pbio.0030085.

53. Valadi, H.; Ekström, K.; Bossios, A.; Sjöstrand, M.; Lee, J.J.; Lötvall, J.O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 2007, 9, 654–659. https://doi.org/10.1038/ncb1596.

54. Mitchell, P.S.; Parkin, R.K.; Kroh, E.M.; Fritz, B.R.; Wyman, S.K.; Pogosova-Agadjanyan, E.L.; Peterson, A.; Noteboom, J.; O'Briant, K.C.; Allen, A.; et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. USA 2008, 105, 10513–10518. https://doi.org/10.1073/pnas.0804549105.

55. Li, Y.; Fang, Y.; Liu, Y.; Yang, X. MicroRNAs in ovarian function and disorders. J. Ovarian Res. 2015, 8, 51. https://doi.org/10.1186/s13048-015-0162-2.

56. Gebremedhn, S.; Salilew-Wondim, D.; Ahmad, I.; Sahadevan, S.; Hossain, M.M.; Hoelker, M.; Rings, F.; Neuhoff, C.; Tholen, E.; Looft, C.; et al. MicroRNA Expression Profile in Bovine Granulosa Cells of Preovulatory Dominant and Subordinate

Follicles during the Late Follicular Phase of the Estrous Cycle. PLoS ONE 2015, 10, e0125912. https://doi.org/10.1371/journal.pone.0125912.

57. Martínez-Peña, A.A.; Lee, K.; Petrik, J.J.; Hardy, D.B.; Holloway, A.C. Gestational exposure to Δ9-THC impacts ovarian follicular dynamics and angiogenesis in adulthood in Wistar rats. J. Dev. Orig. Health Dis. 2021, 12, 865–869. https://doi.org/10.1017/S2040174420001282.

58. Baumgarten, S.C.; Armouti, M.; Ko, C.; Stocco, C. IGF1R Expression in Ovarian Granulosa Cells Is Essential for Steroidogenesis, Follicle Survival, and Fertility in Female Mice. Endocrinology 2017, 158, 2309–2318. https://doi.org/10.1210/en.2017-00146.

59. Zhao, J.; Taverne, M.; van der Weijden, G.; Bevers, M.; Hurk, R.V.D. Immunohistochemical localisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-I) and type I IGF-I receptor, and gene expression of GH and GHR in rat pre-antral follicles. Zygote. 2002, 10, 85–94. https://doi.org/10.1017/s0967199402002125.

60. Mohammed, A.; Alghetaa, H.; Sultan, M.; Singh, N.P.; Nagarkatti, P.; Nagarkatti, M. Administration of Δ9- Tetrahydrocannabinol (THC) Post-Staphylococcal Enterotoxin B Exposure Protects Mice from Acute Respiratory Distress Syndrome and Toxicity. Front. Pharmacol. 2020, 11, 893. https://doi.org/10.3389/fphar.2020.00893.

61. Chandra, L.C.; Kumar, V.; Torben, W.; Vande Stouwe, C.; Winsauer, P.; Amedee, A.; Molina, P.E.; Mohan, M. Chronic administration of Δ9-tetrahydrocannabinol induces intestinal anti-inflammatory microRNA expression during acute simian immunodeficiency virus infection of rhesus macaques. J. Virol. 2015, 89, 1168–1181. https://doi.org/10.1128/JVI.01754-14.

62. Hegde, V.L.; Tomar, S.; Jackson, A.; Rao, R.; Yang, X.; Singh, U.P.; Singh, N.P.; Nagarkatti, P.S.; Nagarkatti, M. Distinct microRNA expression profile and targeted biological pathways in functional myeloid-derived suppressor cells induced by Δ9-tetrahydrocannabinol in vivo: Regulation of CCAAT/enhancer-binding protein α by microRNA-690. J. Biol. Chem. 2013, 288, 36810– 36826. https://doi.org/10.1074/jbc.M113.503037.

63. Zhang, G.; Cui, Z.; Li, J.; Zhang, D.; Li, Z.; Lin, Z.; Yin, H.; Ran, J.; Wang, Y.; Liu, Y. miR-122-5p regulates proliferation and apoptosis of chicken granulosa cells of hierarchal follicles by targeting MAPK3. Gene 2022, 824, 146397. https://doi.org/10.1016/j.gene.2022.146397.

64. Pei, Z.J.; Zhang, Z.G.; Hu, A.X.; Yang, F.; Gai, Y. miR-122-5p inhibits tumor cell proliferation and induces apoptosis by targeting MYC in gastric cancer cells. Die Pharm.-Int. J. Pharm. Sci. 2017, 72, 344–347. https://doi.org/10.1691/ph.2017.6404.

65. Ma, J.; Li, T.; Han, X.; Yuan, H. Knockdown of LncRNA ANRIL suppresses cell proliferation, metastasis, and invasion via regulating miR-122-5p expression in hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2018, 144, 205–214. https://doi.org/10.1007/s00432-017-2543-y.

66. Zhang, X.; Zhang, R.; Hao, J.; Huang, X.; Liu, M.; Lv, M.; Su, C.; Mu, Y.L. miRNA-122-5p in POI ovarian-derived exosomes promotes granulosa cell apoptosis by regulating BCL9. Cancer Med. 2022, 11, 2414–2426. https://doi.org/10.1002/cam4.4615.

67. Menon, B.; Gulappa, T.; Menon, K.M.J. Molecular regulation of LHCGR expression by miR-122 during follicle growth in the rat ovary. Mol. Cell Endocrinol. 2017, 442, 81–89. https://doi.org/10.1016/j.mce.2016.12.002.

68. Menon, K.M.J.; Menon, B.; Gulappa, T. Regulation of Luteinizing Hormone Receptor mRNA Expression in the Ovary: The Role of miR-122. Vitam. Horm. 2018, 107, 67–87. https://doi.org/10.1016/bs.vh.2018.01.010.

69. He, Y.; Liu, X.; Qu, Q.; Chen, J.; Fan, Z.; Zhu, D.; Miao, Y.; Hu, Z. Overexpression of miR-122 promotes apoptosis of dermal papilla cells by directly targeting IGF1R in androgenetic alopecia. Cell Biol. Int. 2022, 46, 185–191. https://doi.org/10.1002/cbin.11723.

70. Xu, Y.; Huang, J.; Ma, L.; Shan, J.; Shen, J.; Yang, Z.; Liu, L.; Luo, Y.; Yao, C.; Qian, C. MicroRNA-122 confers sorafenib resistance to hepatocellular carcinoma cells by targeting IGF-1R to regulate RAS/RAF/ERK signaling pathways. Cancer Lett. 2016, 371, 171– 181. https://doi.org/10.1016/j.canlet.2015.11.034.

71. Wang, B.; Wang, H.; Yang, Z. MiR-122 Inhibits Cell Proliferation and Tumorigenesis of Breast Cancer by Targeting IGF1R. PLoS ONE 2012, 7, e47053. https://doi.org/10.1371/journal.pone.0047053.

72. Zhou, P.; Baumgarten, S.C.; Wu, Y.; Bennett, J.; Winston, N.; Hirshfeld-Cytron, J.; Stocco, C. IGF-I signaling is essential for FSH stimulation of AKT and steroidogenic genes in granulosa cells. Mol. Endocrinol. 2013, 27, 511–523. https://doi.org/10.1210/me.2012-1307.

73. Singh, R.; Chaudhary, P.; Arya, R. Role of IGF-1R in ameliorating apoptosis of GNE deficient cells. Sci. Rep. 2018, 8, 7323. https://doi.org/10.1038/s41598-018-25510-9.

74. Mueller, B.A.; Daling, J.R.; Weiss, N.S.; Moore, D.E. Recreational drug use and the risk of primary infertility. Epidemiology. 1990, 1, 195–200. https://doi.org/10.1097/00001648-199005000-00003.

75. Klonoff-Cohen, H.S.; Natarajan, L.; Chen, R.V. A prospective study of the effects of female and male marijuana use on in vitro fertilization (IVF) and gamete intrafallopian transfer (GIFT) outcomes. Am. J. Obstet. Gynecol. 2006, 194, 369–376. https://doi.org/10.1016/j.ajog.2005.08.020.

76. Mao, Z.; Fan, L.; Yu, Q.; Luo, S.; Wu, X.; Tang, J.; Kang, G.; Tang, L. Abnormality of Klotho Signaling Is Involved in Polycystic Ovary Syndrome. Reprod. Sci. 2018, 25, 372–383. https://doi.org/10.1177/1933719117715129.

77. Manabe, N.; Goto, Y.; Matsuda-Minehata, F.; Inoue, N.; Maeda, A.; Sakamaki, K.; Miyano, T. Regulation mechanism of selective atresia in porcine follicles: Regulation of granulosa cell apoptosis during atresia. J. Reprod. Dev. 2004, 50, 493–514. https://doi.org/10.1262/jrd.50.493.

78. Uniyal, S.; Panda, R.P.; Chouhan, V.S.; Yadav, V.P.; Hyder, I.; Dangi, S.S.; Gupta, M.; Khan, F.A.; Sharma, G.T.; Bag, S.; et al. Expression and localization of insulin-like growth factor system in corpus luteum during different stages of estrous cycle in water buffaloes (Bubalus bubalis) and the effect of insulin-like growth factor I on production of vascular endothelial growth factor and progesterone in luteal cells cultured in vitro. Theriogenology 2015, 83, 58–77. https://doi.org/10.1016/j.theriogenology.2014.07.034.

79. Greenaway, J.; Lawler, J.; Moorehead, R.; Bornstein, P.; Lamarre, J.; Petrik, J. Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). J. Cell Physiol. 2007, 210, 807–818. https://doi.org/10.1002/jcp.20904.

80. Greenaway, J.; Connor, K.; Pedersen, H.G.; Coomber, B.L.; LaMarre, J.; Petrik, J. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. Endocrinology 2004, 145, 2896– 2905. https://doi.org/10.1210/en.2003-1620.

81. Sargent, K.M.; Lu, N.; Clopton, D.T.; Pohlmeier, W.E.; Brauer, V.M.; Ferrara, N.; Silversides, D.W.; Cupp, A.S. Loss of Vascular Endothelial Growth Factor A (VEGFA) Isoforms in Granulosa Cells Using pDmrt-1-Cre or Amhr2-Cre Reduces Fertility by Arresting Follicular Development and by Reducing Litter Size in Female Mice. PLoS ONE 2015, 10, e0116332. https://doi.org/10.1371/journal.pone.0116332.

82. Klein, C.; Karanges, E.; Spiro, A.; Wong, A.; Spencer, J.; Huynh, T.; Gunasekaran, N.; Karl, T.; Long, L.E.; Huang, X.F.; et al. Cannabidiol potentiates Δ9 -tetrahydrocannabinol (THC) behavioural effects and alters THC pharmacokinetics during acute and chronic treatment in adolescent rats. Psychopharmacology 2011, 218, 443–457. https://doi.org/10.1007/s00213-011-2342-0.

83. Schwope, D.M.; Karschner, E.L.; Gorelick, D.A.; Huestis, M.A. Identification of recent cannabis use: Whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. Clin. Chem. 2011, 57, 1406–1414. https://doi.org/10.1373/clinchem.2011.171777.

84. Falcon, M.; Pichini, S.; Joya, J.; Pujadas, M.; Sanchez, A.; Vall, O.; García Algar, O.; Luna, A.; de la Torre, R.; Rotolo, M.C.; et al. Maternal hair testing for the assessment of fetal exposure to drug of abuse during early pregnancy: Comparison with testing in placental and fetal remains. Forensic Sci. Int. 2012, 218, 92–96. https://doi.org/10.1016/j.forsciint.2011.10.022.

85. Natale, B.V.; Gustin, K.N.; Lee, K.; Holloway, A.C.; Laviolette, S.R.; Natale, D.R.C.; Hardy, D.B. Δ9-tetrahydrocannabinol exposure during rat pregnancy leads to symmetrical fetal growth restriction and labyrinth-specific vascular defects in the placenta. Sci. Rep. 2020, 10, 544. https://doi.org/10.1038/s41598-019-57318-6.

86. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001, 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

87. Fereshteh, Z.; Schmidt, S.A.; Al-Dossary, A.A.; Accerbi, M.; Arighi, C.; Cowart, J.; Song, J.L.; Green, P.J.; Choi, K.; Yoo, S.; et al. Murine Oviductosomes (OVS) microRNA profiling during the estrous cycle: Delivery of OVS-borne microRNAs to sperm where miR-34c-5p localizes at the centrosome. Sci. Rep. 2018, 8, 16094. https://doi.org/10.1038/s41598-018-34409-4.

88. Tesfaye, D.; Gebremedhn, S.; Salilew-Wondim, D.; Hailay, T.; Hoelker, M.; Grosse-Brinkhaus, C.; Schellander, K. MicroRNAs: Tiny molecules with a significant role in mammalian follicular and oocyte development. Reproduction. 2018, 155, R121–35. https://doi.org/10.1530/REP-17-0428.

89. Xie, F.; Xiao, P.; Chen, D.; Xu, L.; Zhang, B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol. 2012, 80, 75-84. https://doi.org/10.1007/s11103-012-9885-2.

90. Barnett, G.; Chiang, C.W.; Perez-Reyes, M.; Owens, S.M. Kinetic study of smoking marijuana. J. Pharmacokinet. Biopharm. 1982, 10, 495–506. https://doi.org/10.1007/BF01059033.

Figures and tables

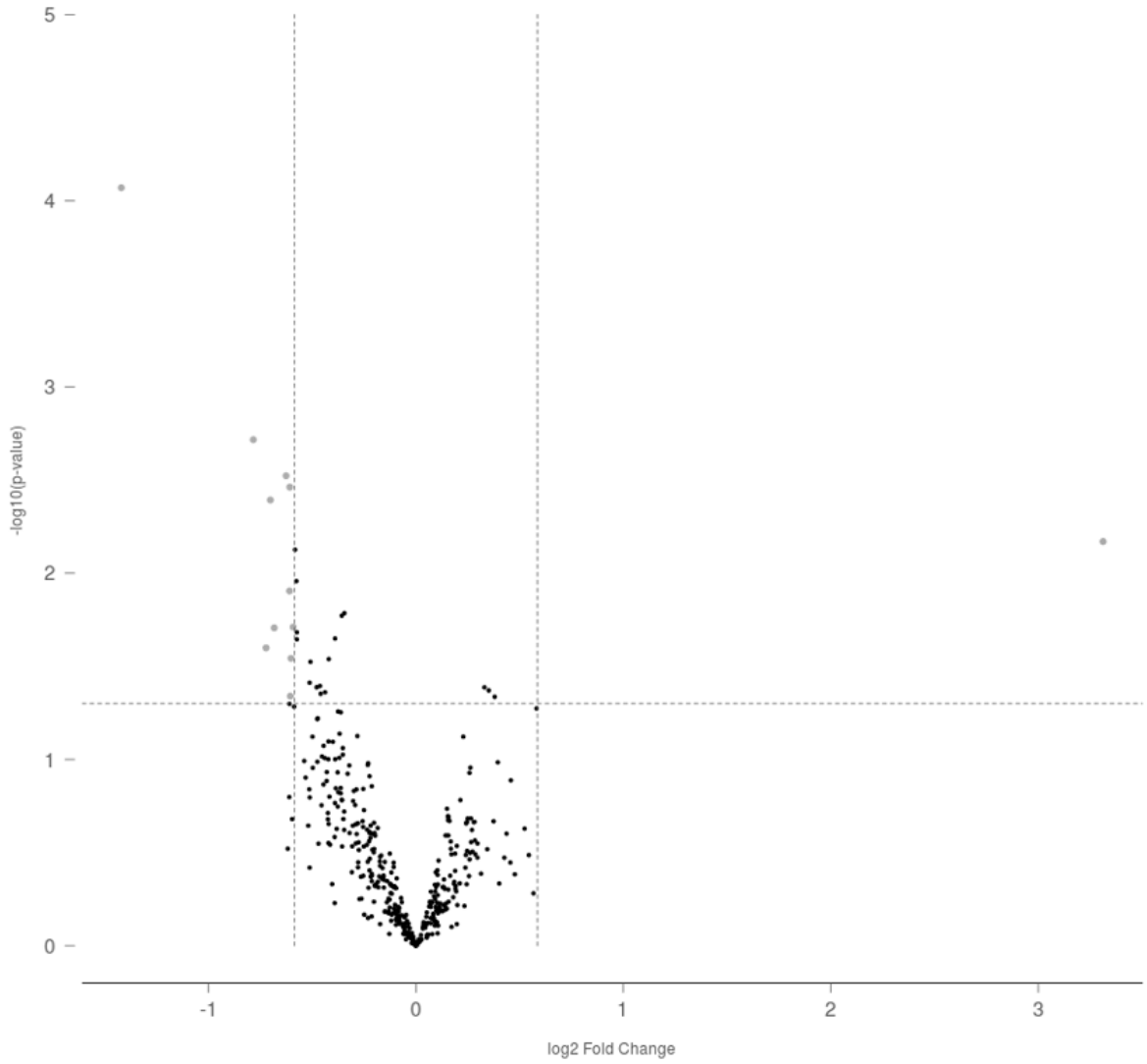

Figure 1. Volcano plot representation of differential miRNA expression as a result of gestational exposure to THC in adult rat ovaries. The dotted lines represent the fold change (≥1.5 or ≤-1.5) and *p*-value (<0.05) cutoff values selected to determine statistical difference.

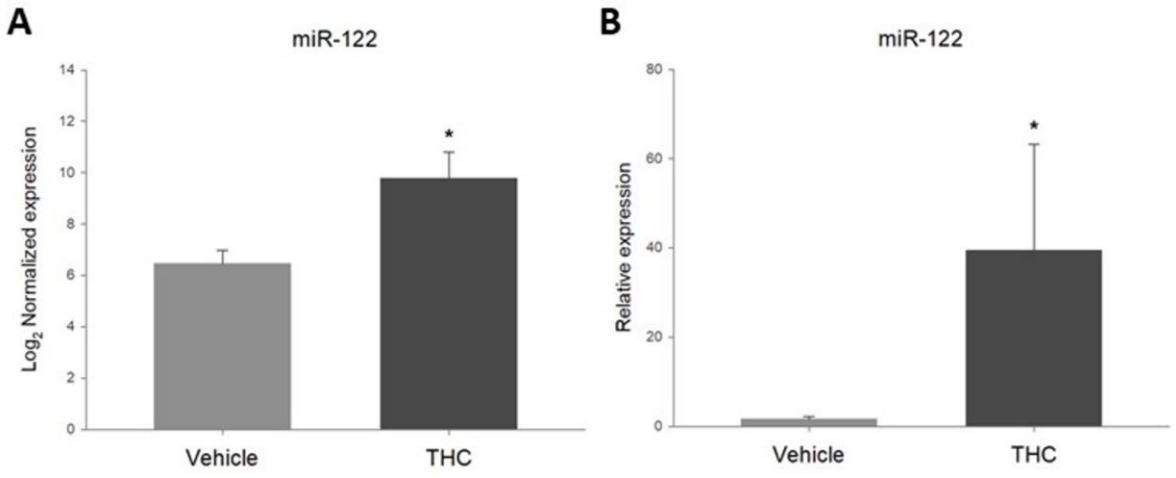

Figure 2. Differential expression of miR-122-5p (miR-122) in adult rat ovaries prenatally exposed to THC analyzed with (**A**): Rat v1.5 miRNA assay (Nanostring Technologies, Seattle, USA), nSolver® and ROSALIND® analysis software, and (**B**): miRCURY™ LNA miRNA PCR assay system (Qiagen, Hilden, Germany) and SigmaPlot® (mean + SEM; $N = 5$, * $p < 0.05$).

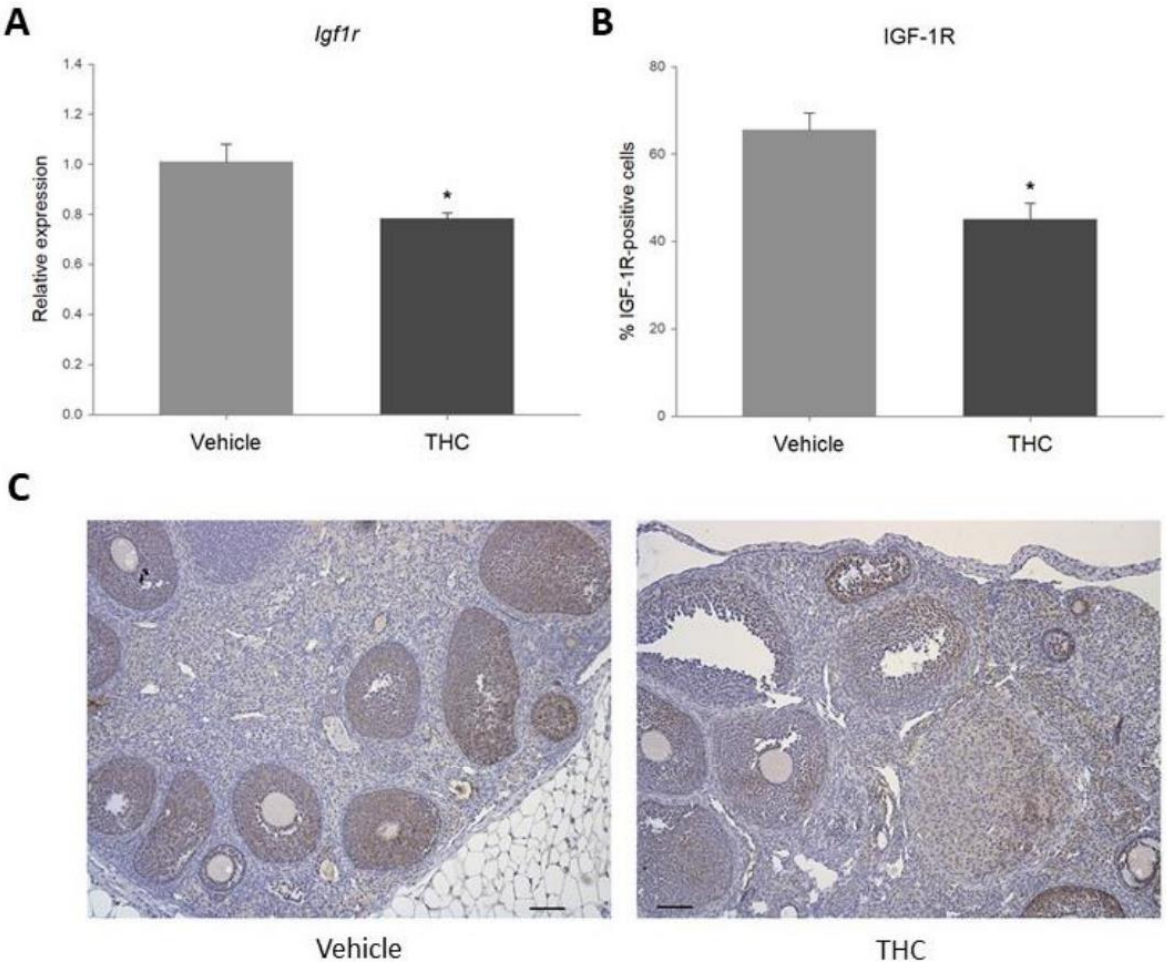

Figure 3. (**A**). Relative expression of *Igf1r* in prenatally THC-exposed adult rat ovaries. (**B**). Percentage of IGF-1R-positive ovarian follicular cells in prenatally THC-exposed adult rat ovaries. (**C**). IGF-1R protein levels in prenatally THC-exposed adult rat ovaries (mean + SEM; $N = 5$, * $p < 0.05$).

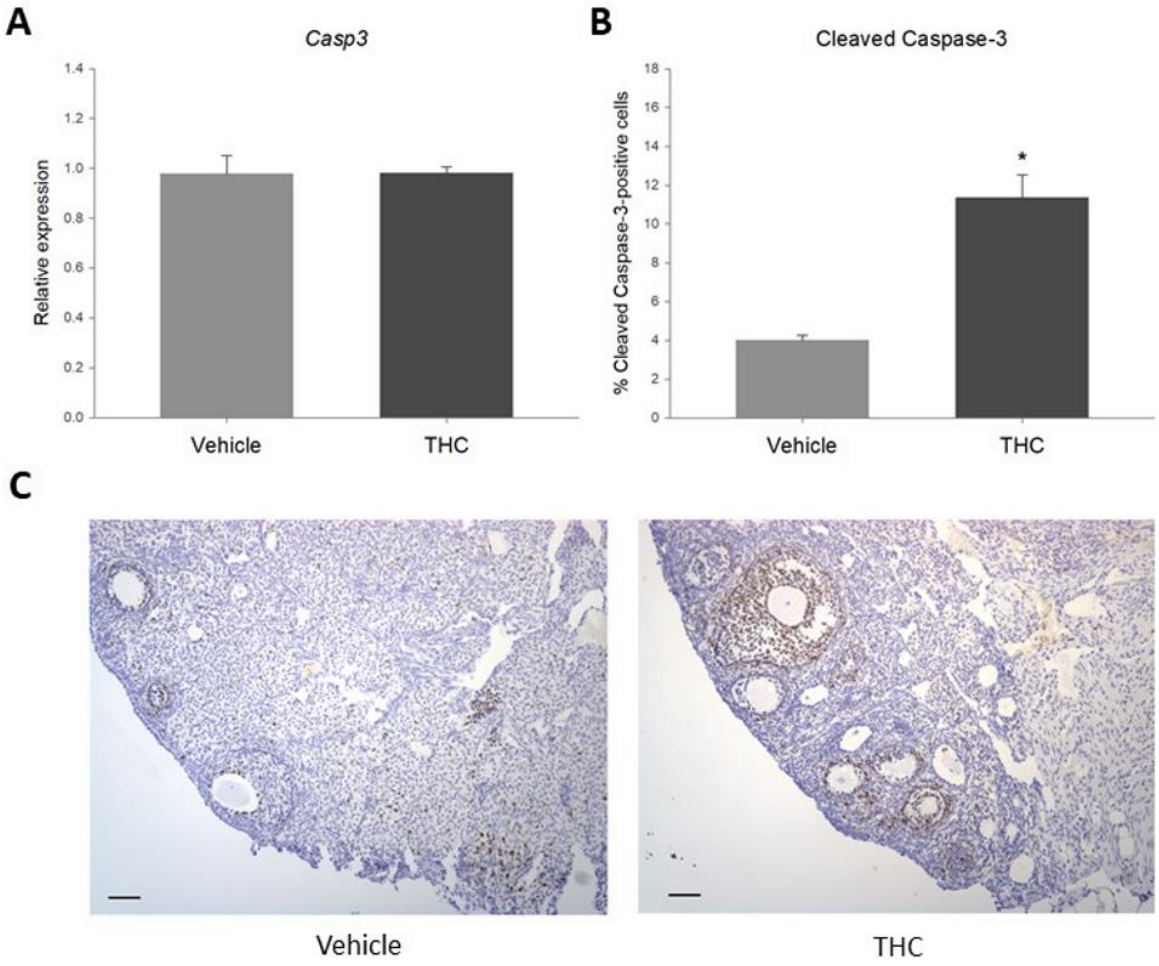

Figure 4. (**A**). Relative expression of *Casp3* in prenatally THC-exposed adult rat ovaries. (**B**). Percentage of cleaved caspase-3-positive ovarian follicular cells in prenatally THC-exposed adult rat ovaries. (**C**). Cleaved caspase-3 protein levels in prenatally THC-exposed adult rat ovaries (mean + SEM; $N = 5$, * $p < 0.05$).

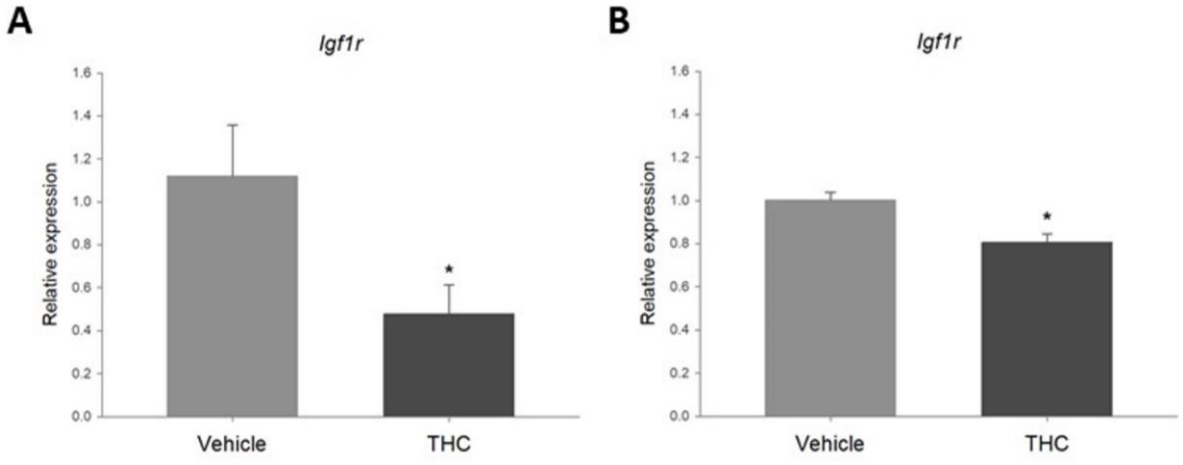

Figure 5. (**A**). Relative expression of *Igf1r* in rat ovary explants exposed to 3 μM THC for 48 h. (**B**). Relative expression of *Igf1r* in spontaneously immortalized rat granulosa cells exposed to 3 μM THC for 48 h (mean + SEM; $N = 5$, * $p < 0.05$).

Table 1. List of differentially expressed miRNA targets in the adult rat ovary as a result of prenatal THC exposure.

Accession Number	Target	Effect	Fold Change	*p* Value
MIMAT0000827	rno-miR-122-5p	Upregulated	9.93741	0.00676
MIMAT0000856	rno-miR-154-5p	Downregulated	−2.67658	8.50×10^{-5}
MIMAT0000885	rno-miR-214-3p	Downregulated	−1.72175	0.00192
MIMAT0017813	rno-miR-3552	Downregulated	−1.65014	0.02515
MIMAT0000787	rno-miR-18a-5p	Downregulated	−1.62661	0.00404
MIMAT0004742	rno-miR-296-3p	Downregulated	−1.60593	0.01964
MIMAT0005284	rno-miR-874-3p	Downregulated	−1.54335	0.00299
MIMAT0000882	rno-miR-211-5p	Downregulated	−1.52523	0.01243
MIMAT0003212	rno-miR-20b-3p	Downregulated	−1.52416	0.00344
MIMAT0005325	rno-miR-598-3p	Downregulated	−1.52143	0.04569
MIMAT0004641	rno-miR-330-5p	Downregulated	−1.51789	0.02862
MIMAT0005302	rno-miR-190b-5p	Downregulated	−1.50745	0.01945

Table 2. Primer sequences for miR-122-5p, RNU5G and miR-191-5p.

Accession Number	RNA	Sequence (5′-)
MIMAT0000421	hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG
NR_002852	RNU5G snRNA	AUACUCUGGUUUCUCUUCAGAUCGCAU-AAAUCUUUCGCCUUUUACUAAAGAU-UUCCGUGGA-GAGGAACAACUCUGAGUCUUAACCCAAUUUUU
MIMAT0000440	hsa-miR-191-5p	UGAGCCUUGCUCCGACAAGGCUA CAACGGAAUCCCAAAAGCAGCUG

Table 3. Primer sequences for *Igf1r*, *Casp3*, *B2m* and *Hprt*.

Accession Number	Gene Name	Symbol	Forward (5′-3′)	Reverse (5′-3′)
NM_052807.2	Insulin-like growth factor 1 receptor	*Igf1r*	GGAATGGGTCGTG-GACAGAT	ACAATCAGCAGGATGG-CAAC
NM_012922.2	Caspase 3	*Casp3*	GAGCTTGGAAC-GCGAAGAAAA	AGAGTCCATCGACTT-GCTTCC
NM_012512.2	Beta-2-microglobulin	*B2m*	AATTCACACCCACCGA-GACC	GCTCCTTCAGAGTGAC-GTGT
NM_012583.2	Hypoxanthine phosphori-bosyltransferase 1	*Hprt1*	GCAG-TACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAA-GCT

CHAPTER 4: DELTA-9-TETRAHYDROCANNABINOL INCREASES VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) SECRETION THROUGH A CYCLOOXYGENASE-DEPENDENT MECHANISM IN RAT GRANULOSA CELLS.

This chapter is a reprint of the original article with the same title, published in Reproductive Toxicology, 2022 August;111:59-67 (doi: 10.1016/j.reprotox.2022.05.004). Author list: Martínez-Peña AA, Petrik JJ, Hardy DB, and Holloway AC. Copyright and licensing can be found in Appendix A.

Cannabis use has been associated with altered gonadotropin levels, menstrual cycle dysregulation, ovulatory issues, fewer and poorer quality oocytes, as well as lower pregnancy rates by IVF in women. Animal studies demonstrated that THC alone can disrupt the HPG axis and decrease ovulation. In addition, THC has been shown to have a direct impact on the ovaries, interfering with gonadotropin response and steroid hormone production. Furthermore, in vitro experiments have suggested that THC has anti-angiogenic effects on several different cell types. Taking into account that prenatal THC-exposure had a significant impact on the expression of VEGF in ovarian granulosa cells (Chapter 2), and that the in vitro model used in chapter 3 showed similar results to those observed after in utero exposure, we hypothesized that direct exposure to THC would affect VEGF production in granulosa cells. The second and third objectives of this chapter were to determine the functional consequences and mechanistic pathways associated with these alterations.

Abstract

While the effects of delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, have been studied extensively in the central nervous system, there is limited knowledge about its effects on the female reproductive system. The aim of this study was to assess the effect of THC on the expression and secretion of the angiogenic factor vascular endothelial growth factor (VEGF) in the ovary, and to determine if these effects were mediated by prostaglandins. Spontaneously immortalized rat granulosa cells (SIGCs) were exposed to THC for 24 h. Gene expression, proliferation and TNFα-induced apoptosis were evaluated in the cells and concentrations of VEGF and prostaglandin E2 (PGE_2), a known regulator of VEGF production, were determined in the media. To evaluate the role of the prostanoid pathway, cells were pre-treated with cyclooxygenase (COX) inhibitors prior to THC exposure. THC-exposed SIGCs had a significant increase in VEGF and PGE_2 secretion, along with an increase in proliferation and cell survival when challenged with an apoptosis-inducing factor. Pre-treatment with COX inhibitors reversed the THC-induced increase in both PGE_2 and VEGF secretion. Alterations in granulosa cell function, such as the ones observed after THC exposure, may impact essential ovarian processes including folliculogenesis and ovulation, which could in turn affect female reproductive health and fertility. With the ongoing increase in cannabis use and potency, further study on the impact of cannabis and its constituents on female reproductive health is required.

Introduction

Cannabis, which is the generic term that denotes several preparations of the plant *Cannabis sativa*, is the most widely used illicit drug in the world [96]. The use of cannabis has grown considerably during the last decades, particularly among developed countries, in which there is a tendency towards the legalization of medicinal and recreational cannabis use [96]. Recent estimates suggest that 15–27% of Canadians over the age of 15 use cannabis [84,85].

In addition to the growing prevalence of cannabis use, the concentration of the psychoactive phytocannabinoid delta-9- tetrahydrocannabinol (THC) [67] in cannabis has increased considerably in the last few decades [11,25,26,68,96]. According to Health Canada, the percentage of THC in dried cannabis has increased from around 3% in the 1980s to an average of 15% in 2018, with some strains containing as much as 30% [39]. Over the past decade, the perception of cannabis as a harmful drug has decreased, particularly amongst adolescents [96]. Moreover, 70% of pregnant and non-pregnant women in the US believe there is little to no harm using cannabis once or twice per week [17,52,87], despite the advice of obstetricians and gynaecologists [93] and considering that there are limited studies on the effect of cannabis on female reproductive health [18]. While some studies have found no significant associations between cannabis consumption and female reproductive health and fertility [10,49,98], others have associated its use with altered luteinizing hormone levels [69], menstrual cycle dysregulation and ovulatory issues [48,72], fewer and poorer quality oocytes, as well as lower pregnancy rates by in vitro

fertilization (IVF) when compared to non-cannabis users [50], pointing out the need for more studies in this area. Since clinical studies are limited by several factors such as ethical considerations and difficulty adjusting for confounding variables related to socio-demographics, sample size, poly-substance use, cannabis potency, frequency and duration of use, which may affect the observable data, the use of animal and in vitro models represent a useful strategy to address the effects of THC on reproductive health.

A previous study from our research group revealed that prenatal exposure to THC resulted in altered follicle dynamics and inhibited ovarian vascularization in the adult rat offspring [61]. Reduced blood vessel density in these ovaries was associated with a decreased expression of the angiogenic factor vascular endothelial growth factor (VEGF) and an increased expression of the anti-angiogenic factor thrombospondin 1 (TSP-1) in granulosa cells. In the ovary, angiogenesis is involved in several processes including folliculogenesis, antrum formation, follicular rupture, ovulation, and corpus luteum formation [1,89, 91]. VEGF is well established as a major regulator of ovarian angiogenesis, since blocking its action within the ovary disrupts follicle rupture, oocyte release, and subsequent luteal function [44,99]. Additionally, as follicles mature, VEGF expression in granulosa and theca cells increases, and VEGF protein in follicular fluid rises [100,40,41]. VEGF expression is regulated by a number of factors including cyclooxygenase (COX) derived prostanoids (i.e., prostaglandins). This is of great interest considering prostaglandins have a vital role in angiogenesis during implantation and decidualization by regulating VEGF

expression [63]. In addition, it has been suggested that the ability of the ovulatory gonadotropin surge to stimulate angiogenesis is mediated by prostaglandin E2 (PGE_2) [94]. Moreover, several in vitro and in vivo studies have reported changes in prostaglandin synthesis as a result of THC exposure [13,15,7]. Given that prenatal exposure to THC resulted in altered expression of VEGF in granulosa cells, and that THC has been shown to affect prostaglandins, which may in turn influence VEGF expression, our goal was to assess the direct effect of THC on VEGF production in granulosa cells and to determine if these changes were prostaglandin-mediated.

Materials and methods

Cell culture

Spontaneously immortalized rat granulosa cells (SIGCs) were cultured in DMEM/F12 media with L-glutamine (Corning Inc., New York, USA) supplemented with 10% fetal bovine serum (FBS) and 2% Penicillin/Streptomycin. For initial treatments, cells were cultured with vehicle or 15 μM THC for 6 and 24 h. This concentration was based on a pharmacokinetic study which reported similar levels in the serum of cannabis users [5]. For experiments with COX-1 and COX-2 inhibitors, cells were pretreated for one hour with either 1 μM SC-560 (a COX-1 inhibitor; Cayman Chemical Company, Michigan, USA) in dimethyl sulfoxide (DMSO), or 5 μM SC-236 (a COX-2 inhibitor; Sigma-Aldrich, Missouri, USA) in ethanol, and then exposed to 15 μM THC for 24 h. The final concentrations of vehicle in the media were 0.1% DMSO in the COX-1 inhibitor experiment and 0.086%

ethanol in the COX-2 inhibitor experiment. For all in vitro assessments, the results of 5 individual experiments are presented.

RNA isolation and quantitative real-time PCR

Treated cells were harvested with TRIzol® Reagent (Thermo Fisher Scientific, Massachusetts, USA) and total RNA was extracted by precipitation with isopropanol and subsequent ethanol washes. RNA concentration and purity were assessed using a NanoDrop One Micro-UV/ Vis Spectrophotometer (Thermo Fisher Scientific) and cDNA was synthesized from 4 µg of total RNA using a High capacity cDNA Reverse transcription kit (Thermo Fisher Scientific). Gene expression was evaluated by RT-qPCR using PerfeCTa SYBR® Green FastMix (Quantabio, Massachusetts, USA) and the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, California, USA). Given that our previous study revealed changes in VEGF protein in granulosa cells as a result of THC exposure, the main target gene in the present study was vascular endothelial growth factor A (*Vegfa*). In order to assess different pathways responsible for the regulation of *Vegfa* expression, the anti-angiogenic factor thrombospondin 1 (*Thbs1*) was evaluated in addition to assessing regulators of prostaglandin biosynthesis. Prostaglandin-endoperoxide synthase 1 and 2 (*Ptgs1* and *Ptgs2*, respectively) represent the rate-limiting step enzymes in prostaglandin synthesis, which has also been shown to affect *Vegf* expression [81]. RT-qPCR results were analyzed with the $2^{-\Delta\Delta Ct}$ method [54] using beta-2-microglobulin (*B2m*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*)

as internal references. The forward and reverse primer sequences can be found in Table 1.

Protein isolation and western blotting

SIGCs were cultured as described above with either vehicle or 15 μM THC for 24 h. Protein was extracted using lysis buffer containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 250 mM sucrose, 1 mM DTT and 1 mM sodium orthovanadate, with 1% Triton X-100 and one tablet of cOmplete™ Protease Inhibitor Cocktail (Roche, Basil, Switzerland) per 50 mL. Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were then normalized and denatured at 95 ◦C for 5 min. 40 μg of total protein from each sample were subjected to SDS-PAGE using a 10% separating gel and then transferred to a PVDF membrane. Membranes were blocked with 5% BSA for one hour before incubating with either rabbit polyclonal anti-VEGFA (1:1000 dilution; Abcam, Cambridge, UK), mouse monoclonal anti-thrombospondin 1 (1:500 dilution; Santa Cruz Biotechnology Inc., Texas, USA) or rabbit polyclonal anti-alpha tubulin (1:1000 dilution; Abcam) overnight in a cold room. After three washes with TBS-T, membranes were incubated with either HRP-conjugated goat anti-rabbit (1:10000 dilution; Abcam) or goat anti-mouse (1:10000 dilution; Abcam) antibodies for one hour. Membranes were washed as described above and protein was detected by chemiluminescence using a ChemiDoc Imaging System (Bio-Rad Laboratories). Densitometric analysis was performed using ImageJ® and VEGF and TSP-1 bands were normalized to α-

tubulin, after confirming THC exposure had no effect on the expression of this protein.

Extracellular VEGF and PGE_2 quantification

Media from THC-exposed SIGCs was collected and extracellular concentrations of VEGF and PGE_2 were determined by enzyme-linked immunosorbent assays (Rat VEGF ELISA kit, Abcam; PGE_2 ELISA kit, Abcam; PGE_2 ELISA Kit, Enzo Life Sciences Inc., New York, USA) according to the manufacturer's instructions.

Detection of apoptosis and proliferation

SIGCs were seeded in a 96-well plate and cultured as described above. At approximately 70% confluency, the media was replaced with serum-free DMEM/F12 with 2% Penicillin/Streptomycin for 24 h. Cells were then cultured with 15 μM THC, 100 ng/mL TNFα (Sigma-Aldrich) or both in serum-free media for another 24 h. In order to detect apoptotic cells, the CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific) was added to each well. Cells were then incubated at 37 ◦C for 30 min and fluorescence intensity was determined with a Synergy H1 microplate reader (Agilent, California, USA).

In order to determine the effects of THC exposure on cell proliferation, SIGCs were cultured on sterile glass coverslips with vehicle or 15 μM THC for 24 h. Cells were fixed with neutral buffered formalin 10% for 1 h at room temperature and stored at 4◦C. Cells were permeabilized using 0.2% Triton X-100, washed with PBS and blocked with 5% BSA at room temperature. Proliferation was detected using anti-

phosphorylated histone H3 antibody (1:400; Abcam) diluted in 5% BSA overnight at 4˚C. Coverslips were then incubated with secondary anti-rabbit antibody (1:100; Sigma-Aldrich) for 1 h at room temperature. Cells were counterstained with DAPI and imaged using an Eclipse E600 research microscope (Nikon Corporation, Tokyo, Japan). The percentage of proliferating cells was calculated as the number of phospho-histone H3 (PHH3)-positive cells compared to the total number of DAPI-positive cells. Immunopositive cell counts were conducted manually by the same individual, who was blinded to the treatment group until all proliferation data had been collected. Cell counts were conducted using integrated morphometry software (MetaMorph Inc., California, USA) and the average of 5 fields of view/coverslip was used to calculate the percentage of immunopositive cells.

Statistical analysis

After checking for normal distribution and equal variance, a one-way ANOVA was used to determine differences between the means of multiple experimental groups. In case a difference between means was detected ($p < 0.05$), a post-hoc Tukey test was performed. A student T test was performed to determine statistical differences between the means of two experimental groups.

Results

THC increases VEGF expression and secretion in granulosa cells

While there were no significant changes in the steady-state mRNA expression of *Vegfa* after 6 h of exposure to THC, there was a significant increase after 24 h (Fig.

1A). Similarly, at 24 h, THC exposure significantly increased VEGF secretion (Fig. 1C) and while the intracellular VEGF protein levels were increased, this did not reach statistical significance ($p = 0.07$) (Fig. 1B).

THC protects granulosa cells from TNFα-induced apoptosis and increases proliferation

It has previously been shown that VEGF has cytoprotective effects not only on endothelial cells, but on granulosa cells as well. In an experiment in which spontaneously immortalized rat granulosa cells were serum-deprived and treated with the apoptosis-inducing factor TNFα, the addition of exogenous VEGF reduced apoptosis and the presence of activated caspase-3 [40]. Since exposure to THC increased VEGF in the SIGCs, we hypothesized that this exposure would have similar cytoprotective effects on these cells. As seen in Fig. 2, the addition of TNFα to serum-deprived SIGCs increased the activated caspase-3/7 signal compared to SIGCs exposed to THC. However, when serum-deprived SIGCs were cultured in the presence of both THC (15 μM) and TNFα (100 ng/mL), the TNFα-induced increase in activated caspase-3/7 was blocked, suggesting that THC has a cytoprotective effect on these cells.

In addition, VEGF has been shown to induce proliferation in endothelial and granulosa cells [22,46,6]. As seen in Fig. 3, exposure to THC increased the percentage of proliferating SIGCs, as determined by the expression of the proliferation marker PHH3.

THC-induced increase in VEGF production is not TSP-1 mediated

One of the mechanisms through which granulosa cells regulate VEGF levels is the production of the anti-angiogenic factor thrombospondin 1 (TSP-1). In fact, TSP-1 has been shown to reduce VEGF expression, inhibit ovarian angiogenesis and induce follicle atresia [36,37]. Since TSP-1 directly binds to VEGF, resulting in its internalization and degradation through the low-density lipoprotein receptor related protein (LRP-1) [42], we hypothesized that the THC-induced increase in VEGF could be related to a decrease in TSP-1. However, there were no significant changes in the mRNA or protein levels of TSP-1 in the THC-exposed SIGCs (Fig. 4), suggesting the increase in VEGF is independent from TSP-1 regulation.

THC increases PGE_2 secretion in granulosa cells

Prostaglandin E2 (PGE_2) has been suggested to play an important role in gonadotropin-induced angiogenesis in the ovary [94], and it has been clearly demonstrated that PGE_2 can induce VEGF expression in several in vitro models such as ovarian cancer cells [35], luteal endothelial cells [81] and luteinized granulosa cells [23]. In addition, THC has been shown to increase PGE_2 secretion in some in vitro models [12, 78]. In order to assess the effect of THC on this signaling pathway, the concentration of PGE_2 was determined in media from THC-exposed SIGCs and the mRNA expression of *Ptgs1* and *Ptgs2*, the rate-limiting enzymes involved in PGE_2 synthesis, was determined.

As depicted in Fig. 5, exposure to THC for 24 h did not significantly alter the steady-state mRNA levels of either *Ptgs1* or *Ptgs2* (Figs. 5B,5C). However, THC exposure resulted in a significant increase in PGE_2 secretion (Fig. 5A) from granulosa cells. The levels of PGE_2 in the media were significantly correlated with VEGF secretion in the same cells ($r = 0.85$; $N = 5$, $p = 0.0019$).

Prostaglandins mediate THC-induced increase in VEGF secretion

To determine if the increase in VEGF secretion was causally related to increased PGE_2 production, SIGCs were pretreated with either a specific COX-1 or COX-2 inhibitor (SC-560 or SC-236, respectively) prior to exposure to THC. Since these enzymes represent the rate limiting step in the prostaglandin synthesis pathway, blocking their activity results in a general decrease in prostaglandin synthesis. As seen in Fig. 6C, pre-treatment with both COX inhibitors blocked the THC-induced increase in PGE_2 secretion.

While neither of the COX inhibitors on their own altered *Vegf* expression or blocked the THC-induced increase in *Vegfa* gene expression (Fig. 6A), concurrent treatment with either COX-1 or COX-2 inhibitor prior to THC exposure, did block the THC-mediated increase in VEGF secretion (Fig. 6B). Collectively, these results suggest that the THC-induced increase in VEGF secretion is at least partially mediated by prostaglandins, and that this regulation is not at the gene expression level.

Discussion

As the use and potency of cannabis and cannabis derived products increase, it is important to understand the effects of this drug and its components on human health. While many studies have looked at the effects of cannabis on the nervous system, its effects on other peripheral cannabinoid-targets, such as the female reproductive system, have received less attention, despite concerns that exposure to cannabis may adversely affect reproductive health [32,66]. Results from this study indicate that THC exposure had profound effects to stimulate mRNA expression and secretion of VEGF from granulosa cells. While some studies have observed cannabinoid-induced anti-angiogenic effects, most of these have assessed other cannabinoids, such as the non-psychoactive cannabidiol (CBD) or the synthetic cannabinoids JWH-133 and WIN-55,212–2 [9,83], which have different affinity and potency at the CB1 and CB2 cannabinoid receptors, relative to THC [2]. Studies with in vitro exposure models more similar to the present one in terms of THC concentration and exposure time, have obtained results that agree with our observations. For example, a study in which human trophoblast cells (BeWo) were exposed to 15 μM THC for 24 h, reported an increase in VEGF expression [55]. Similarly, a recent study with human colorectal cancer cells (HCT116) also reported an increase in VEGF secretion after exposure to THC [58].

While an increase in the secretion of angiogenic factors is a necessary part of follicle development, angiogenesis, ovulation and luteolysis, all of these processes are tightly regulated during the ovarian cycle [1]. VEGF expression, for example,

increases in granulosa cells as the follicle develops, and is thought to play an important role in follicle recruitment into the ovarian cycle and selection of the dominant follicle, which will in turn ovulate [104,22,64]. Therefore, dysregulation of the synthesis and secretion of angiogenic factors, such as the THC-induced increase in VEGF we observed, could result in alterations in ovarian function. Indeed, it has been shown that exogenous VEGF increases the number of small, preantral follicles formed and accelerates follicle growth in rats [19]. TSP-1 null mice are subfertile and have altered ovarian morphology associated with increased vascularization and disrupted follicle dynamics [42]. Since secretion of VEGF from granulosa cells increases significantly in response to the LH surge, prior to ovulation, an increase in this factor as a result of external stimuli may impact this process [82,86]. Interestingly, cannabis use has been associated with menstrual cycle and ovulatory disorders [48,72], and animal studies suggest that THC may exert a direct inhibitory effect on folliculogenesis and ovulation [24].

In addition to the possible disturbances of the ovarian cycle, dysregulation of VEGF has been linked to reproductive disorders such as ovarian hyperstimulation syndrome (OHSS) [28]. This disorder is associated with multiple follicle development and is more common in patients undergoing IVF, as it is enhanced by the surrogate LH surge [47]. In fact, elevated concentrations of VEGF in follicular fluid have been related to decreased conception rates in assisted reproductive technologies [33,60]. Similarly, cannabis use has also been associated with lower pregnancy rates by IVF, as well as less and poorer quality oocytes [50]. Moreover, increased VEGF levels

have been linked to other disorders such as endometriosis [30,34] and polycystic ovary syndrome (PCOS) [77].

Secreted VEGF can bind to its receptors on endothelial cells and induce several processes such as differentiation, survival, migration and proliferation [102]. However, VEGF can also act in an auto and paracrine way, affecting the granulosa cells that produce it in a comparable manner. Since THC exposure resulted in an increase in secreted VEGF from SIGCs, we assessed the effect of this compound on proliferation and apoptosis in these cells. THC exposure increased the percentage of proliferating SIGCs, assessed by the expression of the proliferation marker PHH3, and conferred a cytoprotective effect on the cells when challenged with the pro-apoptotic factor TNFα. These results are consistent with the literature, since VEGF has been shown to increase proliferation and reduce activated caspase-3 and apoptosis in early antral follicles and rat granulosa cells [40,46].

Given that granulosa cell proliferation supports the progression of follicle growth and maturation after recruitment, and that apoptosis is one of the mechanisms underlying follicular atresia, through which the dominant follicle is selected, these events must be tightly regulated in order to maintain the proper balance between the cyclical growth and regression of follicles [62]. As with VEGF, the THC-induced increase in proliferation and decrease in apoptosis observed in our study could therefore interfere with folliculogenesis and ovulation, as it has previously been suggested in clinical and animal studies with cannabis and THC, respectively [48,72,24]. Additionally, increased proliferation and decreased apoptosis may be involved in

ovarian pathologies such as PCOS and cancer [20,53]. Das and co-workers observed that there were significantly more proliferating and significantly less apoptotic granulosa cells in patients with PCOS. The authors demonstrated that granulosa cells from anovulatory PCOS follicles had lower activated caspase-3 levels compared to granulosa cells from healthy ovulatory follicles [20]. Increased proliferation and decreased apoptosis are also often associated with cancer [53]. In accordance, VEGF and its receptors have been shown to be overexpressed in granulosa cell tumours (GCT) [29], and the use of an anti-VEGF antibody was proven to slow tumour development by inhibiting proliferation in a GCT rodent model [95]. To our knowledge, there are no studies that address the effect of THC on the etiology of these conditions. However, it is possible that the THC-mediated increase in VEGF, along with the increase in proliferation and the decrease in apoptosis of granulosa cells, may contribute to the progression of these pathologies in cannabis users.

The influence of THC on proliferation and apoptosis has been widely studied in several different models, obtaining contrasting results. While some report an increase in proliferation and a decrease in apoptosis [21, 43,90] similar to our observations, others report the opposite effects [101,103,65]. These contradictory observations may be a result of the differential expression of cannabinoid receptors [3]. For example, in a study in which a tumour grade-dependent expression of CB1 was observed in human ovarian tumours, the authors suggested that cannabinoids have opposing effects on non-cancerous cells (such as SIGCs), in which they may

activate proliferative pathways, versus cancerous cells, in which they promote anti-proliferative and apoptotic events [70]. Indeed, several studies that report anti-angiogenic, anti-proliferative and pro-apoptotic effects of THC do so in cancer cell lines or tissues such as brain and immune cells, which are typically rich in CB1 and CB2 [8,71]. In addition, granulosa cells have been shown to express not only the canonical cannabinoid receptors CB1 and CB2, but also the non-canonical receptors GPR55 and TRPV1, which are associated with different signaling pathways and downstream cascades than CB1 and CB2 [27,57,97].

To elucidate the mechanism behind the THC-induced increase in VEGF expression and secretion in the granulosa cells, two pathways were evaluated. Since the anti-angiogenic factor thrombospondin 1 (TSP-1) has been shown to be a key regulator of VEGF in the ovary [42], we suspected that reductions in the expression of this protein might be responsible for the increase in VEGF after THC exposure. However, no significant changes were observed in mRNA or protein levels of TSP-1 in the THC-exposed SIGCs, suggesting that the increase in VEGF is not related to altered expression of TSP-1. Interestingly, although we previously demonstrated altered follicle dynamics in adult rat ovaries as a result of prenatal exposure to THC, we also observed a decrease in the percentage of granulosa cells expressing VEGF and an increase in the percentage of granulosa cells expressing TSP-1 [61]. This suggests that THC affects different regulatory mechanisms, depending on the direct or indirect interaction with the tissue, as well as the time and window of exposure.

Prostaglandins have also been proposed as angiogenic regulators in the ovary, and several in vitro models have demonstrated the direct influence of PGE_2 on VEGF expression [56,81,94]. To determine if THC influenced the prostanoid synthesis pathway, the expression of the prostaglandin biosynthetic enzymes PTGS1 (COX-1) and PTGS2 (COX-2) was determined, and PGE_2 concentrations were measured. While there were no significant changes in the expression of either *Ptgs1* or *Ptgs2*, THC exposure resulted in a significant increase in PGE_2 secretion after 24 h. The THC-induced increase in PGE_2 secretion by granulosa cells may affect several ovarian processes. As previously mentioned, since secretion of both VEGF and PGE_2 increases significantly from granulosa cells as a response to the LH surge prior to ovulation [86], an increase in these factors due to external stimuli may result in alterations in this process [75]. Excessive PGE_2 synthesis may also cause inflammatory damage in the ovary [76]. In addition, PGE_2 has been linked to PCOS, since granulosa cells from patients with this condition secrete greater levels of PGE_2 than healthy controls [74]. On the other hand, epithelial ovarian cancers overexpress biosynthetic prostaglandin enzymes and prostaglandin receptors [79], and it has been shown that exposure of epithelial ovarian cancer cells to PGE_2 stimulates proliferation and reduces apoptosis in vitro [73]. Interestingly, Takeda and co-workers reported that the THC-induced proliferation of human breast cancer cells (MCF-7) was diminished by PTGS inhibition and enhanced by the addition of arachidonic acid, the precursor of prostaglandins and a product of endocannabinoid metabolism [90].

The effect of THC on the prostaglandin signaling pathway appears to be largely tissue dependent. Several studies have reported a THC-induced increase in PGE_2 in in vivo models, such as the rodent brain [16,45,7], and in vitro models, such as human lung fibroblast cells [12], trabecular meshwork cells [78] and nonpigmented ciliary epithelium cells [80]. Other studies, however, have reported a THC-induced decrease in PGE_2 [4,15]. In fact, it is not uncommon for cannabis to be used to relieve symptoms associated with inflammation [59].

While in the present study we observed an increase in PGE_2 output in the absence of any changes in the expression of *Ptgs1* or *Ptgs2*, it is possible that there were alterations in other enzymes involved in prostaglandin synthesis or degradation, and/or changes in their substrate levels. Indeed, Burstein and colleagues suggested that the induction of PGE_2 secretion by THC was mediated through the activation of phospholipase A2 (PLA2), which hydrolases membrane phospholipids into arachidonic acid [14]. Furthermore, in a study with bovine endothelial cells, inhibition of PLA2 diminished the glucose-induced elevation of PGE_2 and VEGF, as well as VEGF-induced proliferation [38].

In order to determine if the increase in VEGF was a consequence of increased PGE_2 output, SIGCs were treated with specific COX-1 and COX-2 inhibitors in combination with THC exposure. While neither of the COX inhibitors affected *Vegf* gene expression on their own, nor did they block the THC-induced increase in *Vegf* expression, both inhibitors were able to block the THC-mediated increase in VEGF secretion. These results suggest that the increase in VEGF secretion as a response

to THC exposure is prostaglandin-mediated, and that this regulation is not at the gene expression level. Similar to our observations, a study with human airway smooth muscle cells (HASM) reported a concentration-dependent increase in VEGF secretion as a result of exposure to the pro-inflammatory mediator bradykinin (BK). A non-specific COX inhibitor (indomethacin) and a COX-2 specific inhibitor (NS-398) reduced PGE_2 synthesis and blocked the increase in VEGF secretion. In accordance, treatment of HASM with PGE_2 also resulted in a concentration-dependent increase in VEGF secretion, and the addition of arachidonic acid increased both VEGF and PGE_2 production, an effect that was blocked by indomethacin. Since BK treatment increased VEGF secretion without changing VEGF mRNA levels, the authors suggested a post-transcriptional regulatory mechanism [51]. In the present study, treatment of granulosa cells with COX inhibitors in combination with THC resulted in an increase in *Vegf* gene expression, but not in an increase in VEGF secretion, while treatment with COX inhibitors on their own significantly decreased VEGF secretion without changing *Vegf* gene expression. Therefore, these results also suggest a COX mediated post-transcriptional regulatory mechanism of VEGF secretion. In addition, although it is clear that both COX inhibitors reversed the THC-mediated increase in VEGF secretion compared to the vehicle group, it is plausible that this effect was counteracted rather than abolished, given that treatment with inhibitors alone significantly decreased VEGF secretion. However, more experiments are needed in order to fully elucidate the underlying regulatory mechanisms.

Conclusions

While there are several limitations to a short-term in vitro study such as the present one, exposure of ovarian granulosa cells to delta-9- tetrahydrocannabinol resulted in significant changes in the expression and secretion of important regulating factors, as well as alterations in cell survival and proliferation. THC-induced increase in VEGF and PGE_2 secretion, as well as the increased proliferation and decreased apoptosis, may have an impact on essential ovarian processes such as folliculogenesis and ovulation, which may in turn affect female fertility. In addition, considering increased levels of VEGF and PGE_2 have been associated with gynecological disorders such as polycystic ovary syndrome, endometriosis, ovarian hyperstimulation syndrome and ovarian cancer, it is possible that chronic THC exposure may play a role in these conditions. Given the increasing use of cannabis by reproductive age women and the uncertainties regarding its impact on reproductive health, more studies are urgently required.

Declaration of Competing Interest

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

Acknowledgements

This study was supported by a CIHR (Canadian Institutes of Health Research (Canada)) Project Grant to ACH (PJT-155981) and a HSFC (Heart and Stroke Foundation of Canada (Canada)) Grant-in-Aid to DBH (G-19-0026343). AAMP was sponsored by the Mexican National Council of Science and Technology, MEXICO (CONACYT).

References

[1] O. Abulafia, D.M. Sherer, Angiogenesis of the ovary, Am. J. Obstet. Gynecol. 182 (1 Pt 1) (2000) 240–246, https://doi.org/10.1016/s0002-9378(00)70519-9.

[2] D. An, S. Peigneur, L.A. Hendrickx, J. Tytgat, Targeting cannabinoid receptors: current status and prospects of natural products, Int. J. Mol. Sci. 21 (14) (2020) 5064, https://doi.org/10.3390/ijms21145064.

[3] T. Ayakannu, A.H. Taylor, T.H. Marczylo, J.M. Willets, J.C. Konje, The endocannabinoid system and sex steroid hormone-dependent cancers, Int. J. Endocrinol. 2013 (2013), 259676, https://doi.org/10.1155/2013/259676.

[4] D. Ayalon, I. Nir, T. Cordova, S. Bauminger, M. Puder, Z. Naor, R. Kashi, U. Zor, A. Harell, H.R. Lindner, Acute effect of delta1-tetrahydrocannabinol on the hypothalamo-pituitary-ovarian axis in the rat, Neuroendocrinology 23 (1) (1977) 31–42, https://doi.org/10.1159/000122652.

[5] G. Barnett, C.W. Chiang, M. Perez-Reyes, S.M. Owens, Kinetic study of smoking marijuana, J Pharmacokinet Biopharm. 10 (5) (1982) 495–506, https://doi.org/10.1007/BF01059033.

[6] P.N. Bernatchez, S. Soker, M.G. Sirois, Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent, J. Biol. Chem. 274 (43) (1999) 31047–31054, https://doi.org/10.1074/jbc.274.43.31047.

[7] S.K. Bhattacharya, delta-9-tetrahydrocannabinol (THC) increases brain prostaglandins in the rat, Psychopharmacology 90 (4) (1986) 499–502, https://doi.org/10.1007/BF00174068.

[8] M. Bifulco, V. Di Marzo, Targeting the endocannabinoid system in cancer therapy: a call for further research, Nat. Med. 8 (6) (2002) 547–550, https://doi.org/10.1038/nm0602-547.

[9] C. Blazquez, L. Gonzalez-Feria, L. Alvarez, A. Haro, M.L. Casanova, M. Guzman, Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas, Cancer Res. 64 (16) (2004) 5617–5623, https://doi.org/10.1158/0008-5472. CAN-03-3927.

[10] R.I. Block, R. Farinpour, J.A. Schlechte, Effects of chronic marijuana use on testosterone, luteinizing hormone, follicle stimulating hormone, prolactin and cortisol in men and women, Drug Alcohol Depend 28 (2) (1991) 121–128, https://doi.org/10.1016/0376-8716(91)90068-a.

[11] J.R. Burgdorf, B. Kilmer, R.L. Pacula, Heterogeneity in the composition of marijuana seized in California, Drug Alcohol Depend 117 (1) (2011) 59–61, https://doi.org/10.1016/j.drugalcdep.2010.11.031.

[12] S. Burstein, S.A. Hunter, C. Sedor, S. Shulman, Prostaglandins and cannabis–IX. Stimulation of prostaglandin E2 synthesis in human lung fibroblasts by delta 1-tetrahydrocannabinol, Biochem. Pharmacol. 31 (14) (1982) 2361–2365, https://doi.org/10.1016/0006-2952(82)90530-5.

[13] S. Burstein, S.A. Hunter, K. Ozman, Prostaglandins and cannabis. XII. The effect of cannabinoid structure on the synthesis of prostaglandins by human lung fibroblasts, Mol. Pharmacol. 23 (1) (1983) 121–126.

[14] S. Burstein, J. Budrow, M. Debatis, S.A. Hunter, A. Subramanian, Phospholipase participation in cannabinoid-induced release of free arachidonic acid, Biochem. Pharmacol. 48 (6) (1994) 1253–1264, https://doi.org/10.1016/0006-2952(94) 90163-5.

[15] Y.H. Chang, S.T. Lee, W.W. Lin, Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrophages: involvement of eicosanoids, J. Cell Biochem. 81 (4) (2001) 715–723, https://doi.org/10.1002/jcb.1103.

[16] R. Chen, J. Zhang, N. Fan, Z.Q. Teng, Y. Wu, H. Yang, Y.P. Tang, H. Sun, Y. Song, C. Chen, delta9-THC-caused synaptic and memory impairments are mediated through COX-2 signaling, Cell 155 (5) (2013) 1154–1165, https://doi.org/ 10.1016/j.cell.2013.10.042.

[17] D.J. Corsi, H. Hsu, D. Weiss, D.B. Fell, M. Walker, Trends and correlates of cannabis use in pregnancy: a population-based study in Ontario, Canada from 2012 to 2017, Can. J. Public Health 110 (1) (2019) 76–84, https://doi.org/ 10.17269/s41997-018-0148-0.

[18] D.J. Corsi, M.S.Q. Murphy, J. Cook, The effects of cannabis on female reproductive health across the life course, Cannabis Cannabinoid Res. 6 (4) (2021) 275–287, https://doi.org/10.1089/can.2020.0065.

[19] D.R. Danforth, L.K. Arbogast, S. Ghosh, A. Dickerman, R. Rofagha, C.I. Friedman, Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary, Biol. Reprod. 68 (5) (2003) 1736–1741, https://doi.org/10.1095/ biolreprod.101.000679.

[20] M. Das, O. Djahanbakhch, B. Hacihanefioglu, E. Saridogan, M. Ikram, L. Ghali, M. Raveendran, A. Storey, Granulosa cell survival and proliferation are altered in polycystic ovary syndrome, J. Clin. Endocrinol. Metab. 93 (3) (2008) 881–887, https://doi.org/10.1210/jc.2007-1650.

[21] J.M. Derocq, M. Segui, J. Marchand, G. Le Fur, P. Casellas, Cannabinoids enhance human B-cell growth at low nanomolar concentrations, FEBS Lett. 369 (2–3) (1995) 177–182, https://doi.org/10.1016/0014-5793(95)00746-v.

[22] L.K. Doyle, C.A. Walker, F.X. Donadeu, VEGF modulates the effects of gonadotropins in granulosa cells, Domest. Anim. Endocrinol. 38 (3) (2010) 127–137, https://doi.org/10.1016/j.domaniend.2009.08.008.

[23] W.C. Duncan, J. Nio-Kobayashi, Targeting angiogenesis in the pathological ovary, Reprod. Fertil. Dev. 25 (2) (2013) 362–371, https://doi.org/10.1071/RD12112.

[24] M.R. El-Talatini, A.H. Taylor, J.C. Elson, L. Brown, A.C. Davidson, J.C. Konje, Localisation and function of the endocannabinoid system in the human ovary, PLoS One 4 (2) (2009), e4579, https://doi.org/10.1371/journal.pone.0004579.

[25] M.A. ElSohly, S.A. Ross, Z. Mehmedic, R. Arafat, B. Yi, B.F. Banahan 3rd, Potency trends of delta9-THC and other cannabinoids in confiscated marijuana from 1980-1997, J. Forensic Sci. 45 (1) (2000) 24–30.

[26] M.A. ElSohly, Z. Mehmedic, S. Foster, C. Gon, S. Chandra, J.C. Church, Changes in cannabis potency over the last 2 decades (1995-2014): analysis of current data in the United States, Biol. Psychiatry 79 (7) (2016) 613–619, https://doi.org/ 10.1016/j.biopsych.2016.01.004.

[27] J. Ernst, U. Grabiec, T. Greither, B. Fischer, F. Dehghani, The endocannabinoid system in the human granulosa cell line KGN, Mol. Cell Endocrinol. 423 (2016) 67–76, https://doi.org/10.1016/j.mce.2016.01.006.

[28] L. Fang, Y. Yu, Y. Li, S. Wang, J. He, R. Zhang, Y.P. Sun, Upregulation of AREG, EGFR, and HER2 contributes to increased VEGF expression in granulosa cells of patients with OHSS, Biol. Reprod. 101 (2) (2019) 426–432, https://doi.org/10.1093/biolre/ioz091.

[29] A. Farkkila, M. Pihlajoki, H. Tauriala, R. Butzow, A. Leminen, L. Unkila-Kallio, M. Heikinheimo, M. Anttonen, Serum vascular endothelial growth factor a (VEGF) is elevated in patients with ovarian granulosa cell tumor (GCT), and VEGF inhibition by bevacizumab induces apoptosis in GCT in vitro, J. Clin. Endocrinol. Metab. 96 (2011) E1973–E1981, https://doi.org/10.1210/jc.2011-1812.

[30] A. Fasciani, G. D'Ambrogio, G. Bocci, M. Monti, A.R. Genazzani, P.G. Artini, High concentrations of the vascular endothelial growth factor and interleukin-8 in ovarian endometriomata, Mol. Hum. Reprod. 6 (1) (2000) 50–54, https://doi.org/10.1093/molehr/6.1.50.

[32] B.M. Fonseca, I. Rebelo, Cannabis and Cannabinoids in Reproduction and Fertility: Where We Stand, Reprod Sci (2021), https://doi.org/10.1007/s43032- 021-00588-1.

[33] C.I. Friedman, D.B. Seifer, E.A. Kennard, L. Arbogast, B. Alak, D.R. Danforth, Elevated level of follicular fluid vascular endothelial growth factor is a marker of diminished pregnancy potential, Fertil. Steril. 70 (5) (1998) 836–839, https://doi.org/10.1016/s0015-0282(98)00301-x.

[34] M. García Manero, B. Olartecoechea, M. Auba, J.L. Alcazar, G. Lopez, Angiogenesis and endometriosis, Rev. Med. Univ. Navarra 53 (2) (2009) 8–13.

[35] M.P. Garrido, I. Hurtado, M. Valenzuela-Valderrama, R. Salvatierra, A. Hernandez, M. Vega, A. Selman, A.F.G. Quest, C. Romero, NGF-Enhanced Vasculogenic Properties of Epithelial Ovarian Cancer Cells Is Reduced by Inhibition

of the COX-2/PGE2 Signaling Axis, in: Cancers, 11, Basel, 2019, p. 1970, https://doi.org/10.3390/cancers11121970. Dec 7.

[36] S.A. Garside, C.R. Harlow, S.G. Hillier, H.M. Fraser, F.H. Thomas, Thrombospondin-1 inhibits angiogenesis and promotes follicular atresia in a novel in vitro angiogenesis assay, Endocrinology 151 (3) (2010) 1280–1289, https://doi.org/10.1210/en.2009-0686.

[37] S.A. Garside, J. Henkin, K.D. Morris, S.M. Norvell, F.H. Thomas, H.M. Fraser, A thrombospondin-mimetic peptide, ABT-898, suppresses angiogenesis and promotes follicular atresia in pre- and early-antral follicles in vivo, Endocrinology. 151 (12) (2010) 5905–5915, https://doi.org/10.1210/en.2010- 0283.

[38] Y. Gong, X. Jin, Q.S. Wang, S.H. Wei, B.K. Hou, H.Y. Li, M.N. Zhang, Z.H. Li, The involvement of high mobility group 1 cytokine and phospholipases A2 in diabetic retinopathy, Lipids Health Dis. 13 (2014) 156, https://doi.org/10.1186/1476- 511X-13-156.

[39] Government of Canada, Health Canada. About Cannabis, 2018. Available at: https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/about.html. Last accessed Feb 15, 2022.

[40] J. Greenaway, K. Connor, H.G. Pedersen, B.L. Coomber, J. LaMarre, J. Petrik, Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle, Endocrinology 145 (6) (2004) 2896–2905, https://doi.org/10.1210/en.2003- 1620.

[41] J. Greenaway, P.A. Gentry, J.J. Feige, J. LaMarre, J.J. Petrik, Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development, Biol. Reprod. 72 (5) (2005) 1071–1078, https://doi.org/10.1095/biolreprod.104.031120.

[42] J. Greenaway, J. Lawler, R. Moorehead, P. Bornstein, J. Lamarre, J. Petrik, Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1), J. Cell Physiol. 210 (3) (2007) 807–818, https://doi.org/10.1002/jcp.20904.

[43] S. Hart, O.M. Fischer, A. Ullrich, Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor, Cancer Res. 64 (6) (2004) 1943–1950, https://doi.org/10.1158/0008-5472.can-03-3720.

[44] T.M. Hazzard, F. Xu, R.L. Stouffer, Injection of soluble vascular endothelial growth factor receptor 1 into the preovulatory follicle disrupts ovulation and subsequent luteal function in rhesus monkeys, Biol. Reprod. 67 (4) (2002) 1305–1312, https://doi.org/10.1095/biolreprod67.4.1305.

[45] S.A. Hunter, C.A. Audette, S. Burstein, Elevation of brain prostaglandin E2 levels in rodents by delta 1-tetrahydrocannabinol, Prostaglandins Leukot Essent Fatty Acids 43 (3) (1991) 185–190, https://doi.org/10.1016/0952-3278(91)90167-4.

[46] G. Irusta, D. Abramovich, F. Parborell, M. Tesone, Direct survival role of vascular endothelial growth factor (VEGF) on rat ovarian follicular cells, Mol. Cell Endocrinol. 325 (1–2) (2010) 93–100, https://doi.org/10.1016/j. mce.2010.04.018.

[47] B.N. Jahromi, M.E. Parsanezhad, Z. Shomali, P. Bakhshai, M. Alborzi, N. M. Vaziri, Z. Anvar, Ovarian hyperstimulation syndrome: a narrative review of its pathophysiology, risk factors, prevention, classification, and management, Iran J. Med. Sci. 43 (3) (2018) 248–260.

[48] A.M. Jukic, C.R. Weinberg, D.D. Baird, A.J. Wilcox, Lifestyle and reproductive factors associated with follicular phase length, J. Womens Health 16 (9) (2007) 1340–1347, https://doi.org/10.1089/jwh.2007.0354.

[49] A.M. Kasman, M.E. Thoma, A.C. McLain, M.L. Eisenberg, Association between use of marijuana and time to pregnancy in men and women: findings from the national survey of family growth, Fertil. Steril. 109 (5) (2018) 866–871, https:// doi.org/10.1016/j.fertnstert.2018.01.015.

[50] H.S. Klonoff-Cohen, L. Natarajan, R.V. Chen, A prospective study of the effects of female and male marijuana use on in vitro fertilization (IVF) and gamete intrafallopian transfer (GIFT) outcomes, Am. J. Obstet. Gynecol. 194 (2) (2006) 369–376, https://doi.org/10.1016/j.ajog.2005.08.020.

[51] A.J. Knox, L. Corbett, J. Stocks, E. Holland, Y.M. Zhu, L. Pang, Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism, FASEB J. 15 (13) (2001) 2480–2488, https://doi.org/10.1096/fj.01-0256com.

[52] J.Y. Ko, S.L. Farr, V.T. Tong, A.A. Creanga, W.M. Callaghan, Prevalence and patterns of marijuana use among pregnant and nonpregnant women of reproductive age, Am. J. Obstet. Gynecol. 213 (2) (2015) 201, https://doi.org/ 10.1016/j.ajog.2015.03.021.

[53] J. Li, R. Bao, S. Peng, C. Zhang, The molecular mechanism of ovarian granulosa cell tumors, J. Ovarian Res. 11 (1) (2018) 13, https://doi.org/10.1186/s13048- 018-0384-1.

[54] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[55] T. Lojpur, Z. Easton, S. Raez-Villanueva, S. Laviolette, A.C. Holloway, D.B. Hardy, Δ9-Tetrahydrocannabinol leads to endoplasmic reticulum stress and mitochondrial dysfunction in human BeWo trophoblasts, Reprod. Toxicol. 87 (2019) 21–31, https://doi.org/10.1016/j.reprotox.2019.04.008.

[56] F.L. Lopes, J. Desmarais, S. Ledoux, N.Y. Gevry, P. Lefevre, B.D. Murphy, Transcriptional regulation of uterine vascular endothelial growth factor during early gestation in a carnivore model, Mustela vison, J. Biol. Chem. 281 (34) (2006) 24602–24611, https://doi.org/10.1074/jbc.M602146200.

[57] T. Lowin, R.H. Straub, Cannabinoid-based drugs targeting CB1 and TRPV1, the sympathetic nervous system, and arthritis, Arthritis Res. Ther. 17 (1) (2015) 226, https://doi.org/10.1186/s13075-015-0743-x.

[58] C.K. Luo, P.H. Chou, S.K. Ng, W.Y. Lin, T.T. Wei, Cannabinoids orchestrate crosstalk between cancer cells and endothelial cells in colorectal cancer, Cancer Gene Ther. (2021), https://doi.org/10.1038/s41417-021-00346-0.

[59] C.A. MacCallum, E.B. Russo, Practical considerations in medical cannabis administration and dosing, Eur. J. Intern Med. 49 (2018) 12–19, https://doi.org/10.1016/j.ejim.2018.01.004.

[60] A. Malamitsi-Puchner, A. Sarandakou, S.G. Baka, J. Tziotis, D. Rizos, D. Hassiakos, G. Creatsas, Concentrations of angiogenic factors in follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity and fertilization, Fertil. Steril. 76 (1) (2001) 98–101, https://doi.org/10.1016/s0015-0282(01)01854-4.

[61] A.A. Martínez-Peña, K. Lee, J.J. Petrik, D.B. Hardy, A.C. Holloway, Gestational exposure to Δ9-THC impacts ovarian follicular dynamics and angiogenesis in adulthood in Wistar rats, J. Dev. Orig. Health Dis. 12 (6) (2021) 865–869, https://doi.org/10.1017/S2040174420001282.

[62] T. Maruo, J.B. Laoag-Fernandez, S. Takekida, X. Peng, J. Deguchi, T. Samoto, H. Kondo, H. Matsuo, Regulation of granulosa cell proliferation and apoptosis during follicular development, Gynecol. Endocrinol. 13 (6) (1999) 410–419, https://doi.org/10.3109/09513599909167588.

[63] H. Matsumoto, W.G. Ma, T. Daikoku, X. Zhao, B.C. Paria, S.K. Das, J.M. Trzaskos, S.K. Dey, Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice, J. Biol. Chem. 277 (32) (2002) 29260–29267, https://doi.org/10.1074/jbc.M203996200.

[64] M. Mattioli, B. Barboni, M. Turriani, G. Galeati, A. Zannoni, G. Castellani, P. Berardinelli, P.A. Scapolo, Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension, Biol. Reprod. 65 (4) (2001) 1014–1019, https://doi.org/10.1095/biolreprod65.4.1014.

[65] S.D. McAllister, C. Chan, R.J. Taft, T. Luu, M.E. Abood, D.H. Moore, K. Aldape, G. Yount, Cannabinoids selectively inhibit proliferation and induce death of cultured human glioblastoma multiforme cells, J. Neurooncol. 74 (1) (2005) 31–40, https://doi.org/10.1007/s11060-004-5950-2.

[66] F. Meah, M. Lundholm, N. Emanuele, H. Amjed, C. Poku, L. Agrawal, M. A. Emanuele, The effects of cannabis and cannabinoids on the endocrine system, Rev. Endocr. Metab. Disord. (2021), https://doi.org/10.1007/s11154-021- 09682-w.

[67] R. Mechoulam, Y. Gaoni, A total synthesis of dl-Δ1-tetrahydrocannabinol, the active constituent of hashish, J. Am. Chem. Soc. 87 (1965) 3273–3275, https://doi.org/10.1021/ja01092a065.

[68] Z. Mehmedic, S. Chandra, D. Slade, H. Denham, S. Foster, A.S. Patel, S.A. Ross, I. A. Khan, M.A. ElSohly, Potency trends of Δ9-THC and other cannabinoids in confiscated cannabis preparations from 1993 to 2008, J. Forensic Sci. 55 (5) (2010) 1209–1217, https://doi.org/10.1111/j.1556-4029.2010.01441.x.

[69] J.H. Mendelson, N.K. Mello, J. Ellingboe, A.S. Skupny, B.W. Lex, M. Griffin, Marihuana smoking suppresses luteinizing hormone in women, J. Pharmacol. Exp. Ther. 237 (3) (1986) 862–866.

[70] E.M. Messalli, F. Grauso, R. Luise, A. Angelini, R. Rossiello, Cannabinoid receptor type 1 immunoreactivity and disease severity in human epithelial ovarian tumors, Am. J. Obstet. Gynecol. 211 (3) (2014) 234, https://doi.org/10.1016/j.ajog.2014.04.004.

[71] E. Moreno, M. Cavic, A. Krivokuca, V. Casado, E. Canela, The endocannabinoid system as a target in cancer diseases: are we there yet? Front. Pharmacol. 10 (2019) 339, https://doi.org/10.3389/fphar.2019.00339.

[72] B.A. Mueller, J.R. Daling, N.S. Weiss, D.E. Moore, Recreational drug use and the risk of primary infertility, Epidemiology 1 (3) (1990) 195–200, https://doi.org/10.1097/00001648-199005000-00003.

[73] A.R. Munkarah, R. Morris, P. Baumann, G. Deppe, J. Malone, M.P. Diamond, G. M. Saed, Effects of prostaglandin E(2) on proliferation and apoptosis of epithelial ovarian cancer cells, J. Soc. Gynecol. Investig. 9 (3) (2002) 168–173.

[74] P. Navarra, C.L. Andreani, N. Lazzarin, E. Pierro, A. Mirtella, A. Lanzone, S. Mancuso, Increased production and release of prostaglandin-E2 by human granulosa cells from polycystic ovaries, Prostaglandins 52 (3) (1996) 187–197, https://doi.org/10.1016/s0090-6980(96)00096-2.

[75] J.D. Niringiyumukiza, H. Cai, W. Xiang, Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation, Reprod. Biol. Endocrinol. 16 (1) (2018) 43, https://doi.org/10.1186/s12958-018-0359-5.

[76] C.J. Park, P.C. Lin, S. Zhou, R. Barakat, S.T. Bashir, J.M. Choi, J.A. Cacioppo, O. R. Oakley, D.M. Duffy, J.P. Lydon, C.J. Ko, Progesterone receptor serves the ovary as a trigger of ovulation and a terminator of inflammation, Cell Rep. 31 (2) (2020), 107496, https://doi.org/10.1016/j.celrep.2020.03.060.

[77] P. Peitsidis, R. Agrawal, Role of vascular endothelial growth factor in women with PCO and PCOS: a systematic review, Reprod. Biomed. Online 20 (4) (2010) 444–452, https://doi.org/10.1016/j.rbmo.2010.01.007.

[78] R. Ramer, B. Hinz, Cyclooxygenase-2 and tissue inhibitor of matrix metalloproteinases-1 confer the antimigratory effect of cannabinoids on human trabecular meshwork cells, Biochem. Pharmacol. 80 (6) (2010) 846–857, https:// doi.org/10.1016/j.bcp.2010.05.010.

[79] K. Rask, Y. Zhu, W. Wang, L. Hedin, K. Sundfeldt, Ovarian epithelial cancer: a role for PGE2-synthesis and signalling in malignant transformation and progression, Mol. Cancer 5 (2006) 62, https://doi.org/10.1186/1476-4598-5-62.

[80] S. Rosch, ¨ R. Ramer, K. Brune, B.R. Hinz, (+)-methanandamide and other cannabinoids induce the expression of cyclooxygenase-2 and matrix metalloproteinases in human nonpigmented ciliary epithelial cells, J. Pharmacol. Exp. Ther. 316 (3) (2006) 1219–1228, https://doi.org/10.1124/jpet.105.092858.

[81] T. Sakurai, K. Suzuki, M. Yoshie, K. Hashimoto, E. Tachikawa, K. Tamura, Stimulation of tube formation mediated through the prostaglandin EP2 receptor in rat luteal endothelial cells, J. Endocrinol. 209 (1) (2011) 33–43, https://doi. org/10.1530/JOE-10-0357.

[82] M. Shinagawa, I. Tamura, R. Maekawa, S. Sato, Y. Shirafuta, Y. Mihara, M. Okada-Matsumoto, T. Taketani, H. Asada, H. Tamura, N. Sugino, C/EBPbeta regulates Vegf gene expression in granulosa cells undergoing luteinization during ovulation in female rats, Sci. Rep. 9 (1) (2019) 714, https://doi.org/10.1038/ s41598-018-36566-y.

[83] M. Solinas, P. Massi, A.R. Cantelmo, M.G. Cattaneo, R. Cammarota, D. Bartolini, V. Cinquina, M. Valenti, L.M. Vicentini, D.M. Noonan, A. Albini, D. Parolaro,

Cannabidiol inhibits angiogenesis by multiple mechanisms, Br. J. Pharmacol. 167 (6) (2012) 1218–1231, https://doi.org/10.1111/j.1476-5381.2012.02050.x.

[84] Statistics Canada. National Cannabis Survey, 2020. Available at: https://www 23.statcan.gc.ca/imdb/p2SV.pl?Function=getSurvey&SDDS=5262. Last Accessed Feb 15 2022.

[85] Statistics Canada. Canadian Tobacco, Alcohol and Drugs Survey, 2017. Available at: https://www.canada.ca/en/health-canada/services/canadian-alcohol-dru gs-survey/2017-summary.html Last Accessed Feb 15 2022.

[86] R.L. Stouffer, F. Xu, D.M. Duffy, Molecular control of ovulation and luteinization in the primate follicle, Front. Biosci. 12 (2007) 297–307, https://doi.org/ 10.2741/2065.

[87] Substance Abuse and Mental Health Services Administration, U.S. Department of Health and Human Services. 2019 National Survey on Drug Use and Health: Women. Available at: https://www.samhsa.gov/data/report/2019-nsdu h-women. Last Accessed Feb 15 2022.

[89] T. Suzuki, H. Sasano, R. Takaya, T. Fukaya, A. Yajima, H. Nagura, Cyclic changes of vasculature and vascular phenotypes in normal human ovaries, Hum. Reprod. 13 (4) (1998) 953–959, https://doi.org/10.1093/humrep/13.4.953.

[90] S. Takeda, I. Yamamoto, K. Watanabe, Modulation of Delta9-tetrahydrocannabinol-induced MCF-7 breast cancer cell growth by cyclooxygenase and aromatase, Toxicology 259 (1–2) (2009) 25–32, https://doi. org/10.1016/j.tox.2009.01.024.

[91] C. Tamanini, M. De Ambrogi, Angiogenesis in developing follicle and corpus luteum, Reprod. Domest. Anim. 39 (4) (2004) 206–216, https://doi.org/ 10.1111/j.1439-0531.2004.00505.x.

[93] The society of obstetricians and gynaecologists of Canada. Legal Cannabis Not Worth Risk for Pregnant Women. Available at: https://sogc.org/en/content/fe atured-news/legal-cannabis-not-work-risk.aspx. Last Accessed Feb 15 2022.

[94] H.A. Trau, J.S. Davis, D.M. Duffy, Angiogenesis in the primate ovulatory follicle is stimulated by luteinizing hormone via prostaglandin E2, Biol. Reprod. 92 (1) (2015) 15, https://doi.org/10.1095/biolreprod.114.123711.

[95] M. Tsoi, M.N. Lague, A. Boyer, M. Paquet, M.E. Nadeau, D. Boerboom, AntiVEGFA therapy reduces tumor growth and extends survival in a Murine model of ovarian Granulosa cell tumor, Transl. Oncol. 6 (2013) 226–233, https://doi.org/ 10.1593/tlo.13136.

[96] United Nations, Office on drugs and crime. World drug report, 2021. Available at: https://www.unodc.org/unodc/en/data-and-analysis/wdr2021.html. Last Accessed Feb 15, 2022.

[97] X.F. Wang, E. Galaj, G.H. Bi, C. Zhang, Y. He, J. Zhan, M.H. Bauman, E. L. Gardner, Z.X. Xi, Different receptor mechanisms underlying phytocannabinoidversus synthetic cannabinoid-induced tetrad effects: opposite roles of CB(1) /CB (2) versus GPR55 receptors, Br. J. Pharmacol. 177 (8) (2020) 1865–1880, https://doi.org/10.1111/bph.14958.

[98] L.A. Wise, A.K. Wesselink, E.E. Hatch, K.J. Rothman, E.M. Mikkelsen, H. T. Sørensen, S. Mahalingaiah, Marijuana use and fecundability in a North American preconception cohort study, J. Epidemiol. Community Health 72 (3) (2018) 208–215, https://doi.org/10.1136/jech-2017-209755.

[99] C. Wulff, H. Wilson, S.J. Wiegand, J.S. Rudge, H.M. Fraser, Prevention of thecal angiogenesis, antral follicular growth, and ovulation in the primate by treatment with vascular endothelial growth factor Trap R1R2, Endocrinology 143 (7) (2002) 2797–2807, https://doi.org/10.1210/endo.143.7.8886.

[100] S. Yamamoto, I. Konishi, Y. Tsuruta, K. Nanbu, M. Mandai, H. Kuroda, K. Matsushita, A.A. Hamid, Y. Yura, T. Mori, Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary, Gynecol. Endocrinol. 11 (6) (1997) 371–381, https://doi.org/ 10.3109/09513599709152564.

[101] M. Yebra, T.W. Klein, H. Friedman, Delta 9-tetrahydrocannabinol suppresses concanavalin A induced increase in cytoplasmic free calcium in mouse thymocytes, Life Sci. 51 (2) (1992) 151–160, https://doi.org/10.1016/0024- 3205(92)90009-e.

[102] I. Zachary, VEGF signalling: integration and multi-tasking in endothelial cell biology, Biochem. Soc. Trans. 31 (Pt 6) (2003) 1171–1177, https://doi.org/ 10.1042/bst0311171.

[103] W. Zhu, H. Friedman, T.W. Klein, Delta9-tetrahydrocannabinol induces apoptosis in macrophages and lymphocytes: involvement of Bcl-2 and caspase-1, J. Pharmacol. Exp. Ther. 286 (2) (1998) 1103–1109.

[104] R.C. Zimmermann, E. Xiao, P. Bohlen, M. Ferin, Administration of antivascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey, Endocrinology 143 (7) (2002) 2496–2502, https://doi.org/10.1210/endo.143.7.8896.

Figures and tables

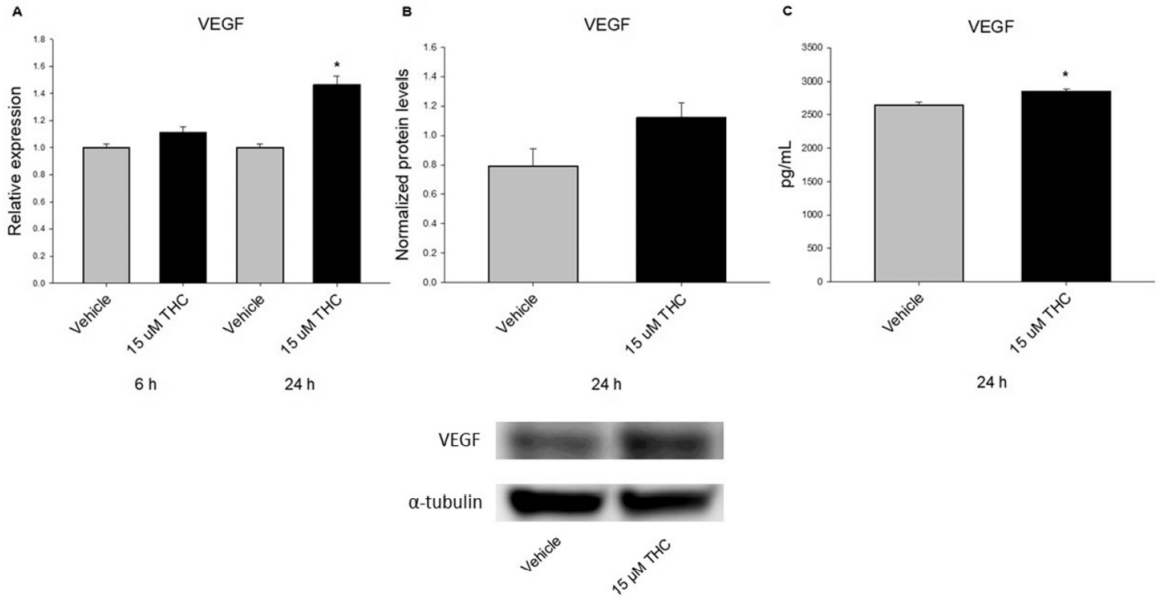

Fig. 1. **A.** Relative expression of *Vegfa* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 6 and 24 h (h). **B.** Intracellular VEGF protein levels relative to α-tubulin in SIGCs exposed to 15 μM THC for 24 h. **C.** Extracellular VEGF concentrations in media from SIGCs exposed to 15 μM THC for 24 h (Mean+SE; $N = 5$, $^*p < 0.05$).

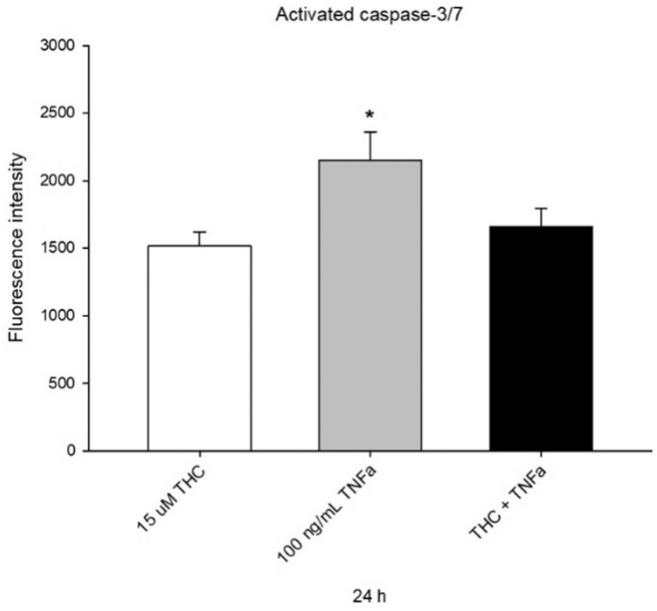

Fig. 2. Activated caspase-3/7 in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC, 100 ng/mL TNFα or both (THC + TNFa) for 24 h (Mean+SE; $N = 5$, $^*p < 0.05$).

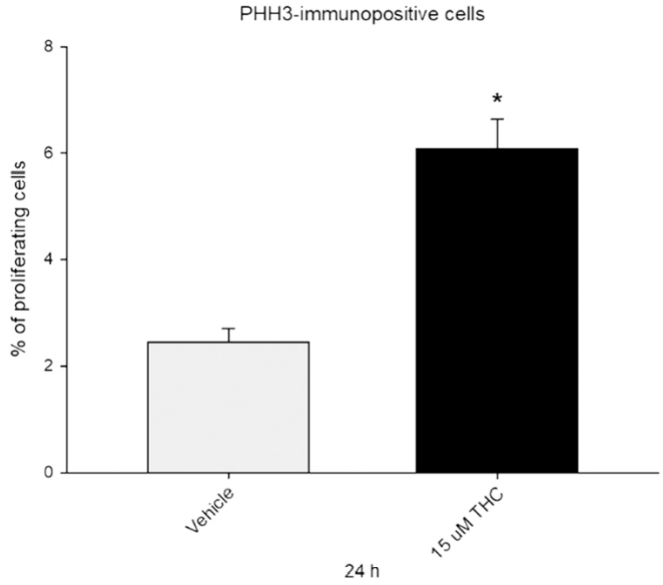

Fig. 3. Percent of proliferating spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 µM THC for 24 h (Mean+SE; $N = 5$, $^*p < 0.05$).

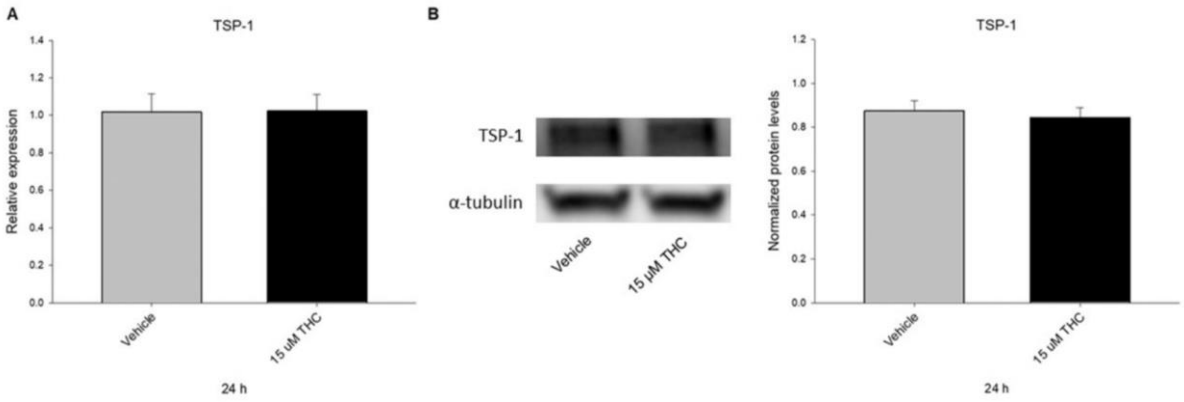

Fig. 4. A. Relative expression of *Thbs1* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 24 h. **B.** Intracellular TSP-1 protein levels relative to α-tubulin in SIGCs exposed to 15 μM THC for 24 h (Mean+SE; *N* = 5).

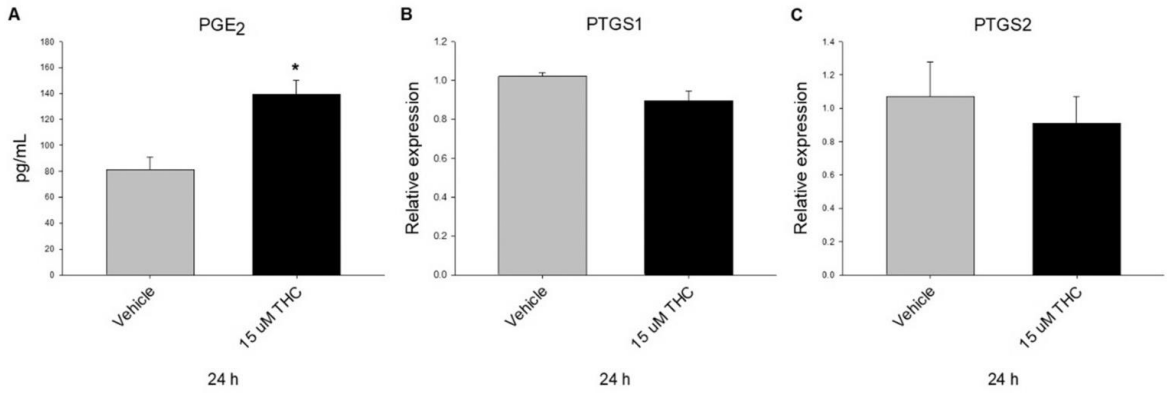

Fig. 5. A. Concentration of PGE_2 in media from spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 µM THC for 24 h. **B.** Relative expression of *Ptgs1* in SIGCs exposed to 15 µM THC for 24 h. **C.** Relative expression of *Ptgs2* in SIGCs exposed to 15 µM THC for 24 h (Mean+SE; $N = 5$, $*p < 0.05$).

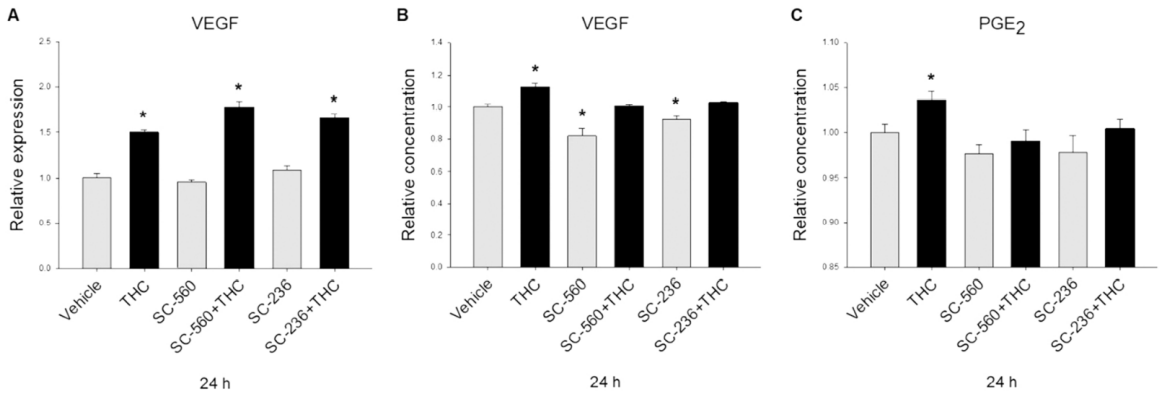

Fig. 6. A. Relative expression of *Vegfa* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 µM THC (THC), 1 µM SC-560 alone (SC-560) or in combination with THC (SC-560 +THC), 5 µM SC-236 alone (SC-236) or in combination with THC (SC-236 +THC) for 24 h. **B.** Concentration of VEGF in media from SIGCs exposed to THC, SC-560, SC-560 +THC, SC-236 or SC-236 +THC for 24 h. **C.** Concentration of PGE_2 in media from SIGCs exposed to THC, SC-560, SC560 +THC, SC-236 or SC-236 +THC for 24 h (Mean+SE; $N = 5$, $^*p < 0.05$).

Table 1. Forward and reverse primer sequences for *Vegfa*, *Thbs1*, *Ptgs1*, *Ptgs2*, *B2m* and *Hprt1*.

Accession number	Gene name	Symbol	Forward (5'–3')	Reverse (5'–3')
NM_031836.3	Vascular endothelial growth factor A	*Vegfa*	TCTCCCAGATCGGTGACAGT	AAGGAATGTGTGGTGGGGAC
NM_001013062.3	Thrombospondin 1	*Thbs1*	GGCAAAGACTGTGTTGGTGATG	GATGTTCCGTTGTGATTG
NM_017043.4	Prostaglandin-endoperoxide synthase 1	*Ptgs1*	AGTCTGGAACGACAGTACCAC	GGACGCCTGTTCTACGGA
NM_017232.3	Prostaglandin-endoperoxide synthase 2	*Ptgs2*	GAAAAGCCTCGTCCAGATGC	TCCGAAGGTGCTAGGTTTCC
NM_012512.2	Beta-2-microglobulin	*B2m*	AATTCACACCCACCGAGACC	GCTCCTTCAGAGTGACGTGT
NM_012583.2	Hypoxanthine phosphoribosyltransferase 1	*Hprt1*	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Summary of the work

The overall goal of this thesis was to evaluate the impact of cannabis constituents on ovarian health and function, with a focus on the long-term effects of prenatal exposure and the underlying molecular mechanisms. The main findings of this research project are that gestational exposure to delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, resulted in altered follicle dynamics, decreased vascularization and increased follicular apoptosis in the adult rat ovary. These alterations were associated with changes in the ovarian miRNA profile, as well as with altered gene expression and protein levels of important factors involved in the regulation of ovarian processes.

Specifically, prenatal THC exposure increased the expression of miR-122-5p and decreased the expression of its validated target gene insulin-like growth factor 1 receptor (*Igf1r*), which plays an important role in the regulation of ovarian steroidogenesis, folliculogenesis, angiogenesis, and granulosa cell proliferation and apoptosis [159]. Prenatally THC-exposed ovaries also had increased follicular apoptosis, determined by the presence of cleaved caspase-3. The reduced vascularization in prenatally THC-exposed ovaries, on the other hand, was associated with a decrease in the percentage of granulosa cells expressing the angiogenic factor vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2), as well as an increase in the percentage of granulosa cells expressing the antiangiogenic factor thrombospondin 1 (TSP-1). The differential expression of

these important growth factors in the ovary, along with the decreased vascularization and increased follicular apoptosis may partially explain the altered follicle dynamics observed in prenatally THC-exposed rats at 6 months of age, as they had accelerated folliculogenesis with follicular development arrest.

In addition, the direct impact of THC on ovarian explants and spontaneously immortalized rat granulosa cells was also evaluated, revealing both similarities and differences between the *in utero*, *ex vivo* and *in vitro* models. This suggests that while the ovary appears to be a target for THC, it is likely that this compound exerts its effects through different molecular mechanisms depending on the direct or indirect interaction with the tissue, as well as the concentration, time, window and duration of exposure.

Alterations in ovarian follicular dynamics

Considering that the rodent embryo expresses the cannabinoid receptors CB1 and CB2, to which THC is known to bind to, and that gestational exposure to THC has previously been shown to result in intrauterine growth restriction [78,160], we suspected that prenatal exposure to this compound may have direct and/or indirect effects on the developing ovary that could manifest during adulthood.

In female mammals, primordial germ cells arise from the yolk sac and migrate through the primitive gut into dorsal mesentery and then laterally to the gonadal ridges, where they proliferate as oogonia and subsequently enter meiosis. Later in development, somatic cells, that will eventually differentiate into granulosa cells,

closely associate with oogonia, that will enlarge and develop into oocytes, and together they form primordial follicles [161]. These primordial follicles are the reproductive units of the ovary, and they remain dormant until being recruited into the growing pool via a process known as activation [162,163]. Folliculogenesis describes the progression of small primordial follicles into large preovulatory follicles. Upon activation, primordial follicles transition into primary follicles, when granulosa cells surrounding the oocyte become cuboidal and undergo extensive proliferation. Primary follicles then grow into secondary follicles, which consist of an oocyte surrounded by multiple granulosa cell layers, basal lamina and a theca cell layer. This is followed by the formation of a fluid-filled antral follicle which can then release the mature oocyte from the ovary in a process known as ovulation [164]. The vast majority of the follicles that are recruited into the growing pool undergo endocrine controlled apoptosis (atresia), regardless of the developmental stage [165,166].

In the present study, exposure of rats to THC from gestation day (GD) 6 to GD22 resulted in altered ovarian follicular dynamics in the adult offspring. At 6 months of age, prenatally THC-exposed rats had a significant increase in the number of follicles transitioning from the primordial stage to the primary stage, which could be a result of an increase in the activation of the dormant primordial follicle pool. The activation of primordial follicles is a complex but orchestrated process regulated by multiple factors and pathways, both in the oocytes and the granulosa cells [167]. At the earliest stages, the oocyte-intrinsic factor FOXO3 plays a role as a suppressor of primordial follicle activation. Phosphorylation of FOXO3 by the PI3K-AKT pathway

suppresses its transcriptional function, while dephosphorylation by PTEN does the opposite. Therefore, PTEN-mutant mice have increased oocyte activation [167,168]. Upstream of PI3K, the granulosa cell-expressed KITL binds to its oocyte-expressed receptor KIT to initiate primordial follicle activation [167,168]. In granulosa cells, the mTOR kinase complex 1 (mTORC1) is involved in oocyte activation, and the deficiency of its inhibitor TSC1 induces the premature awakening of dormant oocytes [168]. Other factors, such as FOXL2, Sohlh1, NOBOX, BMP4, bFGF and Lhx 8 are also involved in the regulation of primordial follicle activation [167]. Sohlh1-deficient ovaries, for example, show defects in the primordial to primary follicle transition [168]. In later stages, the secretion of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) regulate granulosa cell proliferation. Therefore, GDF9 and BMP15 deficient mice show a block in follicular development beyond the primary one-layer follicle stage, with BMP-15 deficient mice having a milder phenotype [168]. Interestingly, ovaries from prenatally THC-exposed animals have a significant decrease in GDF9 and BMP15 expressing follicular cells (Supplementary figure 1), which could be associated with the apparent follicular development arrest beyond this stage. These different signaling pathways and regulating factors remain to be explored in the prenatally THC-exposed animals, as changes in their expression or activity could help elucidate the mechanisms behind the apparent increase in follicle activation.

Beyond the molecular mechanisms regulating primordial follicle activation, however, it has previously been suggested that primordial follicles that are prematurely

recruited into the growing pool are subsequently lost, resulting in no net loss or gain of developed follicles [169]. In support of this hypothesis, we did not observe any differences in the levels of circulating estradiol, progesterone or testosterone, or in the number of developed follicles, which are largely responsible for the synthesis of steroid hormones. In fact, we found no significant differences in the number of follicles of any of the later stages of development (primary, secondary or antral), suggesting that a significant portion of the transitioning follicles did not continue to develop. Considering the stage at which folliculogenesis seemed to diminish, it is possible that the conditions were not appropriate for the transitioning follicles to continue to grow [170]. This could be related to insufficient nutrients, oxygen or an imbalance between hormones and/or growth factors. Indeed, prenatally THC-exposed ovaries had decreased vascularization, along with a decrease in VEGF, VEGFR-2 and IGF-1R, as well as an increase in TSP-1, all important factors involved in the regulation of angiogenesis and folliculogenesis. Gestational exposure to THC has previously been shown to impair placental vascularization, suggested to be the main cause of the fetal growth restriction observed in THC-exposed rodents [78,171]. Interestingly, the glucose transporter GLUT1, known to be regulated by the IGF1/IGF1-R pathway, was also decreased in THC-exposed placenta [78,172], suggesting other fetal tissues may present similar phenotypes. In addition to the follicular developmental arrest, prenatally THC-exposed offspring had increased granulosa cell apoptosis, an important factor of follicular atresia [173]. Although there were no statistically significant differences in the number of atretic follicles in the THC-exposed ovaries ($p = 0.197$), this could be a reflection of the small sample size.

It is also possible that the increase in apoptosis may manifest more clearly as follicle loss as the animal ages [174].

As it is generally accepted that the total population of primordial follicles within the postnatal mammalian ovary will decrease gradually until the cessation of reproductive function is reached [175], factors that accelerate the depletion of the primordial follicle pool result in a shorter reproductive lifespan and premature ovarian failure [176,177]. Therefore, the increase in transitioning follicles, along with the follicular development arrest and increased follicular apoptosis observed in the prenatally THC-exposed animals could lead to a premature loss of the primordial follicle pool and early reproductive senescence. Additionally, considering that the primordial germ cells of these animals were also exposed to THC during gestation, it is not only possible that the quality of the oocytes may have been affected, but also that these effects may be transmitted to their offspring. Indeed, it has been shown that *in utero* insults can result in diminished ovarian health and function in adulthood, and that these alterations can be transmitted to the next generations, most likely through changes in epigenetic mechanisms [146]. It would therefore be an important next step to assess the fertility and reproductive lifespan of prenatally THC-exposed animals, as well as ovarian health and function in their offspring.

Changes in epigenetic regulation

Considering that alterations in prenatally THC-exposed offspring were observed at 6 months of age, which is long after the exposure to THC ceased, we hypothesized

that THC might have affected the regulation of epigenetic pathways in the ovaries. Indeed, prenatal THC exposure altered the expression of 12 out of the 420 evaluated miRNAs, with 11 of them being downregulated and only miR-122-5p (miR-122) being upregulated. As miR-122 is highly conserved among species and had a much higher fold-change when compared to controls, this target was chosen for further evaluation [178]. While the down-regulation of *Igf1r*, a validated target for miR-122, was an interesting and consistent observation that might be involved in the altered follicle dynamics, apoptosis and vascularization observed in these ovaries, other miR-122 targets remain to be explored. For example, the miR-122 validated target genes serum response factor (*Srf*), mitogen-activated protein kinase 3 (*Mapk3*) and B-cell lymphoma 9 protein (*Bcl9*) are also involved in the regulation of important ovarian processes, such as cellular response to gonadotropins, and granulosa cell proliferation and apoptosis [178–181]. Indeed, it has been shown that overexpression of miR-122 significantly inhibited chicken granulosa cell proliferation, as well as decreasing the expression of its target gene *Mapk3* [178]. Interestingly, prenatally THC-exposed ovaries had a non-statistically significant ($p=0.051$) decrease in *Mapk3* expression (Supplementary figure 2). However, given the small p value, it is still worth evaluating MAPK3 protein levels, as well as a proliferation marker in the prenatally THC-exposed ovaries. In addition, using a primary ovarian insufficiency (POI) mouse model, Zhang and colleagues demonstrated that miR-122 promoted granulosa cell apoptosis by targeting *Bcl9* [179]. As prenatally THC-exposed ovaries had increased follicular apoptosis, it would be relevant to assess

Bcl9 expression. The evaluation of other miR-122 targets may provide further insight into the pathways affected by *in utero* THC exposure.

Moreover, given the significant impact of prenatal THC exposure on the expression of miR-122 in the ovary, it is possible that the expression of this miRNA is also affected in other tissues. It was previously thought that miR-122 was liver-specific, as it accounts for a significant portion of all the hepatic miRNA population [180]. In the same cohort of animals as the present study, prenatally THC-exposed male offspring exhibited increased visceral adiposity and higher hepatic triglycerides at 6 months of age [182]. This is relevant considering that miR-122 plays a crucial role in the regulation of cholesterol and fatty acid metabolism in the adult liver [183]. Indeed, it has been shown that inhibition of miR-122 reduced total cholesterol and triglycerides in plasma of healthy mice, and decreased liver steatosis and triglyceride accumulation in high-fat diet mice [184]. In the same study, miR-122 inhibition was associated with a decrease in hepatic fatty-acid synthesis rate, along with reduced expression of genes involved in fatty-acid synthesis including FASN, ACC1, ACC2, SCD1 and ACLY, suggesting that miR-122 plays a significant role in promoting lipid synthesis in the adult liver [184]. Interestingly, prenatally THC-exposed rats had augmented expression of ACC1 and SCD1 at 3 weeks of age [182]. While the THC-induced metabolic alterations were associated with decreased expression of miR-203a-3p and miR-29a/b/c in this study [182], it is also possible that prenatal THC-exposure resulted in an increased expression of miR-122, similar to what was seen in the ovary, and that this dysregulation is implicated in the increased visceral

adiposity and hepatic triglycerides. This hypothesis is currently being evaluated by our research group.

Furthermore, it is important to note that alterations in different epigenetic pathways are not mutually exclusive; namely, although changes in miRNA expression were detected in prenatally THC-exposed animals, alterations in DNA methylation patterns or histone modifications may also be present. In fact, it is possible that the overexpression of miR-122 itself may be regulated by other epigenetic mechanisms [185]. For example, it has been shown that the promoter region of miR-122 is hypermethylated in human hepatocellular cancer cells (HCC) when compared to human primary hepatocytes [186], and treatment of HCC with a DNA methylation inhibitor (5-Aza-CdR) significantly increases the expression of miR-122 [185]. In addition, the upregulation of miR-122 may also affect the expression of other epigenetic factors such as enzymes involved in DNA or histone modification, including the histone methyltransferase G9a [187]. Therefore, and taking into account that gestational THC exposure has previously been shown to affect histone modifications in the adult rat brain [147], it would be relevant to assess the effects of prenatal THC exposure on DNA methylation and histone modifications.

Direct effects of THC on the ovary

In order to elucidate the direct effects of THC on the ovary, *ex vivo* and *in vitro* models were used. Considering the impact of THC on IGF-1R in the prenatally exposed ovaries, the expression of *Igf1r* was evaluated in rat ovarian explants and

spontaneously immortalized rat granulosa cells (SIGCs). Similar to what was seen in the prenatally THC-exposed ovaries, THC reduced the expression of *Igf1r* in both the ovarian explants and SIGCs. The IGF-1R plays a key role in the ovary, as it is involved in the regulation of ovarian steroidogenesis, folliculogenesis, angiogenesis and granulosa cell proliferation and apoptosis [159,188]. Mice with a conditional granulosa cell knockdown of *Igf1r* are sterile, with small ovaries lacking antral follicles, unable to ovulate and unresponsive to gonadotropins [159]. Indeed, it has previously been shown that IGF-1R inactivation reduces gonadotropin-induced follicle growth in rats, and that selective inhibition of IGF-1R activity in rat granulosa cells prevents the FSH-induced expression of *Cyp19* and *Cyp11a1*, both involved in steroidogenesis and important markers of granulosa cell differentiation and follicle growth [189]. The authors proposed that FSH amplifies basal IGF-1R signaling, and that the interaction between the endocrine effect of FSH and the autocrine actions of IGF-1 might play an essential role in follicle growth and dominance [189]. For this reason, it would be interesting to evaluate if exposure to THC impacts granulosa cell response to FSH, either by assessing the expression of differentiation markers, AKT activation or steroid hormone production. This could provide information that might help explain the adverse reproductive outcomes reported in adult cannabis users such as an increased risk of infertility due to ovulatory abnormalities [71], fewer and poorer quality oocytes, as well as lower pregnancy rates by *in vitro* fertilization (IVF) [72].

Moreover, as blood vessel density and granulosa cell expression of VEGF were also significantly affected by gestational THC exposure, the expression of *Vegfa* was evaluated in spontaneously immortalized rat granulosa cells (SIGCs) cultured with THC. Interestingly, exposure to THC increased the expression and secretion of VEGF in rat granulosa cells, contrary to what was seen in the prenatally exposed adult ovaries. As expected with the increase in VEGF, THC-exposed granulosa cells also had increased proliferation and cell survival when challenged with the apoptosis-inducing factor TNFα. Given that these alterations were not associated with changes in TSP-1 production, and that it has been clearly demonstrated that prostaglandin E2 (PGE_2) plays an important role in gonadotropin-induced angiogenesis and in the regulation of VEGF expression in the ovary [56,190–192], the prostanoid pathway was evaluated. THC exposure increased secretion of PGE_2 without affecting the expression of COX1 and COX2, the rate-limiting enzymes involved in prostanoid synthesis. Pre-treatment of SIGCs with COX inhibitors, prior to THC exposure, blocked the THC-induced increase in PGE_2 and VEGF secretion, without affecting *Vegfa* expression.

These results were interesting considering that cannabinoids and cannabis in general are thought to be anti-angiogenic, anti-proliferative and anti-inflammatory [171,193–196]. Nevertheless, it is important to note that these effects seem to depend on the cannabinoid used and specific tissue type. For example, Blázquez and colleagues reported the anti-angiogenic effects of the synthetic cannabinoids WIN-55,212–2 and JWH-133 in mouse glioma [197]. However, these synthetic

cannabinoids have a higher affinity and potency at the CB1 and CB2 receptors, relative to THC [198]. In addition, not only is the brain a tissue known to be rich in CB1 [199], but it has also been shown that there is an overexpression of cannabinoid receptors in cancerous tissue when compared to its healthy counterpart [193,200]. Several studies that report anti-angiogenic, anti-proliferative and pro-apoptotic effects of THC do so in cancer cell lines or tissues such as brain and immune cells, which are typically rich in CB1 and CB2 [193,196]. Indeed, in a study in which a tumour grade-dependent expression of CB1 was observed in human ovarian tumours, the authors suggested that cannabinoids have opposing effects on non-cancerous cells, in which they may activate proliferative pathways, versus cancerous cells, in which they promote anti-proliferative and apoptotic events [200].

Granulosa cells have been shown to express not only the canonical cannabinoid receptors CB1 and CB2, but also the non-canonical receptors GPR55 and TRPV1, which are associated with different signaling pathways and downstream cascades than CB1 and CB2 [158,201,202]. For example, the activation of GPR55 by one of its natural agonists, lysophosphatidylinositol (LPI), has been associated with angiogenesis and endothelial cell proliferation in ovarian cancer [203]. Therefore, it is possible that THC exerts its effects on VEGF and PGE_2 production through a different mechanism than by binding to and activating the canonical CB1 and CB2 receptors in the SIGCs. In support of this hypothesis, when SIGCs were pretreated with CB1 and CB2 antagonists (1 μM Rimonabant hydrochloride and 0.1 μM SR144528, respectively) for 1 hour prior to THC exposure, the THC-induced

increase in *Vegfa* expression was not blocked (Supplementary figure 3). Additionally, these effects were seen after exposure to 15 µM THC, a concentration that has been shown to increase VEGF expression in human trophoblast cells [204] and that is 5 times higher than the concentration of THC that downregulated *Igf1r* and had no effect on *Vegfa* (i.e., 3 µM), suggesting different concentrations of this compound may activate different pathways. Taking this into account, it would be relevant to repeat this experiment using specific antagonists for other cannabinoid receptors, such as GPR55, in order to determine the mechanism through which THC enhances *Vegfa* expression in the SIGCs.

Overall, results from this thesis suggest that *in utero* exposure to THC may have detrimental effects on ovarian health in adulthood that could lead to subfertility and/or early reproductive senescence, and that these effects are regulated by changes in epigenetic mechanisms. On the other hand, THC exposure in adulthood may interfere with folliculogenesis and ovulation, and/or be involved in the progression of gynaecological disorders such as ovarian hyperstimulation syndrome, polycystic ovary syndrome and ovarian cancer [72,205–216]. It is possible to conclude that THC affects different regulatory mechanisms, depending on the concentration, the direct or indirect interaction with the tissue, as well as the time and window of exposure. It is still clear, however, that exposure to THC had a profound impact on several key pathways that regulate ovarian function. Some of these pathways (e.g., regulation of apoptosis) are also important for the maintenance of tissue function in other organs, highlighting the need to explore the effects of fetal exposure to THC in

other organs and systems, although this remains to be evaluated. Given the increasing use of cannabis by pregnant women and women of reproductive age, more studies are required in order to have a better understanding of the effects of cannabis and its constituents on female reproductive health and fertility.

Limitations

While several important conclusions can be drawn from this body of work, there are still limitations and further questions which remain to be explored. The standardization of cannabis exposure, for example, continues to be a challenge. All of the studies presented in this thesis evaluated the effects of delta-9-tetrahydrocannabinol (THC) alone. However, THC is rarely consumed on its own [4]. As mentioned, the cannabis plant contains over 500 compounds from several different chemical classes, and these compounds may interact with each other and possibly act through different mechanistic pathways in the body [217,218].

Of particular concern is the non-psychoactive phytocannabinoid cannabidiol (CBD), as it is increasingly being advocated as a therapeutic resource for several conditions ranging from neurological, psychiatric and psychological disorders [219–222], to posttraumatic stress disorder [223], opioid use disorder [224], social anxiety/stress [225,226] and insomnia [227], as well as other physiological conditions such as ulcerative colitis/inflammation [228,229], nausea and emesis [230]. It is therefore not surprising that CBD is increasingly being used by pregnant women as an apparently safer alternative to treat pregnancy-related symptoms [231]. Although little is known

regarding the effect of CBD on female reproductive health, studies suggest that this phytocannabinoid may affect the homeostasis of the endocannabinoid system [232], as well as the process of decidualization, through which the endometrium becomes receptive to embryo implantation [217,233]. Moreover, adequate data on the developmental risks associated with the use of CBD in pregnant women is currently insufficient [231]. Animal studies have shown that perinatal exposure to high concentrations of CBD (150 or 250 mg/kg/day) resulted in increased embryofetal mortality, as well as decreased growth, delayed sexual maturation, neurobehavioural changes and adverse effects on male reproductive organ development and fertility [234]. More studies are needed in order to assess the potential effects of CBD on female reproductive health and its impact on the developing fetus when used during pregnancy.

Furthermore, while the use of three different experimental models in this thesis provides greater insight into the effects of THC on the ovary and allows the study of different mechanistic pathways, each of these models comes with its own advantages and limitations. For example, *ex vivo* and *in vitro* models facilitate the evaluation of direct exposure of the tissue and cells to the compound, with greater control over experimental conditions. The use of ovarian explants represents a microenvironment and cell diversity that more closely resemble *in vivo* conditions, while the use of spontaneously immortalized granulosa cells provides information on the effect of THC only on this specific cell type. However, both of these models represent short-term exposure and may not reflect the impact of long-term cannabis

or THC exposure on the ovary. In addition, cannabis is more commonly smoked or ingested [4] and ovarian exposure is a mixture of the parent compound as well as metabolites, an effect which is not accounted for in these models. Finally, THC and other cannabis components reach other organs and systems that could indirectly affect the ovary. In particular, it has been shown that cannabis and THC can alter components of the HPG axis, such as levels of gonadotropin releasing hormone and/or gonadotropins, which could impact ovarian function [69,73–77].

The *in utero* exposure model, on the other hand, allows the evaluation of the long-term effects of THC on the developing ovary. Although this model is closer to human prenatal cannabis exposure, there are other limitations to be considered. In the animal model described in this thesis, a single dose of THC was administered by IP injection. Since cannabis strains differ in THC content, and the amount and frequency of consumption vary widely from user to user [4], a large dose range would be necessary in order to encompass all of human exposure. In addition, the ovaries from prenatally THC-exposed offspring were evaluated at a single time point, limiting the knowledge about the effects that could have been observed prior to or after that point. Therefore, there are still questions regarding the affected pathways in the neonatal and prepubertal stages, as well as the potential loss of follicles and premature reproductive senescence in later stages of life.

Despite these limitations, the use of several models and techniques allowed the assessment of different reproductive parameters, pathways and regulating mechanisms affected by delta-9-tetrahydrocannabinol in the ovary. The data

obtained in this thesis represents a significant contribution to the literature regarding the developmental and reproductive toxicological properties of cannabis and its constituents.

REFERENCES

[1] LABOR UNOODA. WORLD DRUG REPORT 2021 (SET OF 5 BOOKLETS). S.l.: UNITED NATIONS; 2022.

[2] Parnes JE, Smith JK, Conner BT. Reefer madness or much ado about nothing? Cannabis legalization outcomes among young adults in the United States. Int J Drug Policy 2018;56:116–20. https://doi.org/10.1016/j.drugpo.2018.03.011.

[3] Fischer B, Lee A, Robinson T, Hall W. An overview of select cannabis use and supply indicators pre- and post-legalization in Canada. Subst Abuse Treat Prev Policy 2021;16:77. https://doi.org/10.1186/s13011-021-00405-7.

[4] Canada H. Canadian Cannabis Survey 2021: Summary 2021. https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/research-data/canadian-cannabis-survey-2021-summary.html (accessed July 13, 2022).

[5] Canada H. Canadian Tobacco, Alcohol and Drugs (CTADS) Survey: 2017 detailed tables 2018. https://www.canada.ca/en/health-canada/services/canadian-alcohol-drugs-survey/2017-summary/2017-detailed-tables.html (accessed July 13, 2022).

[6] Corsi DJ, Hsu H, Weiss D, Fell DB, Walker M. Trends and correlates of cannabis use in pregnancy: a population-based study in Ontario, Canada from 2012 to 2017. Can J Public Health Rev Can Santé Publique 2018;110:76–84. https://doi.org/10.17269/s41997-018-0148-0.

[7] Leemaqz SY, Dekker GA, McCowan LM, Kenny LC, Myers JE, Simpson NAB, et al. Maternal marijuana use has independent effects on risk for spontaneous preterm birth but not other common late pregnancy complications. Reprod

Toxicol Elmsford N 2016;62:77–86. https://doi.org/10.1016/j.reprotox.2016.04.021.

[8] 2019 National Survey on Drug Use and Health: Women | CBHSQ Data n.d. https://www.samhsa.gov/data/report/2019-nsduh-women (accessed June 4, 2022).

[9] Ko JY, Farr SL, Tong VT, Creanga AA, Callaghan WM. Prevalence and patterns of marijuana use among pregnant and nonpregnant women of reproductive age. Am J Obstet Gynecol 2015;213:201.e1-201.e10. https://doi.org/10.1016/j.ajog.2015.03.021.

[10] Moore DG, Turner JD, Parrott AC, Goodwin JE, Fulton SE, Min MO, et al. During pregnancy, recreational drug-using women stop taking ecstasy (3,4-methylenedioxy-N-methylamphetamine) and reduce alcohol consumption, but continue to smoke tobacco and cannabis: initial findings from the Development and Infancy Study. J Psychopharmacol (Oxf) 2010;24:1403–10. https://doi.org/10.1177/0269881109348165.

[11] Bayrampour H, Asim A. Cannabis Use During the Pre-Conception Period and Pregnancy After Legalization. J Obstet Gynaecol Can JOGC J Obstet Gynecol Can JOGC 2021;43:740–5. https://doi.org/10.1016/j.jogc.2021.02.119.

[12] Singh S, Filion KB, Abenhaim HA, Eisenberg MJ. Prevalence and outcomes of prenatal recreational cannabis use in high-income countries: a scoping review. BJOG Int J Obstet Gynaecol 2020;127:8–16. https://doi.org/10.1111/1471-0528.15946.

[13] Kaarid KP, Vu N, Bartlett K, Patel T, Sharma S, Honor RD, et al. Assessing the prevalence and correlates of prenatal cannabis consumption in an urban Canadian population: a cross-sectional survey. CMAJ Open 2021;9:E703–10. https://doi.org/10.9778/cmajo.20200181.

[14] Lozano J, García-Algar O, Marchei E, Vall O, Monleon T, Giovannandrea RD, et al. Prevalence of gestational exposure to cannabis in a Mediterranean city by meconium analysis. Acta Paediatr Oslo Nor 1992 2007;96:1734–7. https://doi.org/10.1111/j.1651-2227.2007.00535.x.

[15] Metz TD, Silver RM, McMillin GA, Allshouse AA, Jensen TL, Mansfield C, et al. Prenatal Marijuana Use by Self-Report and Umbilical Cord Sampling in a State With Marijuana Legalization. Obstet Gynecol 2019;133:98–104. https://doi.org/10.1097/AOG.0000000000003028.

[16] Volkow ND, Han B, Compton WM, McCance-Katz EF. Self-reported Medical and Nonmedical Cannabis Use Among Pregnant Women in the United States. JAMA 2019;322:167–9. https://doi.org/10.1001/jama.2019.7982.

[17] Westfall RE, Janssen PA, Lucas P, Capler R. Survey of medicinal cannabis use among childbearing women: Patterns of its use in pregnancy and retroactive self-assessment of its efficacy against 'morning sickness.' Complement Ther Clin Pract 2006;12:27–33. https://doi.org/10.1016/j.ctcp.2005.09.006.

[18] Roberson EK, Patrick WK, Hurwitz EL. Marijuana Use and Maternal Experiences of Severe Nausea During Pregnancy in Hawai'i. Hawaii J Med Public Health 2014;73:283–7.

[19] Metz TD, Borgelt LM. Marijuana Use in Pregnancy and While Breastfeeding. Obstet Gynecol 2018;132:1198–210. https://doi.org/10.1097/AOG.0000000000002878.

[20] Legal Cannabis Not Worth Risk for Pregnant Women. Why Risk It? n.d. https://sogc.org/en/en/content/featured-news/legal-cannabis-not-work-risk.aspx (accessed July 13, 2022).

[21] Marijuana Use During Pregnancy and Lactation n.d. https://www.acog.org/en/clinical/clinical-guidance/committee-opinion/articles/2017/10/marijuana-use-during-pregnancy-and-lactation (accessed July 13, 2022).

[22] Jarlenski M, Tarr JA, Holland CL, Farrell D, Chang JC. Pregnant Women's Access to Information About Perinatal Marijuana Use: A Qualitative Study. Womens Health Issues Off Publ Jacobs Inst Womens Health 2016;26:452–9. https://doi.org/10.1016/j.whi.2016.03.010.

[23] Pang RD, Dormanesh A, Hoang Y, Chu M, Allem J-P. Twitter Posts About Cannabis Use During Pregnancy and Postpartum:A Content Analysis. Subst Use Misuse 2021;56:1074–7. https://doi.org/10.1080/10826084.2021.1906277.

[24] Vastis V, Vincent S, Metz TD, Shea AK. Are Canadian Cannabis Dispensaries Counselling Pregnant Women Appropriately? J Obstet Gynaecol Can 2021;43:506-510.e2. https://doi.org/10.1016/j.jogc.2020.08.011.

[25] Dickson B, Mansfield C, Guiahi M, Allshouse AA, Borgelt LM, Sheeder J, et al. Recommendations From Cannabis Dispensaries About First-Trimester Cannabis Use. Obstet Gynecol 2018;131:1031–8. https://doi.org/10.1097/AOG.0000000000002619.

[26] Appendino G, Chianese G, Taglialatela-Scafati O. Cannabinoids: occurrence and medicinal chemistry. Curr Med Chem 2011;18:1085–99. https://doi.org/10.2174/092986711794940888.

[27] Elsohly MA, Slade D. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life Sci 2005;78:539–48. https://doi.org/10.1016/j.lfs.2005.09.011.

[28] Dewey WL. Cannabinoid pharmacology. Pharmacol Rev 1986;38:151–78.

[29] Mechoulam R, Gaoni Y. A TOTAL SYNTHESIS OF DL-DELTA-1-TETRAHYDROCANNABINOL, THE ACTIVE CONSTITUENT OF HASHISH. J Am Chem Soc 1965;87:3273–5. https://doi.org/10.1021/ja01092a065.

[30] Lucas CJ, Galettis P, Schneider J. The pharmacokinetics and the pharmacodynamics of cannabinoids. Br J Clin Pharmacol 2018;84:2477–82. https://doi.org/10.1111/bcp.13710.

[31] Johansson E, Agurell S, Hollister LE, Halldin MM. Prolonged apparent half-life of delta 1-tetrahydrocannabinol in plasma of chronic marijuana users. J Pharm Pharmacol 1988;40:374–5. https://doi.org/10.1111/j.2042-7158.1988.tb05272.x.

[32] Carlier J, Huestis MA, Zaami S, Pichini S, Busardò FP. Monitoring Perinatal Exposure to Cannabis and Synthetic Cannabinoids. Ther Drug Monit 2020;42:194–204. https://doi.org/10.1097/FTD.0000000000000667.

[33] Burgdorf JR, Kilmer B, Pacula RL. Heterogeneity in the composition of marijuana seized in California. Drug Alcohol Depend 2011;117:59–61. https://doi.org/10.1016/j.drugalcdep.2010.11.031.

[34] Freeman TP, Craft S, Wilson J, Stylianou S, ElSohly M, Di Forti M, et al. Changes in delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) concentrations in cannabis over time: systematic review and meta-analysis. Addict Abingdon Engl 2021;116:1000–10. https://doi.org/10.1111/add.15253.

[35] Mehmedic Z, Chandra S, Slade D, Denham H, Foster S, Patel AS, et al. Potency trends of Δ9-THC and other cannabinoids in confiscated cannabis preparations from 1993 to 2008. J Forensic Sci 2010;55:1209–17. https://doi.org/10.1111/j.1556-4029.2010.01441.x.

[36] Canada H. About cannabis 2018. https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/about.html (accessed July 13, 2022).

[37] Kilaru A, Chapman KD. The endocannabinoid system. Essays Biochem 2020;64:485–99. https://doi.org/10.1042/EBC20190086.

[38] Walker OS, Holloway AC, Raha S. The role of the endocannabinoid system in female reproductive tissues. J Ovarian Res 2019;12:3. https://doi.org/10.1186/s13048-018-0478-9.

[39] Fonseca BM, Costa MA, Almada M, Correia-da-Silva G, Teixeira NA. Endogenous cannabinoids revisited: a biochemistry perspective. Prostaglandins Other Lipid Mediat 2013;102–103:13–30. https://doi.org/10.1016/j.prostaglandins.2013.02.002.

[40] Hourani W, Alexander SPH. Cannabinoid ligands, receptors and enzymes: Pharmacological tools and therapeutic potential. Brain Neurosci Adv 2018;2:2398212818783908. https://doi.org/10.1177/2398212818783908.

[41] Taylor AH, Amoako AA, Bambang K, Karasu T, Gebeh A, Lam PMW, et al. Endocannabinoids and pregnancy. Clin Chim Acta Int J Clin Chem 2010;411:921–30. https://doi.org/10.1016/j.cca.2010.03.012.

[42] Sun X, Dey SK. Endocannabinoid signaling in female reproduction. ACS Chem Neurosci 2012;3:349–55. https://doi.org/10.1021/cn300014e.

[43] Bagavandoss P, Grimshaw S. Temporal and spatial distribution of the cannabinoid receptors (CB1, CB2) and fatty acid amide hydroxylase in the rat ovary. Anat Rec Hoboken NJ 2007 2010;293:1425–32. https://doi.org/10.1002/ar.21181.

[44] El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC, Konje JC. Localisation and function of the endocannabinoid system in the human ovary. PloS One 2009;4:e4579. https://doi.org/10.1371/journal.pone.0004579.

[45] Horne AW, Phillips JA, Kane N, Lourenco PC, McDonald SE, Williams ARW, et al. CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy. PloS One 2008;3:e3969. https://doi.org/10.1371/journal.pone.0003969.

[46] Brighton PJ, Marczylo TH, Rana S, Konje JC, Willets JM. Characterization of the endocannabinoid system, CB(1) receptor signalling and desensitization in human myometrium. Br J Pharmacol 2011;164:1479–94. https://doi.org/10.1111/j.1476-5381.2011.01425.x.

[47] Taylor AH, Abbas MS, Habiba MA, Konje JC. Histomorphometric evaluation of cannabinoid receptor and anandamide modulating enzyme expression in the human endometrium through the menstrual cycle. Histochem Cell Biol 2010;133:557–65. https://doi.org/10.1007/s00418-010-0695-9.

[48] Habayeb OMH, Taylor AH, Bell SC, Taylor DJ, Konje JC. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 2008;149:5052–60. https://doi.org/10.1210/en.2007-1799.

[49] Lewis SEM, Rapino C, Di Tommaso M, Pucci M, Battista N, Paro R, et al. Differences in the endocannabinoid system of sperm from fertile and infertile men. PloS One 2012;7:e47704. https://doi.org/10.1371/journal.pone.0047704.

[50] Taylor AH, Ang C, Bell SC, Konje JC. The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. Hum Reprod Update 2007;13:501–13. https://doi.org/10.1093/humupd/dmm018.

[51] El-Talatini MR, Taylor AH, Konje JC. The relationship between plasma levels of the endocannabinoid, anandamide, sex steroids, and gonadotrophins during the menstrual cycle. Fertil Steril 2010;93:1989–96. https://doi.org/10.1016/j.fertnstert.2008.12.033.

[52] Navarrete F, García-Gutiérrez MS, Jurado-Barba R, Rubio G, Gasparyan A, Austrich-Olivares A, et al. Endocannabinoid System Components as Potential Biomarkers in Psychiatry. Front Psychiatry 2020;11.

[53] Whirledge S, Cidlowski JA. Glucocorticoids, stress, and fertility. Minerva Endocrinol 2010;35:109–25.

[54] Tetsuka M, Milne M, Simpson GE, Hillier SG. Expression of 11beta-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. Biol Reprod 1999;60:330–5. https://doi.org/10.1095/biolreprod60.2.330.

[55] Matsumoto H, Ma W-G, Daikoku T, Zhao X, Paria BC, Das SK, et al. Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice. J Biol Chem 2002;277:29260–7. https://doi.org/10.1074/jbc.M203996200.

[56] Trau HA, Davis JS, Duffy DM. Angiogenesis in the primate ovulatory follicle is stimulated by luteinizing hormone via prostaglandin E2. Biol Reprod 2015;92:15. https://doi.org/10.1095/biolreprod.114.123711.

[57] Correa F, Wolfson ML, Valchi P, Aisemberg J, Franchi AM. Endocannabinoid system and pregnancy. Reprod Camb Engl 2016;152:R191–200. https://doi.org/10.1530/REP-16-0167.

[58] Maccarrone M, Bab I, Bíró T, Cabral GA, Dey SK, Di Marzo V, et al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci 2015;36:277–96. https://doi.org/10.1016/j.tips.2015.02.008.

[59] Malenczyk K, Keimpema E, Piscitelli F, Calvigioni D, Björklund P, Mackie K, et al. Fetal endocannabinoids orchestrate the organization of pancreatic islet microarchitecture. Proc Natl Acad Sci U S A 2015;112:E6185-6194. https://doi.org/10.1073/pnas.1519040112.

[60] Oltrabella F, Melgoza A, Nguyen B, Guo S. Role of the endocannabinoid system in vertebrates: Emphasis on the zebrafish model. Dev Growth Differ 2017;59:194–210. https://doi.org/10.1111/dgd.12351.

[61] Paria BC, Das SK, Dey SK. The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. Proc Natl Acad Sci U S A 1995;92:9460–4. https://doi.org/10.1073/pnas.92.21.9460.

[62] Wang H, Xie H, Guo Y, Zhang H, Takahashi T, Kingsley PJ, et al. Fatty acid amide hydrolase deficiency limits early pregnancy events. J Clin Invest 2006;116:2122–31. https://doi.org/10.1172/JCI28621.

[63] Paria BC, Deutsch DD, Dey SK. The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. Mol Reprod Dev 1996;45:183–92. https://doi.org/10.1002/(SICI)1098-2795(199610)45:2<183::AID-MRD11>3.0.CO;2-2.

[64] Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, et al. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 2001;276:20523–8. https://doi.org/10.1074/jbc.M100679200.

[65] Berrendero F, Sepe N, Ramos JA, Di Marzo V, Fernández-Ruiz JJ. Analysis of cannabinoid receptor binding and mRNA expression and endogenous cannabinoid contents in the developing rat brain during late gestation and early postnatal period. Synap N Y N 1999;33:181–91. https://doi.org/10.1002/(SICI)1098-2396(19990901)33:3<181::AID-SYN3>3.0.CO;2-R.

[66] Maccarrone M, Finazzi-Agrò A. Anandamide hydrolase: a guardian angel of human reproduction? Trends Pharmacol Sci 2004;25:353–7. https://doi.org/10.1016/j.tips.2004.05.002.

[67] Corsi DJ, Murphy MSQ, Cook J. The Effects of Cannabis on Female Reproductive Health Across the Life Course. Cannabis Cannabinoid Res 2021;6:275–87. https://doi.org/10.1089/can.2020.0065.

[68] Canada H. Is cannabis safe during preconception, pregnancy and breastfeeding? 2018. https://www.canada.ca/en/health-canada/services/publications/drugs-health-products/is-cannabis-safe-during-preconception-pregnancy-breastfeeding.html (accessed July 14, 2022).

[69] Mendelson JH, Mello NK, Ellingboe J, Skupny AS, Lex BW, Griffin M. Marihuana smoking suppresses luteinizing hormone in women. J Pharmacol Exp Ther 1986;237:862–6.

[70] Jukic AMZ, Weinberg CR, Baird DD, Wilcox AJ. Lifestyle and reproductive factors associated with follicular phase length. J Womens Health 2002 2007;16:1340–7. https://doi.org/10.1089/jwh.2007.0354.

[71] Mueller BA, Daling JR, Weiss NS, Moore DE. Recreational drug use and the risk of primary infertility. Epidemiol Camb Mass 1990;1:195–200. https://doi.org/10.1097/00001648-199005000-00003.

[72] Klonoff-Cohen HS, Natarajan L, Chen RV. A prospective study of the effects of female and male marijuana use on in vitro fertilization (IVF) and gamete intrafallopian transfer (GIFT) outcomes. Am J Obstet Gynecol 2006;194:369–76. https://doi.org/10.1016/j.ajog.2005.08.020.

[73] Chakravarty I, Shah PG, Sheth AR, Ghosh JJ. Mode of action of delta-9-tetrahydrocannabinol on hypothalamo-pituitary function in adult female rats. J Reprod Fertil 1979;57:113–5. https://doi.org/10.1530/jrf.0.0570113.

[74] Kumar AM, Haney M, Becker T, Thompson ML, Kream RM, Miczek K. Effect of early exposure to delta-9-tetrahydrocannabinol on the levels of opioid peptides, gonadotropin-releasing hormone and substance P in the adult male rat brain. Brain Res 1990;525:78–83. https://doi.org/10.1016/0006-8993(90)91322-8.

[75] ASCH RH, SMITH CG, SILER-KHODR TM, PAUERSTEIN CJ. Effects of Δ9-Tetrahydrocannabinol during the Follicular Phase of the Rhesus Monkey (Macaca mulatto)*. J Clin Endocrinol Metab 1981;52:50–5. https://doi.org/10.1210/jcem-52-1-50.

[76] Nir I, Ayalon D, Tsafriri A, Cordova T, Lindner HR. Suppression of the Cyclic Surge of Luteinizing Hormone Secretion and of Ovulation in the Rat by Δ1-Tetrahydrocannabinol. Nature 1973;243:470–1. https://doi.org/10.1038/243470a0.

[77] Wenger T, Tóth BE, Martin BR. Effects of anandamide (endogen cannabinoid) on anterior pituitary hormone secretion in adult ovariectomized rats. Life Sci 1995;56:2057–63. https://doi.org/10.1016/0024-3205(95)00189-d.

[78] Natale BV, Gustin KN, Lee K, Holloway AC, Laviolette SR, Natale DRC, et al. Δ9-tetrahydrocannabinol exposure during rat pregnancy leads to symmetrical

fetal growth restriction and labyrinth-specific vascular defects in the placenta. Sci Rep 2020;10:544. https://doi.org/10.1038/s41598-019-57318-6.

[79] Dalterio S, Bartke A. Fetal testosterone in mice: effect of gestational age and cannabinoid exposure. J Endocrinol 1981;91:509–14. https://doi.org/10.1677/joe.0.0910509.

[80] Rosenkrantz H, Grant RJ, Fleischman RW, Baker JR. Marihuana-induced embryotoxicity in the rabbit. Fundam Appl Toxicol Off J Soc Toxicol 1986;7:236–43. https://doi.org/10.1016/0272-0590(86)90153-3.

[81] Abel EL, Tan SE. Effects of delta-9-tetrahydrocannabinol, chlordiazepoxide (Librium), and their combination on pregnancy and offspring in mice [correction of rats]. Reprod Toxicol Elmsford N 1987;1:37–40. https://doi.org/10.1016/0890-6238(87)90069-4.

[82] Reich R, Laufer N, Lewysohn O, Cordova T, Ayalon D, Tsafriri A. In vitro effects of cannabinoids on follicular function in the rat. Biol Reprod 1982;27:223–31. https://doi.org/10.1095/biolreprod27.1.223.

[83] Lewysohn O, Cordova T, Nimrod A, Ayalon D. The suppressive effect of delta-1-tetrahydrocannabinol on the steroidogenic activity of rat granulosa cells in culture. Horm Res 1984;19:43–51. https://doi.org/10.1159/000179858.

[84] Adashi EY, Jones PB, Hsueh AJ. Direct antigonadal activity of cannabinoids: suppression of rat granulosa cell functions. Am J Physiol 1983;244:E177-185. https://doi.org/10.1152/ajpendo.1983.244.2.E177.

[85] Treinen KA, Sneeden JL, Heindel JJ. Specific inhibition of FSH-stimulated cAMP accumulation by delta 9-tetrahydrocannabinol in cultured rat granulosa cells. Toxicol Appl Pharmacol 1993;118:53–7. https://doi.org/10.1006/taap.1993.1008.

[86] Luke S, Hutcheon J, Kendall T. Cannabis Use in Pregnancy in British Columbia and Selected Birth Outcomes. J Obstet Gynaecol Can JOGC J Obstet Gynecol Can JOGC 2019;41:1311–7. https://doi.org/10.1016/j.jogc.2018.11.014.

[87] Varner MW, Silver RM, Rowland Hogue CJ, Willinger M, Parker CB, Thorsten VR, et al. Association between stillbirth and illicit drug use and smoking during pregnancy. Obstet Gynecol 2014;123:113–25. https://doi.org/10.1097/AOG.0000000000000052.

[88] Hayatbakhsh MR, Flenady VJ, Gibbons KS, Kingsbury AM, Hurrion E, Mamun AA, et al. Birth outcomes associated with cannabis use before and during pregnancy. Pediatr Res 2012;71:215–9. https://doi.org/10.1038/pr.2011.25.

[89] Hurd YL, Wang X, Anderson V, Beck O, Minkoff H, Dow-Edwards D. Marijuana impairs growth in mid-gestation fetuses. Neurotoxicol Teratol 2005;27:221–9. https://doi.org/10.1016/j.ntt.2004.11.002.

[90] Warshak CR, Regan J, Moore B, Magner K, Kritzer S, Van Hook J. Association between marijuana use and adverse obstetrical and neonatal outcomes. J Perinatol Off J Calif Perinat Assoc 2015;35:991–5. https://doi.org/10.1038/jp.2015.120.

[91] Campbell EE, Gilliland J, Dworatzek PDN, Vrijer BD, Penava D, Seabrook JA. SOCIOECONOMIC STATUS AND ADVERSE BIRTH OUTCOMES: A POPULATION-BASED CANADIAN SAMPLE. J Biosoc Sci 2018;50:102–13. https://doi.org/10.1017/S0021932017000062.

[92] El Marroun H, Tiemeier H, Steegers EAP, Jaddoe VWV, Hofman A, Verhulst FC, et al. Intrauterine Cannabis Exposure Affects Fetal Growth Trajectories: The Generation R Study. J Am Acad Child Adolesc Psychiatry 2009;48:1173–81. https://doi.org/10.1097/CHI.0b013e3181bfa8ee.

[93] Gabrhelík R, Mahic M, Lund IO, Bramness J, Selmer R, Skovlund E, et al. Cannabis Use during Pregnancy and Risk of Adverse Birth Outcomes: A Longitudinal Cohort Study. Eur Addict Res 2021;27:131–41. https://doi.org/10.1159/000510821.

[94] Conner SN, Carter EB, Tuuli MG, Macones GA, Cahill AG. Maternal marijuana use and neonatal morbidity. Am J Obstet Gynecol 2015;213:422.e1-4. https://doi.org/10.1016/j.ajog.2015.05.050.

[95] Mark K, Desai A, Terplan M. Marijuana use and pregnancy: prevalence, associated characteristics, and birth outcomes. Arch Womens Ment Health 2016;19:105–11. https://doi.org/10.1007/s00737-015-0529-9.

[96] van Gelder MMHJ, Reefhuis J, Caton AR, Werler MM, Druschel CM, Roeleveld N, et al. Characteristics of pregnant illicit drug users and associations between cannabis use and perinatal outcome in a population-based study. Drug Alcohol Depend 2010;109:243–7. https://doi.org/10.1016/j.drugalcdep.2010.01.007.

[97] Smith A, Kaufman F, Sandy MS, Cardenas A. Cannabis Exposure During Critical Windows of Development: Epigenetic and Molecular Pathways Implicated in Neuropsychiatric Disease. Curr Environ Health Rep 2020;7:325–42. https://doi.org/10.1007/s40572-020-00275-4.

[98] Fried PA, Watkinson B. 36- and 48-month neurobehavioral follow-up of children prenatally exposed to marijuana, cigarettes, and alcohol. J Dev Behav Pediatr JDBP 1990;11:49–58.

[99] Day NL, Richardson GA, Goldschmidt L, Robles N, Taylor PM, Stoffer DS, et al. Effect of prenatal marijuana exposure on the cognitive development of offspring at age three. Neurotoxicol Teratol 1994;16:169–75. https://doi.org/10.1016/0892-0362(94)90114-7.

[100] Goldschmidt L, Richardson GA, Willford J, Day NL. Prenatal marijuana exposure and intelligence test performance at age 6. J Am Acad Child Adolesc Psychiatry 2008;47:254–63. https://doi.org/10.1097/CHI.0b013e318160b3f0.

[101] Richardson GA, Ryan C, Willford J, Day NL, Goldschmidt L. Prenatal alcohol and marijuana exposure: effects on neuropsychological outcomes at 10 years. Neurotoxicol Teratol 2002;24:309–20. https://doi.org/10.1016/s0892-0362(02)00193-9.

[102] Smith AM, Fried PA, Hogan MJ, Cameron I. Effects of prenatal marijuana on visuospatial working memory: An fMRI study in young adults. Neurotoxicol Teratol 2006;28:286–95. https://doi.org/10.1016/j.ntt.2005.12.008.

[103] Smith AM, Fried PA, Hogan MJ, Cameron I. Effects of prenatal marijuana on response inhibition: an fMRI study of young adults. Neurotoxicol Teratol 2004;26:533–42. https://doi.org/10.1016/j.ntt.2004.04.004.

[104] Nashed MG, Hardy DB, Laviolette SR. Prenatal Cannabinoid Exposure: Emerging Evidence of Physiological and Neuropsychiatric Abnormalities. Front Psychiatry 2020;11:624275. https://doi.org/10.3389/fpsyt.2020.624275.

[105] Campolongo P, Trezza V, Ratano P, Palmery M, Cuomo V. Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. Psychopharmacology (Berl) 2011;214:5–15. https://doi.org/10.1007/s00213-010-1892-x.

[106] Gillies R, Lee K, Vanin S, Laviolette SR, Holloway AC, Arany E, et al. Maternal exposure to Δ9-tetrahydrocannabinol impairs female offspring glucose homeostasis and endocrine pancreatic development in the rat. Reprod Toxicol Elmsford N 2020;94:84–91. https://doi.org/10.1016/j.reprotox.2020.04.070.

[107] De Domenico E, Todaro F, Rossi G, Dolci S, Geremia R, Rossi P, et al. Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads and impairs ovarian reserve. Cell Death Dis 2017;8:e3085. https://doi.org/10.1038/cddis.2017.496.

[108] Lee K, Hardy DB. Metabolic Consequences of Gestational Cannabinoid Exposure. Int J Mol Sci 2021;22:9528. https://doi.org/10.3390/ijms22179528.

[109] Castel P, Barbier M, Poumerol E, Mandon-Pépin B, Tassistro V, Lepidi H, et al. Prenatal cannabinoid exposure alters the ovarian reserve in adult offspring of rats. Arch Toxicol 2020;94:4131–41. https://doi.org/10.1007/s00204-020-02877-1.

[110] Gibson E, Mahdy H. Anatomy, Abdomen and Pelvis, Ovary. StatPearls, Treasure Island (FL): StatPearls Publishing; 2022.

[111] Banu SK, Stanley JA, Sivakumar KK, Arosh JA, Barhoumi R, Burghardt RC. Identifying a novel role for X-prolyl aminopeptidase (Xpnpep) 2 in CrVI-induced adverse effects on germ cell nest breakdown and follicle development in rats. Biol Reprod 2015;92:67. https://doi.org/10.1095/biolreprod.114.125708.

[112] Pepe GJ, Lynch TJ, Albrecht ED. Regulation of baboon fetal ovarian development by placental estrogen: onset of puberty is delayed in offspring deprived of estrogen in utero. Biol Reprod 2013;89:132. https://doi.org/10.1095/biolreprod.112.107318.

[113] Smith P, Wilhelm D, Rodgers RJ. Development of mammalian ovary. J Endocrinol 2014;221:R145–61. https://doi.org/10.1530/JOE-14-0062.

[114] Goswami D, Conway GS. Premature ovarian failure. Hum Reprod Update 2005;11:391–410. https://doi.org/10.1093/humupd/dmi012.

[115] Kramer MS. Determinants of low birth weight: methodological assessment and meta-analysis. Bull World Health Organ 1987;65:663–737.

[116] Cooper C, Kuh D, Egger P, Wadsworth M, Barker D. Childhood growth and age at menarche. Br J Obstet Gynaecol 1996;103:814–7. https://doi.org/10.1111/j.1471-0528.1996.tb09879.x.

[117] Sloboda DM, Hart R, Doherty DA, Pennell CE, Hickey M. Age at menarche: Influences of prenatal and postnatal growth. J Clin Endocrinol Metab 2007;92:46–50. https://doi.org/10.1210/jc.2006-1378.

[118] Ibáñez L, de Zegher F. Puberty and prenatal growth. Mol Cell Endocrinol 2006;254–255:22–5. https://doi.org/10.1016/j.mce.2006.04.010.

[119] Hackman E, Emanuel I, van Belle G, Daling J. Maternal birth weight and subsequent pregnancy outcome. JAMA 1983;250:2016–9.

[120] Tom SE, Cooper R, Kuh D, Guralnik JM, Hardy R, Power C. Fetal environment and early age at natural menopause in a British birth cohort study. Hum Reprod Oxf Engl 2010;25:791–8. https://doi.org/10.1093/humrep/dep451.

[121] Bernal AB, Vickers MH, Hampton MB, Poynton RA, Sloboda DM. Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. PloS One 2010;5:e15558. https://doi.org/10.1371/journal.pone.0015558.

[122] Franks S. Do animal models of polycystic ovary syndrome help to understand its pathogenesis and management? Yes, but their limitations should be recognized. Endocrinology 2009;150:3983–5. https://doi.org/10.1210/en.2009-0652.

[123] Risti N, Nestorovi N, Manojlovi-Stojanoski M, Trifunovi S, Ajdžanovi V, Filipovi B, et al. Prenatal dexamethasone exposure and developmental programming of the ovary of the offspring: a structural study in the rat. Reprod Fertil Dev 2021;33:245–55. https://doi.org/10.1071/RD20164.

[124] Luderer U, Meier MJ, Lawson GW, Beal MA, Yauk CL, Marchetti F. In Utero Exposure to Benzo[a]pyrene Induces Ovarian Mutations at Doses That Deplete Ovarian Follicles in Mice. Environ Mol Mutagen 2019;60:410–20. https://doi.org/10.1002/em.22261.

[125] Santamaría C, Durando M, Muñoz de Toro M, Luque EH, Rodriguez HA. Ovarian dysfunctions in adult female rat offspring born to mothers perinatally exposed to low doses of bisphenol A. J Steroid Biochem Mol Biol 2016;158:220–30. https://doi.org/10.1016/j.jsbmb.2015.11.016.

[126] Gill S, Brehm E, Leon K, Chiu J, Meling DD, Flaws JA. Prenatal exposure to an environmentally relevant phthalate mixture alters ovarian steroidogenesis and folliculogenesis in the F1 generation of adult female mice. Reprod Toxicol 2021;106:25–31. https://doi.org/10.1016/j.reprotox.2021.09.013.

[127] Pampanini V, Jahnukainen K, Sahlin L, Germani D, Puglianiello A, Cianfarani S, et al. Impact of uteroplacental insufficiency on ovarian follicular pool in the rat. Reprod Biol Endocrinol RBE 2019;17:10. https://doi.org/10.1186/s12958-019-0453-3.

[128] de Bruin JP, Dorland M, Bruinse HW, Spliet W, Nikkels PG, Te Velde ER. Fetal growth retardation as a cause of impaired ovarian development. Early Hum Dev 1998;51:39–46. https://doi.org/10.1016/s0378-3782(97)00073-x.

[129] Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet Lond Engl 1986;1:1077–81. https://doi.org/10.1016/s0140-6736(86)91340-1.

[130] Barker DJ. The intrauterine origins of cardiovascular and obstructive lung disease in adult life. The Marc Daniels Lecture 1990. J R Coll Physicians Lond 1991;25:129–33.

[131] Barker DJP, Eriksson JG, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol 2002;31:1235–9. https://doi.org/10.1093/ije/31.6.1235.

[132] Holness MJ, Sugden MC. Epigenetic regulation of metabolism in children born small for gestational age. Curr Opin Clin Nutr Metab Care 2006;9:482–8. https://doi.org/10.1097/01.mco.0000232912.69236.e0.

[133] Jazwiec PA, Sloboda DM. Nutritional adversity, sex and reproduction: 30 years of DOHaD and what have we learned? J Endocrinol 2019;242:T51–68. https://doi.org/10.1530/JOE-19-0048.

[134] Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429:457–63. https://doi.org/10.1038/nature02625.

[135] Goyal D, Limesand SW, Goyal R. Epigenetic responses and the developmental origins of health and disease. J Endocrinol 2019;242:T105–19. https://doi.org/10.1530/JOE-19-0009.

[136] Zhang Y, Sun Z, Jia J, Du T, Zhang N, Tang Y, et al. Overview of Histone Modification. In: Fang D, Han J, editors. Histone Mutat. Cancer, Singapore: Springer; 2021, p. 1–16. https://doi.org/10.1007/978-981-15-8104-5_1.

[137] Kim VN. Small RNAs: classification, biogenesis, and function. Mol Cells 2005;19:1–15.

[138] Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol 2005;3:e85. https://doi.org/10.1371/journal.pbio.0030085.

[139] Wadhwa PD, Buss C, Entringer S, Swanson JM. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Semin Reprod Med 2009;27:358–68. https://doi.org/10.1055/s-0029-1237424.

[140] Legoff L, Dali O, D'Cruz SC, Suglia A, Gely-Pernot A, Hémery C, et al. Ovarian dysfunction following prenatal exposure to an insecticide, chlordecone, associates with altered epigenetic features. Epigenetics Chromatin 2019;12:29. https://doi.org/10.1186/s13072-019-0276-7.

[141] Lite C, Ahmed SSSJ, Santosh W, Seetharaman B. Prenatal exposure to bisphenol-A altered miRNA-224 and protein expression of aromatase in ovarian granulosa cells concomitant with elevated serum estradiol levels in F1 adult offspring. J Biochem Mol Toxicol 2019;33:e22317. https://doi.org/10.1002/jbt.22317.

[142] Sui S, Jia Y, He B, Li R, Li X, Cai D, et al. Maternal Low-protein Diet Alters Ovarian Expression of Folliculogenic and Steroidogenic Genes and Their Regulatory MicroRNAs in Neonatal Piglets. Asian-Australas J Anim Sci 2014;27:1695–704. https://doi.org/10.5713/ajas.2014.14335.

[143] Liu J, Liao J, Zhang C, Zeng L, Zong C, Lv Y, et al. The role of miRNAs in regulating the effect of prenatal cadmium exposure on ovarian granulosa cells in a transgenerational manner in female rats. Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc 2021;150:112062. https://doi.org/10.1016/j.fct.2021.112062.

[144] Qin Y, Wang Y, Zhao H, Yang Z, Kang Y. Aberrant miRNA-mRNA regulatory network in polycystic ovary syndrome is associated with markers of insulin sensitivity and inflammation. Ann Transl Med 2021;9:1405. https://doi.org/10.21037/atm-21-1288.

[145] Zama AM, Uzumcu M. Targeted Genome-Wide Methylation and Gene Expression Analyses Reveal Signaling Pathways Involved in Ovarian Dysfunction after Developmental EDC Exposure in Rats. Biol Reprod 2013;88:52. https://doi.org/10.1095/biolreprod.112.104802.

[146] Gong X, Zhang J, Ge C, Yi Y, Dai S, Fan G, et al. miRNA320a-3p/RUNX2 signal programming mediates the transgenerational inheritance of inhibited ovarian estrogen synthesis in female offspring rats induced by prenatal dexamethasone exposure. Pharmacol Res 2021;165:105435. https://doi.org/10.1016/j.phrs.2021.105435.

[147] DiNieri JA, Wang X, Szutorisz H, Spano SM, Kaur J, Casaccia P, et al. Maternal cannabis use alters ventral striatal dopamine D2 gene regulation in the offspring. Biol Psychiatry 2011;70:763–9. https://doi.org/10.1016/j.biopsych.2011.06.027.

[148] Murphy SK, Itchon-Ramos N, Visco Z, Huang Z, Grenier C, Schrott R, et al. Cannabinoid exposure and altered DNA methylation in rat and human sperm. Epigenetics 2018;13:1208–21. https://doi.org/10.1080/15592294.2018.1554521.

[149] Prini P, Penna F, Sciuccati E, Alberio T, Rubino T. Chronic Δ^8-THC Exposure Differently Affects Histone Modifications in the Adolescent and Adult Rat Brain. Int J Mol Sci 2017;18:E2094. https://doi.org/10.3390/ijms18102094.

[150] Pucci M, Rapino C, Di Francesco A, Dainese E, D'Addario C, Maccarrone M. Epigenetic control of skin differentiation genes by phytocannabinoids. Br J Pharmacol 2013;170:581–91. https://doi.org/10.1111/bph.12309.

[151] Todd SM, Zhou C, Clarke DJ, Chohan TW, Bahceci D, Arnold JC. Interactions between cannabidiol and Δ9-THC following acute and repeated dosing: Rebound hyperactivity, sensorimotor gating and epigenetic and neuroadaptive changes in the mesolimbic pathway. Eur Neuropsychopharmacol J Eur Coll Neuropsychopharmacol 2017;27:132–45. https://doi.org/10.1016/j.euroneuro.2016.12.004.

[152] Yang X, Hegde VL, Rao R, Zhang J, Nagarkatti PS, Nagarkatti M. Histone modifications are associated with Δ9-tetrahydrocannabinol-mediated alterations in antigen-specific T cell responses. J Biol Chem 2014;289:18707–18. https://doi.org/10.1074/jbc.M113.545210.

[153] Watson CT, Szutorisz H, Garg P, Martin Q, Landry JA, Sharp AJ, et al. Genome-Wide DNA Methylation Profiling Reveals Epigenetic Changes in the Rat Nucleus Accumbens Associated With Cross-Generational Effects of Adolescent THC Exposure. Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol 2015;40:2993–3005. https://doi.org/10.1038/npp.2015.155.

[154] Sido JM, Yang X, Nagarkatti PS, Nagarkatti M. Δ9-Tetrahydrocannabinol-mediated epigenetic modifications elicit myeloid-derived suppressor cell activation via STAT3/S100A8. J Leukoc Biol 2015;97:677–88. https://doi.org/10.1189/jlb.1A1014-479R.

[155] Chiarlone A, Börner C, Martín-Gómez L, Jiménez-González A, García-Concejo A, García-Bermejo ML, et al. MicroRNA let-7d is a target of cannabinoid CB1 receptor and controls cannabinoid signaling.

Neuropharmacology 2016;108:345–52. https://doi.org/10.1016/j.neuropharm.2016.05.007.

[156] Juknat A, Gao F, Coppola G, Vogel Z, Kozela E. miRNA expression profiles and molecular networks in resting and LPS-activated BV-2 microglia-Effect of cannabinoids. PloS One 2019;14:e0212039. https://doi.org/10.1371/journal.pone.0212039.

[157] Fuchs Weizman N, Wyse BA, Szaraz P, Defer M, Jahangiri S, Librach CL. Cannabis alters epigenetic integrity and endocannabinoid signalling in the human follicular niche. Hum Reprod Oxf Engl 2021;36:1922–31. https://doi.org/10.1093/humrep/deab104.

[158] Ernst J, Grabiec U, Greither T, Fischer B, Dehghani F. The endocannabinoid system in the human granulosa cell line KGN. Mol Cell Endocrinol 2016;423:67–76. https://doi.org/10.1016/j.mce.2016.01.006.

[159] Baumgarten SC, Armouti M, Ko C, Stocco C. IGF1R Expression in Ovarian Granulosa Cells Is Essential for Steroidogenesis, Follicle Survival, and Fertility in Female Mice. Endocrinology 2017;158:2309–18. https://doi.org/10.1210/en.2017-00146.

[160] Buckley NE, Hansson S, Harta G, Mezey E. Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. Neuroscience 1998;82:1131–49. https://doi.org/10.1016/s0306-4522(97)00348-5.

[161] Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, et al. A new model of development of the mammalian ovary and follicles. PloS One 2013;8:e55578. https://doi.org/10.1371/journal.pone.0055578.

[162] Lee HN, Chang EM. Primordial follicle activation as new treatment for primary ovarian insufficiency. Clin Exp Reprod Med 2019;46:43–9. https://doi.org/10.5653/cerm.2019.46.2.43.

[163] Wright CJ, Cari EL, Sandoval J, Bales E, Sam PK, Zarate MA, et al. Control of Murine Primordial Follicle Growth Activation by IκB/NFκB Signaling. Reprod Sci Thousand Oaks Calif 2020;27:2063–74. https://doi.org/10.1007/s43032-020-00225-3.

[164] Chan CJ, Bevilacqua C, Prevedel R. Mechanical mapping of mammalian follicle development using Brillouin microscopy. Commun Biol 2021;4:1–10. https://doi.org/10.1038/s42003-021-02662-5.

[165] Kaipia A, Hsueh AJ. Regulation of ovarian follicle atresia. Annu Rev Physiol 1997;59:349–63. https://doi.org/10.1146/annurev.physiol.59.1.349.

[166] Hirshfield AN. Size-frequency analysis of atresia in cycling rats. Biol Reprod 1988;38:1181–8. https://doi.org/10.1095/biolreprod38.5.1181.

[167] Chen Y, Yang W, Shi X, Zhang C, Song G, Huang D. The Factors and Pathways Regulating the Activation of Mammalian Primordial Follicles in vivo. Front Cell Dev Biol 2020;8.

[168] Nagamatsu G. Regulation of primordial follicle formation, dormancy, and activation in mice. J Reprod Dev 2021;67:189–95. https://doi.org/10.1262/jrd.2021-040.

[169] Tsoulis MW, Chang PE, Moore CJ, Chan KA, Gohir W, Petrik JJ, et al. Maternal High-Fat Diet-Induced Loss of Fetal Oocytes Is Associated with Compromised Follicle Growth in Adult Rat Offspring. Biol Reprod 2016;94:94. https://doi.org/10.1095/biolreprod.115.135004.

[170] Fraser HM, Wulff C. Angiogenesis in the primate ovary. Reprod Fertil Dev 2001;13:557–66. https://doi.org/10.1071/rd01055.

[171] Chang X, Li H, Li Y, He Q, Yao J, Duan T, et al. RhoA/MLC signaling pathway is involved in Δ^9-tetrahydrocannabinol-impaired placental angiogenesis. Toxicol Lett 2018;285:148–55. https://doi.org/10.1016/j.toxlet.2017.12.031.

[172] Borges MH, Pullockaran J, Catalano PM, Baumann MU, Zamudio S, Illsley NP. Human placental GLUT1 glucose transporter expression and the fetal insulin-like growth factor axis in pregnancies complicated by diabetes. Biochim Biophys Acta Mol Basis Dis 2019;1865:2411–9. https://doi.org/10.1016/j.bbadis.2019.06.002.

[173] Manabe N, Goto Y, Matsuda-Minehata F, Inoue N, Maeda A, Sakamaki K, et al. Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. J Reprod Dev 2004;50:493–514. https://doi.org/10.1262/jrd.50.493.

[174] Sargent KM, Lu N, Clopton DT, Pohlmeier WE, Brauer VM, Ferrara N, et al. Loss of Vascular Endothelial Growth Factor A (VEGFA) Isoforms in Granulosa Cells Using pDmrt-1-Cre or Amhr2-Cre Reduces Fertility by Arresting Follicular Development and by Reducing Litter Size in Female Mice. PLOS ONE 2015;10:e0116332. https://doi.org/10.1371/journal.pone.0116332.

[175] Maidarti M, Anderson RA, Telfer EE. Crosstalk between PTEN/PI3K/Akt Signalling and DNA Damage in the Oocyte: Implications for Primordial Follicle Activation, Oocyte Quality and Ageing. Cells 2020;9:E200. https://doi.org/10.3390/cells9010200.

[176] Kawamura K, Kawamura N, Hsueh AJW. Activation of dormant follicles: a new treatment for premature ovarian failure? Curr Opin Obstet Gynecol 2016;28:217–22. https://doi.org/10.1097/GCO.0000000000000268.

[177] Kallen A, Polotsky AJ, Johnson J. Untapped Reserves: Controlling Primordial Follicle Growth Activation. Trends Mol Med 2018;24:319–31. https://doi.org/10.1016/j.molmed.2018.01.008.

[178] Zhang G, Cui Z, Li J, Zhang D, Li Z, Lin Z, et al. miR-122-5p regulates proliferation and apoptosis of chicken granulosa cells of hierarchal follicles by targeting MAPK3. Gene 2022;824:146397. https://doi.org/10.1016/j.gene.2022.146397.

[179] Zhang X, Zhang R, Hao J, Huang X, Liu M, Lv M, et al. miRNA-122-5p in POI ovarian-derived exosomes promotes granulosa cell apoptosis by regulating BCL9. Cancer Med 2022. https://doi.org/10.1002/cam4.4615.

[180] Nakao K, Miyaaki H, Ichikawa T. Antitumor function of microRNA-122 against hepatocellular carcinoma. J Gastroenterol 2014;49:589–93. https://doi.org/10.1007/s00535-014-0932-4.

[181] Russell DL, Doyle KMH, Gonzales-Robayna I, Pipaon C, Richards JS. Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3’,5’-monophosphate regulatory element binding protein, serum response factor, sp1, and early growth response factor-1. Mol Endocrinol Baltim Md 2003;17:520–33. https://doi.org/10.1210/me.2002-0066.

[182] Oke SL, Lee K, Papp R, Laviolette SR, Hardy DB. In Utero Exposure to Δ9-Tetrahydrocannabinol Leads to Postnatal Catch-Up Growth and Dysmetabolism in the Adult Rat Liver. Int J Mol Sci 2021;22:7502. https://doi.org/10.3390/ijms22147502.

[183] Bandiera S, Pfeffer S, Baumert TF, Zeisel MB. miR-122--a key factor and therapeutic target in liver disease. J Hepatol 2015;62:448–57. https://doi.org/10.1016/j.jhep.2014.10.004.

[184] Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 2006;3:87–98. https://doi.org/10.1016/j.cmet.2006.01.005.

[185] Song K, Han C, Zhang J, Lu D, Dash S, Feitelson M, et al. Epigenetic regulation of MicroRNA-122 by peroxisome proliferator activated receptor-gamma and hepatitis b virus X protein in hepatocellular carcinoma cells. Hepatology 2013;58:1681–92. https://doi.org/10.1002/hep.26514.

[186] Jung CJ, Iyengar S, Blahnik KR, Ajuha TP, Jiang JX, Farnham PJ, et al. Epigenetic modulation of miR-122 facilitates human embryonic stem cell self-renewal and hepatocellular carcinoma proliferation. PloS One 2011;6:e27740. https://doi.org/10.1371/journal.pone.0027740.

[187] Yuan L-T, Lee W-J, Yang Y-C, Chen B-R, Yang C-Y, Chen M-W, et al. Histone Methyltransferase G9a-Promoted Progression of Hepatocellular Carcinoma Is Targeted by Liver-Specific Hsa-miR-122. Cancers 2021;13:2376. https://doi.org/10.3390/cancers13102376.

[188] Zhao J, Taverne M a. M, van der Weijden GC, Bevers MM, van den Hurk R. Immunohistochemical localisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-I) and type I IGF-I receptor, and gene expression of GH and GHR in rat pre-antral follicles. Zygote Camb Engl 2002;10:85–94. https://doi.org/10.1017/s0967199402002125.

[189] Zhou P, Baumgarten SC, Wu Y, Bennett J, Winston N, Hirshfeld-Cytron J, et al. IGF-I signaling is essential for FSH stimulation of AKT and steroidogenic genes in granulosa cells. Mol Endocrinol Baltim Md 2013;27:511–23. https://doi.org/10.1210/me.2012-1307.

[190] Duncan WC, Nio-Kobayashi J. Targeting angiogenesis in the pathological ovary. Reprod Fertil Dev 2013;25:362–71. https://doi.org/10.1071/RD12112.

[191] Garrido MP, Hurtado I, Valenzuela-Valderrama M, Salvatierra R, Hernández A, Vega M, et al. NGF-Enhanced Vasculogenic Properties of Epithelial Ovarian Cancer Cells Is Reduced by Inhibition of the COX-2/PGE2 Signaling Axis. Cancers 2019;11:E1970. https://doi.org/10.3390/cancers11121970.

[192] Sakurai T, Suzuki K, Yoshie M, Hashimoto K, Tachikawa E, Tamura K. Stimulation of tube formation mediated through the prostaglandin EP2 receptor in rat luteal endothelial cells. J Endocrinol 2011;209:33–43. https://doi.org/10.1530/JOE-10-0357.

[193] Moreno E, Cavic M, Krivokuca A, Casadó V, Canela E. The Endocannabinoid System as a Target in Cancer Diseases: Are We There Yet? Front Pharmacol 2019;10:339. https://doi.org/10.3389/fphar.2019.00339.

[194] MacCallum CA, Russo EB. Practical considerations in medical cannabis administration and dosing. Eur J Intern Med 2018;49:12–9. https://doi.org/10.1016/j.ejim.2018.01.004.

[195] Ruhaak LR, Felth J, Karlsson PC, Rafter JJ, Verpoorte R, Bohlin L. Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from Cannabis sativa. Biol Pharm Bull 2011;34:774–8. https://doi.org/10.1248/bpb.34.774.

[196] Bifulco M, Di Marzo V. Targeting the endocannabinoid system in cancer therapy: a call for further research. Nat Med 2002;8:547–50. https://doi.org/10.1038/nm0602-547.

[197] Blázquez C, González-Feria L, Alvarez L, Haro A, Casanova ML, Guzmán M. Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas. Cancer Res 2004;64:5617–23. https://doi.org/10.1158/0008-5472.CAN-03-3927.

[198] An D, Peigneur S, Hendrickx LA, Tytgat J. Targeting Cannabinoid Receptors: Current Status and Prospects of Natural Products. Int J Mol Sci 2020;21:E5064. https://doi.org/10.3390/ijms21145064.

[199] Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 1997;74:129–80. https://doi.org/10.1016/s0163-7258(97)82001-3.

[200] Messalli EM, Grauso F, Luise R, Angelini A, Rossiello R. Cannabinoid receptor type 1 immunoreactivity and disease severity in human epithelial ovarian tumors. Am J Obstet Gynecol 2014;211:234.e1-6. https://doi.org/10.1016/j.ajog.2014.04.004.

[201] Lowin T, Straub RH. Cannabinoid-based drugs targeting CB1 and TRPV1, the sympathetic nervous system, and arthritis. Arthritis Res Ther 2015;17:226. https://doi.org/10.1186/s13075-015-0743-x.

[202] Wang X-F, Galaj E, Bi G-H, Zhang C, He Y, Zhan J, et al. Different receptor mechanisms underlying phytocannabinoid- versus synthetic cannabinoid-induced tetrad effects: Opposite roles of CB1 /CB2 versus GPR55 receptors. Br J Pharmacol 2020;177:1865–80. https://doi.org/10.1111/bph.14958.

[203] Hofmann NA, Yang J, Trauger SA, Nakayama H, Huang L, Strunk D, et al. The GPR 55 agonist, L-α-lysophosphatidylinositol, mediates ovarian carcinoma cell-induced angiogenesis. Br J Pharmacol 2015;172:4107–18. https://doi.org/10.1111/bph.13196.

[204] Lojpur T, Easton Z, Raez-Villanueva S, Laviolette S, Holloway AC, Hardy DB. Δ9-Tetrahydrocannabinol leads to endoplasmic reticulum stress and mitochondrial dysfunction in human BeWo trophoblasts. Reprod Toxicol Elmsford N 2019;87:21–31. https://doi.org/10.1016/j.reprotox.2019.04.008.

[205] Fang L, Yu Y, Li Y, Wang S, He J, Zhang R, et al. Upregulation of AREG, EGFR, and HER2 contributes to increased VEGF expression in granulosa cells of patients with OHSS†. Biol Reprod 2019;101:426–32. https://doi.org/10.1093/biolre/ioz091.

[206] Niringiyumukiza JD, Cai H, Xiang W. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. Reprod Biol Endocrinol RBE 2018;16:43. https://doi.org/10.1186/s12958-018-0359-5.

[207] Stouffer RL, Xu F, Duffy DM. Molecular control of ovulation and luteinization in the primate follicle. Front Biosci J Virtual Libr 2007;12:297–307. https://doi.org/10.2741/2065.

[208] Friedman CI, Seifer DB, Kennard EA, Arbogast L, Alak B, Danforth DR. Elevated level of follicular fluid vascular endothelial growth factor is a marker of diminished pregnancy potential. Fertil Steril 1998;70:836–9. https://doi.org/10.1016/s0015-0282(98)00301-x.

[209] Malamitsi-Puchner A, Sarandakou A, Baka SG, Tziotis J, Rizos D, Hassiakos D, et al. Concentrations of angiogenic factors in follicular fluid and oocyte-cumulus complex culture medium from women undergoing in vitro fertilization: association with oocyte maturity and fertilization. Fertil Steril 2001;76:98–101. https://doi.org/10.1016/s0015-0282(01)01854-4.

[210] Das M, Djahanbakhch O, Hacihanefioglu B, Saridogan E, Ikram M, Ghali L, et al. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. J Clin Endocrinol Metab 2008;93:881–7. https://doi.org/10.1210/jc.2007-1650.

[211] Li J, Bao R, Peng S, Zhang C. The molecular mechanism of ovarian granulosa cell tumors. J Ovarian Res 2018;11:13. https://doi.org/10.1186/s13048-018-0384-1.

[212] Färkkilä A, Pihlajoki M, Tauriala H, Bützow R, Leminen A, Unkila-Kallio L, et al. Serum vascular endothelial growth factor A (VEGF) is elevated in patients with ovarian granulosa cell tumor (GCT), and VEGF inhibition by bevacizumab induces apoptosis in GCT in vitro. J Clin Endocrinol Metab 2011;96:E1973-1981. https://doi.org/10.1210/jc.2011-1812.

[213] Tsoi M, Laguë M-N, Boyer A, Paquet M, Nadeau M-È, Boerboom D. Anti-VEGFA Therapy Reduces Tumor Growth and Extends Survival in a Murine Model of Ovarian Granulosa Cell Tumor. Transl Oncol 2013;6:226–33. https://doi.org/10.1593/tlo.13136.

[214] Navarra P, Andreani CL, Lazzarin N, Pierro E, Mirtella A, Lanzone A, et al. Increased production and release of prostaglandin-E2 by human granulosa cells from polycystic ovaries. Prostaglandins 1996;52:187–97. https://doi.org/10.1016/s0090-6980(96)00096-2.

[215] Munkarah AR, Morris R, Baumann P, Deppe G, Malone J, Diamond MP, et al. Effects of prostaglandin E(2) on proliferation and apoptosis of epithelial ovarian cancer cells. J Soc Gynecol Investig 2002;9:168–73.

[216] Rask K, Zhu Y, Wang W, Hedin L, Sundfeldt K. Ovarian epithelial cancer: a role for PGE2-synthesis and signalling in malignant transformation and progression. Mol Cancer 2006;5:62. https://doi.org/10.1186/1476-4598-5-62.

[217] Almada M, Amaral C, Oliveira A, Fernandes PA, Ramos MJ, Fonseca BM, et al. Cannabidiol (CBD) but not tetrahydrocannabinol (THC) dysregulate in vitro decidualization of human endometrial stromal cells by disruption of estrogen

signaling. Reprod Toxicol Elmsford N 2020;93:75–82. https://doi.org/10.1016/j.reprotox.2020.01.003.

[218] Laprairie RB, Bagher AM, Kelly MEM, Denovan-Wright EM. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. Br J Pharmacol 2015;172:4790–805. https://doi.org/10.1111/bph.13250.

[219] Thiele EA, Marsh ED, French JA, Mazurkiewicz-Beldzinska M, Benbadis SR, Joshi C, et al. Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet Lond Engl 2018;391:1085–96. https://doi.org/10.1016/S0140-6736(18)30136-3.

[220] Devinsky O, Cross JH, Laux L, Marsh E, Miller I, Nabbout R, et al. Trial of Cannabidiol for Drug-Resistant Seizures in the Dravet Syndrome. N Engl J Med 2017;376:2011–20. https://doi.org/10.1056/NEJMoa1611618.

[221] Leehey MA, Liu Y, Hart F, Epstein C, Cook M, Sillau S, et al. Safety and Tolerability of Cannabidiol in Parkinson Disease: An Open Label, Dose-Escalation Study. Cannabis Cannabinoid Res 2020;5:326–36. https://doi.org/10.1089/can.2019.0068.

[222] Leweke FM, Piomelli D, Pahlisch F, Muhl D, Gerth CW, Hoyer C, et al. Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. Transl Psychiatry 2012;2:e94. https://doi.org/10.1038/tp.2012.15.

[223] Elms L, Shannon S, Hughes S, Lewis N. Cannabidiol in the Treatment of Post-Traumatic Stress Disorder: A Case Series. J Altern Complement Med N Y N 2019;25:392–7. https://doi.org/10.1089/acm.2018.0437.

[224] Gonzalez-Cuevas G, Martin-Fardon R, Kerr TM, Stouffer DG, Parsons LH, Hammell DC, et al. Unique treatment potential of cannabidiol for the prevention of relapse to drug use: preclinical proof of principle. Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol 2018;43:2036–45. https://doi.org/10.1038/s41386-018-0050-8.

[225] Bergamaschi MM, Queiroz RHC, Zuardi AW, Crippa JAS. Safety and side effects of cannabidiol, a Cannabis sativa constituent. Curr Drug Saf 2011;6:237–49. https://doi.org/10.2174/157488611798280924.

[226] Crippa JAS, Derenusson GN, Ferrari TB, Wichert-Ana L, Duran FLS, Martin-Santos R, et al. Neural basis of anxiolytic effects of cannabidiol (CBD) in generalized social anxiety disorder: a preliminary report. J Psychopharmacol Oxf Engl 2011;25:121–30. https://doi.org/10.1177/0269881110379283.

[227] Shannon S, Lewis N, Lee H, Hughes S. Cannabidiol in Anxiety and Sleep: A Large Case Series. Perm J 2019;23:18–041. https://doi.org/10.7812/TPP/18-041.

[228] Irving PM, Iqbal T, Nwokolo C, Subramanian S, Bloom S, Prasad N, et al. A Randomized, Double-blind, Placebo-controlled, Parallel-group, Pilot Study of Cannabidiol-rich Botanical Extract in the Symptomatic Treatment of Ulcerative Colitis. Inflamm Bowel Dis 2018;24:714–24. https://doi.org/10.1093/ibd/izy002.

[229] Lehmann C, Fisher NB, Tugwell B, Szczesniak A, Kelly M, Zhou J. Experimental cannabidiol treatment reduces early pancreatic inflammation in type 1 diabetes. Clin Hemorheol Microcirc 2016;64:655–62. https://doi.org/10.3233/CH-168021.

[230] Rock EM, Limebeer CL, Pertwee RG, Mechoulam R, Parker LA. Therapeutic Potential of Cannabidiol, Cannabidiolic Acid, and Cannabidiolic Acid Methyl

Ester as Treatments for Nausea and Vomiting. Cannabis Cannabinoid Res 2021;6:266–74. https://doi.org/10.1089/can.2021.0041.

[231] Ochiai W, Kitaoka S, Kawamura T, Hatogai J, Harada S, Iizuka M, et al. Maternal and Fetal Pharmacokinetic Analysis of Cannabidiol during Pregnancy in Mice. Drug Metab Dispos Biol Fate Chem 2021;49:337–43. https://doi.org/10.1124/dmd.120.000270.

[232] Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, et al. Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 2001;134:845–52. https://doi.org/10.1038/sj.bjp.0704327.

[233] Neradugomma NK, Drafton K, Mor GG, Mao Q. Marijuana-derived cannabinoids inhibit uterine endometrial stromal cell decidualization and compromise trophoblast-endometrium cross-talk. Reprod Toxicol Elmsford N 2019;87:100–7. https://doi.org/10.1016/j.reprotox.2019.05.064.

[234] EPIDIOLEX (cannabidiol) oral solution n.d.:30.

SUPPLEMENTARY FIGURES

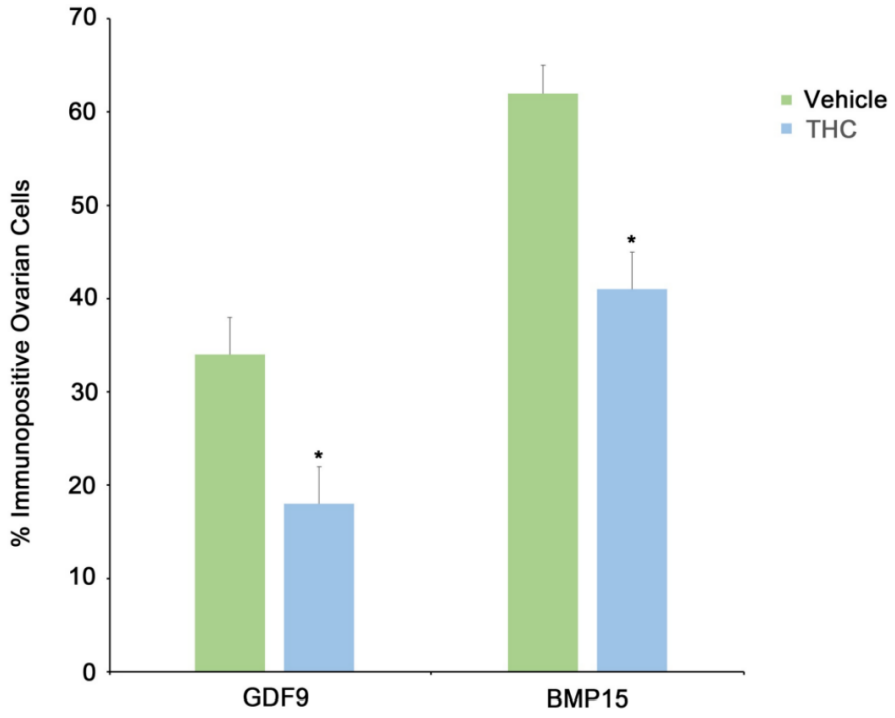

Figure 1. Percentage of GDF9 and BMP15-positive ovarian follicular cells in prenatally THC-exposed adult rat ovaries (Mean+SEM; *N*=5, **p*<0.05).

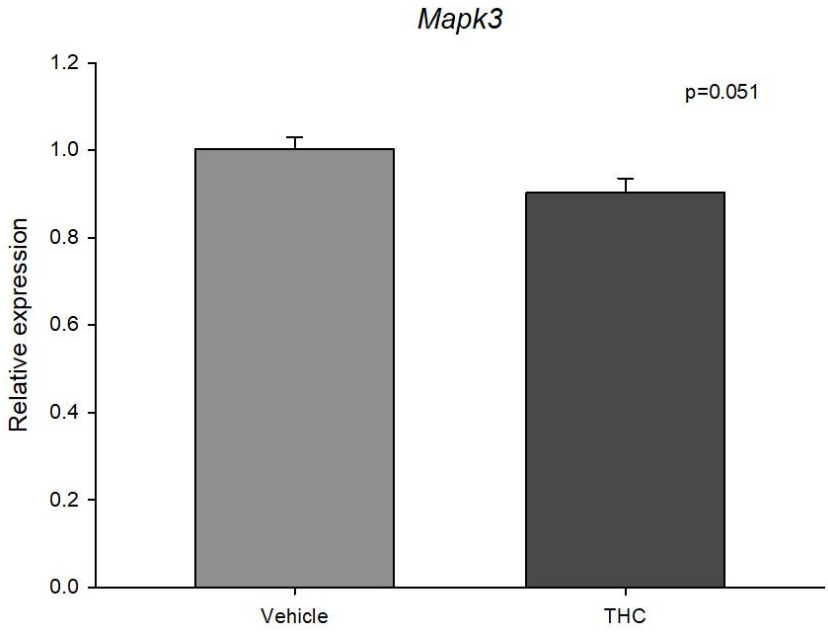

Figure 2. Relative expression of *Mapk3* in prenatally THC-exposed adult rat ovaries (Mean+SEM; *N*=5).

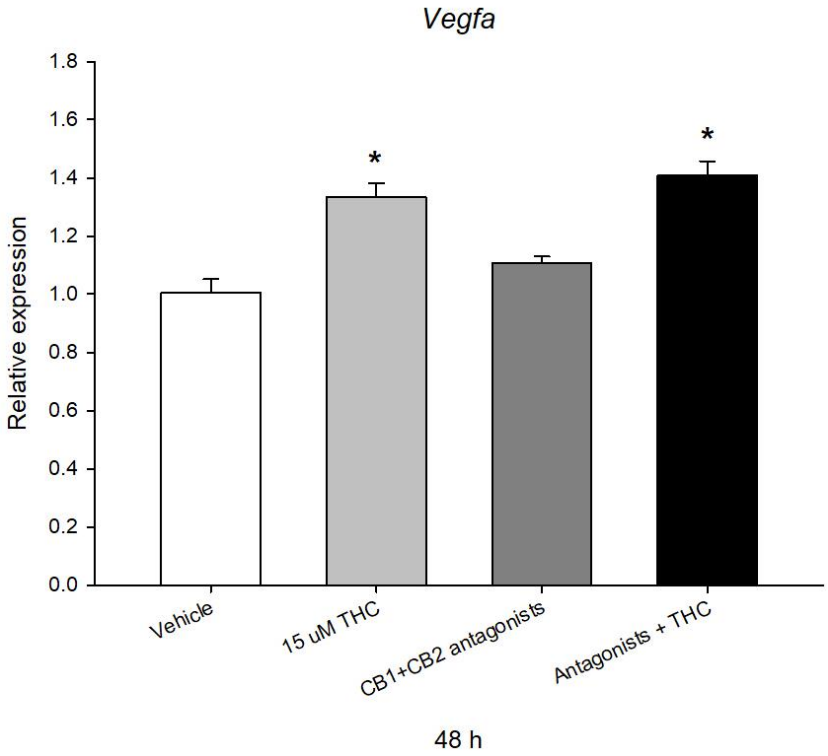

Figure 3. Relative expression of *Vegfa* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC, 1 μM Rimonabant hydrochloride and 0.1 μM SR144528 (CB1+CB2 antagonists), or all three compounds (Antagonists + THC) for 48 h (Mean+SEM; *N*=6, *p<0.05).

APPENDIX A

Chapter 2 was published in Journal of Developmental Origins of Health and disease, which does not require a license for use of content in a doctoral dissertation:

Gestational exposure to Δ9-THC impacts ovarian follicular dynamics and angiogenesis in adulthood in Wistar rats

Author: Annia A. Martínez-Peña, Kendrick Lee, James J. Petrik, Daniel B. Hardy, Alison C. Holloway
Publication: Journal of Developmental Origins of Health and Disease
Publisher: Cambridge University Press
Date: Jan 7, 2021

Copyright © © The Author(s), 2021. Published by Cambridge University Press in association with International Society for Developmental Origins of Health and Disease

License Not Required

Permission is granted at no cost for use of content in a Master's Thesis and/or Doctoral Dissertation. If you intend to distribute or sell your Master's Thesis/Doctoral Dissertation to the general public through print or website publication, please return to the previous page and select 'Republish in a Book/Journal' or 'Post on intranet/password-protected website' to complete your request.

BACK CLOSE

Chapter 3 and the review article from which sections were included in chapter 1 were published in International Journal of Molecular Sciences, an open access journal in which copyright is retained by the authors:

Copyright and Licensing

For all articles published in MDPI journals, copyright is retained by the authors. Articles are licensed under an open access Creative Commons CC BY 4.0 license, meaning that anyone may download and read the paper for free. In addition, the article may be reused and quoted provided that the original published version is cited. These conditions allow for maximum use and exposure of the work, while ensuring that the authors receive proper credit.

In exceptional circumstances articles may be licensed differently. If you have specific condition (such as one linked to funding) that does not allow this license, please mention this to the editorial office of the journal at submission. Exceptions will be granted at the discretion of the publisher.

Chapter 4 was published in Reproductive Toxicology, that establishes that the author retains the right to include the article in their thesis:

Delta-9-tetrahydrocannabinol increases vascular endothelial growth factor (VEGF) secretion through a cyclooxygenase-dependent mechanism in rat granulosa cells

Author: Annia A. Martínez-Peña,James J. Petrik,Daniel B. Hardy,Alison C. Holloway
Publication: Reproductive Toxicology
Publisher: Elsevier
Date: August 2022

© 2022 Elsevier Inc. All rights reserved.

Journal Author Rights

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: https://www.elsevier.com/about/our-business/policies/copyright#Author-rights

BACK CLOSE WINDOW