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ARTICLE

Cannabidiol as a potential novel treatment for endometriosis by its anti-inflammatory, antioxidative and antiangiogenic effects in an experimental rat model

BIOGRAPHY

Berkem Okten, MD, completed his Ob/Gyn residency at Trakya University. He then completed his education in assisted reproductive technologies (ART) at Istanbul Memorial Hospital, ART and Genetics Center. His main fields of scientific interest are ART, infertility and endometriosis. He is currently working at Acibadem Kozyatagi Hospital, IVF Center, Istanbul.

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KEY MESSAGE

This is the first study in the literature that evaluates the effects of CBD on endometriosis in a rat model. With its anti-inflammatory, antioxidative and antiangiogenic effects and favorable safety and tolerability profile, it might be a candidate for a novel treatment in endometriosis.

ABSTRACT

Research question: Can cannabidiol (CBD) be used in the treatment of endometriosis for its anti-inflammatory, antioxidative and antiangiogenic effects?

Design: Endometrial implants were surgically induced in 36 female Wistar albino rats. After confirmation of endometriotic foci, the rats were randomized into four groups. In the leuprolide acetate group, rats were given a single 1 mg/kg s.c. leuprolide acetate injection. The other groups were 5 mg/kg CBD (CBD5), saline solution and 20 mg/kg CBD (CBD20); daily i.p. injections were administered for 7 days. After 21 days, the rats were euthanised, and total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) measurements in blood and peritoneal fluid samples, and immunohistochemical staining for TNF-α, IL-6 and vascular endothelial growth factor (VEGF) of endometriotic tissues were evaluated.

Results: Significant reductions in the endometriotic implant surface area ($P = 0.0213$), serum TOS ($P = 0.0491$), OSI ($P = 0.0056$), IL-6 ($P = 0.0236$), TNF-α ($P = 0.0083$) and peritoneal fluid OSI ($P = 0.0401$), IL-6 ($P = 0.0205$) and TNF-α ($P = 0.0045$) concentrations were observed in the CBD5 group when compared with the saline solution group. Compared with the saline solution group, increased TAS concentrations in serum ($P = 0.0012$) and peritoneal fluid ($P = 0.0145$) were found in the CBD5 group. The CBD5 and leuprolide acetate groups were similar regarding inflammatory and oxidative stress parameters of serum and peritoneal fluid samples. The CBD5 group showed significantly lower mean intensity in both surface epithelium and stromal cells for VEGF (both $P = 0.002$) and only in surface epithelium cells for IL-6 ($P = 0.0108$), when compared with the leuprolide acetate group.

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KEYWORDS

Angiogenesis
Cannabidiol
Endometriosis treatment
Inflammation
Oxidative stress
Phytocannabinoids

Conclusion: Due to its anti-inflammatory, antioxidative and antiangiogenic effects, CBD might be a therapeutic agent candidate for endometriosis.

INTRODUCTION

Endometriosis is a chronic inflammatory condition defined as the presence of endometrial cells outside the uterine cavity; it affects ovaries, Fallopian tubes, pelvic peritoneum and other organs (*Johnson et al., 2017*). The condition may lead to problems such as dysmenorrhoea, dyspareunia, chronic pelvic pain and infertility due to its chronic inflammatory nature (*Burney and Giudice, 2012*; *Santulli et al., 2016*). About 10% of women of reproductive age are affected by endometriosis, while the prevalence increases to 49% and 75% for women with chronic pelvic pain and women with chronic pelvic pain unresponsive to medical treatment, respectively (*Shafrir et al., 2018*). The aetiology of endometriosis remains unclear; over time, several different theories have been proposed. Of these theories, retrograde menstruation is the most widely supported, with some scientific evidence; lymphatic or haematogenous spread, metaplasia of the mesothelium or other cell types, immune dysregulation and a polygenic–multifactorial pattern of inheritance are the other candidate factors that might be involved in the pathophysiology of the disease (*Sampson, 1927*; *Zondervan et al., 2020*).

The pathogenesis and pathophysiological features of endometriosis are complex but it has been well documented that interacting biological effects such as localized inflammatory response, vascularization and oxidative stress are involved in the establishment and maintenance of the lesions (*Zondervan et al., 2020*). Parallel to these key mechanisms that are involved in the pathogenesis of the disease, endometriosis patients have been found to have significantly higher concentrations of inflammatory markers such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) in their peritoneal fluid (*Eisermann et al., 1988*; *Harada et al., 1997*) compared to healthy women. As inflammation and oxidative stress are closely linked events, elevated concentrations of oxidative stress indicators in the peritoneal fluid of endometriosis-affected women have also been reported (*Gupta et al., 2006*). The concentration of TNF-α in the peritoneal fluid is found to be positively correlated with severity of the disease and also the number and size of the lesions (*Bedaiwy et al., 2002*). This proinflammatory cytokine is also associated with the adhesion and proliferation of endometrial cells and induction of metalloprotease expression, which in turn promotes invasion and angiogenesis (*Braun et al., 2002*). Furthermore, previous immunohistochemical studies have shown the pivotal role of vascular endothelial growth factor (VEGF) in maintenance of the endometriotic implants (*Donnez et al., 1998*; *McLaren, 2000*).

Hormonal drugs have been routinely used to suppress the endometriotic lesions and their symptoms, but in long-term management they have many systemic side effects and symptoms can reoccur after cessation of the drugs. The need for an optimal medical treatment, which would prevent endometriotic lesion growth, recurrence and the symptoms of endometriosis, while allowing a healthy ovulation, has led researchers to focus on alternative novel drugs (*Vercellini et al., 2009*). As inflammation, oxidative stress and angiogenesis are among the crucial steps involved in the growth of endometriotic implants, numerous different molecules targeting these steps have been studied (*Delbandi et al., 2013*; *Leconte et al., 2010*; *May and Becker, 2008*; *Oktem et al., 2014*; *Ozcan Cenksoy et al., 2015*).

The endocannabinoid system (ECS) comprises cannabinoid type 1 and 2 receptors (CB1 and CB2) and a variety of endogenous bioactive lipids which activate them. The CB1 receptors are mainly expressed in the cerebellum, basal ganglia, cortex, hippocampus, amygdala, thalamus, hypothalamus, pons and medulla areas of the brain, which are involved in the control of motor activity, cognition and memory, emotions, and perception of senses (*Venance et al., 2004*). Other than the brain, CB1 receptors are found in other tissues such as the uterus, testis, vascular endothelium, spleen and eye (*Howlett et al., 2004*). The CB2 receptors are expressed mostly in tissues and cells involved in the immune system, such as lymph nodes and spleen (*Gardner et al., 2002*; *Pertwee, 1997*); uterus, intestines, pancreas and lungs are among the other organs where CB2 receptors are expressed (*Onaivi et al., 2002*).

A wide variety of physiological and pathological events in the body are linked to changes in the ECS. The up-regulation of ECS plays a protective role in some diseases like multiple sclerosis and inhibits disease progression, whereas abnormal modulation of cannabinoid receptor expression is linked to diseases such as colorectal cancer and liver fibrosis (*Miller and Devi, 2011*). Because there are endocannabinoids and their receptors throughout all of the female reproductive tract, dysregulation in the ECS might be a potential cause of endometriosis (*Di Blasio et al., 2013*; *Sanchez et al., 2012*). Recent studies have focused on the ECS to uncover the mystery of endometriosis and suggest that modulators of the ECS might have a role in the inflammation, cell migration and proliferation mechanisms in the occurrence and maintenance of endometriosis, which make cannabinoids a potential drug of choice (*Castaneto et al., 2014*; *Kis et al., 2019*; *Luschnig and Schicho, 2019*; *Sanchez et al., 2017*).

Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the two main constituents of the plant *Cannabis sativa*. Unlike THC, CBD is a cannabinoid with no psychoactive effect. It acts indirectly upon the CB1 receptor and as an inverse agonist of the CB2 receptor, and also exhibits its effects through the serotonin 1A receptor, transient receptor potential cation channel subfamily V member 1, G protein–coupled receptor 18 and G protein–coupled receptor 55 (*McPartland et al., 2015*). By its antiproliferative, antiangiogenic, anti-inflammatory and antioxidative effects and positive safety and tolerability profile, CBD has attracted interest as a potential treatment agent for a diverse range of diseases (*Iffland and Grotenhermen, 2017*; *Kis et al., 2019*; *Millar et al., 2019*).

This study aimed to investigate whether a phytocannabinoid (CBD) has any therapeutic effects on endometriosis in a rat model through anti-inflammatory, antioxidative and antiangiogenic markers from serum, peritoneal washing fluid and tissue samples by comparing the results with a treatment group (leuprolide acetate, which is being used for endometriosis treatment and exerts its 'therapeutic effects' by hormonal suppression) and a negative control group (saline solution).

MATERIALS AND METHODS

Animals

Thirty-six mature cycling female Wistar albino rats weighing 250–300 g and provided by Bezmialem Vakif University were caged four per cage at constant temperature (22 ± 2°C) with 12 h light/dark cycles. Standard rat feed and reverse osmosis-purified water were provided *ad libitum*. All rats were allowed to acclimatize to this environment for 1 week before the experiments. All the experiments were carried out in accordance with current guidelines for the care of laboratory animals and procedures involving animal handling were subject to approval from the Institutional Animal Care and Use Committee of Bezmialem University (permit no: 2020/59, 18 June 2020).

Surgical procedures, randomization and drug administrations

All rats went through three laparotomies. Before every surgery, the rats were anaesthetized with an i.m. administration of 50 mg/kg of ketamine hydrochloric acid (Ketalar; Eczacibasi Warner-Lambert Ilac, Sanayi, Turkey) and 7 mg/kg of xylazine hydrochloric acid (Rompun; Bayer Sisli, Turkey). Using the aseptic technique, a ventral midline incision was made to expose the reproductive organs. The surgeries are detailed below.

First surgery

The surgical technique used to induce endometriotic lesions in rats is as described by *Rajkumar et al. (1990)*. Briefly, the cervical end and utero-tubal junction of the right uterine horn was ligated and resected. The resected horn was excised and opened in isotonic sterile saline and a 5 × 5 mm section was cut out. The excised piece was then transplanted onto the peritoneal cavity of the right ventrolateral body wall, adjacent to a large vessel with the endometrial side of the piece facing the peritoneum, with 6.0 polypropylene sutures. After fascia and skin incision closure, all rats were observed in their cages for 21 days, with no medication, until the second surgery.

Second surgery

This procedure was an exploratory laparotomy to confirm endometriotic implant formation and measure the endometrial implant diameters. After the endometrial implants were measured (length × width = mm^2) and noted for each rat (FIGURE 1), the laparotomies were closed. After the second surgery, all rats were allowed a resting period of 1 day before injections. On the day of injections, rats were randomized into four groups: (i) the leuprolide acetate group (Group I, $n = 9$); (ii) the 5 mg/kg CBD group (Group II, $n = 9$); (iii) the saline solution group (Group III, $n = 9$); and (iv) the 20 mg/kg CBD group (Group IV, $n = 9$). Rats in the leuprolide acetate group were given a single 1 mg/kg s.c. injection of leuprolide acetate depot formulation (Lucrin; Abbott, Istanbul, Turkey), a dose that has been reported to be effective in treating endometriosis in rats (*Oktem et al., 2014*). Rats in the 5 mg/kg CBD and 20 mg/kg CBD groups were given daily i.p. injections of these doses of CBD for 7 days; CBD dosage was chosen based on a previous study (*Durst et al., 2007*). CBD was kindly provided by Reakiro Company (Poland) in powder form and dissolved in dimethylsulphoxide: saline (1:3) before experimental injection. The control (saline solution) group were given saline solution (saline with equivalent dimethylsulphoxide; 1:3) in the same manner.

Third surgery

Third laparotomies were performed 21 days after the second. First, the abdominal cavities were washed with 2 ml saline solution and the peritoneal fluid samples were collected to assess and compare TNF-α, IL-6, total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) between groups. Then the endometrial implants were measured (length × width = mm^2) and noted for each rat. The implants were then excised and fixed in 10% formalin for histopathological and immunohistochemical examination for VEGF, TNF-α and IL-6. Finally, the rats were euthanized and intracardiac blood samples were collected for measurement of IL-6,

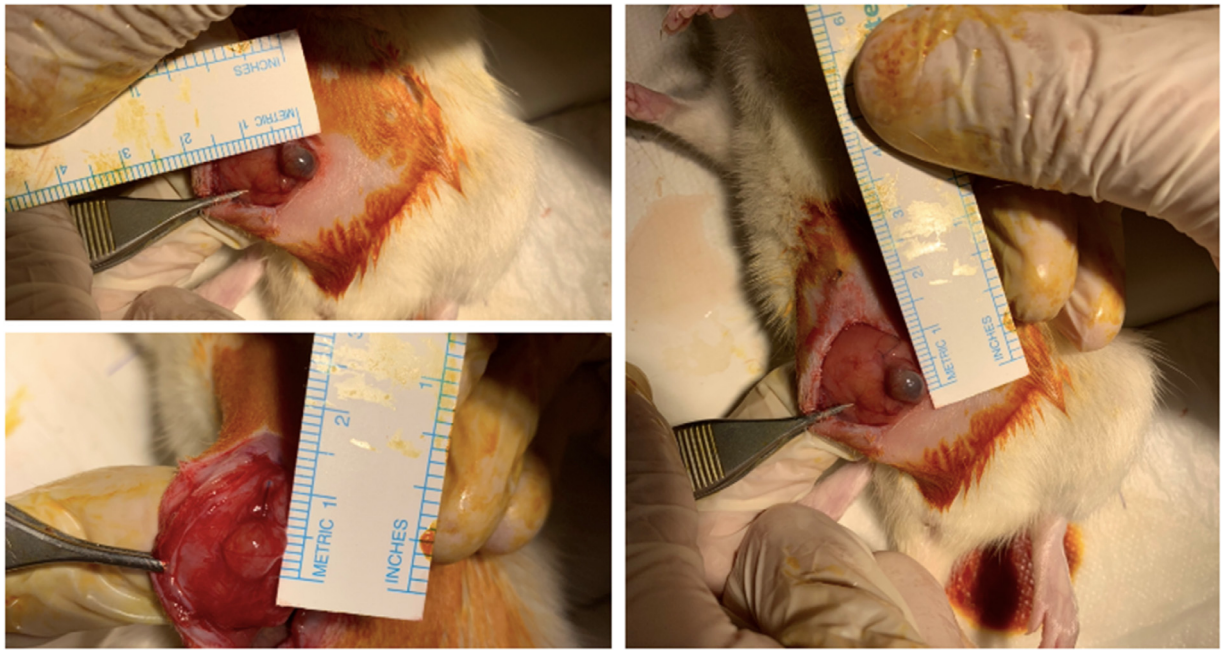

FIGURE 1 New endometriotic implants observed during the second surgery. Representative images shown before randomization.

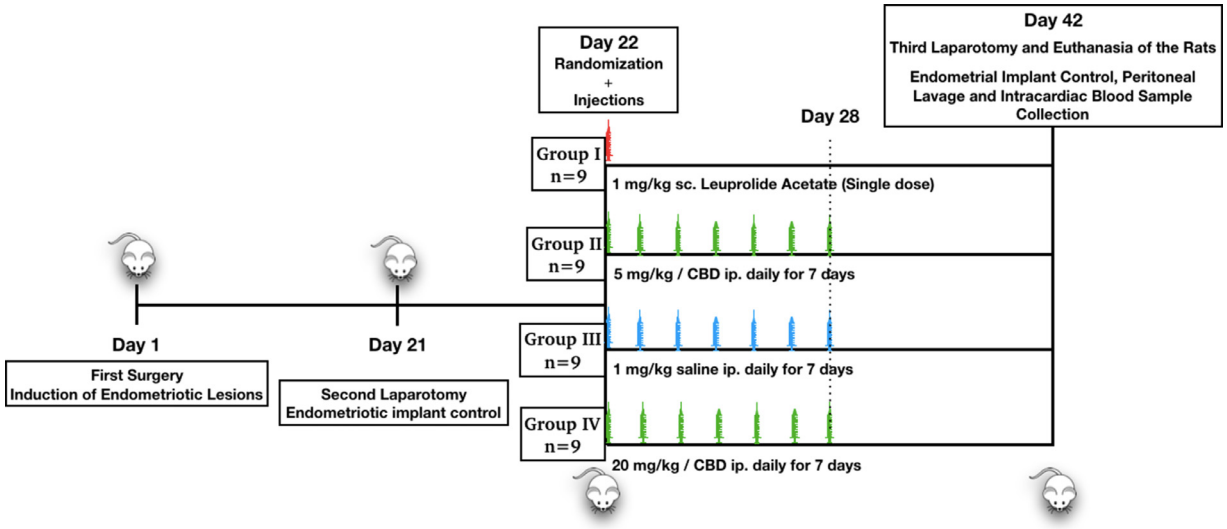

FIGURE 2 Surgical procedures, randomization and drug administrations.

TNF-α, TAS, TOS and OSI. All procedures were performed by two clinicians blinded to which groups the rats were in. Surgical procedures, randomization and drug administrations are all shown in FIGURE 2.

Measurement of inflammatory cytokines and oxidative stress biomarkers

Serum and intraperitoneal fluid IL-6 (pg/ml [mg protein]; Elabscience, Shanghai, China, E-EL-R0015), TNF-α (pg/ml [mg protein]; Elabscience, E-EL-R0019), TAS (mmol ascorbate eq/l [mg/protein]; Rel Assay, Mega Tıp, Gaziantep, Turkey, RL0024) and TOS (μmol H_2O_2 eq/l [mg/protein]; Rel Assay, Turkey, RL0017) were analysed using commercial ELISA kits. Results were obtained by the spectrophotometric method and units are expressed as specified in each kit's protocol for serum and intraperitoneal fluid samples. The OSI value was calculated by a mathematical equation (OSI = TOS/TAS).

Histopathological examination and VEGF, TNF-α and IL-6 immunohistochemistry

The excised endometriotic implants from the rats were fixed in 10% neutral formalin for 72 h. They were then rinsed with water and fully dehydrated in a graded series of ethanol (70%, 90%, 96% and 100%) and cleared in xylene. Following that, the samples were immersed in paraffin overnight at 60° C. The implants were then sectioned at 5 μm thickness from the paraffin blocks and placed on positively charged slides for immunohistochemical analysis.

Tissue sections were deparaffinized with xylene and rehydrated in descendant grades of ethanol. After deparaffinization, slices were treated in 3% hydrogen peroxide in methanol for 10 min to prevent endogenous enzyme inhibition. Following washing in phosphate-buffered saline (PBS), the sections were microwaved at 200 W with citrate buffer pH 6.1 for 20 min for antigen retrieval. After washing with PBS, they were incubated in protein blocking solution (1% bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide in 0.01 mmol/l PBS, pH 7.2–7.4) for 20 min to prevent non-specific antibody binding and then incubated with anti-TNF-α (1:200, Novus Biologicals, cat no: NBP1-67821), anti-IL-6 (1:400, Novus Biologicals, cat no: NBP2-16957) and anti-VEGF-A (1:100, Biorbyt, cat no: orb642460) primary antibodies at 4°C, overnight. Non-specific staining was checked by using diluent buffer instead of primary antibodies for negative control staining; non-immune rabbit serum (Agilent Dako, cat no: RUO X0902) at the same concentration of the primary antibodies was added to the diluent solution, because all the antibodies used were rabbit polyclonal. Secondary antibody staining was performed using an UltraTek HRP Anti-Polyvalent IHC Detection Kit (cat no: UHP 125, ScyTek) following the manufacturer's protocol. After washing, sections were incubated with streptavidin–peroxidase (ready-to-use) for 10 min at room temperature, followed by incubation with 3,3′-diaminobenzidine (DAB) for 5 min. The sections were finally counterstained with Mayer's Hematoxylin.

The immunohistochemically stained sections were examined under a laser scanning confocal microscope (LSM 880, Zeiss, Germany) and digital histological score (D-HSCORE) was measured using image analysis software (ImageJ, National Institutes of Health, USA) with colour deconvolution method from taken digital images. In each image, the software calculated the mean intensity of DAB, ranging from 0 (black) to 255 (total white). The final DAB intensity was calculated according to the formula $f = 255 - i$, where f = final DAB intensity, i = mean DAB intensity obtained from the software; i ranges from 0 (zero = deep brown, highest expression) to 255 (total white) (*Fuhrich et al., 2013*).

Statistical analysis

A sample size and power calculation determined that sufficient statistical power required nine rats for each group (power = 0.80, type 1 error = 0.05 and type 2 error = 0.20). The power calculation was based on immunohistochemical staining scores of endometriotic implants for VEGF, which was performed in a previous study (*Ozcan Cenksoy et al., 2015*). The data were analysed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) by one-way ANOVA followed by the Tukey test. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

The mean surface area of endometriotic implants at the second surgery, prior to medical treatment, were 22.88 ± 11.30, 29.88 ± 26.35, 66.44 ± 63.45 and 64.66 ± 72.38 mm^2 in the leuprolide acetate, 5 mg/kg CBD, saline solution and

20 mg/kg CBD groups, respectively ($P = 0.2$). There was no significant difference between groups regarding the mean surface area of endometriotic implants at the second surgery, prior to medical treatment. However, there was a statistically significant difference between groups in terms of the mean surface area of endometriotic implants at the third surgery after medical treatment (5.75 ± 3.24 in the leuprolide acetate, 7.55 ± 5.29 in the 5 mg/kg CBD, 34.25 ± 28.32 in the saline solution and 13.87 ± 11.31 in the 20 mg/kg CBD groups, respectively, $P < 0.001$). The leuprolide acetate and 5 mg/kg CBD groups were similar ($P = 1$), whereas both groups showed significantly smaller mean surface area of endometriotic implants at the third surgery after medical treatment compared with the saline solution group ($P < 0.001$). The mean surface area of endometriotic implants for the 20 mg/kg CBD group compared with the saline solution group was not significant ($P = 0.208$). The percentage reduction rates in the mean surface area of endometriotic implants after treatment were also calculated and compared between groups. The reduction rates for the leuprolide acetate, 5 mg/kg CBD, saline solution and 20 mg/kg CBD groups were 70.86 ± 17.42%, 76.93 ± 16.87%, 28.12 ± 24.48% and 68.39 ± 22.64%, respectively ($P = 0.025$). The 5 mg/kg CBD group showed a significant reduction rate in the mean surface area of endometriotic implants after treatment when compared with the saline solution group ($P = 0.0213$). The reduction rates in the mean endometriotic implant surface area after treatment for the leuprolide acetate and 20 mg/kg CBD groups when compared with the saline solution group were not significant ($P = 0.148$ and $P = 0.1$, respectively; FIGURE 3).

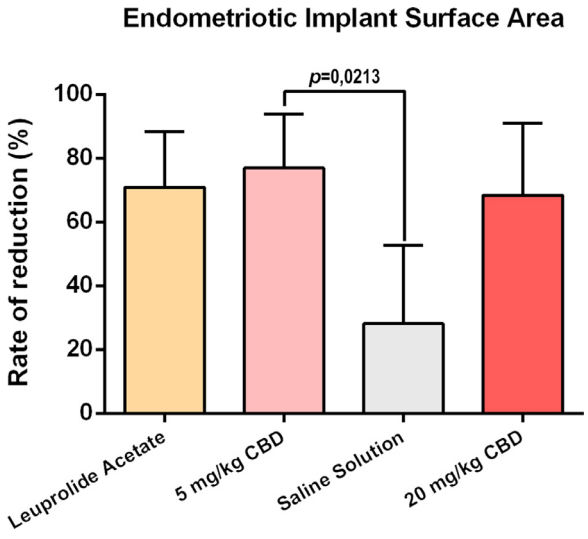

FIGURE 3 Reduction rate in the surface areas of endometriotic implants after medical treatment during third surgery and comparison between groups. Data shown as mean ± SD, $n = 9$ per group. One-way analysis of variance and Tukey test were used.

Expression of TNF-α was strongly observed in the whole surface epithelium (arrow) of endometriotic implants in the saline solution and 20 mg/kg CBD groups. In the leuprolide acetate and 5 mg/kg CBD groups, the TNF-α expression was decreased and weakly observed in certain regions of the surface epithelium (arrow) (FIGURE 4). A significant difference was found in the mean intensity of surface epithelium of endometriotic implants for TNF-α between groups (80.13 ± 20.77 in the leuprolide acetate group; 74.25 ± 13.29 in the 5 mg/kg CBD group; 135.8 ± 8.97 in the saline solution group and 121.7 ± 12.71 in the 20 mg/kg CBD group; $P < 0.0001$) (FIGURE 5). The leuprolide acetate and 5 mg/kg CBD groups were similar ($P = 0.9$) and showed the lowest TNF-α intensity of surface epithelium of endometriotic implants and both groups showed significantly lower scores when compared

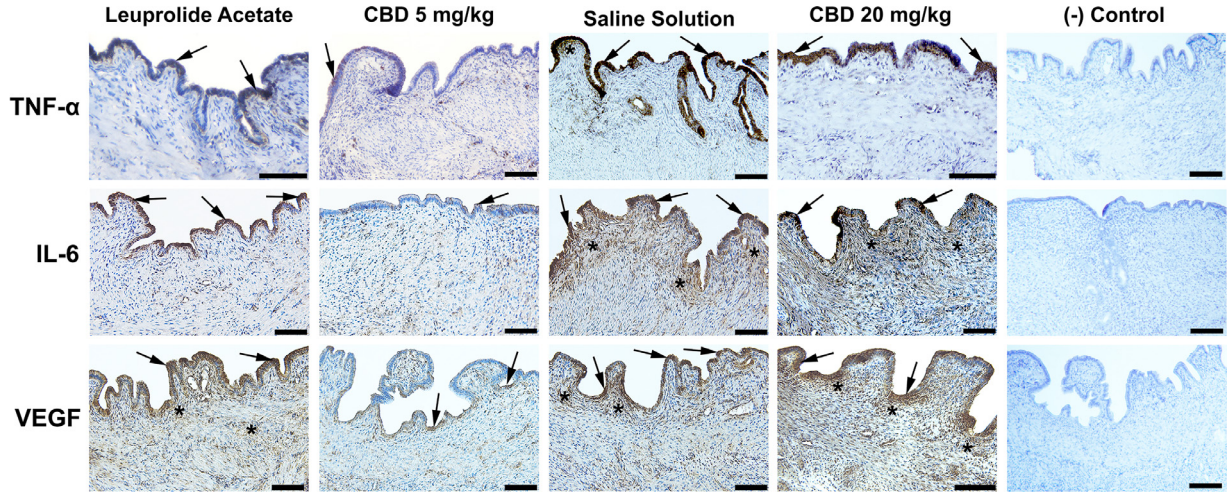

FIGURE 4 Immunohistochemical evaluation of tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) in surface epithelium and stromal cells of endometriotic implants in all groups. Positive staining (brown) shown in surface epithelium (arrow) and stromal cells (asterisk). In the right column, negative controls (non-immune serum) are seen for each antibody. Counterstained with Mayer's Hematoxylin (blue). Scale bar = 100 μm.

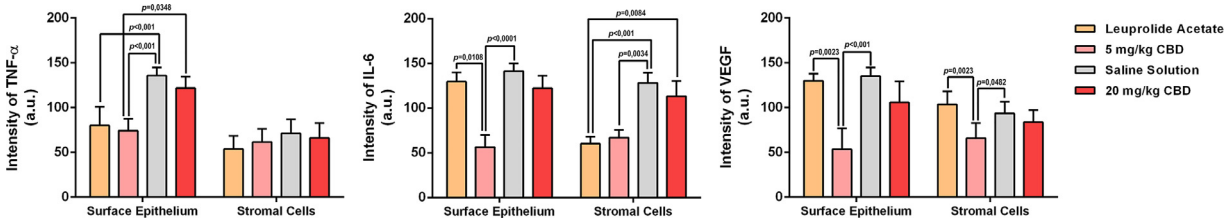

FIGURE 5 Intensity of tumour necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) immunohistochemical staining in surface epithelium and stromal cells of endometriotic implants compared between the four groups. Data shown as mean ± SD, $n = 9$ per group. One-way analysis of variance and Tukey test were used.

with the saline solution group ($P < 0.001$ and $P < 0.0003$, respectively). In all groups, expression of TNF-α was weakly observed in stromal cells of endometriotic implants. There was no significant difference in the mean intensity of stromal cells of endometriotic implants for TNF-α between all groups ($P = 0.285$) (FIGURE 5).

A significant difference was found in the mean intensity of surface epithelium and stromal cells of endometriotic implants for IL-6 between all groups ($P < 0.001$ and $P < 0.001$, respectively). Expression of IL-6 was strongly observed in the whole surface epithelium (arrow) of endometriotic implants in LA, SS and 20 mg/kg CBD groups. In the 5 mg/kg CBD group, expression of IL-6 was decreased and weakly observed in certain regions of surface epithelium (arrow) (FIGURE 4). The 5 mg/kg CBD group showed the lowest mean intensity of IL-6 in the surface epithelium of implants, which was significant when compared with the leuprolide acetate and saline solution groups ($P = 0.0108$ and $P < 0.0001$, respectively). There was no significant difference between the leuprolide acetate and saline solution groups regarding IL-6 expression in the surface epithelium. Expression of IL-6 was strongly observed in the stromal cells (asterisk) close to the surface epithelium in the saline solution and 20 mg/kg CBD groups. In the leuprolide acetate and 5 mg/kg CBD groups, expression of IL-6 was decreased and moderately observed in stromal cells (FIGURE 4). The mean intensity of stromal cells of implants for IL-6 were similar between the leuprolide acetate and 5 mg/kg CBD groups, and both had a significantly lower intensity when compared with the saline solution group ($P = 0.0001$ and $P = 0.0034$, respectively) (FIGURE 5).

A significant difference was found in the mean intensity of surface epithelium and stroma of endometriotic implants for VEGF between groups ($P < 0.001$ and $P = 0.002$, respectively). Expression of VEGF was strongly observed in the whole surface epithelium (arrow) of endometriotic implants in the leuprolide acetate, saline solution and 20 mg/kg CBD groups. In the 5 mg/kg CBD group, expression of VEGF was decreased and moderately observed in certain regions of surface epithelium (arrow) (FIGURE 4). Compared with the leuprolide acetate and saline solution groups, the mean intensity of VEGF for surface epithelium was significantly lower in the 5 mg/kg CBD group ($P = 0.002$ and $P = 0.0001$, respectively) (FIGURE 5). Expression of VEGF was moderately observed in the stromal cells (asterisk) close to the surface epithelium in the leuprolide acetate, saline solution and 20 mg/kg CBD groups. In the 5 mg/kg CBD group, expression of VEGF was decreased and weakly observed in stromal cells (FIGURE 4). Also, for the stromal cells of the endometriotic implants, compared with the leuprolide acetate and saline solution groups, the mean intensity of VEGF was significantly lower in the 5 mg/kg CBD group ($P = 0.002$ and $P = 0.04$, respectively) (FIGURE 5).

According to the results of the serum analysis, there were statistically significant differences regarding serum TAS, TOS and OSI between groups ($P < 0.001$) (FIGURE 6). Moreover, there was a significant difference regarding serum IL-6 and TNF-α between groups ($P < 0.001$) (FIGURE 7). The leuprolide acetate and 5 mg/kg CBD groups were similar in terms of serum TAS, TOS, OSI, IL-6 and TNF-α ($P = 1$, $P = 0.7$, $P = 0.46$, $P = 0.23$ and $P = 0.36$, respectively). Serum TOS, OSI, IL-6 and TNF-α were lowest and serum TAS was highest in the leuprolide acetate and 5 mg/kg CBD groups. Serum TOS, OSI, IL-6 and TNF-α values were significantly lower, and serum TAS was significantly higher in the 5 mg/kg CBD group when compared with the saline solution group ($P = 0.049$, $P = 0.006$, $P = 0.023$, $P = 0.008$ and $P = 0.001$, respectively).

A significant difference was found in all parameters of peritoneal fluid between groups ($P < 0.001$). The leuprolide acetate and 5 mg/kg CBD groups showed the highest TAS and the lowest OSI, IL-6 and TNF-α values in the peritoneal fluid samples. The leuprolide acetate and 5 mg/kg CBD groups were similar in terms of TAS, OSI, IL-6 and TNF-α values in peritoneal fluid ($P = 0.4$, $P = 0.3$, $P = 0.9$ and $P = 0.62$, respectively). Peritoneal fluid OSI, IL-6 and TNF-α values were significantly lower and serum TAS was significantly higher in the 5 mg/kg CBD group when compared with the saline solution group ($P = 0.04$, $P = 0.02$, $P = 0.004$ and $P = 0.014$, respectively). Mean values of TOS in the peritoneal fluid were similar between the 5 mg/kg CBD, 20 mg/kg CBD and saline solution groups ($P = 1$), whereas the mean TOS for the leuprolide acetate group in the peritoneal fluid was significantly lower than other groups ($P = 0.032$ for 5 mg/kg CBD, $P = 0.0015$ for saline solution and $P = 0.004$ for 20 mg/kg CBD) (FIGURES 6 and 7).

DISCUSSION

In the past, cannabis was widely used to ease dysmenorrhoea and other gynaecological pains (*Russo, 2002*). More recent survey studies also report the efficacy of cannabis products in easing endometriosis pain (*Armour et al., 2019*; *Reinert and Hibner, 2019*; *Sinclair et al., 2020*). However, data for these studies were collected from online surveys with self-reported measures, which may lead to recall bias, were not standardized (dosage, method of administration, duration of usage), and the sample sizes were small, thus providing low-quality evidence. Also, the fact that cannabis contains many cannabinoids other than THC and CBD

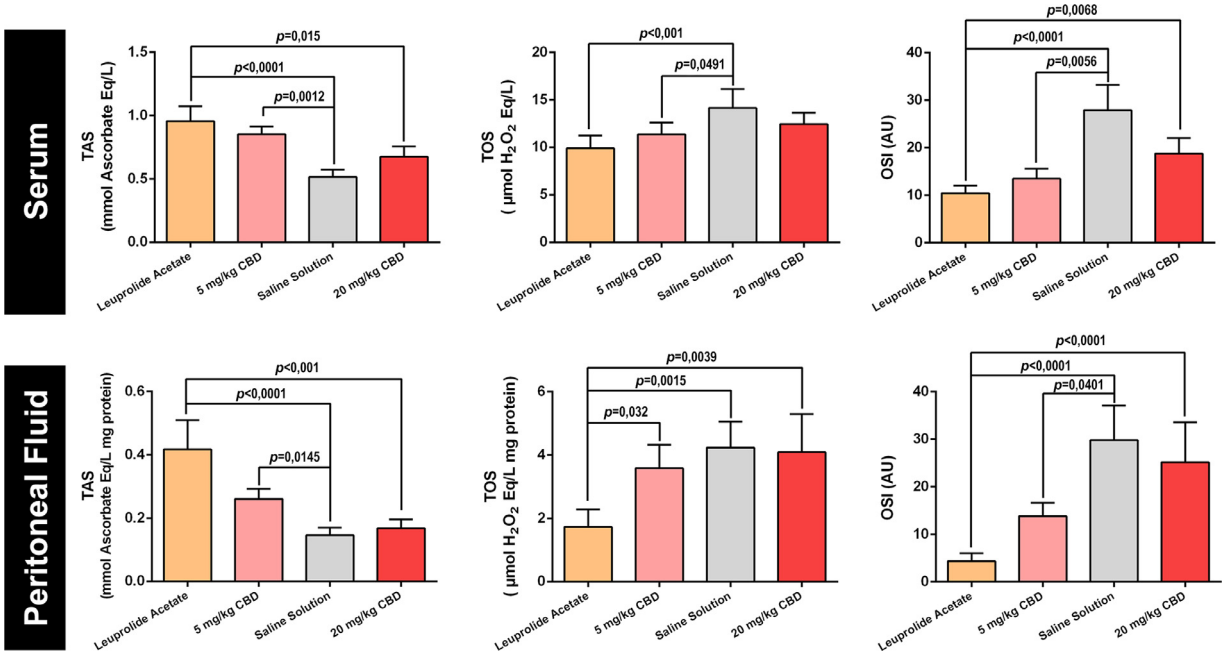

FIGURE 6 Serum and peritoneal fluid values of total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) among groups after treatment. Data shown as mean ± SD, $n = 9$ per group. One-way analysis of variance and Tukey test were used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

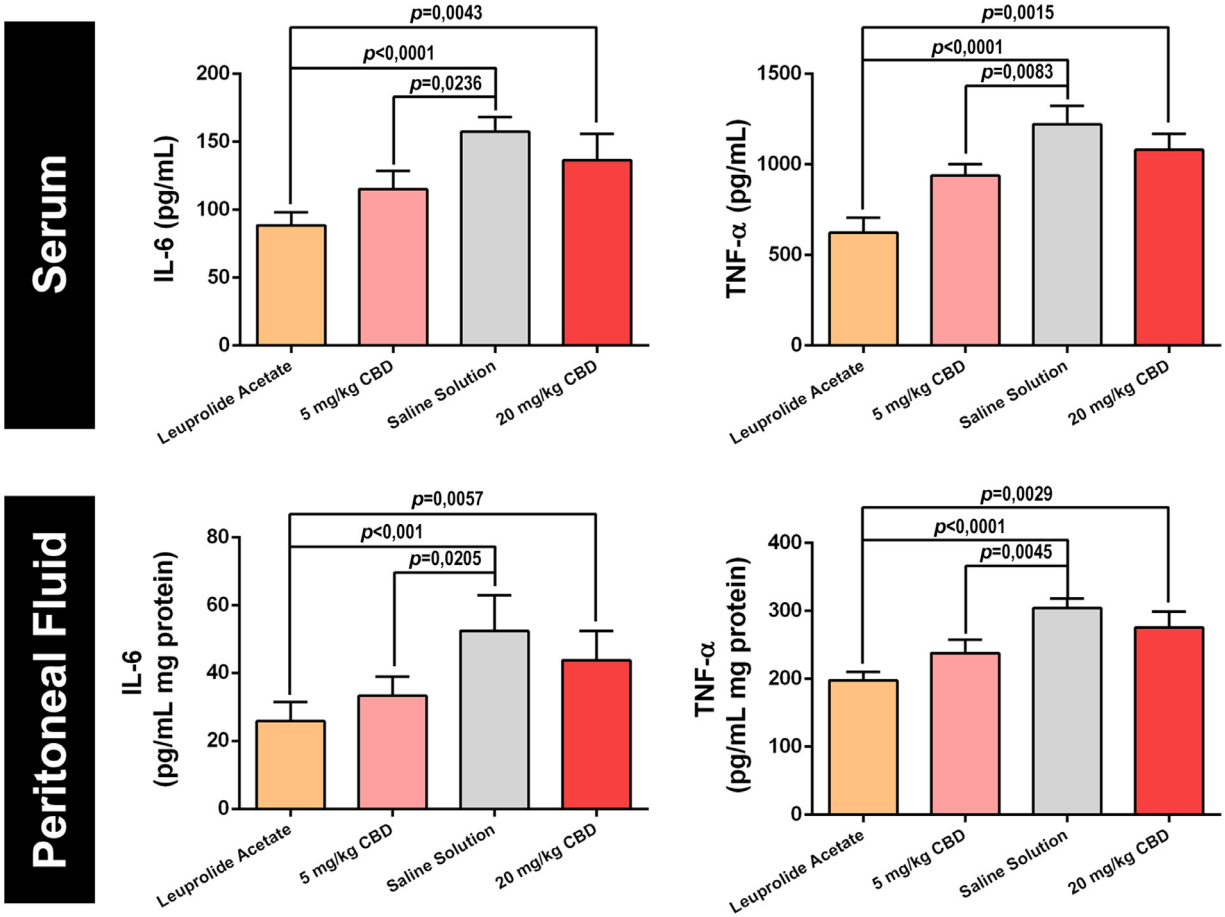

FIGURE 7 Serum and peritoneal fluid values of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) among groups after treatment. Data shown as mean ± SD, $n = 9$ per group. One-way analysis of variance and Tukey test were used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

means that it is necessary to investigate which cannabinoids are responsible for this positive effect on endometriosis, and by which mechanism.

To the best of our knowledge, this is the first study to investigate the effects of CBD, a natural cannabinoid, on endometriosis by means of inflammatory, oxidative and angiogenic markers in an experimental rat model. Promising results were observed, comparable to the conventional gonadotrophin-releasing hormone agonist (leuprolide acetate) treatment.

Key strengths of the study include that it compared the effects of CBD with a treatment group as well as a placebo group. It also demonstrated the dose-dependent effect of CBD by also comparing the high-dose and low-dose groups in between.

Regarding endometriotic implant size, serum values of TAS, TOS, OSI, IL-6, TNF-α and peritoneal fluid sample values of TAS, OSI, IL-6, TNF-α in the 5 mg/kg CBD group showed similar results to the leuprolide acetate group, while the results of both groups were significantly better when compared with the saline solution group. It is noteworthy that the reduction rate in the mean surface area of endometriotic implants after treatment was highest in the 5 mg/kg CBD group, which was also the only group to show statistical significance compared with the saline solution group regarding this parameter ($P = 0.0213$).

On the other hand, when compared with the saline solution group, the high-dose CBD group (20 mg/kg) also presented smaller endometriotic implants (13.87 ± 11.31 versus 34.25 ± 28.32 mm^2), a higher reduction in the surface area of the implants after treatment (68.39 versus 28.12%) and apparently higher TAS and lower OSI, IL-6 and TNF-α values in both serum and peritoneal fluid samples, yet none of them reached statistical significance.

A separate histopathological examination of the surface epithelium and stromal cells of the endometriotic implants was conducted to determine the mean immunohistochemical staining scores for VEGF, TNF-α and IL-6. The mean immunohistochemical staining scores of the leuprolide acetate and 5 mg/kg CBD groups were similar for TNF-α expression in the surface epithelium and IL-6 expression in stromal cells of the lesions and both were significantly lower when compared with the saline solution group. Strikingly, the mean immunohistochemical staining scores for VEGF in the surface epithelium and the stromal cells and the mean immunohistochemical staining scores for IL-6 in the surface epithelium were significantly lower in the 5 mg/kg CBD group when compared with the leuprolide acetate group. Apart from IL-6 expression in the stromal cells, the leuprolide acetate group showed no significant superiority in any of the immunohistochemical staining scores when compared to the 20 mg/kg CBD group. According to recent studies, endometriotic lesions contain a special microenvironment. Immune and hormonal pathways control the communication between immune cells found in the peritoneal fluid and epithelial and stromal compartments of the lesion (*Symons et al., 2018*). So, this immunohistochemical staining superiority of CBD to leuprolide acetate may be explained by its action of targeting dysregulated immune pathways rather than a hormonal pathway.

The peritoneal fluid of endometriosis patients contains immune cells along with numerous cytokines secreted from them, which are associated with the inflammatory and increased oxidative nature of the disease. It was also reported that TNF-α and IL-6 are released by macrophages, which in turn cause the overexpression of VEGF from infiltrating neutrophils and macrophages in a mouse model of endometriosis (*Lin et al., 2006*). The expressions of CB1 and CB2 receptor mRNA in immune cells such as CD4 and CD8 leukocytes, B cells, monocytes, neutrophils and natural killer cells have been well documented in previous studies (*Lee et al., 2001*; *Parolaro, 1999*). In this respect, *Raborn et al. (2008)* demonstrated the inhibition of peritoneal macrophage chemotaxis to RANTES/CCL5 by THC (the psychoactive component of marijuana) via the CB2 receptor in a murine model. Another study had shown the anti-inflammatory effect of rimonabant (a synthetic cannabinoid) via inhibition of TNF-α-induced IκB kinase a/b phosphorylation, IL-6 production and IκBα degradation (*Huang et al., 2010*). In the current study, in parallel to the literature, lower concentrations of TNF-α and IL-6 were observed in the serum and peritoneal fluid samples of the CBD group when compared with the saline solution group.

Along with inflammation, the oxidative stress level also rises in endometriosis. Many studies conducted with CBD for different diseases demonstrated the antioxidative effect of CBD by reducing the production of reactive oxygen species (ROS) and lipid peroxidation (*Borrelli et al., 2009*; *Izzo et al., 2009*). Parallel to the literature, this study also observed a significant reduction in TOS and an increase in TAS after treatment with CBD 5 mg/kg.

It is generally believed that the primary factor stimulating the increased vessel permeability and angiogenesis in endometriosis is VEGF (*Taylor et al., 2002*). In a rat model of peritoneal endometriosis, *Machado et al. (2010)* found higher expression of VEGF and its receptors in endometriotic lesions when compared with eutopic endometrium. Many cannabinoids exhibit their antiangiogenic effect by inhibiting vascular endothelial cell survival and migration by binding to CB1 and/or CB2 receptors (*Freimuth et al., 2010*; *Kogan et al., 2006*). It has been suggested that the development and growth of endometriotic lesions are highly associated with the balance between local anti- and pro-angiogenic factors, just as happens in tumour growth (*Folkman, 1971*; *Machado et al., 2010*).

Optimal results were observed in the 5 mg/kg CBD group rather than with 20 mg/kg CBD. Some studies conducted with CBD also report this dose-dependent effect and better results using the lower dose (*Aviello et al., 2012*; *Borrelli et al., 2009*). Although most of them did not reach statistical significance, most parameters in the 20 mg/kg CBD group appeared to show better results when compared with the saline solution group. This might be interpreted as a higher dose than needed has neither hazardous effects nor benefits, but this needs further investigation.

Many studies that have been conducted for many different diseases have shown the anti-inflammatory, antioxidative, antiproliferative and antiangiogenic effects of cannabinoids, which are also key factors in the pathogenesis of endometriosis. This had led researchers to focus on the association between the ECS and endometriosis in order to find a novel treatment for the disease (*Clemenza et al., 2018*; *Sanchez et al., 2012*).

There have been several studies conducted *in vitro* or using animal models that have investigated the molecular connections between the cannabinoid system and endometriosis, with conflicting results, most of which were performed using synthetic cannabinoids (*Bilgic et al., 2017*; *Han et al., 2017*; *Leconte et al., 2010*).

Gentilini et al. (2010) observed endometrial stromal cell migration that was stimulated by methanandamide via the CB1 receptor. This dose-dependent effect was conducted through the activation of ERK1/2 and PI3K/Akt pathways and also increased the electrical signal produced by K^+ channels. The same team also observed a bimodal manner in the effect of methanandamide in high doses (10^{-5} mmol/l) on endometrial cell proliferation in which prolonged exposure (>48 h) led to apoptosis, whereas increased cell proliferation occurred in the first 24 h (*Di Blasio et al., 2013*).

Some studies observed reduced cannabinoid receptor expression in the endometrial tissue of patients with endometriosis, which they defined as 'endocannabinoid deficiency'. *Resuehr et al. (2012)* compared endometrial biopsy samples of women with and without endometriosis and reported reduced expression of the CB1 receptor in mRNA and protein levels. This decreased expression has been linked to the potential of interleukin-1a and persistent environmental toxins to disturb the expression of the normal endometrial CB1 receptor by inducing a progesterone resistance phenotype in disease-affected individuals. The authors suggested that the increased proliferative capacity of endometriotic lesions might be explained by this diminished cannabinoid signalling. *Bilgic et al. (2017)* analysed adenomyotic and endometriotic tissues and also reported reduced levels of CB1 and CB2 receptors when compared with the healthy group. They also demonstrated the dose-dependent apoptotic and antiproliferative effects of synthetic cannabinoid agonists on endometriotic cells *in vitro* and suggested cannabinoid agonists as a potential treatment agent. Unlike others, *Sanchez et al. (2017)* reported that the cannabinoid receptor CB1 contributes to the development of ectopic lesions in a mouse model of endometriosis.

Leconte et al. (2010) transplanted human deep-infiltrating endometriosis nodules to nude mice and observed the effect of synthetic cannabinoid agonist WIN 55212-2. They reported significantly reduced implant size in the treated group when compared with the untreated mice. Also, in the same study, they observed the effect of WIN 55212-2 treatment *in vitro* and reported its antiproliferative effect, which was achieved through a mechanism involving a decrease in the formation of ROS and inhibition of the Akt pathway activation. They also observed higher inhibition of O_2 and H_2O_2 production with the higher doses of WIN 55212-2 in deep-infiltrating stromal cells. *Dmitrieva et al. (2010)* also used synthetic cannabinoid agonist WIN 55212-2 to treat surgically endometriosis-induced rats and observed a significant decrease in endometriosis-associated hyperalgesia, whereas an increase was observed when treated with CB1 antagonist AM251. They reported high expression of CB1 receptors on the sympathetic and sensory neurons that innervate the growth of the endometriotic lesion.

Instead of synthetic cannabinoids, a recent study conducted with THC reported a significant reduction in mechanical hypersensitivity and pain unpleasantness, as well as similar results to this study regarding inhibition of endometrial cyst development, in a mouse model of surgically-induced endometriosis (*Escudero-Lara et al., 2020*). As CBD has a favourable safety and tolerability profile, a randomized controlled clinical trial has been designed to investigate its effect on endometriosis pain, which compares two different doses of CBD and placebo and has been ongoing since 2020 with an expected completion date of June 2023 (https://clinicaltrials.gov, NCT04527003).

Modulators of the ECS appear to be a candidate for the treatment of endometriosis as they are involved in proliferation, apoptosis, cell migration, inflammation, angiogenesis, oxidative stress and pain mechanisms of the disease (*Sanchez et al., 2012*). Although this study has one limitation as it is an experimental rat model, it reveals the therapeutic effects of CBD, targeting the crucial pathogenetic steps of endometriosis, together with the apparent need for further investigation of its precise mechanisms of action.

AUTHOR ROLES

SB Okten developed the study idea and design, wrote the draft and the manuscript, performed the surgeries and finalized the manuscript. C Cetin contributed to the drafting of the manuscript, data entry, and reviewed the final manuscript. OE Tok performed the immunohistochemical staining of tissue samples, contributed to statistical analysis and interpretation of the data and reviewed the final manuscript. EM Guler performed the biochemical analyses of serum and peritoneal fluid samples, contributed to statistical analysis and interpretation of the data, and reviewed the final manuscript. SH Taha performed surgeries and contributed to data entry. P Ozcan developed the study design, performed the surgeries, interpreted the data, and edited, reviewed and finalized the manuscript. C Ficicioglu participated in the study design and drafting of the manuscript, and reviewed and finalized the manuscript. All authors were involved in the decision to publish the paper and in critical revisions of the manuscript and take responsibility for the integrity of the data.

DATA AVAILABILITY

Data will be made available on request.

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