RESEARCH ARTICLE / *Araştırma Makalesi*

Protective Effect of Cannabinoid Type 2 Receptor Agonist Against Ovarian Ischemia/reperfusion Injury in Rats

Kannabinoid Tip 2 Reseptör Agonistinin Sıçanlarda Over İskemi/reperfüzyon Hasarına Karşı Koruyucu Etkisi

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Abstract

Objective: The aim of this study is to prove whether or not the cannabinoid (CB)2 receptor agonist, which is known to be effective in anti-inflammatory effects and tried in various organ and/or tissue ischemia/reperfusion (I/R) injuries, works in the treatment of I/R injury, which is a replication of the damage caused by clinical ovarian torsion.

Materials and Methods: Rats were divided into five groups (n=6). After anesthesia, an approximately 2-cm incision was made in the lower abdominal region of the rats and ovaries, uterine horns, and adnexa were occluded with a clamp for 30 min, except for the sham control group. After 3 h, ovaries were surgically removed. Blood was collected for the measurement of cytokine levels in the serum.

Results: The induction of ovaries by I/R injury resulted in an increase in interleukin 1 β, tumor necrosis factor alpha (TNF-α), and malondialdehyde (MDA) levels and decrease in glutathione (GSH) levels in ovarian tissues and serum. The pretreatment of the CB2 receptor agonist decreased these cytokines levels in both ovarian tissues and serum. Both ovarian tissue and serum GSH levels decreased due to I/R injury. CB2 agonist caused an increase in GSH level. However, the antagonist reversed the healing effect of the agonist on GSH level.

Conclusion: The results of this study support that the CB2 receptor agonist can be used to treat I/R injury, which is an imitation of ovarian torsion.

Keywords: Ischemia Reperfusion; CB2 Agonist; Ovarian Torsion; Cytokines; CB2 Antagonist.

Öz

Amaç: Bu çalışmanın amacı, anti-enflamatuar etkili olduğu bilinen ve çeşitli organ ve/veya doku iskemisi/reperfüzyon (I/R) yaralanmalarında denenen kannabinoid (CB) 2 reseptör agonistinin klinik over torsiyonunun neden olduğu hasarın bir göstergesi olan I/R hasarının tedavisinde etkisinin olup olmadığını kanıtlamaktır.

Gereç ve Yöntemler: Ratlar beş gruba ayrıldı (n=6). Anestezi sonrası, sıçanların alt karın bölgesinde yaklaşık 2 cm'lik bir insizyon yapıldı ve yumurtalıklar, rahim boynuzu ve adneks, şam kontrol grubu hariç 30 dakika boyunca bir klemp ile kapatıldı. 3 saat sonra, yumurtalıklar cerrahi olarak çıkarıldı. Serumdaki sitokin seviyelerinin ölçümü için kan alındı.

Bulgular: Yumurtalıkların I/R hasarı ile indüklenmesi, interlökin 1 beta (IL-1β), tümör nekroz faktörü alfa (TNF-α) ve malondialdehit (MDA) seviyelerinde bir artışa ve yumurtalık dokularında ve serumda glutatyon (GSH) seviyelerinde bir azalmaya neden olmuştur. CB2 reseptörü agonistinin ön tedavisi, hem over dokularında hem de serumda bu sitokin seviyelerini düşürmüştür. Hem over dokusu hem de serum GSH düzeyleri I/R hasarı nedeniyle azaldı. CB2 agonisti GSH seviyesinde bir artışa neden oldu. Ancak, antagonist, agonistin GSH seviyesi üzerindeki iyileştirici etkisini tersine çevirdi.

Sonuç: Bu çalışmanın sonuçları CB2 reseptörü agonistinin, yumurtalık torsiyonunun bir imitasyonu olan I/R hasarını tedavi etmek için kullanılabileceğini desteklemektedir.

Anahtar Kelimeler: İskemi Reperfüzyon; Kannabinoid 2 Reseptör Agonist; Ovaryum Torsiyonu; Sitokinler; Kannabinoid 2 Reseptör Antagonist.

INTRODUCTION

The development of ischemia in the vessels the ovary is one of the serious health problems in the world, leading to infertility and psychological problems. Ovarian cysts, pregnancy, polycystic ovary syndrome, and transient or permanent obstruction of the ovarian artery may cause ischemia. The most common pathological condition is ovarian torsion. Fertilization can be maintained if medical and surgical intervention is performed quickly and appropriately.[1] Ovarian torsion is an emergency in the gynecology clinic. It is particularly important when diagnosed during the reproductive period. Ischemia/reperfusion (I/R) injury can damage ovarian tissues and also reduce ovarian reserve.[2] Therefore, the duration of diagnosis is important. In addition, reperfusion may reduce ischemic injury to ovarian tissues, but it may paradoxically cause reperfusion injury.[3]

I/R injury is a series of interrelated, complex, cellular, and humoral events. There are four main factors responsible for the pathophysiology of I/R injury: (1) free oxygen radicals,[2] polymorphonuclear leukocytes (PNL), (3) complement system, and [(4)] endothelial cells. These four factors are responsible for reactive oxygen species (ROS) formation.[4] Furthermore, cytokines and chemokines such as interleukin 1 (IL-1), IL-6, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) released by PNL and proteases such as elastase and collagenase lead to more inflammatory cell aggregation and increased ROS formation in the region.[5] In addition, PNL create microvascular obstructions by forming a cell population within the vessel and adhering to the endothelium with active platelets.[6] Lipid peroxide radicals initiate new reactions by removing hydrogen atoms from other polyunsaturated fatty acid molecules, and malondialdehyde (MDA) is formed as the final product.[7]

Inflammation-induced improvements in vascular permeability result in the accumulation of more fluid in endothelial cells than can be absorbed.[8] In previously studies on cannabinoid (CB) agonists, they have been found to reduce the antinociceptive and anti-inflammatory effects of CB agonists.[3,9,10] In particular, they have been found to mediate the effects of antiedema and anti-inflammatory through peripheral CB2 receptors.[11]

In the ovarian I/R model, experimentally, tadalafil,[12] ghrelin,[13] various antioxidants,2 Vaccinium myrtillus extract,[14] Ginkgo biloba extract,[15] curcumin,[16] and magnesium[17] were used. However, CBs have never been used for this purpose. Therefore, CBs use for ovarian torsion seems to be reasonable in theory because of their anti-inflammatory effects.

MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA), (R,S)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole (AM1241), 6-iodo-2-methyl-1-(2-(4-morpholinyl)ethyl)-1H-indol-3-yl)(4-methoxyphenyl)methanone (AM630), thiobarbituric acid (TBA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Na2HPO4, and dimethylsulfoxide (DMSO) used in this study were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Animals

In this study, 28 female Wistar albino rats weighing 200–250 g were used. The study was started after obtaining an approval from the Ethics Committee of Adıyaman University, Adıyaman, Turkey (2018/024). The rats were randomly divided into five groups, with five to six rats in each group. The rats were maintained at room temperature (23°C ± 3°C) under 12-h light–dark cycles and fed with commercial rat feed and tap water ad libitum.

Experimental design and experimental groups

Group 1: Sham control group. The rats were anesthetized; an incision of approximately 2 cm was made in the lower abdomen; and ovaries, uterine horns, and adnexa were observed for 1 min and then the abdominal wall was clo-

sed with 3–0 silk sutures. After 3 h, relaparotomy was performed, and ovaries were surgically removed. Blood was drawn from the heart for the measurement of cytokine levels in the serum.

Group 2: I/R group. The rats were anesthetized; an incision of approximately 2 cm was made in the lower abdomen; and ovaries, uterine horns, and adnexa were clamped for 30 min. After 2.5 h of reperfusion, ovaries were surgically removed.

Group 3: CB2 receptor agonist + I/R. The CB2 receptor agonist was administered, 3 mg/kg intraperitoneally (IP) daily, starting 3 days prior to the surgery. After the last application, the rats were anesthetized and treated as in Group 2.

Group 4: CB2 receptor antagonist + agonist + I/R. At 10-min intervals, the CB2 receptor antagonist and agonist were administered IP at doses of 1 and 3 mg/kg, respectively, starting 3 days prior to the surgery. After the last application, the rats were anesthetized and treated as in Group 2.

Group 5: I/R + DMSO (in order to solve the CB2 receptor agonist and antagonist). DMSO was administered, 2 ml IP daily, starting 3 days prior to the surgery. After the last application, the rats were anesthetized and treated as in Group 2.

Collection of ovarian tissues

The ovarian tissue was weighed after washing with +4 degrees of saline and drying. After weighing, it was divided into four parts and put into Eppendorf tubes and stored at – 86°C until examination.

Measurement of cytokine levels in ovarian tissues and serum

The IL-1β and TNF-α levels were measured in ovarian homogenate and serum, using enzyme-linked immunosorbent assay kits for rats (Thermo Fisher Scientific, Inc.). This process was performed twice, as per the manufacturer's instructions,[18,19] and the results were expressed as pg/ml serum and pg/g wet tissue.

Measurement of MDA levels in ovarian tissues and serum

The lipid peroxide levels in ovarian tissues were MDA concentrations according to the previously described method.[9] The tissue samples were briefly homogenized in an ice-cold bath. Cold TCA was homogenized by adding 10 ml of 10% TCA per g tissue with an ultrasonic tissue homogenizer. After centrifugation at 3000 g for 15 min, 0.5 ml of the supernatant was mixed with an equal volume of 0.67% TBA and heated to 100°C for 15 min. The absorbances of the samples were then measured spectrophotometrically at 535 nm. Each experiment was performed twice.

Measurement of glutathione levels in ovarian tissues and serum

The glutathione (GSH) levels in ovarian tissues and serum were measured using the modified Ellman method, with minor changes.[20] Na2HPO4 solution (2 ml of 0.3 M) was added to 0.5 ml of the supernatant obtained using the same homogenization procedure as described above. DTNB solution (0.2 ml) was added to the mixture and the absorbance at 412 nm was measured immediately after vortexing. Each experiment was repeated twice.

Statistical analyses

All values are reported as mean ± standard deviation. Statistical analysis was performed using the one-way analysis of variance (ANOVA), following the Bonferroni post hoc test. Statistical significance was set at a p-value of less than 0.05. Data analysis was performed using Prism 7.0 software (GraphPad, San Diego, CA, USA).

RESULTS

Amount of cytokine levels in ovarian tissues and serum

In this study, the IL-1β and TNF-α levels in both ovarian tissues and serum were measured. As shown in Figure

1(a), the induction of ovaries by I/R injury significantly increased the TNF-α levels (3066.67 ± 381.66 pg/ml) in the serum, compared to those in the sham control group (823.33 ± 206.56 pg/ml). The agonist significantly reduced ovarian I/R injury in terms of serum TNF-α (1434.67 ± 400.91 pg/ml). The administration of the antagonist reversed the effect of agonist on reducing ovarian I/R injury. When the sham control group was compared with the I/R group, the difference was found to be statistically significant and the p-value was less than 0.001. Furthermore, when the agonist and antagonist groups were compared, the p-value was 0.0029.

As shown in Figure 1(b), the TNF-α level in ovarian tissues was significantly increased in the I/R group (107.01 ± 17.72 pg/g; p = 0.0002) when compared to the sham control group (56.02 ± 9.16 pg/g). The agonist (70.54 ± 12.82 pg/g) significantly reduced the TNF-α level in ovarian tissues, but the antagonist (100.71 ± 11.07 pg/g; p = 0.039) inhibited this reducing effect of the agonist.

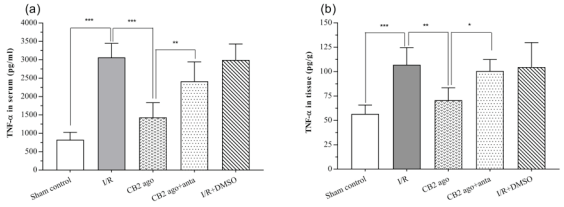

*Figure 1. Tumor necrosis factor alpha (TNF-α) levels in (a) serum and (b) ovarian tissues. Cannabinoid (CB)2 receptor agonist dose is 3 mg/kg; antagonist dose is 1 mg/kg. * p < 0.05; ** p < 0.01; *** p < 0.001. The one-way analysis of variance (ANOVA) test, followed by the Bonferroni post hoc test, was used to determine statistical differences. Data are expressed as mean ± standard deviation (n = 5, 6).*

IL-1β is another important marker associated with inflammation. Increased levels of this cytokine in both ovarian tissues and serum are a measure of the severity of inflammation. In this context, IL-1β levels were measured to obtain the extent of ovarian I/R injury. In the serum, there was a statistically significant difference between the sham control and I/R groups (204.33 ± 68.84 and 1106.81 ± 222.62 pg/ml, respectively; p < 0.001). The agonist (631.17 ± 68.92 pg/ml; p 0.0058) was found to play a role in decreasing these IL-1β levels, which increased as a result of I/R injury, and the antagonist inhibited the reducing effect of the agonist (Figure 2(a)).

As illustrated in Figure 2(b), significant differences were found in the IL-1β levels in ovarian tissues when compared with the sham control (7.11 ± 1.87 pg/g) and I/R (15.73 ± 3.23 pg/g) groups (p < 0.001). When the agonist (8.92 ± 1.33 pg/g) and antagonist (13.01 ± 2.45 pg/g) groups were compared, statistical significance was observed (p = 0.046).

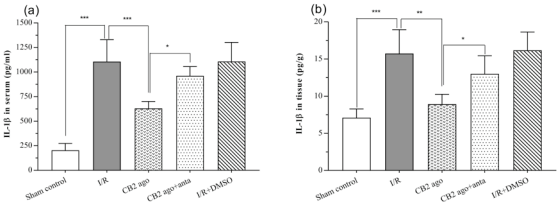

*Figure 2. Interleukin 1 beta (IL-1β) levels in (a) serum and (b) ovarian tissues. Cannabinoid (CB)2 receptor agonist dose is 3 mg/kg; antagonist dose is 1 mg/kg. * p < 0.05; ** p < 0.01; *** p < 0.001. The one-way analysis of variance (ANOVA) test, followed by the Bonferroni post hoc test, was used to determine statistical differences. Data are expressed as mean ± standard deviation (n = 5, 6).*

Oxidation and antioxidation levels in ovarian tissues and serum

Th induction of ovaries by I/R injury (7.51 ± 0.75 nmol/ml) was found to increase the MDA concentration in the serum, compared to the sham control group (2.91 ± 0.39 nmol/ml; p<0.001). In the agonist group (4.14 ± 1.11 nmol/ml), the MDA concentration in the serum was significantly lower than that in the I/R, agonist + antagonist (6.56 ± 1.24 nmol/ml), and DMSO (7.18 ± 0.87 nmol/ml) groups. The antagonist reversed the beneficial effect of the

agonist (p = 0.001; Figure 3(a)).

It was found that the MDA levels in ovarian tissues were significantly increased in the I/R group when compared with the sham control group (109.06 ± 11.04 and 77.66 ± 12.28 nmol/g, respectively; p = 0.010). The CB2 receptor agonist (86.50 ± 16.32 nmol/g) caused a significant decrease in the MDA levels. When the agonist group was compared with the antagonist group (99.11 ± 5.07 nmol/g; p = 0.39), the difference was not found to be statistically significant (Figure 3(b)).

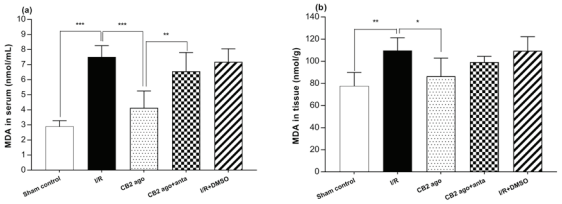

*Figure 3. Malondialdehyde (MDA) levels in (a) serum and (b) ovarian tissues. Cannabinoid (CB)2 receptor agonist dose is 3 mg/kg; antagonist dose is 1 mg/kg. * p < 0.05; ** p < 0.01; *** p < 0.001. The one-way analysis of variance (ANOVA) test, followed by the Bonferroni post hoc test, was used to determine statistical differences. Data are expressed as mean ± standard deviation (n = 5, 6).*

Another criterion for determining the degree of ovarian damage is the determination of GSH levels in ovarian tissues and serum. In this context, as shown in Figure 4(a, b), when the I/R (24.51 ± 6.06 mg/100 ml, 0.56 ± 0.25 µmol/g) and sham control (38.06 ± 5.08 mg/100 ml, 1.33 ± 0.07 µmol/g) groups were compared in terms of GSH levels, it was found that the GSH levels decreased significantly in ovarian tissues and serum. When the agonist (34.31 ± 4.27 mg/100 ml, 1.07 ± 0.21 µmol/g) and I/R groups were compared in terms of GSH levels, the GSH levels in ovarian tissues and serum were significantly increased.

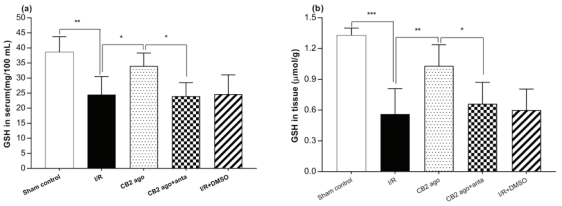

*Figure 4. Glutathione (GSH) levels in (a) serum and (b) ovarian tissues. Cannabinoid (CB)2 receptor agonist dose is 3 mg/kg; antagonist dose is 1 mg/kg. * p < 0.05; ** p < 0.01; *** p < 0.001. The one-way analysis of variance (ANOVA) test, followed by the Bonferroni post hoc test, was used to determine statistical differences. Data are expressed as mean ± standard deviation (n = 5, 6)..*

DISCUSSION

Although uterine torsion with a period of more than 45 degrees around its long axis is one of the rare cases, it can progress to infertility if left untreated.[21] Ovarian injury caused by uterine torsion is similar to I/R injury.[22] Besides ROS, MDA, GSH, and cytokines such as IL-1β and TNF-α also play a role in the pathogenesis of ovarian tissues.[23] ROS is produced by cells as a result of biochemical reactions. It also causes ROS to increase as a result of the accumulation of neutrophils because of I/R injury.[22]

The results of this study demonstrated that the CB2 receptor has a major therapeutic role in ovarian I/R injury in rats and emphasized the pharmacological responses of the CB2 receptor to decreased cytokine levels in ovarian tissues post–I/R injury. Moreover, it has been shown that the depletion of antioxidant defenses, peroxidation of cell membrane lipid, and overexpression of proinflammatory cytokines, as well as migration of PNL in ovarian damage.[24] In addition, similar to the results of previous studies, the results of this study showed that the administration of CB2 receptor agonist lipid peroxidation production reversed the GSH consumed, inhibited the expression of TNF-α and IL-1β, and significantly reduced MDA formation in ovarian I/R injury in rats.[10,25] The lack of investigation into

the contraction responses of the uterine smooth muscle with acetylcholine (from 10−8 to 10−3) and electrical stimulation in an isolated organ bath and the absence of histological examination are the limitations of this study. Because this study does not include in vitro studies and receptor mechanism interactions between the CB2 receptor and cholinergic and adrenergic systems, the CB2 receptor agonist was administered IP. In addition, the activation of the CB2 receptor agonist has been shown to have beneficial effects on physiological factors, such as microcirculation in defense systems, and has anti-inflammatory and antioxidant effects.

By preadministration of the CB2 receptor agonist, the MDA levels in ovarian tissues and serum decreased almost to the MDA levels in the sham control group. The results of this study show that I/R injury causes decreased GSH content in ovarian tissues and serum. Moreover, preadministration of the CB2 receptor agonist led to increased GSH levels in ovarian tissues and serum. Hence, as evidenced in previous studies, the CB2 receptor agonist once again proved to reduce oxidative stress.[26] In ovarian I/R injury, all or part of the blood circulation may be temporarily blocked in surgical interventions, especially caesarean section, or in traumatic events.[27] Although ovarian damage has occurred during ischemia, it has been shown in previous studies that the main damage takes place during reperfusion.[28] In line with the results of previous studies, the results of this study show that reperfusion increases the harmful effects of ischemic damage because of migration of active neutrophils and ROS formation.[29]

As in a previous study on ileum tissue, it has been noted in this study that the lipid peroxidation level in I/R injury was significantly inhibited by the CB2 receptor agonist.[3] This study demonstrated that the activation of the CB2 receptor prevented I/R injury and reversed the depletion of GSH in the ovarian cells exposed to I/R injury. GSH, which is one of the most important nonenzymatic antioxidants found in almost all cells, is well known to the researchers. It plays a role in the detoxification of ROS, which is involved in the pathogenesis of injury to living cells.[30] In this study, one of the defense mechanisms of ovarian I/R injury was shown to be becasue of the activation of the CB2 receptor agonist. Therefore, the CB2 receptor agonists in the experimental ovarian I/R model prove to be promising as antioxidants for improving the I/R injury.[12,31]

Many studies have shown that the activation of the CB2 receptor causes immunomodulatory effects.[24,32] As in previous studies with paracrine,[33] it was observed that the cytokine levels produced by ovarian damage were reduced by the activation of the CB2 receptor agonist. Recent studies with the activation of the CB2 receptor agonist on some diseases such as asthma, colitis, rheumatoid arthritis, rat paw inflammation, and inflammatory bowel disease show that it inhibits proinflammatory cytokine production, such as TNF-α and IL-1β, and protects it from ovarian injuries.[3,9,10,34,35] Our results show that the ovarian tissue is protected from I/R injury as it inhibits IL-1β and TNF-α levels in both ovarian tissues and serum by the activation of the CB2 receptor agonist.

Conclusions

As a result of this study, it is concluded that the activation of the CB2 receptor decreases anoxia, results in the balance of impaired oxidant/antioxidant redox system, and inhibits the production of provocative and proinflammatory cytokines, such as TNF-α and IL-lβ, in I/R injuries. Further research in this area is, however, necessary for a more detailed discussion of the molecular pathways of ovarian I/R injury. Furthermore, although the role of the activation of the CB1 receptor in many ovarian I/R injuries was investigated, the CB1 receptor agonist was not investigated in this study. In this context, the effect of the CB1 receptor agonist on ovarian I/R injury may be investigated in the future.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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