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Investigation of the Protective Effects of Cannabidiol on Rat Maternal and Fetal Brain Tissues in Lipopolysaccharide-induced Pregnancy Inflammation Model

Kannabidiolün Lipopolisakkarit ile İndüklenen Gebelik Enflamasyon Modelindeki Sıçan Maternal ve Fetal Beyin Dokuları Üzerindeki Koruyucu Etkilerinin İncelenmesi

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ABSTRACT

Aim: Preterm labor (PE) is one of the most common causes of neonatal death world-wide and inflammation during pregnancy is thought to be one of the underlying causes of PE. In this study, inflammation and oxidative stress-mediated protective effects of Cannabidiol (CBD) isolated from *Cannabis sativa* L. were investigated in a lipopolysaccharide (LPS)-induced systemic inflammation model in pregnancy.

Materials and Methods: Adult Wistar albino pregnant rats (n=30) were randomly divided into 5 groups; 1) Control, 2) LPS, 3) LPS + CBD 5 mg/kg, 4) LPS + CBD 10 mg/kg and 5) LPS + CBD 30 mg/kg. On days 15, 16 and 17 of gestation, intraperitoneal (i.p.) CBD injections at doses of 5 mg/kg, 10 mg/kg and 30 mg/kg were performed in the three treatment groups. Following the last CBD injection, 1 mg/kg LPS (i.p.) injection was performed. Fetal and maternal brain tissues were collected 6 hours after LPS injection. To understand the effect of CBD on inflammation and oxidative stress-mediated mechanisms in collected tissues, hematoxylin-eosin staining, immunohistochemical analysis, ELISA and biochemical methods were used to evaluate the levels of inflammatory markers interleukin 1β (IL-1β), hypoxia-induced factor-1α (HIF-1α), immün cell activation marker CD45 and oxidative stress parameters; total antioxidant level (TAS), total oxidant level (TOS) and oxidative stress index (OSI).

Results: CBD administration decreased increased IL-1β levels and CD45 expression levels in maternal brain tissue due to LPS-mediated inflammation. Furthermore, CBD treatment increased TAS levels and decreased TOS and OSI values in maternal and fetal brain tissues. In parallel with oxidative stress parameters, CBD treatment decreased HIF-1α expression levels in maternal and brain tissues.

Conclusion: CBD may have a protective effect on oxidative stress in the maternal and fetal brain due to inflammation in the LPS-induced pregnancy inflammation model. These results suggest that CBD may be a potential agent in the prevention and treatment of PE and related complications.

Keywords: Cannabidiol, inflammation, oxidative stress, pregnancy, preterm labor

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ÖZ

Amaç: Preterm eylem (PE), dünya genelinde en yaygın yeni doğan ölüm nedenlerinden biridir ve gebelikte gelişen enflamasyonun, PE'in altında yatan sebeplerden biri olabileceği düşünülmektedir. Bu çalışma kapsamında, *Cannabis sativa* L. bitkisinden izole edilen Kannabidiol (CBD)'ün gebelikte lipopolisakkarit (LPS) ile indüklenen sistemik enflamasyon modelinde, enflamasyon ve oksidatif stres aracılı koruyucu etkileri araştırılmıştır.

Gereç ve Yöntem: Yetişkin Wistar albino gebe sıçanlar (n=30) rastgele 5 gruba ayrıldı; 1) Kontrol, 2) LPS, 3) LPS + CBD 5 mg/kg, 4) LPS + CBD 10 mg/kg ve 5) LPS + CBD 30 mg/kg. Deney protokolüne göre; gebeliğin 15, 16 ve 17. günlerinde, üç tedavi grubuna 5 mg/kg, 10 mg/kg ve 30 mg/kg dozlarında intraperitoneal (i.p.) CBD enjeksiyonları gerçekleştirildi. Son CBD enjeksiyonunu takiben 1 mg/kg LPS (i.p.) enjeksiyonu gerçekleştirildi. LPS enjeksiyonundan 6 saat sonra fetal ve maternal beyin dokuları alındı. Alınan dokularda, CBD'nin enflamasyon ve oksidatif stres aracılı mekanizma üzerindeki etkisini anlamak amacıyla, hematoksilen-eozin boyama, immünohistokimyasal analiz, ELISA ve biyokimyasal yöntemler kullanılarak enflamatuvar belirteçler; interlökin 1β (IL-1β), hipoksi ile induklenen faktor-1α (HIF-1α), immün hücre aktivasyonun göstergesi olan CD45 ve oksidatif stres parametreleri; total antioksidan seviyesi (TAS), total oksidan seviyesi (TOS) ve oksidatif stres indeksi (OSİ) düzeyleri değerlendirildi.

Bulgular: CBD uygulaması maternal beyin dokusunda LPS'ye bağlı olarak artan IL-1β düzeylerini ve CD45 ifade düzeylerini azaltmıştır. Ayrıca, CBD tedavisi; maternal ve fetal beyin dokularında TAS düzeylerini artırmıştır, TOS ve OSİ değerlerini azaltmıştır. CBD tedavisi oksidatif stres parametreleriyle paralel şekilde, maternal ve beyin dokularında HIF-1α ifade düzeylerini azaltmıştır.

Sonuç: CBD'nin LPS ile indüklenen gebelikte enflamasyon modelinde, enflamasyona bağlı olarak maternal ve fetal beyinde gelişen oksidatif stres üzerinde koruyucu etkisi olabileceği düşünülmektedir. Bu sonuçlar, CBD'nin PE ve buna bağlı komplikasyonların önlenmesinde ve tedavisinde potansiyel bir ajan olabileceğini göstermektedir.

Anahtar Kelimeler: Kannabidiol, enflamasyon, oksidatif stres, gebelik, preterm eylem

INTRODUCTION

Preterm labor (PE) is defined as delivery before 37 weeks of gestation and is known to affect more than 10% of pregnancies worldwide[1]. PE is also one of the leading causes of neonatal death. In surviving infants, it may pose a high risk for infection, neurodevelopmental and cardiometabolic disorders later in life[2-4]. Several factors such as twin pregnancies, chorioamnionitis, genetic factors, maternal diseases, prior PE are known risk factors for preterm delivery[5-7]. In addition, increasing evidence suggests that inflammation and oxidative stress also play an important role in PE[8,9].

To elucidate the inflammatory process that occurs during pregnancy, it is considered necessary to understand the impairment of feto-maternal immune tolerance that occurs during the inflammatory process and is associated with pregnancy complications[10,11]. Many factors with an inflammatory process have been found to contribute to maternal immune activation, including obesity, gestational diabetes, pre-eclampsia, smoking, exposure to environmental pollution, low socioeconomic status, depression, stress, autoimmune diseases, asthma and infection[12]. The main risk factors for PE remain unclear, but increasing evidence supports a central role for dysregulated inflammatory response and oxidative stress in PE[9]. Regulation of inflammation and oxidative stress is an important component of a healthy pregnancy. The changes that occur during maternal inflammation help to maintain both maternal and fetal health[13]. Healthy pregnancy is characterized as an oxidant period during which the production of reactive oxygen species (ROS) occurs, oxidative stress characterizes, plasma levels of free anti-oxidants are reduced, and purine catabolism is increased[14]. These oxidant features observed in many pregnancy-related disorders, including PE, may be exacerbated. The oxidative stress that occurs in PE can lead to cell damage by causing an increase in the production of cytokines such as TNF-α and interleukin (IL)-6 as an inflammatory response and a decrease in the production of anti-inflammatory cytokines such as IL-10[15,16].

Oxidative stress is defined as an imbalance between the production of ROS or reactive nitrogen species and the protective capacity of defensive anti-oxidants. It can be produced through the anti-oxidant system responsible for the control of reactive species and oxidative damage in cells or through enzymatic or non-enzymatic endogenous mechanisms[17]. Oxidative stress develops from early pregnancy. Higher lipid peroxidation is observed in pregnant women compared to non-pregnant women[18]. Overproduction of free reactive radicals is known to cause damage to cell structure and consequently increase the risk of pregnancy disorders, including first trimester abortion, pre-eclampsia and intrauterine growth retardation[19-21].

It is thought that reducing maternal and fetal pro-inflammatory responses during pregnancy may be beneficial in protecting the fetus from inflammation. Studies have shown that agents such as magnesium sulfate, folic acid, melatonin and N-acetyl cysteine may have neuroprotective effects in order to prevent PE and related neurodevelopmental disorders such as cerebral palsy and autism spectrum disorder[22-25]. However, there is still controversy about the possible neuroprotective effects of existing agents on the fetal brain and the safety of their use. Therefore, the need for reliable molecules, especially those isolated and developed from natural sources, continues.

Cannabidiol (CBD), one of the primary non-euphoric components in the *Cannabis sativa* L. plant, is used therapeutically in patients with Lennox-Gastaut syndrome

and Dravet syndrome. Recent studies have drawn attention to the anti-inflammatory, anti-oxidant and neuroprotective properties of cannabinoid molecules such as CBD and Δ^9-tetrahydrocannabinol (Δ^9-THC)[26,27]. Although various disease-related mechanisms are hypothesized in the pathogenesis of PE, where oxidative stress and the inflammatory process are thought to be interconnected, it is known that current treatments alleviate term-action-related consequences but are not sufficient to prevent progression.

Based on all this information shared, this research study aimed to investigate the possible protective effects of CBD, which has been proven to have anti-oxidant and anti-inflammatory effects, on the oxidative stress process caused by inflammation in the fetal and maternal brain in a lipopolysaccharide (LPS)-induced inflammation model in pregnant rats.

MATERIALS AND METHODS

Chemicals

CBD was obtained from Süleyman Demirel University-Natural Products Application and Research Center. The source of CBD is *Cannabis sativa* L. extract. CBD content was >99.9% and THC content was <0.01%, residual alcohol and heavy metal limits are in accordance with USP and EU pharmacopoeia. CBD is dissolved in 100% ethanol (Merck Chemicals, ≥ 99.9%). LPS from *Escherichia coli* O: 127:B8 (#L3129) was obtained from Sigma Aldrich. Mouse monoclonal anti-CD45 (#M0701) was purchased from Dako and mouse polyclonal anti- hypoxia-induced factor 1α (HIF-1α) (#MC0224) was purchased from Medaysis.

Experiment Protocol

All animal care and experimental procedures in this study were conducted in accordance with the animal research guidelines of the National Institutes of Health and approved by the Animal Research Committee of Süleyman Demirel University (decision no: 209, date: 21.09.2023). Thirty female Wistar albino rats weighing 250-300 g were housed in Euro type-IV cages with two males and one female in each cage at 22-24 °C temperature, 55-60% humidity and 12 h light/12 h dark conditions. Vaginal smear were taken from the female rats after 12 hours to confirm mating and the presence of spermatozoa was considered as day zero of pregnancy for the female rats. Rats with confirmed pregnancy were randomly divided into 5 groups.

Groups

A total of 30 pregnant rats, 1) Control, 2) LPS, 3) LPS + CBD 5 mg/kg, 4) LPS + CBD 10 mg/kg, 5) LPS + CBD 30 mg/kg were divided into 5 groups (n=6 per group). On days 15, 16 and 17 of gestation, intraperitoneal (i.p.) CBD injections at doses of 5 mg/kg, 10 mg/kg and 30 mg/kg were performed in the three treatment groups. On day 17, after the last CBD injection, a systemic inflammation model was induced with 0.5 mL volume of LPS (i.p.) at a dose of 1 mg/kg. Maternal and fetal brain tissues were removed by hysterotomy following abdominal incision with ketamine (90 mg/kg)/xylazine (8-10 mg/kg) 6 hours after LPS injection. LPS (#L2630) was obtained from Sigma-Aldrich.

Hematoxylin-Eosin (H&E) Application

Formalin-fixed paraffin blocks were sectioned at 4-5 µm and H&E-stained slides were examined to visualize gross morphology.

Immunohistochemical Application

Blocks of fetal brain and maternal brain tissues were treated with clinically validated CD45 (Dako, #M0701) and HIF-1α (Medaysis, #MC0224) antibodies. Antibody dilutions were prepared according to the manufacturer's instructions. Dako Omnis fully automated sample preparation and staining system was used. Tissue samples were cut from formalin-fixed paraffin-embedded blocks and 4 µm thick sections of human tonsil tissue were taken as positive control. For antigen recovery, tissues were incubated with Envision-FLEX (Carpinteria, CA, USA), high pH solution at 97 °C for 30 minutes and then rinsed with wash buffer for two minutes. After the antigen retrieval step, CD45 and HIF-1α primary antibody incubation was performed for 30 minutes. The slides were then rinsed with washing buffer for 2 minutes. Next, Envision-FLEX peroxidase blocking solution was applied for 3 minutes and rinsed. Before the 20-minute Envision-FLEX/HRP incubation step, the slides were incubated with secondary antibody. Washing steps were then performed. Envision substrate working solution was incubated in chromogen for 5 minutes and washed. Finally, hematoxylin was applied for 3 minutes for counterstaining.

Histopathologic Evaluation

Tissue morphology and cells in H&E sections were evaluated by comparison with immunohistochemical method. Semi-quantitative scoring was performed in immunohistochemical examination. Cells stained with CD45 at 200X magnification were considered negative (score: 0), 5-25 cells (score: 1), 26-50 cells (score: 2) and >50 cells (score: 3). For HIF-1α, negative staining was considered as 0, >0% and ≤25% tissue expression as weak (score: 1), >25% and ≤50% tissue expression as moderate (score: 2), and >50% tissue expression as high (score: 3)[28,29].

Determination of Total Oxidant Levels (TOS)

Serum TOS was determined using Rel Assay (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Türkiye) kit, an automated

measurement method developed by Erel[30], following the protocol steps recommended by the manufacturer. Oxidants present in the sample oxidize the iron ion o-dianisidine complex to Fe^{+3}. The oxidation reaction is enhanced by glycerol molecules present in the reaction medium. The Fe^{+3} ion forms a colored complex with xylenol orange in acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the amount of oxidant molecules present in the sample. According to the manufacturer's instructions, 45 µL of sample or standard or H_2O was added to 96-well plates. Then, 300 µL of reagent 1 solution containing buffer solution and health effects of sulfuric acid (H_2SO_4) was added to the wells and after 30 s of incubation, the first absorbance value was measured on a spectrophotometer at 530 nm. Following this step, 15 µL of reagent 2 solution consisting of substrate solution, H_2SO_4, ferrous ion and o-dianisidine was added. After incubation at 37 ºC for 5 min, the second absorbance value was measured at 530 nm on a spectrophotometer. Hydrogen peroxide (H_2O_2) was used for calibration and the results were expressed in micromolar H_2O_2 equivalents per liter (µmoL H_2O_2 eq/L)[31]. The results were plotted as fold change and ± standard deviation (SD).

Determination of Total Antioxidant Levels (TAS)

Serum TAS was determined using Rel Assay (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Türkiye), an automated measurement method developed by Erel[32], following the protocol steps recommended by the manufacturer. First, 18 µL of sample or standard or H_2O was added to 96-well plates. Then 300 µL of reagent 1 consisting of buffer solution and acetate buffer was added and after 30 s the initial absorbance was measured at 660 nm in a spectrophotometer. After the measurement, 45 µL of reagent 2 containing prochromogen and ABTS was added to each well and after incubation at 37 ºC for 5 min, the second absorbance value was measured at 660 nm in a spectrophotometer. According to this method, the amount of antioxidants in the sample was determined against the strong free radical reactions initiated by the hydroxyl radical produced. The results were expressed as millimoles of Trolox equivalents per liter (eq/L)[31]. The results are presented in the graph as fold change and ± SD.

Calculation of Oxidative Stress Index (OSI)

OSI ratio was calculated as the ratio of TOS to TAS level and expressed as a percentage. For this calculation, TAS units were evaluated as mmol/L and OSI value was calculated according to the formula [TOS (µM H_2O_2 eq/L)/TAS(mmol Trolox eq/L) x100][30,31]. The results are presented in the graph as fold change and ± SD.

Determination of Interleukin-1 beta (IL-1β) Levels by ELISA

After sacrification, fetal and maternal brain tissues obtained from rats were portioned in the range of 50-100 mg. 1xPBS was added at a ratio of 1:10 and homogenized by sonication. A commercially available ELISA kit (Cloud Clone Corp, USCN, #L211201990) was used for IL-1β quantification in maternal and fetal brain tissues. Following the manufacturer's instructions, 100 µL of tissue samples or standards homogenized 1:9 with 1xPBS were added to 96-well plates coated with anti-IL-1β antibody and incubated at 37 ºC for 1 hour. After incubation, substrate solution and reaction terminator solutions were added to the wells, respectively. Subsequently, optical absorbance was measured at 450 nm on a spectrophotometer (BioTek, Epoch2). IL-1β levels in tissue samples were calculated using the line equation on the graph generated using the optical density values of the standards.

Statistical Analysis

Data were analyzed using GraphPad Prism 8.0 software. One-way ANOVA analysis of variance and Tukey's post-hoc t-test were used to evaluate the statistical significance of differences between the control and experimental groups. Differences were considered significant for $p<0.05$.

RESULTS

Investigation of Oxidative Stress Parameters in Fetal Brain Tissues

In order to evaluate inflammation-mediated oxidative stress levels in the LPS-induced inflammation model in pregnancy, changes in TAS, TOS and OSI levels in fetal brain tissues were evaluated. According to our results, there was a decrease in TAS levels in the LPS group compared to the control group in fetal brain tissues (Figure 1A). However, this change was not statistically significant. Although an increase in TAS levels was observed in the LPS + CBD 5 mg/kg and LPS + CBD 10 mg/kg groups compared to the LPS group, these changes were not statistically significant (Figure 1A). There was a statistically significant increase in TAS levels in the LPS + CBD 30 mg/kg group compared to the LPS group ($p<0.05$) (Figure 1A).

There was a significant increase in TOS levels due to LPS administration compared to the control group ($p<0.05$) (Figure 1B). When LPS group was compared with LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, it was determined that TOS levels in LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups showed a statistically significant decrease (Figure 1B).

When analyzed in terms of OSI values, it was determined that there was a significant increase in OSI levels due to LPS administration compared to the control group (Figure 1C). When the LPS group was compared with the LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, it was determined that OSI levels decreased statistically in inverse proportion with increasing dose of CBD ($p<0.05$) (Figure 1C). Our results showed that CBD treatment decreased oxidative stress parameters in fetal brain tissues in LPS-induced systemic inflammation model in pregnancy (Figures 1A-C).

Investigation of Oxidative Stress Parameters in Maternal Brain Tissues

Changes in TAS, TOS and OSI levels were evaluated to assess oxidative stress parameters in maternal brain tissues of the inflammation model. In parallel with fetal brain tissues, there was a decrease in TAS levels in maternal brain tissues in the LPS group compared to the control group (Figure 2A). However, this decrease was not statistically significant. Although an increase in TAS levels was observed in the LPS + CBD 5 mg/kg and LPS + CBD 10 mg/kg groups compared to the LPS group, these changes were not statistically significant (Figure 2A). There was a statistically significant increase in TAS levels in the LPS + CBD 30 mg/kg group compared to the LPS group ($p<0.05$) (Figure 2A).

There was a statistically significant increase in TOS levels due to LPS administration compared to the control group ($p<0.05$) (Figure 2B). In the LPS + CBD 5 mg/kg group, there was a decrease in TOS levels compared to the LPS group, but this change was not statistically significant. Compared to the LPS group, TOS levels of LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups showed a statistically significant decrease ($p<0.05$) (Figure 2B).

In OSI values, a significant increase in OSI levels was observed due to LPS administration compared to the control group (Figure 2C). There was a statistically significant decrease in the OSI values of LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups in inverse proportion to the increasing dose of CBD compared to the LPS group ($p<0.05$) (Figure 2C). The results showed that CBD administration decreased oxidative stress parameters in maternal brain tissues in parallel

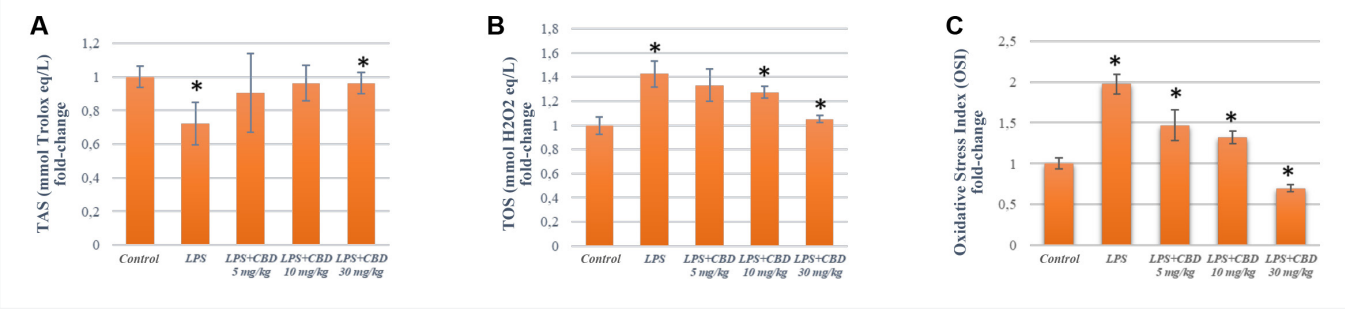

Figure 1. (A) TAS, (B) TOS and (C) OSI values of fetal brain tissues. One-way ANOVA and Tukey's multiple comparison tests were used

'' p<0.05 was considered statistically significant. TAS: Total antioxidant levels, TOS: Total oxidant levels, OSI: Oxidative stress index, LPS: Lipopolysaccharide, CBD: Cannabidiol, H_2O_2: Hydrogen peroxide*

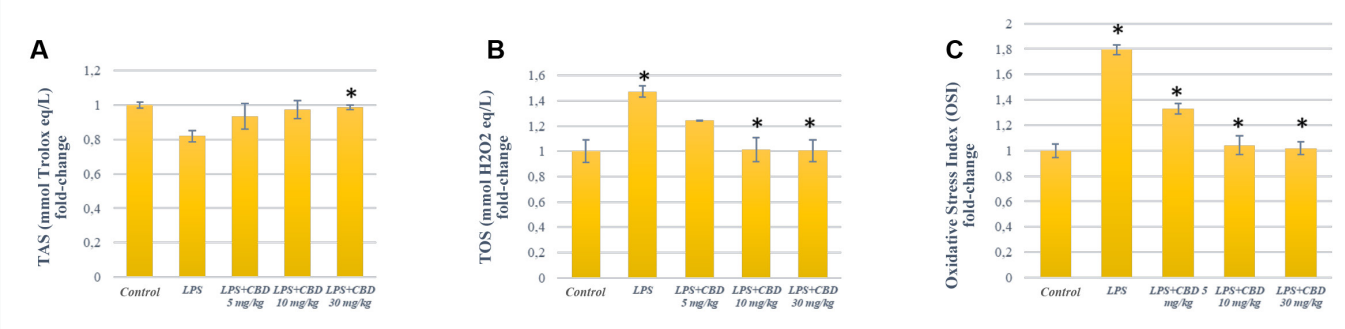

Figure 2. (A) TAS, (B) TOS and (C) OSI values of maternal brain tissues. One-way ANOVA and Tukey's multiple comparison tests were used

'(p<0.05 was considered statistically significant. TAS: Total antioxidant levels, TOS: Total oxidant levels, OSI: Oxidative stress index, LPS: Lipopolysaccharide, CBD: Cannabidiol, H_2O_2: Hydrogen peroxide*

with fetal brain in LPS-induced systemic inflammation model (Figures 2A-C).

Determination of IL-1β levels by ELISA

In order to understand the effect of CBD on maternal brain tissue in the systemic inflammation model induced by LPS, changes in IL-1β levels, which are known to increase in inflammatory processes, were examined by ELISA. It was determined that there was a statistical increase in IL-1β levels in maternal brain tissues due to LPS administration compared to the control group. IL-1β levels in CBD 10 mg/kg and CBD 30 mg/kg groups were significantly decreased compared to the LPS group (Figure 3).

Histopathologic Findings in Maternal and Fetal Brain Tissues

H&E staining was performed to examine the effect of inflammation at the maternal and fetal tissue level in the LPS-mediated pregnancy inflammation model. In the maternal brain tissues of the LPS group, an intense presence of lymphocytes was observed compared to the control group. In the LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, there was a decrease in lymphocyte levels in the opposite direction with increasing dose compared to the LPS group, and the presence of lymphocytes was similar to the control group (Figure 4A).

In fetal brain tissue samples, an intense amount of congestion was observed in the LPS group compared to the control group. In the LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, there was a decrease in congestion findings inversely proportional to the increasing dose (Figure 4B).

Immunohistochemical Determination of CD45 and HIF-1α Levels in Maternal Brain Tissues

CD45 and HIF-1α levels were immunohistochemically determined to evaluate inflammation-mediated oxidative

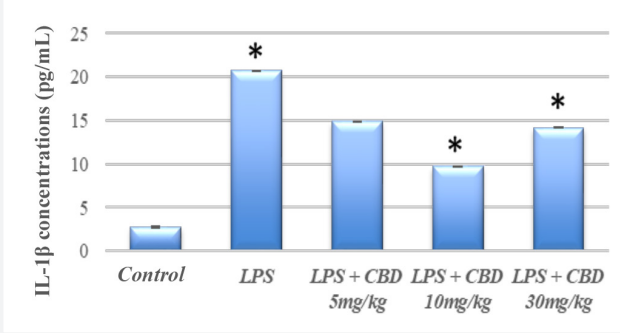

Figure 3. Evaluation of IL-1β concentrations in maternal brain tissues by ELISA method. Analyzed by One-way ANOVA and Tukey's multiple comparison tests

'' p<0.05 was considered statistically significant. LPS: Lipopolysaccharide, CBD: Cannabidiol, IL-1b: Interleukin-1 beta*

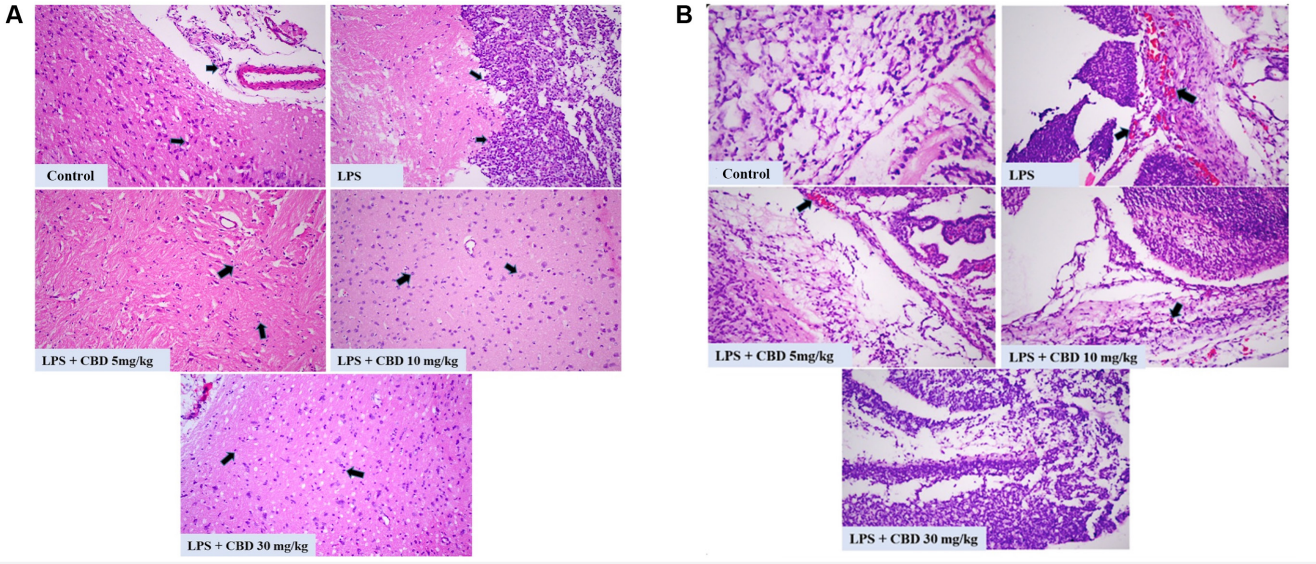

Figure 4. A) Lymphocytes, microglial cells and glial cells are shown with arrows by Hematoxylin-Eosin staining in Control, LPS, LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg, LPS + CBD 30 mg/kg groups of maternal brain tissues. B) Fetal brain tissues of Control, LPS, LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg, LPS + CBD 30 mg/kg groups in which congestion was observed by Hematoxylin-Eosin staining, glial cells and meningeal areas are shown with arrows (H&E, 200X)

LPS: Lipopolysaccharide, CBD: Cannabidiol, H&E: Hematoxylin-eosin

stress in maternal brain tissues. It was observed that CD45 levels were higher in the LPS group compared to the control group (Figure 5A). These findings indicate that the presence of microglial cells and lymphocytes was statistically significantly more intense in the LPS group compared to the control group (Figure 5A). In the LPS + CBD 5 mg/kg and LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, a statistically significant dose-dependent decrease in CD45 levels was observed compared to the LPS group (Figures 5A, 5C). These results indicate that CBD treatment decreased the presence of microglial cells and lymphocytes in maternal brain tissues in an inversely proportional manner with increasing dose compared to the LPS group (Figures 5A, 5C).

HIF-1α, which is an important regulator of gene expression related to hypoxia response, is also known to play a role in inflammation[33]. In the LPS-induced pregnancy inflammation model, HIF-1α levels in maternal brain tissues were examined by immunohistochemistry. Although there was some increase in HIF-1α levels in microglial cells, lymphocytes and tissue (prominent in vascular endothelium) in the control group, a statistically significant increase in HIF-1α levels in microglial cells, lymphocytes and tissue was observed in the LPS group compared to the control group (Figure 5B). In the LPS + CBD 5 mg/kg and LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, a dose-dependent decrease in HIF-1α levels was determined in the LPS + CBD 30 mg/kg group compared to the LPS group, which was statistically significant (Figures 5B, 5C). These findings showed that oxidative stress was reversed with CBD treatment in parallel with LPS-induced inflammation (Figures 5B, 5C).

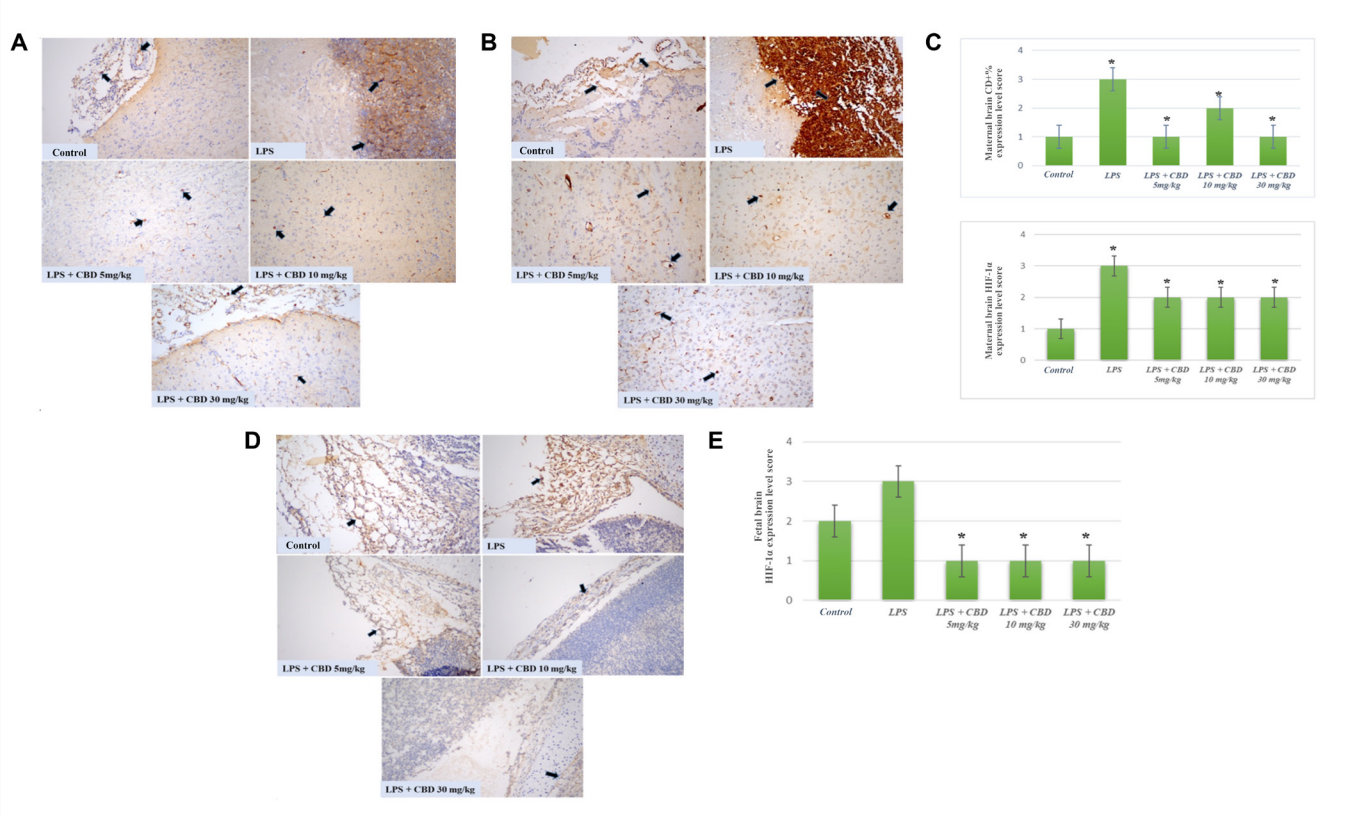

Figure 5. Maternal brain tissues in Control, LPS, LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg, LPS + CBD 30 mg/kg groups A) Immunohistochemical CD45 levels in lymphocytes and microglial cells are shown with arrows. B) HIF-1α expression levels in lymphocytes, microglial cells and tissue are shown with arrows by immunohistochemical method (IHC, 200X). C) Statistical evaluation of the scores of maternal brain CD45 and HIF-1α expression levels. One-way ANOVA and Tukey's multiple comparison tests were used

'' p<0.05 was considered statistically significant. D) In fetal brain tissues, HIF-1α expression levels were determined in meninges and glial tissues in Control, LPS, LPS + CBD 5 mg/kg, LPS + CBD 10 mg/kg, LPS + CBD 30 mg/kg groups. The areas of HIF-1α expression are indicated by arrows (IHC, 200X). E) Statistical evaluation of the scores of fetal brain HIF-1α expression levels. One-way ANOVA and Tukey's multiple comparison tests were used. '*' p<0.05 was considered statistically significant. LPS: Lipopolysaccharide, CBD: Cannabidiol, HIF-1α: Hypoxia-induced factor-1α*

Determination of HIF-1α Levels in Fetal Brain Tissues by Immunohistochemical Method

HIF-1α levels in fetal brain tissues were examined immunohistochemically to evaluate the effects of oxidative stress on fetal brain tissues in the inflammation model induced during pregnancy. Although a small increase in HIF-1α levels was observed in meningeal and glial tissues in the control group, a statistically significant increase in HIF-1α levels was observed in the LPS group (Figure 5D). In the LPS + CBD 5 mg/kg and LPS + CBD 10 mg/kg and LPS + CBD 30 mg/kg groups, a statistically significant decrease in HIF-1α levels was observed compared to the dose-dependent LPS group (Figures 5D, 5E). These results showed that HIF-1α levels increased in fetal brain tissues due to oxidative stress in parallel with maternal brain tissues (Figures 5D, 5E).

DISCUSSION

The cannabis plant has long been the focus of much research due to its medicinal effects. Cannabinoids derived from hemp are called phytocannabinoids. Among them, THC and CBD are the most studied phytocannabinoids[34]. CBD, one of the naturally occurring compounds in cannabis, has become increasingly popular for the treatment of various disorders[35]. The lack of psychoactive effects and low side effect profile compared to THC are among the advantages of CBD and support it as a reliable therapeutic agent. For this reason, the use of CBD has become widespread in conditions such as acute and chronic pain, anxiety, seizure disorders, osteoarthritis, migraine, insomnia and cancer[36]. In addition, commercial preparations containing CBD are used therapeutically in conditions such as Lennox-Gastaut syndrome and Dravet syndrome[26,27]. Although there is insufficient evidence on the safety of CBD use during pregnancy, CBD has been used in pregnant women for symptoms such as nausea, insomnia, anxiety and chronic pain[37]. In this study, we aimed to investigate the possible protective effects of CBD on fetal neuroinflammation-mediated oxidative stress in an LPS-induced systemic inflammation model of pregnancy.

PE is the leading cause of death in children under 5 years of age, accounting for approximately 11% of births worldwide[38]. Preterm infants are at risk of a range of health complications, including respiratory and gastrointestinal disorders, particularly cerebral palsy[39,40]. PE is thought to be a syndrome that can be triggered by multiple mechanisms, including infection or inflammation, uteroplacental ischemia or hemorrhage, stress and other immunologically mediated processes[39,41]. Since most of the risk factors that cause PE lead to increased systemic inflammation, it has been suggested that increased stimulation of infection and inflammation may be associated with multiple risk factors in the mechanisms underlying PE[42]. However, the presence of inflammatory mediators in the uterus has been associated with fetal damage, particularly affecting the fetal lungs and brain[43,44]. Our histopathology findings showed an increased presence of lymphocytes in maternal brain tissues with LPS administration (Figure 4A). Similarly, in the fetal brain tissues, intense congestion was observed in the LPS group due to inflammation, indicating that the inflammation process negatively affected fetal and maternal brain tissues (Figure 4B). On the other hand, CBD administration resulted in a dose-dependent decrease in maternal brain lymphocyte levels and fetal brain congestion findings (Figures 4A, 4B).

IL-1 cytokines; IL-1α, IL-1β and IL-1Ra are known to play an important role in processes such as immune system regulation and inflammation[45]. IL-1β, which is included in the IL-1 family consisting of 11 members, has been included as a therapeutic target for systemic and local inflammatory conditions known as autoinflammatory diseases, the incidence of which is increasing[46]. Our serologic measurements, which we performed to understand the effect of the systemic inflammation model in pregnancy on maternal brain tissue, showed that IL-1β levels, which increased with LPS administration, decreased dose-dependently with CBD administration (Figure 3).

CD45 is a transmembrane glycoprotein and protein tyrosine phosphatase with a molecular weight of 180-220 kDa expressed on all leukocytes. It is also known that CD45 constitutes approximately 10% of cell surface antigens[47,48]. There is increasing evidence that CD45 is involved in the regulation of the immune system[49]. Immunohistochemical studies we performed to evaluate the effects of maternal inflammatory response on the maternal brain in case of systemic inflammation show that the increase in CD45 levels in microglial cells and lymphocytes of maternal brain tissues of the LPS group was reversed by CBD administration in a dose-dependent manner (Figures 5A, 5C).

Hypoxia is known as a state of oxygen deficiency and increased adenosine triphosphate production in cells of metabolically active organs. In a state of hypoxia, the oxygen consumption of the biological system cannot be met[50] and this can impair cellular functions and prevent the maintenance of normal homeostasis[50,51]. Oxygen deficiency or hypoxia causes oxidative stress and the formation of HIF and ROS. HIF is a transcription factor involved in cell physiological responses to hypoxia[52] and HIF-mediated signaling mechanisms are involved in important processes such as cell survival, signaling, migration, anaerobic metabolism and vasodilation[53-55]. HIF is a heterodimer composed of any of three α subunits and a β subunit[56]. HIF-1α is known to be a master regulator of oxygen homeostasis and affects the transcription of genes involved in oxygen homeostasis[57]. Preclinical and clinical studies suggest that maternal oxidative

stress and immune activation have a negative impact on fetal neurodevelopmental process. However, HIF-1 has been shown to be associated with brain development, neurogenesis and neuroprotection[58-60]. Abnormal HIF-1α activation has been observed in pathological conditions such as neurodegenerative diseases and traumatic brain injury[60,61]. Kletkiewicz et al.[62] reported that CBD reduces hypoxia-induced oxidative stress due to its antioxidant activity.

In this study, we examined oxidative stress parameters and fetal and maternal brain HIF-1α levels by immunohistochemistry to evaluate the effects of CBD on inflammation-induced oxidative stress in the maternal and fetal brain. In the fetal brain and maternal brain tissues, it was determined that there was a dose-dependent increase in TAS levels with CBD treatment compared to the LPS group (Figure 1A, Figure 2A), whereas TOS levels decreased inversely with increasing doses of CBD compared to the LPS group (Figure 1B, Figure 2B). Immunohistochemical evaluations revealed that CBD caused a dose-dependent decrease in HIF-1α levels in fetal and maternal brain tissues compared to the LPS group (Figures 5B, 5D). Our results suggest that CBD reversed the inflammation-induced oxidative stress parameters in fetal and maternal brain tissues in the LPS-induced inflammation model. In addition, in maternal tissues, HIF-1α levels, which were observed to increase in the LPS group, decreased due to CBD administration, suggesting that CBD may have a protective effect on oxidative stress, which is thought to occur due to neuroinflammation.

Study Limitations

The main limitation of our study is that further comprehensive studies are needed to more precisely understand the mechanism of the protective roles of CBD administration in inflammation during pregnancy.

CONCLUSION

Collectively, our results suggest that CBD, one of the most important components of Cannabis, may have a protective effect on fetal and maternal brain oxidative stress that develops due to systemic inflammation during pregnancy.

Ethics

Ethics Committee Approval: All animal care and experimental procedures in this study were conducted in accordance with the animal research guidelines of the National Institutes of Health and approved by the Animal Research Committee of Süleyman Demirel University (decision no: 209, date: 21.09.2023).

Informed Consent: Animal experiment.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: D.Ç., Concept: Y.E., Design: Y.E., Data Collection or Processing: D.Ç., Y.E., O.E., S.S., Analysis or Interpretation: D.Ç., Y.E., O.E., S.S., Literature Search: D.Ç., Y.E., O.E., Writing: D.Ç., Y.E.

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