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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.

1. 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

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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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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.

2.5. 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

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

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.

2.6. 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.

3. Results

3.1. 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).

3.2. 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

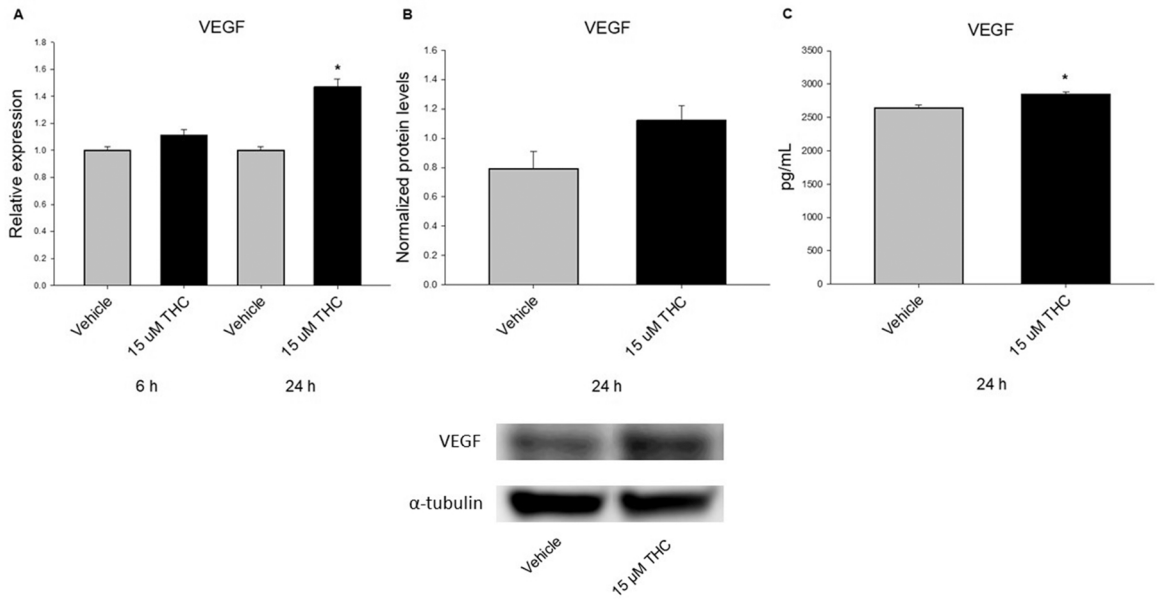

Fig. 1. A. Relative expression of *Vegfa* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 6 and 24 h (h). B. Intracellular VEGF protein levels relative to α-tubulin in SIGCs exposed to 15 μM THC for 24 h. C. Extracellular VEGF concentrations in media from SIGCs exposed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

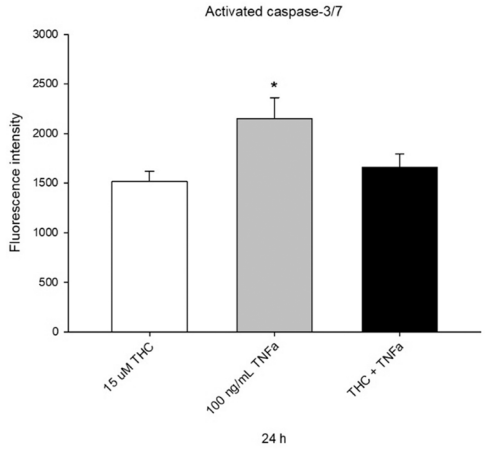

Fig. 2. Activated caspase-3/7 in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC, 100 ng/mL TNFα or both (THC + TNFa) for 24 h (Mean+SE; N = 5, *p < 0.05).

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.

3.3. 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.

3.4. 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.

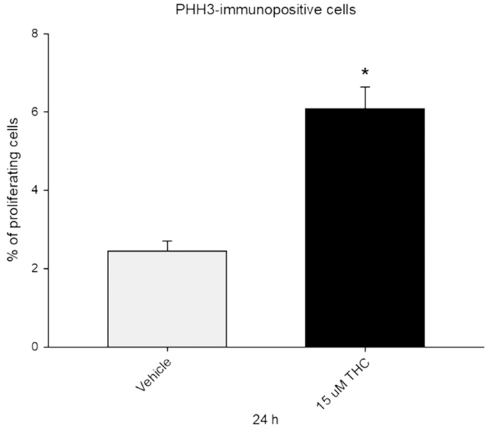

Fig. 3. Percent of proliferating spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 24 h (Mean+SE; N = 5, *p < 0.05).

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 ($r = 0.85$; $N = 5$, $p = 0.0019$).

3.5. 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, pretreatment 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.

4. 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

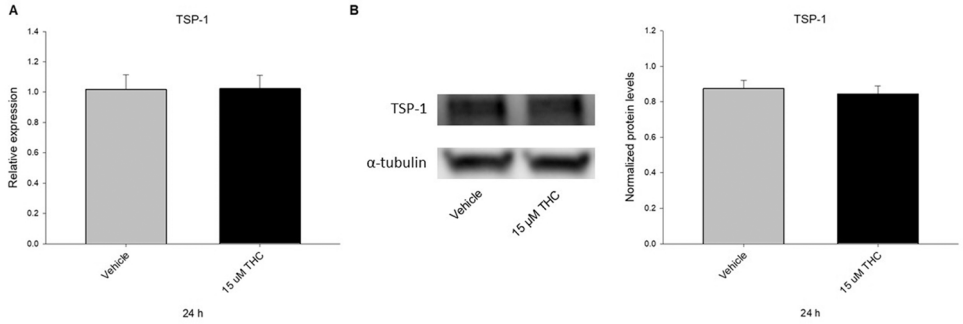

Fig. 4. A. Relative expression of *Thbs1* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 24 h. B. Intracellular TSP-1 protein levels relative to α-tubulin in SIGCs exposed to 15 μM THC for 24 h (Mean+SE; N = 5).

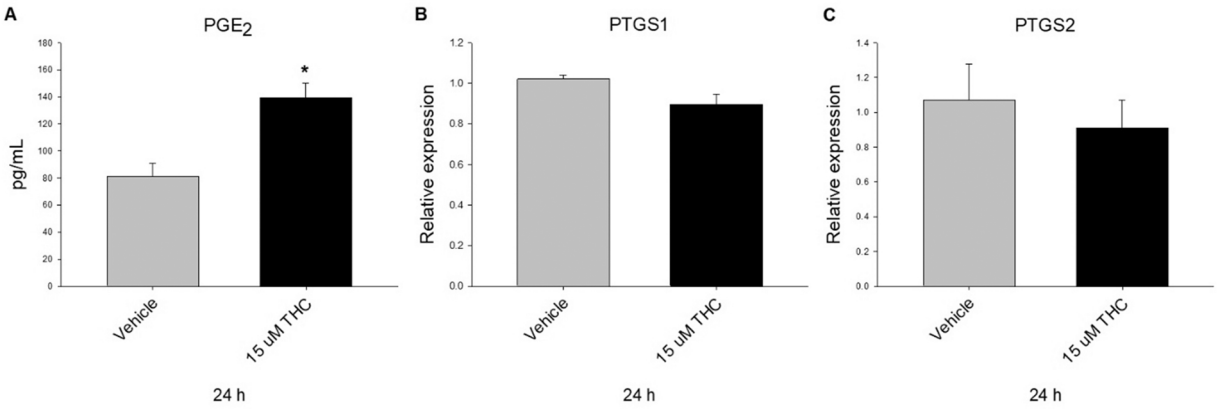

Fig. 5. A. Concentration of PGE_2 in media from spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC for 24 h. B. Relative expression of *Ptgs1* in SIGCs exposed to 15 μM THC for 24 h. C. Relative expression of *Ptgs2* in SIGCs exposed to 15 μM THC for 24 h (Mean+SE; N = 5, *p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

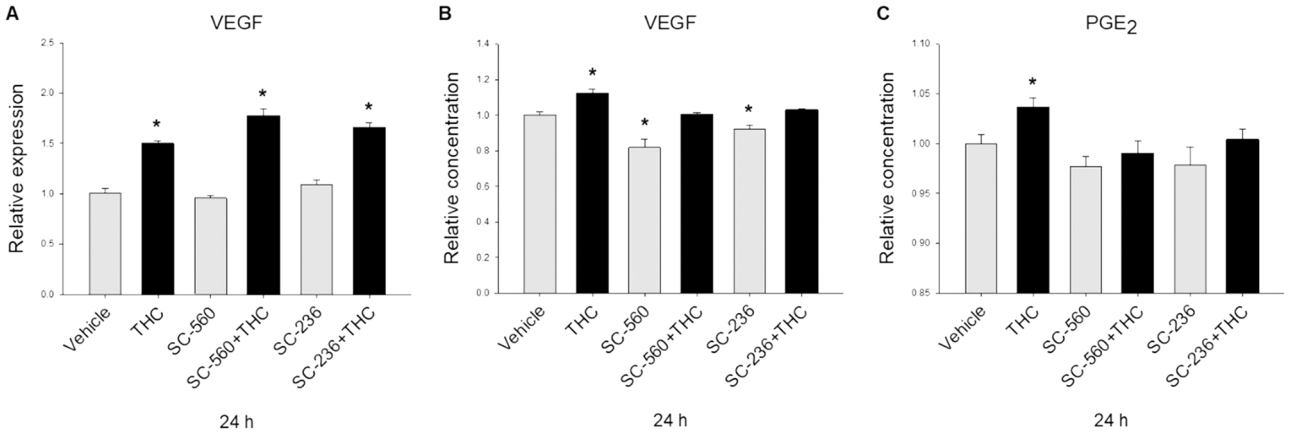

Fig. 6. A. Relative expression of *Vegfa* in spontaneously immortalized rat granulosa cells (SIGCs) exposed to 15 μM THC (THC), 1 μM SC-560 alone (SC-560) or in combination with THC (SC-560 +THC), 5 μM SC-236 alone (SC-236) or in combination with THC (SC-236 +THC) for 24 h. B. Concentration of VEGF in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

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.

5. 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.

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