Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Use of cannabis in pregnancy; effect on placental functions

Diploma thesis

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STATEMENT OF AUTHORSHIP

I hereby declare that I am the sole author of this diploma thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution to obtain a degree.

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ABSTRAKT

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Název diplomové práce: Využití konopí v těhotenství; vliv na placentární funkce

Stále častější užívání kanabinoidů během těhotenství, zejména tetrahydrokanabinolu, hexahydrokanabinolu, kanabidiolu, kanabigerolu a kanabinolu, se stalo hlavním důvodem k obavám kvůli možným nežádoucím účinkům na zdraví plodu. Expozice konopí v prenatálním období byla spojena s kognitivními poruchami, poruchami pozornosti a paměti u vyvíjejícího se plodu, přestože mechanismy, které jsou základem těchto účinků, nejsou doposud zcela objasněny. Placenta, která je nejdůležitějším orgánem pro vývoj plodu, imunitní ochranu a regulaci prozánětlivých cytokinů, může hrát roli v souvislosti mezi užíváním konopí během těhotenství a neurovývojovými vadami plodu. Záněty během těhotenství, ať už způsobené infekcemi nebo jinými zdroji, mohou narušit funkci placenty a dále zvýšit riziko výše zmíněných poruch u dítěte. Navzdory rostoucímu počtu důkazů o dysfunkci placenty a abnormálním neurologickém vývoji plodu v důsledku prenatální expozice konopí není o jejich vzájemném vztahu mnoho známo. Cílem této studie bylo posoudit farmakologické účinky exokanabinoidů na specifické molekuly související se zánětem pomocí *ex vivo* modelu zdravých lidských placentárních explantátů. Placentární explantáty byly vystaveny působení exokanabinoidů po dobu 48 hodin a následně byly ošetřeny lipopolysacharidem (LPS), hlavní složkou buněčných stěn gramnegativních bakterií, která vyvolává akutní zánětlivou reakci. Studie analyzovala genovou expresi a hladiny prozánětlivých cytokinů IL-1β, IL-6, IL-18 a TNF-α po léčbě. Naše výsledky naznačují, že psychoaktivní i nepsychoaktivní kanabinoidy zabránily zvýšení zánětlivé reakce stimulované LPS, což naznačuje potenciální imunomodulační účinek. Očekáváme, že tato zjištění podnítí další výzkum a vyvolají další otázky ohledně bezpečnosti užívání konopí během těhotenství.

ABSTRACT

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The increasing use of cannabinoids among pregnant women, including tetrahydrocannabinol, hexahydrocannabinol, cannabidiol, cannabigerol, and cannabinol, has become a major cause for concern due to the potential adverse effects on fetal health. Prenatal cannabis exposure has been linked to cognitive, attention, and memory deficits in the developing fetus, although the mechanisms underlying these effects are not fully understood. The placenta, a vital organ for fetal development, immune protection, and regulating inflammatory cytokines, may play a role in the relationship between cannabis use during pregnancy and fetal neurodevelopmental disorders. Inflammation during pregnancy, whether from infections or other sources, can impair placental function and further increase the risk of the above-mentioned disorders in the child. Despite the growing evidence of placental dysfunction and abnormal fetal neurodevelopment due to prenatal cannabis exposure, little is known about the relationship between them. This study aimed to assess the pharmacological effects of exocannabinoids on specific inflammation-related molecules using an *ex vivo* model of healthy human placental explants. Placental explants were exposed to exocannabinoids for 48 hours, followed by treatment with lipopolysaccharide (LPS), a principal component of Gram-negative bacteria cell walls that elicits an acute inflammatory response. The study analyzed the gene expression, as well as the pro-inflammatory cytokine levels of IL-1β, IL-6, IL-18, and TNF-α following treatment. Our results suggest that both psychoactive and non-psychoactive cannabinoids prevented the increase in LPS-stimulated inflammatory response, indicating a potential immunomodulatory effect. These findings are expected to inspire further research and raise questions about the safety of cannabis use during pregnancy.

CONTENTS

1. LIST OF FIGURES

2. LIST OF ABBREVIATIONS

Abs	Absorbance
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoassay
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Canabinol
HHC	Hexahydrocannabinol
LDH	Lactate dehydrogenase
MTT	Thiazolyl blue tetrazolium bromide
OTCs	Over-the-counter products
PAMPs	Pathogen-associated molecular patterns
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Oxygen-containing reactive species
TBARS	Thiobarbituric acid-reactive substances
THC	Tetrahydrocannabinol

3. INTRODUCTION

In the realm of prenatal care, one topic that has garnered increasing attention is the use of cannabis during pregnancy and its potential impact on placental functions. Cannabis, renowned as the leading drug of abuse among pregnant women, has notably gained traction as a remedy for morning sickness due to its antiemetic properties [1]. The therapeutic potential of marijuana was reported 181 years ago [2]; however, the persistent classification of marijuana as a type 1 controlled substance limits biomedical research [3]. In recent times, the landscape surrounding marijuana has shifted dramatically, with overwhelming propaganda emphasizing its purported beneficial effects [4-6]. This surge in promotion has led to a surge in its usage among pregnant women, who are now exposed to modified varieties of marijuana containing as much as 55.7% tetrahydrocannabinol (THC), the psychoactive compound of this plant [7, 8]. Notwithstanding the potential positive health effects of marijuana [9-12] no evidence associates the increase in THC percentage with greater health improvement. Likewise, there has been a boom in the unregulated consumption of cannabidiol (CBD) -despite its not elucidated mechanism of action - due to the plethora of supposed health benefits that it possesses [13-16].

It is worth contemplating those initial studies examining the effects of marijuana during pregnancy primarily focused on plants containing THC percentages ranging from 1% to 3% [17-19] However, in light of the scarcity of research surrounding the emerging consumption methods of exocannabinoids [20] such as over-the-counter products (OTCs), edibles, shampoos, vapes, soaps, and body lotions, it becomes plausible to postulate that the pharmacological knowledge derived from these early investigations requires resizing and adjustment to account for the new concentrations and routes of administration with unknown effects on preconception, pregnancy, and fetal development.

In this intricate landscape where the ancient knowledge of cannabis meets the modern advancements in consumption methods, it becomes imperative to explore the intricate relationship between cannabis use during pregnancy and its impact on placental functions. By delving into this uncharted territory, we can uncover invaluable insights that will contribute to the holistic understanding of maternal health, prenatal care, and ultimately, the well-being of future generations.

4. BACKGROUND

4.1 A brief history of cannabis

Cannabis, an annual and dioecious herb, stands as an intriguing botanical entity renowned for its distinctive characteristics. Its leaves, possessing a palmately compound structure with serrate leaflets, contribute to its recognizable allure. Taxonomically speaking, cannabis finds its place within the family *Cannabaceae*, which encompasses a diverse array of plants. However, the precise number of cannabis species continues to be a topic of vigorous debate among experts. Currently, as depicted in Figure 1, the scientific consensus recognizes three main species: *Cannabis sativa, C. indica*, and *C. ruderalis*. Each of these species presents unique attributes and genetic profiles. Among these species, *Cannabis sativa* takes center stage, regarded as the indigenous progenitor hailing from the heart of central Asia. This plant has demonstrated an exceptional capacity for adaptation, facilitating its rapid proliferation across diverse geographical regions throughout the world. Such adaptability has allowed Cannabis sativa to thrive in various climates, altering its growth patterns, chemical composition, and overall morphology in response to environmental factors. While *C. sativa* holds its position as the primary species within the genus, its counterparts, *C. indica* and *C. ruderalis*, contribute to the rich tapestry of cannabis diversity. *C. indica*, often associated with strains that exhibit relaxing and sedating effects, has traditionally been linked to regions such as the Indian subcontinent, Afghanistan, and neighbouring areas. On the other hand, *C. ruderalis*, often regarded as the lesser-known sibling, possesses distinct characteristics such as auto flowering tendencies and a propensity for colder climates [21].

The taxonomy of cannabis is an area of continuous exploration and refinement, as scientists strive to better understand the genetic relationships and delineate the boundaries between these species. With advancements in molecular biology and genomics, researchers have delved deeper into the intricate DNA profiles of cannabis, shedding light on the phylogenetic relationships and unraveling the complex web of its evolutionary history [21].

Humans have been using cannabis for thousands of years and there is evidence of its cultivation and consumption in various regions of the world. The origins of cannabis use can be traced to Central Asia, specifically in what is now modern-day China and Mongolia. Ancient archaeological findings indicate that cannabis was cultivated as early as 4000 BCE for its fibers

and seeds, which were utilized for textiles, food, and medicinal purposes [22]. Cannabis use gradually spread across different civilizations and cultures, including the ancient Egyptians, who incorporated cannabis into their religious practices and medicinal treatments. From there, its usage expanded to other regions of the world, such as the Indian subcontinent, where it played a significant role in religious and cultural traditions. In India, cannabis, known as "ganja" or "bhang," became deeply intertwined with the Hindu religion and was associated with the worship of Lord Shiva. Cannabis was believed to have spiritual and medicinal properties, and its consumption was part of religious ceremonies and festivals. It was also used for its therapeutic effects in Ayurvedic medicine, a traditional Indian system of healing [23].

During the 1st millennium CE, cannabis use began to spread to the Middle East, Africa, and Europe through trade routes and cultural exchanges. The Islamic world, in particular, embraced the use of cannabis for medicinal purposes and recreational enjoyment [24]. In the Americas, cannabis was introduced by European explorers and colonizers. The English colonies in North America cultivated hemp, a non-psychoactive variety of cannabis, for its strong fibers, which were used in shipbuilding, textiles, and paper production. Hemp played a crucial role in the early American economy [25]. In the 19th century, cannabis started to gain attention in the Western medical community, with physicians recognizing its potential therapeutic properties. Cannabis extracts were prescribed for various ailments, including pain relief, muscle spasms, and digestive issues [26]. However, in the early 20th century, the attitudes towards cannabis began to change due to political and social factors. The increasing stigmatization of cannabis culminated in its classification as a controlled substance in the United States in the 1970s [27]. In the realm of cannabis research, understanding the taxonomic intricacies of this remarkable plant is of paramount importance. Accurate classification and identification of cannabis species not only contribute to our scientific knowledge but also hold practical implications in various fields, including agriculture, horticulture, and medicinal cannabis cultivation. Furthermore, a comprehensive understanding of cannabis taxonomy provides a solid foundation for the study of its diverse chemical constituents, pharmacological properties, and potential therapeutic applications [28].

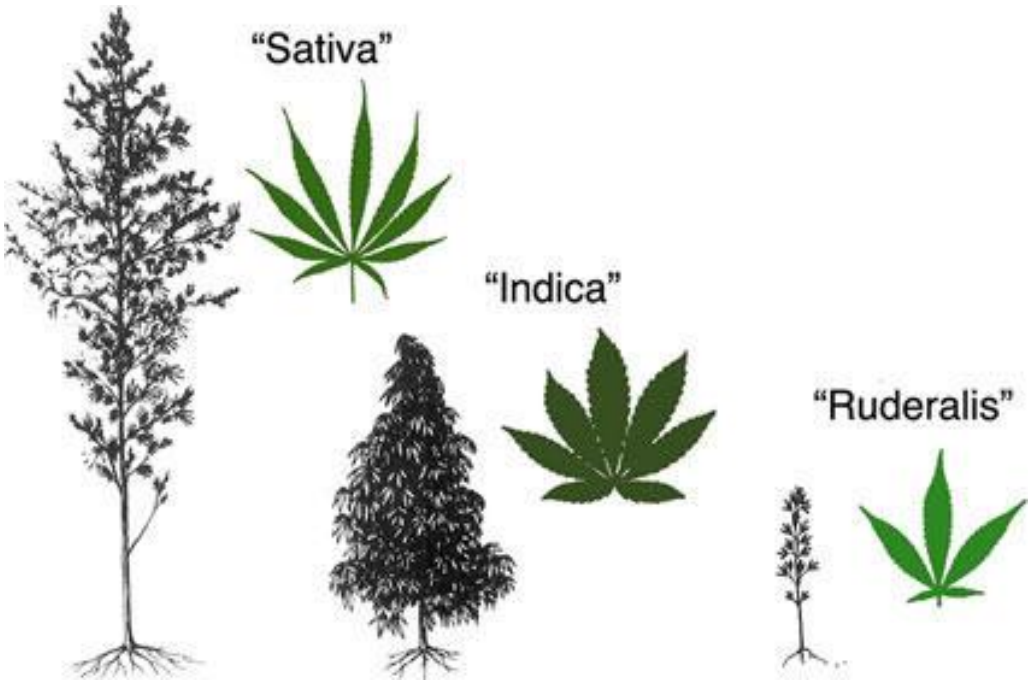

Figure 1. Macro-descriptive taxonomy of cannabis shrubs and leaves: insights from three distinct species. Adapted from McPartland, 2018 [21]).

4.2 Cannabinoids

Cannabinoids, a diverse group of compounds found abundantly within the cannabis plant, encompass a staggering array of over 500 substances, with approximately 100 of them classified as cannabinoids [29]. These remarkable molecules hold immense potential, as they possess the ability to interact with the body's endocannabinoid system, yielding both therapeutic and psychoactive effects [30]. It is within this intricate interplay that cannabinoids orchestrate their intricate symphony of actions, shaping the physiological and psychological responses within the human body. Presently, the most important cannabinoids from a medical perspective are THC and CBD; Figure 2 illustrates the chemical structures of both cannabinoids.

Among the vast array of cannabinoids, several hold particular significances due to their profound impact and intricate mechanisms of action. THC, celebrated for its psychoactive properties, takes center stage, captivating the imagination with its ability to induce euphoria and alter perception. However, it also raises controversy due to its effects on human health [31]. This cannabinoid serves as a beacon, drawing attention to the intriguing world of cannabis [32].

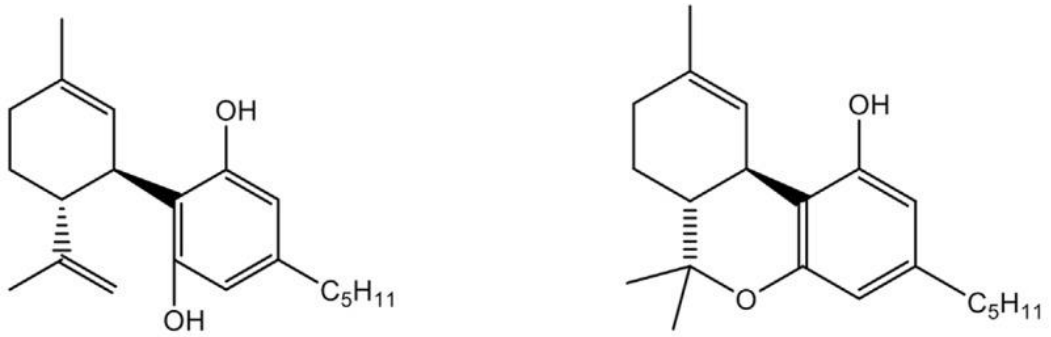

Figure 2. Chemical structures of Cannabidiol and Δ-9-Tetrahidrocannbinol. Adapted from [33].

In the realm of therapeutic potential, CBD has emerged as a star player, garnering immense attention for its remarkable non-psychoactive properties. CBD, devoid of intoxicating effects, has exhibited promise in diverse areas, ranging from its potential to alleviate pain and inflammation to its neuroprotective and anxiolytic qualities [34]. This multifaceted compound continues to captivate scientists and clinicians alike, as they unravel its intricate mechanisms and explore its broad therapeutic applications. Another striking cannabinoid, hexahydrocannabinol (HHC), contributes to the intricate tapestry of cannabis chemistry. Its distinct profile and potential therapeutic properties have sparked curiosity among researchers, as they delve into its unique effects and interactions within the endocannabinoid system [35].

Furthermore, there has been a surging interest in cannabigerol (CBG) and cannabinol (CBN) in recent research [36]. Cannabigerol (CBG), often regarded as the precursor to other cannabinoids, contributes to the intricate mosaic of cannabis chemistry. While its exact mechanisms of action and therapeutic potential are still being elucidated, preliminary research suggests that CBG may possess anti-inflammatory, neuroprotective, and antimicrobial properties. The exploration of this enigmatic cannabinoid offers a glimpse into the rich tapestry of cannabis chemistry [37]. Cannabinol (CBN), another intriguing cannabinoid, has emerged from the shadows to pique the interest of researchers. Although present in smaller quantities compared to other cannabinoids, CBN exhibits unique attributes and potential therapeutic benefits. From its sedative effects to its potential role in managing pain and inflammation, CBN adds depth and complexity to the cannabinoid landscape [38]. As the scientific community

delves deeper into the world of cannabinoids, the profound interplay between these compounds and the endocannabinoid system unfolds.

4.3 Effects of cannabinoids on human health

Cannabinoids exhibit a range of pharmacological effects, extending beyond their well-known psychotropic properties. These compounds possess diverse qualities, such as antiemetic, analgesic, anticonvulsant, antispasmodic, neuroprotective, antipsychotic, anti-asthmatic, anti-glaucomatous, immunosuppressive, and anti-inflammatory effects [39]. The search for a cannabinoid substance with beneficial pharmacological effects devoid of side effects remains a focus of intensive research. However, cannabinoids demonstrate a dualistic nature, with low and high doses potentially yielding opposite effects, rendering their utilization complex [40].

Among the numerous cannabinoids present in cannabis, THC and CBD have emerged as the most important and extensively studied compounds. Understanding their critical aspects is crucial for advancing our knowledge of cannabinoid pharmacology and harnessing their therapeutic potential [41].

THC, the primary psychoactive component of cannabis, binds to cannabinoid receptors type 1 and 2 (CB1 and CB2), mimicking the actions of endogenous cannabinoids. This interaction leads to the characteristic psychotropic effects associated with cannabis use [42]. CBD, on the other hand, modulates the endocannabinoid system in a more complex manner, influencing receptor activity and interacting with other non-cannabinoid receptors [43]. These distinct mechanisms of action contribute to the divergent pharmacological properties of THC and CBD [44].

The pharmacokinetic properties of THC and CBD play a crucial role in determining their absorption, distribution, metabolism, and elimination within the body. THC is rapidly absorbed through inhalation and oral routes, with peak plasma concentrations occurring within minutes to hours. It undergoes extensive hepatic metabolism, resulting in the formation of active metabolites. CBD, on the other hand, exhibits lower bioavailability but a longer elimination half-life [45]. Both compounds undergo metabolic transformations that impact their duration of action and potential drug interactions.

4.4 Cannabis use during pregnancy

The use of cannabis, both for recreational and medicinal purposes, has experienced a remarkable surge in recent years, captivating the attention of society at large [46]. However, this upward trajectory has sparked a cascade of concerns surrounding its potential impact on various aspects of health, particularly when it involves vulnerable populations such as pregnant women [47]. Cannabis, a complex plant, harbors numerous compounds, including cannabinoids, which have the capacity to interact with the body's endocannabinoid system, giving rise to a multitude of physiological effects [48]. It is within this intricate framework that the use of cannabis during pregnancy has emerged as a subject of growing interest and meticulous scrutiny within the medical and scientific communities.

As previously mentioned, cannabis stands as the most commonly used illicit drug during pregnancy, with the number of female cannabis users doubling over the last decade [20]. The prevalence of cannabis consumption in this population is alarming and warrants serious attention due to the inherent risks it poses. Despite the increasing legalization and normalization of cannabis use in many regions [49], the potential risks and effects of cannabis on pregnancy continue to be a topic of intense debate and extensive investigation. Pregnant women may turn to cannabis for a variety of reasons, including the management of pregnancy-related symptoms such as nausea, pain, and anxiety [50]. Additionally, some individuals may continue using cannabis recreationally without fully comprehending the potential implications for both maternal and fetal health.

One aspect that has garnered significant attention is the use of cannabis for alleviating nausea and vomiting during pregnancy. More than 90% of women have reported cannabis as being effective in combating these symptoms. However, it is important to note that certain statistics have indicated a higher risk of experiencing nausea during pregnancy when consuming cannabis prior to conception. This raises concerns about the potential influence of cannabis on the delicate equilibrium of pregnancy and the developing fetus [21].

Emerging research has suggested that exposure to cannabis in utero can lead to negative birth outcomes, including reduced birth weight, an increased risk of neurodevelopmental disorders, higher rates of prematurity, and an elevated likelihood of neonatal intensive care unit admissions [51]. Nevertheless, it is crucial to approach the interpretation of these study results

with caution, as they may be susceptible to bias. Women who utilize marijuana during pregnancy are more likely to engage in the consumption of other substances, such as tobacco, alcohol, or more potent drugs [21-23]. Therefore, disentangling the specific impact of cannabis from the potential confounding effects of co-occurring substance use becomes a challenging task.

Amidst this intricate landscape of cannabis use during pregnancy, it is vital to recognize the need for comprehensive research that takes into account the multifaceted factors at play [52]. By conducting rigorous studies that employ robust methodologies, it becomes possible to disentangle the specific effects of cannabis on maternal and fetal health. Additionally, understanding the intricate interplay between cannabis use and the consumption of other substances during pregnancy is crucial for developing comprehensive interventions and guidelines to ensure the well-being of both mothers and their offspring.

4.5 The placenta

The placenta, a remarkable organ that emerges during pregnancy, plays a crucial role in fetal growth and development. It arises during embryo implantation within the maternal uterus, orchestrating the intricate interplay between the maternal and fetal systems [53]. With its diverse anatomical features [54] and physiological functions, the placenta ensures the supply of vital nutrients, oxygen, and molecules while safeguarding the developing fetus [55]. It acts as a selective barrier, allowing passage of essential substances while blocking harmful ones [56]. Additionally, the placenta produces key hormones and possesses immunological properties, protecting the fetus and supporting maternal well-being [57]. Understanding the placenta's intricacies is vital for diagnosing and managing pathologies, optimizing maternal-fetal health, and preventing adverse outcomes.

Figure 3 depicts the intricately structured morphology of the placenta, a design elegantly crafted to enhance its functions throughout pregnancy. The placenta is a discoid organ that weighs approximately 500g. It is about 20 cm large and 3 cm wide [58]. The fetus is connected to the placenta through the umbilical cord (*umbilicus*); there are two umbilical arteries (*arteria umbilicalis*) and one umbilical vein (*vena umbilicalis*). The umbilical arteries drain deoxygenated blood and waste materials from the fetus. In turn, the umbilical vein brings

oxygenated blood and nutrients through the placenta to the fetus [59]. The placenta consists of two main components: the fetal portion, known as the chorionic villi, and the maternal part, referred to as the decidua. The chorionic villi, characterized by numerous branching structures, are the primary sites of nutrient and gas exchange between the maternal and fetal circulations. These villi are surrounded by a network of blood vessels that facilitate the transfer of oxygen and nutrients from the maternal bloodstream to the developing fetus [60].

The decidua, on the other hand, refers to the specialized lining of the uterus that undergoes significant changes to support the establishment and maintenance of the placenta. It provides a rich vascular network to supply the developing placenta with oxygen and nutrients [61]. The intricate connection between the decidua and the chorionic villi ensures a proper anchorage and vascularization of the placenta, allowing for efficient exchange of substances between the maternal and fetal systems [62].

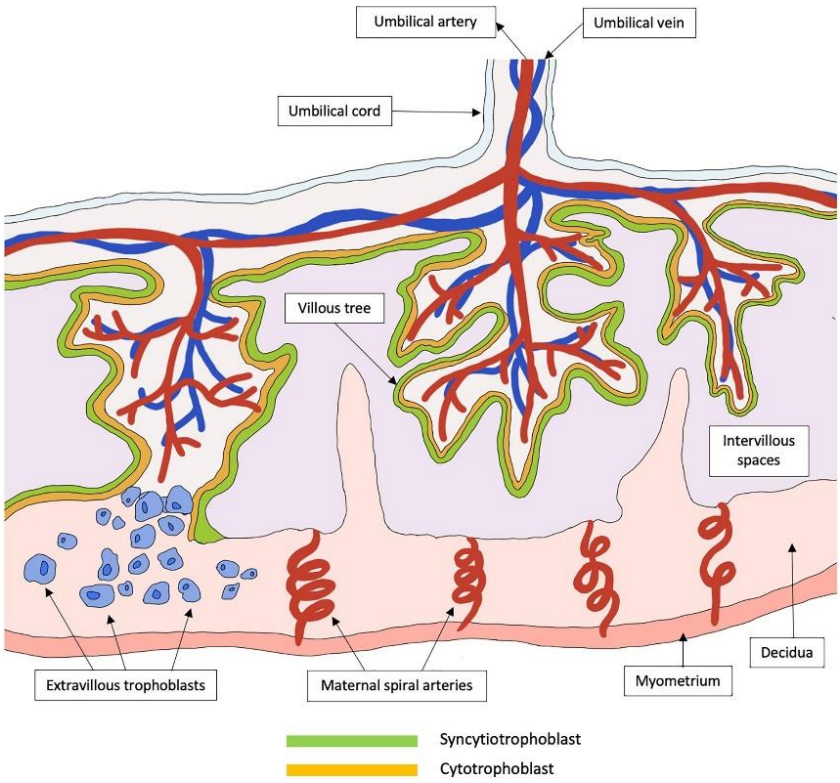

Figure 3. Schematic depiction of the placenta. Adopted from [63].

4.6 Pregnancy and inflammation

Inflammation plays a vital role during pregnancy, acting as a defensive immune response against pathogens and injury [64]. Imbalances in immune regulation can have significant implications for fetal development [65]. Excessive inflammation can disrupt the functioning of the placenta, compromising the supply of vital nutrients and oxygen to the growing fetus, thereby leading to growth restrictions and developmental abnormalities [66]. Moreover, inflammatory mediators can directly impact fetal tissues, perturbing the normal process of organogenesis and giving rise to long-term health consequences [67].

Uncontrolled inflammation during pregnancy has been associated with a range of complications, including preterm birth, preeclampsia, gestational diabetes, and fetal loss [68]. These inflammatory disruptions can disturb the delicate balance of hormones that are essential for maintaining a healthy pregnancy, consequently resulting in adverse outcomes. Furthermore, chronic inflammation can contribute to maternal systemic conditions, such as cardiovascular disease and metabolic disorders, which can have lasting effects on both the mother and the child [69].

It is crucial to recognize that inflammation is a natural and tightly Th1/Th2 regulated process throughout pregnancy. Figure 4 illustrates that a healthy pregnancy involves a sequence of inflammatory events: inflammation during implantation, anti-inflammation throughout gestation, and another phase of inflammation during parturition. From the onset of pregnancy, an orchestrated inflammatory response is necessary for successful implantation and the establishment of the maternal-fetal interface [70]. Disturbances in these intricate processes can have detrimental effects on fetal development and maternal well-being.

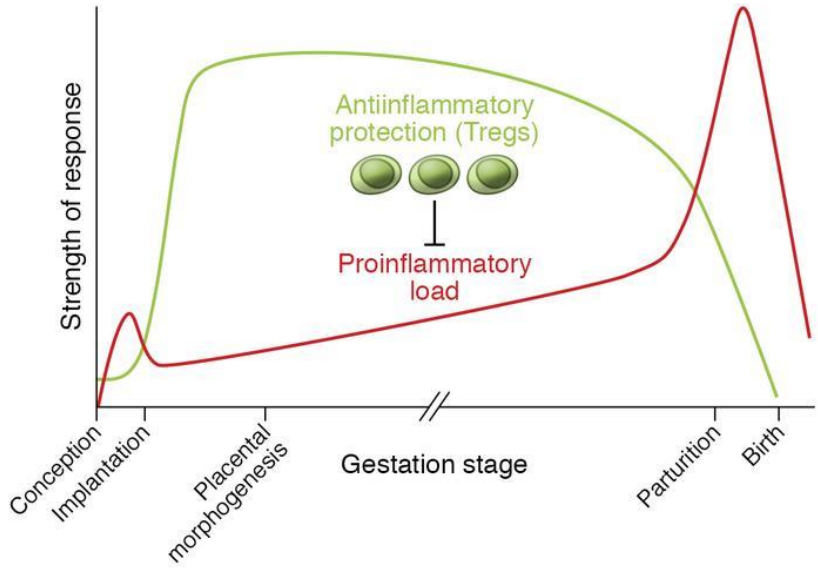

Figure 4. The natural inflammatory processes during pregnancy (adopted from [71]). The red line represents the initial inflammatory response required for successful conception and implantation. This process is then carefully regulated by T cell regulators (Tregs – green line) to maintain a balanced immune environment. Finally, a controlled spike in inflammatory actions is necessary for proper parturition, ensuring a safe and successful delivery.

4.7 Cannabis and inflammation

Cannabinoids have the potential to interact with the immune system, potentially disrupting the delicate balance of inflammation regulation during pregnancy. However, the specific mechanisms by which cannabis and its constituents modulate inflammation in the context of pregnancy are not yet fully understood. It is crucial to investigate how cannabis use may disrupt the intricate interactions between cannabinoids and the immune system, potentially leading to imbalances in inflammation regulation.

One particular concern is the use of cannabis by pregnant women to alleviate symptoms such as nausea. Nevertheless, the impact of cannabis on inflammation homeostasis during pregnancy remains largely unexplored. The complex interplay between cannabinoids and the immune system raises significant questions regarding how cannabis use may disturb the delicate balance of inflammation regulation during this critical period. To fully comprehend the potential consequences and ensure the optimal health of both mother and fetus, further scientific investigation is warranted.

Comprehending the implications of cannabis use on inflammation during pregnancy is of utmost importance in safeguarding the well-being of both the mother and fetus. Extensive scientific research is needed to fully grasp the potential consequences. Rigorous studies should examine the effects of cannabis on inflammation markers, immune cell function, and overall pregnancy outcomes. By addressing the existing knowledge gaps, evidence-based guidelines and recommendations can be established to support pregnant women in making informed decisions about cannabis use, carefully considering the potential risks and benefits for both maternal and fetal health.

4.8 The inflammasome and pregnancy

The inflammasome is an essential multiprotein complex of the innate immune system and plays a crucial role in mediating the inflammatory response during pregnancy. The NLRP3 inflammasome, a unique constituent among inflammasomes, responds to diverse stimuli, including DAMPs (Damage-associated molecular patterns), PAMPs (Pathogen-associated molecular patterns), and bacterial toxins. Comprised of NLRP, ASC (apoptosis-associated speck-like protein with a caspase recruitment domain), and caspase-1 (the IL-1β-converting enzyme), activation of NLRP3 induces the formation of ASC prion-like filaments through PYD-PYD interactions. Linear ubiquitination of ASC enables assembly, while caspase-1 generates its own prion-like filaments via CARD-CARD interactions. Pro-IL-1β and pro-IL-18 are cleaved by caspase-1, leading to the release of active IL-1β and IL-18. Active caspase-1 also initiates pyroptosis, an inflammatory lytic cell death, through the cleavage of GSDMD [72]. Its presence and functionality have been demonstrated in the human placenta [73].

Inflammasome activation involves both transcriptional and post-transcriptional processes, as illustrated in figure 5. NLRP3, moderately expressed in various cell types, necessitates a "signal I" for activation [74]. This signal can originate from microbial ligands, cytokines, or reactive oxygen species (ROS). For example, LPS stimulates NF-κB, resulting in the upregulation of NRLP3 and IL-1β mRNA [75]. Deubiquitination of NLRP3 expedites rapid induction of the NLRP3 inflammasome, enabling the transcription of pro-IL-1β and subsequent cleavage [76]. Notably, cathepsin B release from lysosomes, prompted by endogenous DAMPs, plays a pivotal role in NLRP3 inflammasome activation [77]. Furthermore, cellular and mitochondrial ROS also serve as activators of the NLRP3 inflammasome [78].

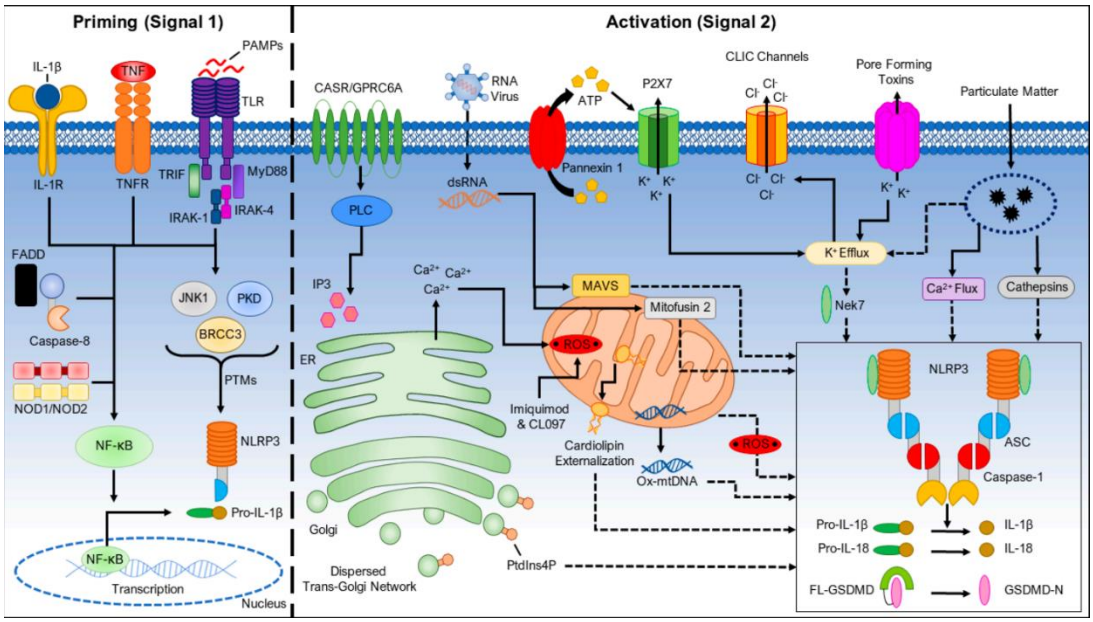

Figure 5. Schematic representation outlining the key mechanisms associated with the activation of the NLRP3 inflammasome. Adopted from [75].

The inflammasome activation is tightly regulated to ensure an appropriate immune response without causing excessive inflammation [79]. Inflammation is a normal and necessary process during pregnancy, contributing to successful implantation, placental development, and immune tolerance [80]. However, dysregulation of the inflammasome can lead to detrimental outcomes, including pregnancy complications and fetal developmental abnormalities [81, 82]. The use of cannabis during pregnancy has raised concerns regarding its impact on the immune function [83]. Cannabinoids present in cannabis, such as THC, can modulate the inflammasome activation. Studies suggest that THC can either enhance or suppress inflammasome activity depending on the context and cell type. This modulation of inflammasome function by cannabis may have implications for pregnancy outcomes [84]. However, the precise mechanisms by which cannabis affects the inflammasome during pregnancy are not yet fully understood. Further research is needed to elucidate the complex interactions between cannabinoids and the inflammasome, as well as their consequences for maternal and fetal health.

5. AIM OF STUDY

The aim of this study is to investigate the impact of psychoactive THC and non-psychoactive CBD cannabinoids on the inflammasome and key antioxidant enzymes in the human placenta, utilizing a bacterial inflammation model. The research aims to elucidate the modulatory capacity of these cannabinoids on the inflammatory response and oxidative stress in placental tissue. This study seeks to contribute to the existing pharmacological and toxicological understanding of these molecules, which have gained popularity as potential therapeutic agents. The findings from this research project will enhance our knowledge regarding the biological effects of cannabinoids and their potential implications for placental health.

6. EXPERIMENTAL METHODS

6.1 Human placental samples

The study was conducted in accordance with the principles outlined in the Declaration of Helsinki by the World Medical Association [85]. Written informed consent was obtained from pregnant women who underwent elective caesarean section delivery at the University Hospital in Hradec Kralove, Czech Republic. The collection of human placenta samples was limited to healthy individuals within the gestational age range of 38 to 40 weeks. Prior to the commencement of the study, ethical approval for the research protocol (201006 S15P) was obtained from the University Hospital Research Ethics Committee, ensuring strict adherence to ethical standards and the protection of human subjects. These stringent measures were implemented to guarantee the ethical integrity of our investigation and the well-being of the participants involved.

6.2 Cultivation of human placenta term explants

Human placenta cotyledons were carefully separated using mechanical detachment techniques to exclude the chorionic plate and decidua, following established protocols. Villous tissue samples, approximately 0.5 cm x 0.5 cm in size, were dissected into 30 mg explants using a

randomized sampling approach, ensuring the removal of large vessels and blood clots. The explants were then rinsed with sterile saline solution and placed in 12-well plates. For cultivation, a culture medium consisting of 2 ml of DMEM-F12 medium supplemented with 10% fetal bovine serum and a combination of antibiotics (penicillin: 100 U/ml, streptomycin: 0.1 mg/ml, and amphotericin B: 2.5 µg/ml) was used. Each well contained three explants, resulting in an approximate total mass of 100 mg per well. The cultures were incubated at 37°C in a sterile environment with oxygen levels maintained at 8%, carbon dioxide at 5%, and nitrogen at 87% for 18 to 24 hours. This incubation period aimed to establish equilibrium and facilitate recovery from the isolation process. After equilibration, the explants were treated with varying concentrations of CBD (0.1-2.5-5-10-20-40 µg/ml) for 48 hours. Medium and treatment replenishment were performed at the 24-hour mark. Following the 48-hour incubation, inflammation was induced by supplementing fresh media with the respective cannabinoid treatment and 1 µg/ml of LPS for 4 hours (Abad *et al* in press). Subsequently, the tissue was processed to prepare homogenates, and RNA isolation was carried out for subsequent analyses. The supernatant obtained from the process was collected and stored at -80°C for future use.

6.3 Assessment of viability of placenta explants exposed to CBD or THC

The viability of the placenta explants was evaluated by two strategies to determine the metabolic function and the integrity of the plasmatic membrane. Metabolic viability was determined after 48 treatment, by measuring the MTT (thiazolyl blue tetrazolium bromide) reduction assay after cellular incorporation [86]. Figure 6 provides a schematic representation of this methodology. This method allows for corroborating the proper function of mitochondria. Briefly, treated placenta explants were washed with Opti-MEM™ and incubated with 0.5 mg/ml of MTT solution at 37 ° C for 1 h, the tissue was transferred to a new well containing 1 ml of DMSO and incubated for 5 minutes with shaking at 25 ºC. Formazan production was measured in the supernatant at 570 and 690 nm wavelengths. The results are expressed as the difference between Abs 570 and Abs 690 / g of tissue.

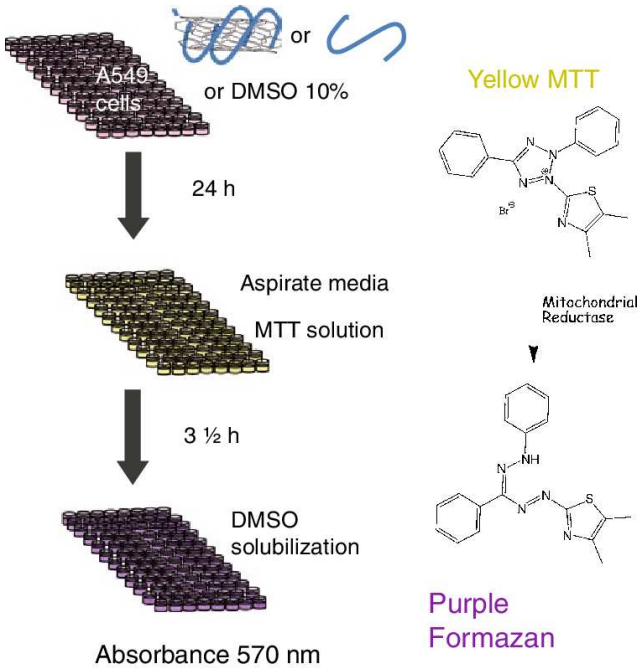

Figure 6. MTT methodology diagram. Adopted from [87].

The plasma membrane integrity of explants was assessed using a Sigma-Aldrich colorimetric LDH activity assay kit (St. Louis, MO, USA) [88], as illustrated in Figure 7. LDH enzymatic activity was standardized to milligrams of explants. Results are expressed as nanomoles of NADH/ ml*min/mg tissue. Quantification of the maximum amount of LDH release to the media was carried out by culture explants with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 12.7 mM EDTA, 1 mM EGTA, 4 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100 and protease inhibitor cocktail, pH 6.8) for 15 min at 37 °C as a positive control.

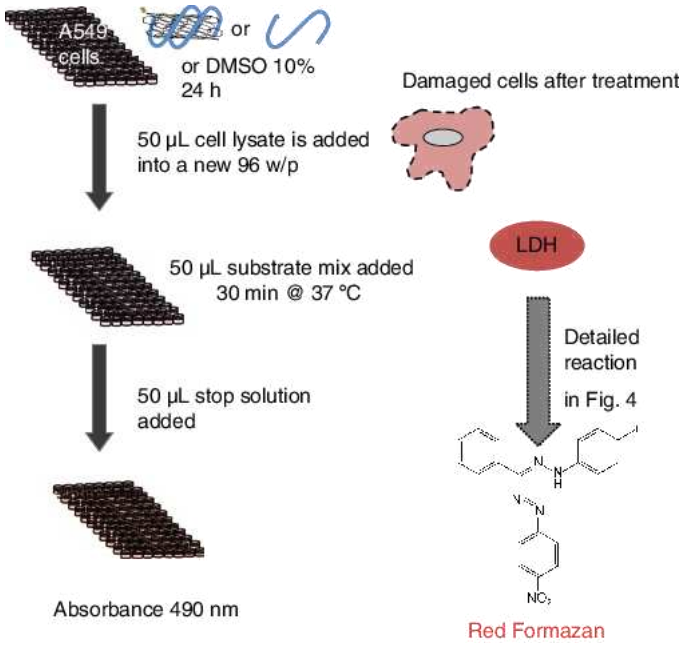

Figure 7. Methodological scheme for the LDH assay to determine viability based on plasma membrane integrity. Adopted from [87].

6.4 RNA isolation and cDNA generation

RNA isolation was conducted following standardized protocols established in our laboratory [89]. A summary of the method is represented in figure 8 and figure 9. Briefly, total RNA was isolated from tissue samples using TriReagent™ solution, according to the manufacturer's instructions. The purity of RNA was assessed by measuring the A260/A280 ratio, while the A260/230 ratio was used to evaluate potential contamination by organic solvents. Absorbance ratios were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the total RNA concentration was calculated based on absorbance at A260. The integrity of the RNA was confirmed by electrophoresis on a 1.5% agarose gel. Reverse transcription (RT) was performed using the iScript Advanced cDNA Synthesis Kit and T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA).

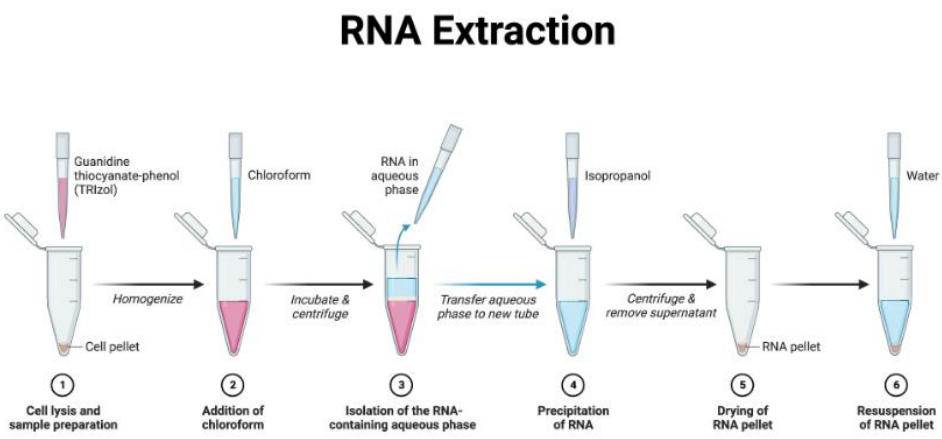

Figure 8. Schematic representation of RNA isolation from homogenized term human placenta samples. The figure was taken from Biorender's bank of templates.

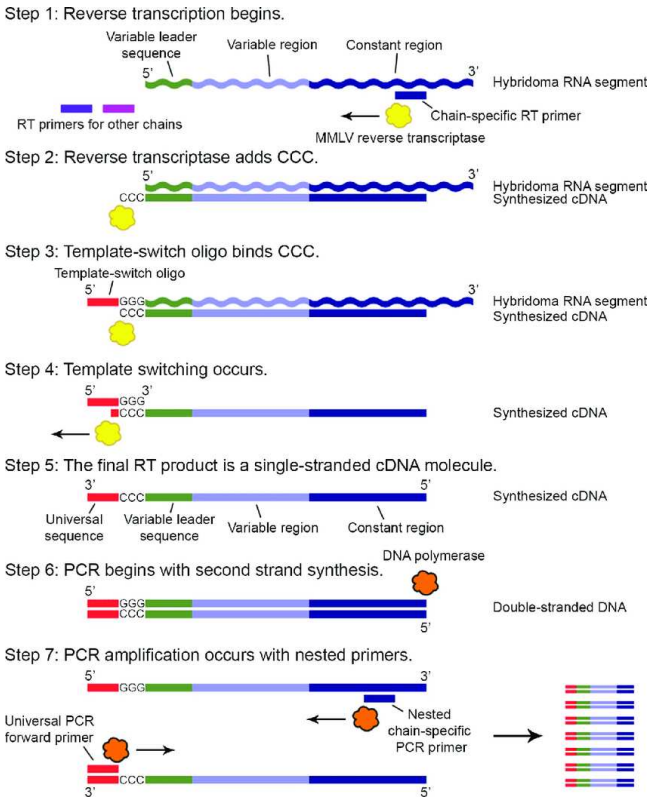

Figure 9. Illustrative scheme for the synthesis of cDNA. Adapted from [90].

6.5 Quantitative PCR

Quantitative gene expression (qPCR) was performed using QuantStudio TM 6 (Thermo Fisher Scientific, Waltham, MA, USA) Figure 10 presents a didactic representation of the method. Briefly, cDNA (12.5 ng/µl) was amplified using TaqMan® Universal Master Mix II without UNG (Thermo Fisher Scientific, Waltham, MA, USA) in 5 µL/well final volume with predesigned TaqMan® Real-Time Expression PCR assays, following 'manufacturer's instructions. Relative gene expressions were normalized to the geometric mean of Ubiquitin *(UBC)*, DNA topoisomerase I (*TOP1*), Eukaryotic translation initiating factor 4A2 *(EIF4A2)*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), and β2 microglobulin (*B2M*) as reference genes. These values were used to generate a heat map of gene expression, using the freely available Web server Heatmapper (http://www.heatmapper.ca/).

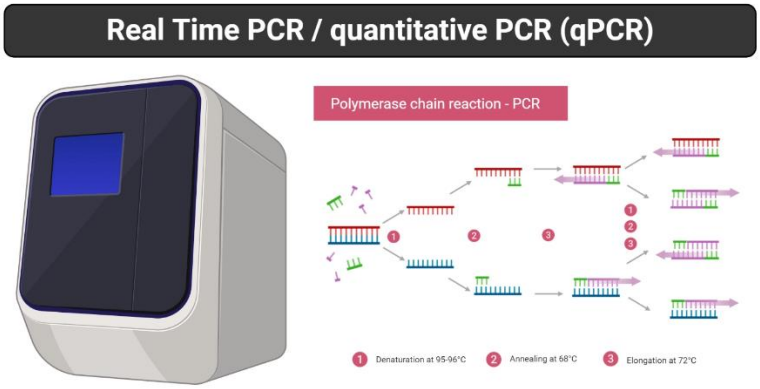

Figure 10. Illustrating the qPCR amplification process. The figure was taken from Biorender's bank of templates.

6.6 Cytokine quantification by indirect ELISA

The cytokine concentrations were determined using the methodology previously published by our laboratory [91]. Pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-18 were quantified in cell-free culture media using Thermo Fisher Scientific ELISA kits, following the manufacturer's instructions. Cytokine concentrations were normalized to tissue weight. Figure 11 displays an artistic illustration of the ELISA methodology.

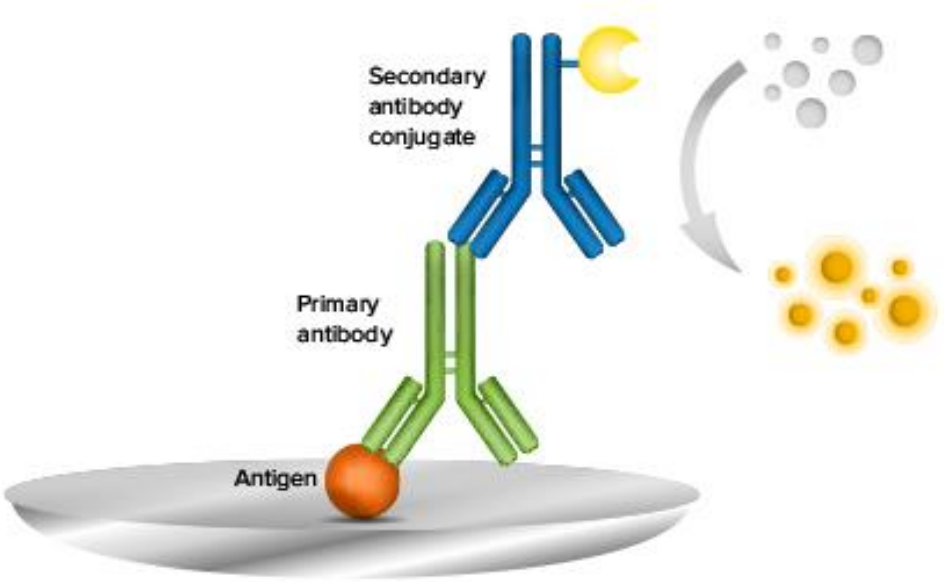

Figure 11. Schematic picture of indirect ELISA for quantifying pro-inflammatory cytokine concentration. The figure was taken from Biorender's bank of templates.

6.7 Lipid peroxidation measurements

Lipid peroxidation, reflecting plasma membrane damage, was assessed by quantifying thiobarbituric acid-reactive substances (TBARS). The TBARS assay followed the protocol outlined by Feix *et al* [92]. Results are presented as nmoles of malondialdehyde per mg of protein. Figure 12 illustrates the chemical foundation of the method and the colorimetric visualization process.

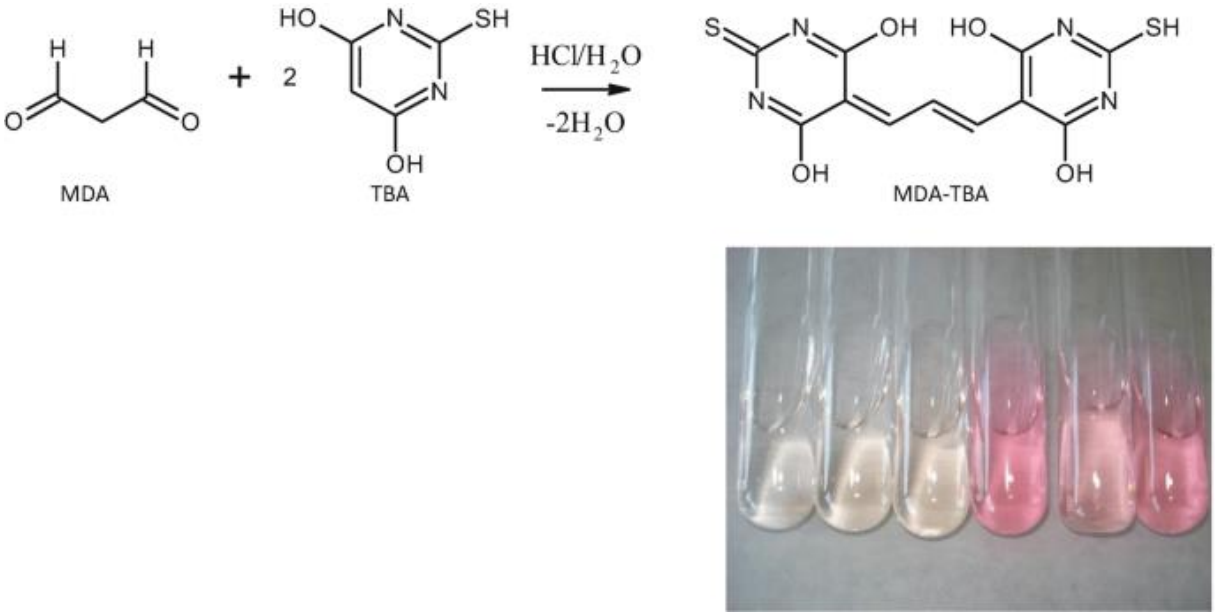

Figure 12. Assessing Lipid Peroxidation with the TBAR Assay. Lipid peroxidation was measured indirectly using the TBAR assay. The figure shows the method's fundamentals. An intermediary MDA-TBA species indicates peroxidation status. In the inset, media turns pink in the presence of lipid peroxidation, while the control remains colorless. Adopted from [93].

6.8 Statistical analysis

Statistical evaluations were performed utilizing non-parametric tests, including the Mann-Whitney test for pairwise comparisons between two groups, and the Kruskal-Wallis test followed by Dunn's multiple comparisons test for comparisons involving more than two groups. The statistical analyses were carried out using GraphPad Prism 8.3.1 software (GraphPad Software, Inc.). In the figures, significance levels were represented by asterisks, with * indicating $p \leq 0.05$, ** indicating $p \leq 0.01$, and *** indicating $p \leq 0.001$.

7. RESULTS

7.1 Assessing placental explant viability: effects of CBD and THC

In our toxicological study investigating the effects of cannabis on placental explants, we utilized the MTT viability assays to assess mitochondrial health and the LDH assay to evaluate plasma membrane integrity. Our results indicate (figure 13) that neither CBD nor THC significantly affected mitochondrial function or plasma membrane integrity at the tested concentrations. Additionally, we conducted toxicity tests on the solvent (ethanol) and found no detrimental effects.

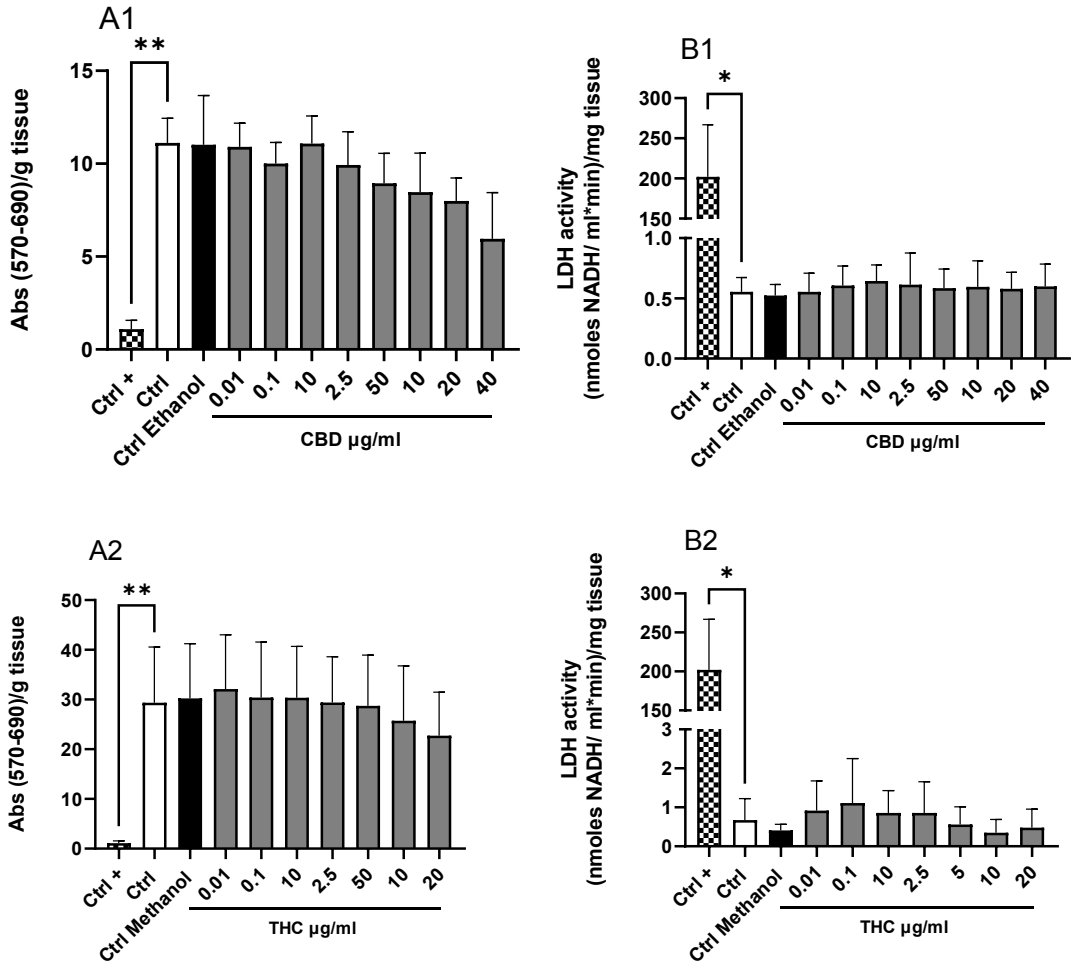

Figure 13. Viability assessment of human placenta explants treated with CBD or THC. The metabolic functionality of human explants was determined using the MTT assay (A1 CBD – A2 THC), while membrane integrity was evaluated using the LDH assay (B1 CBD – B2 THC). Data are presented as the median ± SD; n = 3. Statistical significance was analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$)

7.2 Effects of CBD on pro-inflammatory cytokines, inflammasome, gene, and protein expression in inflammatory challenged human placental explants

The aim of this study is to explore the immunomodulatory potential of psychoactive and non-psychoactive cannabinoids in the human placenta. CBD concentrations were carefully selected based on existing literature, ensuring no adverse effects on viability tests. A 48-hour treatment with CBD was conducted, with medium replacement every 24 hours following the described methodology. Subsequently, the medium was replaced, and a challenge with 1μg/ml LPS was introduced in the presence of CBD.

Figure 14 unveils the remarkable impact of CBD on the normalized gene expression of pro-inflammatory cytokines (IL-1β, IL-18, IL-6, TNF-α) and crucial inflammasome proteins (NLRP-3 and Caspase-1). Notably, a consistent downregulation pattern emerges across the entire spectrum of proteins examined, regardless of CBD concentration. The significance of these findings is accentuated at higher CBD concentrations, underlining the profound regulatory potential of this compound.

Figure 15 illustrates the quantification of pro-inflammatory cytokine protein levels using the enzyme-linked immunosorbent assay (ELISA) technique. Notably, treatment with CBD demonstrates a significant impact on the acute inflammatory response required to counter lipopolysaccharide (LPS) stimulation, as observed across all examined cytokines. This underscores the remarkable immunomodulatory potential of a 48-hour CBD treatment, effectively influencing both the genetic and protein aspects of the immune system.

Although the specific effects of CBD treatment on inflammasome proteins (NLRP3 and Caspase-1) are not presented in this study due to ongoing method standardization, it is important to note the crucial role of the inflammasome in the maturation of IL-1β and IL-18. Based on the observed results for these cytokines, it can be inferred that the enzymatic activity of this intricate macromolecular complex is diminished by the CBD treatment, further supporting its immunomodulatory properties.

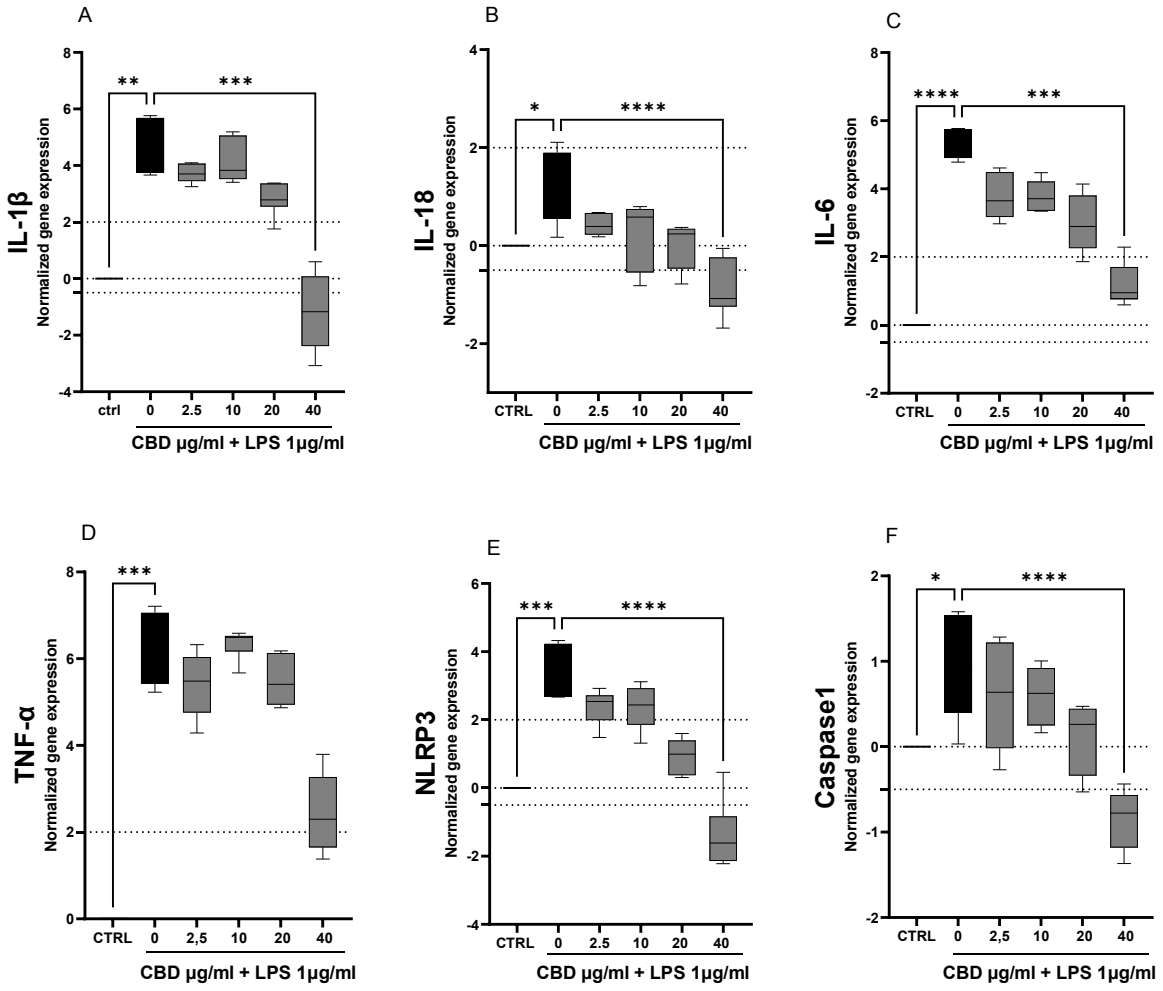

Figure 14. CBD effects on gene expression analysis of pro-inflammatory cytokines and inflammasome proteins in human placental explants. CBD treatment was administered for 48 hours, followed by LPS-induced inflammation for 4 hours in the presence of CBD. qPCR assessed the expression levels of IL-1β (A), IL-18 (B), IL-6 (C), TNF-α (D), NLRP3 (E), and caspase-1 (F). Relative expression was normalized to EIF4A2, TBP, TOP1, and UBC, and compared to control inflammation using standard culture media. Data are presented as Tukey boxplots (1.5-times IQR); n = 6. Statistical significance was evaluated using the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test; * ($p \leq 0.05$), ** ($p \leq 0.01$), ***($p \leq 0.001$) and ****($p \leq 0.0001$).

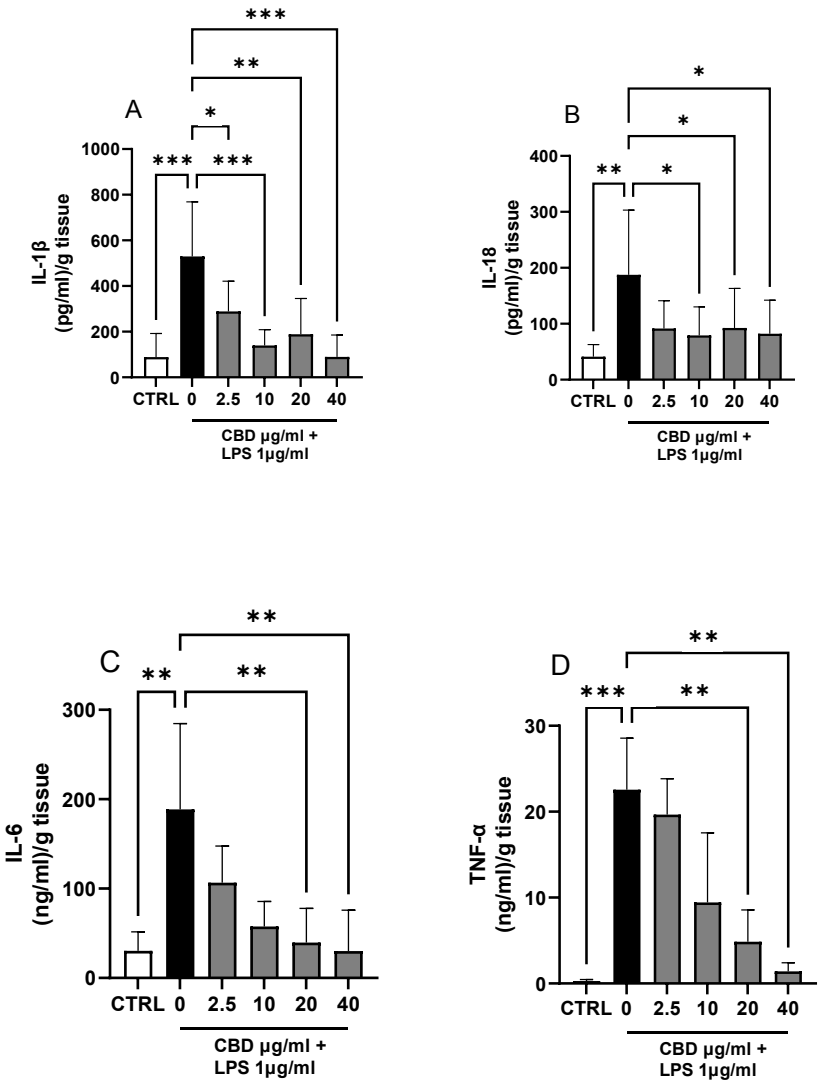

Figure 15. Protein levels of pro-inflammatory cytokines in human term placenta explants treated with CBD and exposed to LPS. The levels of IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D) released into the culture media were measured using ELISA. Villous explants were cultured with CBD for 48 hours, followed by a 4-hour exposure to LPS. Cytokine concentrations in the conditioned media were adjusted for the wet weight of the explant tissue. Data are presented as the median ± SD; n = 6. Statistical significance was analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$).

7.3 Effect of THC treatment protein expression of pro-inflammatory cytokines on human placental explants exposed to inflammatory challenge

In a similar manner, placental explants were treated with THC for 48 hours, followed by a 4-hour challenge with LPS. The supernatant of these explants was assessed using ELISA to quantify pro-inflammatory cytokine protein levels, as described in the materials and methods section. Figure 16 illustrates a substantial decrease in the expression of IL-1β and IL-18, both known to be activated by the inflammasome, compared to the control conditions of experimental inflammation. Notably, a higher THC concentration is required to impact IL-1β activity. This reduction in IL-1β and IL-18 production suggests a potential modulation of the inflammasome's capacity for cytokine maturation by THC treatment. Moreover, the pro-inflammatory cytokines IL-6 and TNF-α show a significant decline in protein expression, particularly at the higher THC concentration of 20 μg/ml. Collectively, these findings indicate that THC primarily affects cytokines dependent on inflammasome signaling.

It is important to highlight that marijuana is classified as a Schedule I substance under the Controlled Substances Act. As a result, the acquisition of this product experienced significant delays, and therefore, only preliminary results ($n \geq 3$) are presented for protein level determination. Assessments of the genetic expression of these cytokines will be conducted in future studies by other members of our laboratory.

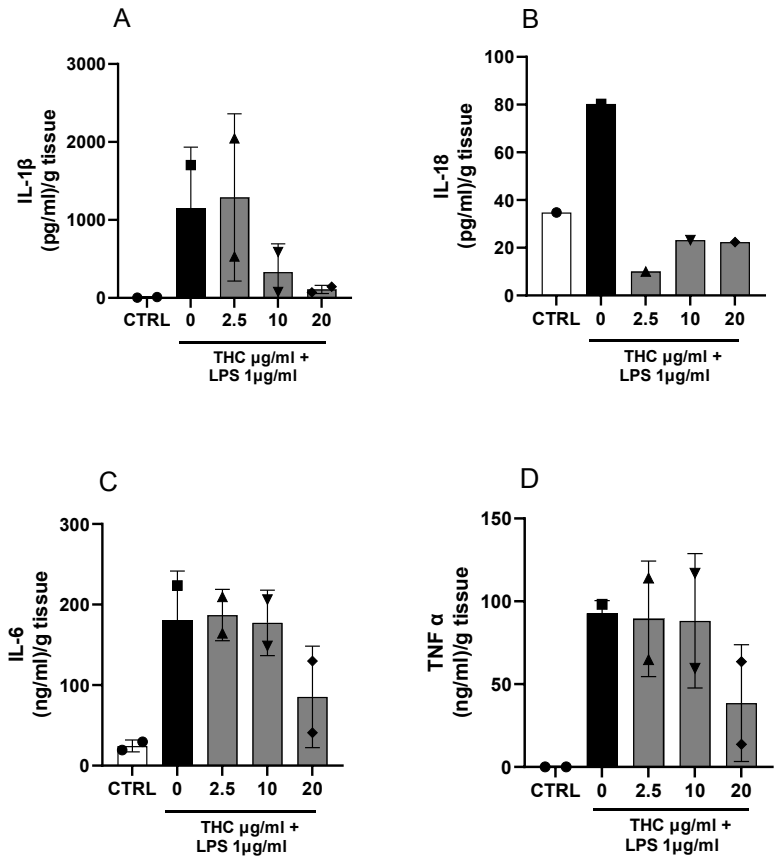

Figure 16. Protein levels of pro-inflammatory cytokines in human term placenta explants treated with THC and exposed to LPS. The levels of IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D) released into the culture media were measured using ELISA. Villous explants were cultured with THC for 48 hours, followed by a 4-hour exposure to LPS. Cytokine concentrations in the conditioned media were adjusted for the wet weight of the explant tissue. Limited sample size precludes statistical analysis: Preliminary findings from a small-scale study ($n \geq 2$).

7.4 Effect of CBD and THC on lipid peroxidation in placental explants exposed to inflammatory challenge

Oxidative stress serves as a crucial defense mechanism against bacterial infections and activates the inflammasome. Considering the reported antioxidant capacity of cannabis, we investigated the potential of CBD and THC in modulating oxidative stress and inflammasome activity. Placental explants were treated with CBD or THC and exposed to a 4-hour inflammatory challenge using 1 μg/ml LPS. Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS) in the tissue, expressed as nmol of malondialdehyde (MDA) per mg of tissue.

Figure 17 (A-CBD, B-THC) illustrates the robust antioxidant properties of both cannabinoids. They effectively reduced oxidative stress levels, surpassing the reductions induced by LPS alone. CBD exhibited a remarkable decrease of up to 90%, while THC showed a significant reduction of up to 84% compared to the control condition without inflammatory stimuli. These findings highlight the capacity of CBD and THC to modulate oxidative stress.

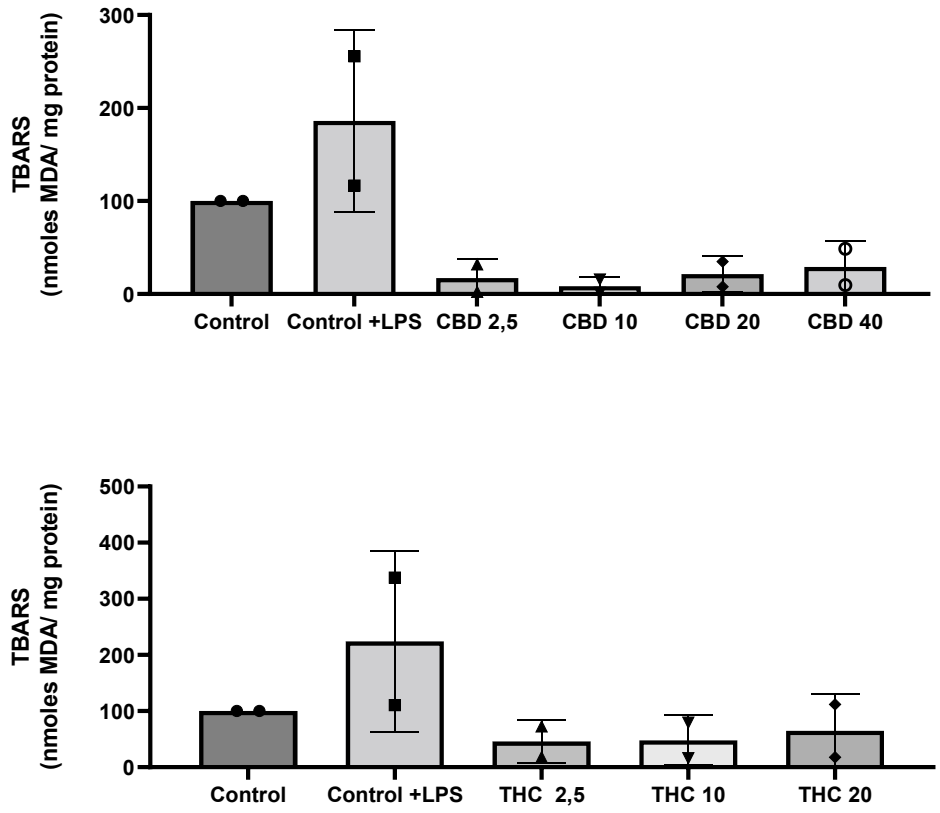

Figure 17. Evaluation of lipid peroxidation of human placenta explants treated with cannabinoids and exposed to LPS. Measurement of thiobarbituric acid reactive substances (TBARS) in placental explant homogenates. Placental explants were cultured for 48 hours with or without varying concentrations of THC, followed by a 4-hour induction of inflammation with LPS. TBARS levels were determined according to the described method. Limited sample size precludes statistical analysis: Preliminary findings from a small-scale study (n=2).

8. DISCUSSION

The relaxation of punitive controls on marijuana and its portrayal as a panacea have led to concerns [94]. Of specific concern is the rising trend of cannabis use during pregnancy to alleviate nausea and vomiting symptoms, despite extensive literature demonstrating its detrimental effects on fetal development and long-term consequences into adulthood [95, 96]. Recognizing the allegedly immunomodulatory properties of cannabis and the importance of the natural immune response during pregnancy, our study aimed to examine the influence of CBD and THC on the placental inflammasome and inflammatory response using term human placenta explants.

The toxicological assessment of marijuana poses challenges due to its recreational and therapeutic use. Despite the unattainable LD50 of 1270 mg/kg for casual or regular users, the misconception persists that cannabis is harmless. To address this, we carefully selected CBD and THC concentrations within the reported literature ranges. Our objective was to examine the effects of these concentrations on explant viability. In Figure 13, both CBD and THC demonstrated no detrimental impact on the metabolic function or plasma membrane integrity of the explants. Our viability results demonstrate that explants exhibit greater tolerance to high concentrations of cannabinoids compared to trophoblast lamellar cultures [97], which can be attributed to the distinct 3D topological conformation of the explants [98]. These viability tests confirm the safety of our chosen cannabinoid concentration range, enabling us to proceed with subsequent methodological strategies.

The pivotal role of inflammation in pregnancy and its impact on the fetoplacental unit is well-established. LPS has been shown to elevate levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β in various placental models [91, 99]. A significant decrease in all examined pro-inflammatory cytokines was observed, evident at both the gene and protein levels following CBD and THC treatments. Recent literature highlights CBD's ability to downregulate pro-inflammatory cytokines in various biological models, while THC's anti-inflammatory effects are reported to rely on CBD as a co-component [100]. Our findings confirm the anti-inflammatory efficacy of CBD. Notably, we also report the anti-inflammatory activity of THC without the need for CBD as a co-component. Moreover, our findings suggest that a mere 48-hour treatment can induce significant changes in gene and protein expression, aligning with reports highlighting the capacity of cannabinoids to exert epigenetic modifications [101, 102].

Considering the placenta's limited capacity to metabolize cannabis, occasional cannabis consumption during pregnancy may have enduring detrimental effects on the fetus with long-term repercussions in adult life.

Oxidative stress plays a dual role in inflammation and in inflammasome activation, sometimes acting as a trigger and sometimes as a consequence [103, 104]. In this study, our aim was to investigate the potential of THC and CBD to reduce or regulate oxidative stress, although in a preliminary way. Our findings indicate that both cannabinoids possess a strong ability to externally reduce LPS-induced oxidative stress, similar to what has been reported in other models [105, 106]. The literature on the impact of CBD on oxidative stress is limited and often contradictory. Although CBD has shown antioxidant effects in vivo and in cell lines exposed [107-109], there are also reports that CBD generates free radicals [110, 111]. Our study supports CBD's antioxidant capacity reported in the placental epithelial BeWo cell line, commonly used to study pregnancy metabolism [97]. CBD antioxidant effects depend on the specific cell type, dose, and duration of treatment [112].

Our understanding of THC's antioxidant potential is quite limited, likely due to strict acquisition regulations [113]. Consistent with our findings, previous studies have reported that THC exhibits antioxidant capacity in trophoblasts and SH-SY5Y neuroblastoma cells [114, 115]. However, there is also conflicting evidence indicating that THC may act as a pro-oxidant agent in BeWo and trophoblast cells [116-118]. To resolve this conflicting information, further research in this area is needed. Therefore, it is important to exercise caution when considering cannabis as a substitute for established therapeutic medications.

Our data strongly support the idea that CBD and THC possess both anti-inflammatory and antioxidant properties, which is initially perceived as positive from a practical standpoint. *However, we must emphasize that during pregnancy, the natural immune response necessitates the expression of pro-inflammatory cytokines at critical stages* [119]. Women using cannabis to reduce anxiety may not be fully aware of the importance of natural inflammation during pregnancy. Interrupting this process can have adverse effects on both successful pregnancy outcomes and the long-term health of the offspring, potentially contributing to the development of autoimmune diseases.

9. CONCLUSION

The present diploma thesis provides preliminary insights into the capacity of CBD and THC to modulate inflammation processes within the placenta, particularly by influencing the inflammasome. Moreover, cannabinoids demonstrate the potential to regulate the antioxidant status of the placenta, which plays a vital role in the inflammatory response. It is crucial to emphasize the distinction between acute and chronic inflammation, as acute inflammation serves as a defensive mechanism while chronic inflammation poses significant risks. Disturbing the natural inflammatory response hinders the achievement of successful pregnancy outcomes. The utilization of explants derived from the human term placentas as a pharmacological model offers numerous advantages, including the evaluation of drug actions in a three-dimensional tissue topology and the assessment of population heterogeneity. Further investigation is warranted to expand upon these preliminary findings and deepen our understanding of the intricate interplay between cannabis, inflammation, and placental functions. Such knowledge is imperative for advancing our understanding of the pharmacological and toxicological properties of cannabinoids, given their emergence as a promising area of research with potential biomedical implications. Psychotropic and non-psychotropic cannabinoids show promise in mitigating immune responses, suggesting their efficacy in managing uncontrolled inflammation processes. However, caution must be exercised, acknowledging that clinical reports highlighting the benefits of cannabis usage have primarily focused on life-threatening conditions where alternative options were limited. While pregnancy itself is not life-threatening, discomforts such as nausea, although significant, can typically be managed without substantial health risks. Therefore, until comprehensive research provides a thorough understanding, the use of cannabis and its cannabinoids is not recommended at any stage of pregnancy.

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