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EFFECT OF RAMELTEON ON LIPOPOLYSACCHARIDE INDUCED HIPPOCAMPAL TOXICITY

LİPOPOLİSAKKARİT İLE İNDÜKLENMİŞ HİPOKAMPAL TOKSİSİTEDE RAMELTEON'UN ETKİSİ

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Öz

Amaç
Tıptaki gelişmelere rağmen sepsis dünya çapında hala önemli bir sağlık sorunu olmaya devam etmektedir ve beyin dokusu sepsisin erken döneminde hasar gören yapılardan biridir. Nöroinflamasyon (NI), septik beyin hasarında ana mekanizma olarak kabul edilir. Ramelteon (RML), seçici olmayan (MT1/MT2) bir melatonin reseptör agonistidir ve 2005 yılında uykusuzluk endikasyonu için FDA tarafından onaylanmıştır. RML, diğer melatonerjik agonist ilaçlar arasında her iki reseptör alt tipi için nispeten daha yüksek afinite gösterir.

Gereç ve Yöntem
RML'nin lipopolisakarit (LPS) ile indüklenen NI üzerindeki koruyucu etkisini araştırmak için yirmi sekiz erkek Wistar Albino sıçan kullanıldı. Kontrol, LPS (5 mg/kg, intraperitoneal), RML (8 mg/kg, oral) ve LPS + RML (LPS'den 45 dakika önce) grupları oluşturuldu. Son ilaç uygulamasından 6 saat sonra sıçanlar sakrifiye edildi. Hemogram analizi için kan, histopatolojik değerlendirme için kortikal ve hipokampal dokular toplandı.

Bulgular
LPS, lökosit ve nötrofil/lenfosit oranını (NLR) artırırken, lenfosit ve trombosit sayısını azalttı. Buna karşın RML ile, NLR'de anlamlı bir azalma ve trombosit sayısında anlamlı bir artış izlendi. Histokimyasal değerlendirmede LPS grubuna ait hipokampal ve kortikal alanlarda belirgin inflamatuar hücre infiltrasyonu ve apoptoz gözlendi. RML, bu alanlarda inflamatuar yanıtı ve apoptotik cisimleri azalttı.

Sonuç
RML, hipokampusta gözlenen LPS'ye bağlı NI'de antiinflamatuar ve antiapoptotik mekanizmalar aracılığıyla koruyucu olabilir.

Anahtar Kelimeler: Lipopolisakkarit, Hipokampus, Ramelteon, Nöroinflamasyon

Abstract

Objective
Despite the advances in medicine, sepsis still remains a major health problem worldwide and brain tissue is one of the structures damaged in the early period of sepsis. Neuroinflammation (NI) is considered as the main mechanism in septic brain injury. Ramelteon (RML) is a non-selective (MT1 / MT2) melatonin receptor agonist and was approved by the FDA in 2005 with the indication of insomnia. RML shows relatively higher affinity for both receptor subtypes among other melatonergic agonist drugs.

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Material and Method
Twenty-eight male Wistar Albino rats were used to investigate the protective effect of RML on lipopolysaccharide (LPS) induced NI. Control, LPS (5 mg/kg, intraperitoneally), RML (8 mg/kg, orally) and LPS + RML (45 minutes before LPS) groups were created. Six hours following the last drug administration, rats were sacrificed. Blood for hemogram analysis and cortical and hippocampal tissues for histopathological evaluation were collected.

Results
LPS increased white blood cell and neutrophil/lymphocyte ratio (NLR) while it decreased lymphocyte and platelet counts. RML decreased NLR and increased platelet counts significantly. In histochemical evaluation, marked inflammatory cell infiltration and apoptosis were observed in both hippocampal and cortical areas of LPS group. RML decreased the inflammatory response and apoptotic bodies in these areas.

Conclusion
RML may be protective on LPS-induced NI observed in hippocampus via anti-inflammatory and anti-apoptotic mechanisms.

Keywords: Hippocampus, Lipopolysaccharide, Neuroinflammation Ramelteon,

Introduction

The central nervous system (CNS) is affected by damaging mechanisms such as oxidative stress and inflammation that occur in the systemic circulation for various reasons. (1). In the case of inflammation, cytokines circulating in the blood, increase the blood-brain barrier permeability (2). Increased permeability contributes to the development of neuroinflammation (NI) by causing the brain tissue to be more exposed to these stressor effects (3-5). Therefore, NI emerges as a response to the damage in the CNS, involving all cells of the brain tissue, including macroglia and microglia (6, 7). Inflammatory cells with increased infiltration into the tissue due to developing NI cause changes in neuronal plasticity in the brain, neuronal loss as a result of decreased neurogenesis, permanent behavioral changes secondary to the effects on hippocampal tissue, deterioration in cognitive performance, and decrease in quality of life (8). NI, a critical pathological process occurring in the brain tissue, is also accepted as one of the main causes of neurodegenerative (ND) diseases such as Alzheimer's, Parkinson's, and Multiple Sclerosis (9, 10).

Lipopolysaccharide (LPS), which is an important component of the cell wall of gram-negative bacteria, is used in experimental studies to mimic systemic inflammation (11-13). It has been shown that a single dose of LPS administered at an adequate dose intraperitoneally (i.p.) produces neurodegenerative effects secondary to NI in mouse or rat brain tissue (14-16). It is known that in addition to inflammation and oxidative stress, which are the main damage mechanisms, apoptosis, a cell death mechanism, also plays an important role in tissue damage during NI (17-20).

Melatonin (MEL) is the main hormone produced by the pineal gland and its level decreases with age in humans (21-23). Ramelteon (RML), a tricyclic synthetic MEL analog (24), acts on the MT1 and MT2 receptors with a 3 to 16 times higher affinity than that of endogenous MEL (25-27). Also, RML shows its effect without interacting with other receptors in the hippocampus (GABA receptor complex or neuropeptide, serotonin, dopamine, noradrenaline, acetylcholine, and opioid receptors) by means of no side effects due to the activation of these receptors. (28). In many studies, RML has been shown to have significant anti-inflammatory and antioxidant effects (29-32).

In this study, we aimed to evaluate the effects of RML on LPS-induced hippocampal damage. The results of this study will provide new scientific data on both the reduction of LPS-induced hippocampal damage and the effect of RML on the hippocampus and the possible mechanisms of this effect.

Material and Method

Animals and Ethical Approval

All experiments were performed under the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research of Suleyman Demirel University, Isparta (Approval No: 08.07.2020/04-04).

Totally twenty-eight Wistar albino male rats weighing 300-350 grams and provided from Suleyman Demirel University Experimental Animals and Medical Research Application and Research Center were housed at a controlled room temperature (21-22 °C), maintained under a 12-hour light/12-hour dark

cycle and humidity (60 ± 5%). During the study, experimental animals were subjected to standard feeding (Korkuteli Yem, Antalya, TURKIYE), housing and care conditions.

Experimental design: Rats were divided into 4 groups of 7 animal in each as below:

1. Control (n=7): Fourty five minutes after oral administration of normal salin (SF) in 1 ml volume, 1 ml of SF was given i.p.

2. LPS (n=7): 45 minutes after oral administration of SF in 1 ml volume, LPS (048 K4126, Sigma Aldrich, Sweden) in 5 mg/kg dose was given i.p. (33).

3. LPS+RML (n=7): 45 minutes after oral administration of 8 mg/kg RML (Ramelda, Abdi Ibrahim Ilac, Turkey) dissolved in 1 ml SF, LPS in 5 mg/kg dose was given i.p (33, 34).

4. RML (n=7): 45 minutes after oral administration of 8 mg/kg RML dissolved in 1 ml SF, salin in 1 ml volume was given i.p. (34).

Six hours after the all administrations, rats were sacrificed under i.p. injection of 90 mg/kg ketamine (Alfamin, Alfasan IBV) and 10 mg/kg xylazine (Alfazin, Alfasan IBV) anesthesia. Blood samples were collected from inferior vena cava for surgical exanguination and hematological analyses. A hippocampus extraction mechanism was created by placing filter papers soaked in cold phosphate buffer on ice packs while harvesting the hippocampus. Then, cortical and hippocampal tissues were taken into containers containing 10% formaldehyde for routine Hematoxylin-Eosin (HE) staining.

Hematological Analyses

Red blood cell (RBC), hemoglobin (Hb), white blood cell (WBC), neutrophil (N), lymphocyte (L), and platelet (PLT) counts and percentages were measured from heparinized blood samples taken into anticoagulant tubes. An automated hematology analyzer (Code No: CR163712, Sysmex, Japan) were used to caunt these cells. The ratio of N count to L count was considered as Neutrophil/Lymphocyte Ratio (NLR) (35).

Histopathological Analyses

Cortical and hippocampal tissues fixed in 10% neutral formalin solution were routinely processed by an automatic tissue processor equipment (Leica ASP300S, Wetzlar, Germany), then embedded into paraffin wax and sections in 5 µm thickness cut by a rotary microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany) were obtained. These pieces were stained by HE, covered with a coverslip with a mounting medium and examine under a light microscope (36).

Statistical Analyses

Variables were presented as mean + standard deviation. Analysis of variance (with a post hoc Bonferroni test) was used to compare hematological and histopathologic scores between the groups. SPSS 18.0 program pack was used for statistical calculations $P < 0.05$ was set as the value for significance.

Results

Hematological Findings

Hematological results of the study groups were shown in Table 1. While WBC and NLR were significantly increased in LPS group compared to control (p:0.044 and p<0.001, respectively) RML treatment decreased both values, but only the decrease in NLR was statically significant (p>0.05 and p<0.001, respectively). LPS caused a significant decreament in L and PLT counts compared to controls (p<0.001 for both). The increaments by RML added to LPS seen in L and PLT count was only significant for PLT, compared to LPS (p>0.05 and p.0.003, respectively). There were no significant differences between the groups by means of RBC or Hb

Histopathological Findings

Cortical and hippocampal tissues stained with HE were evaluated under an imaging-assisted binocular light microscope for histopathological evaluation and photographs were obtained. Pathological findings were scored as: (-): no pathological finging, (+): low level pathological finging, (++): moderate level pathological finging, (+++): severe level pathological finging. Scores of pathological finding belonging to the cortical and hippocampal areas were shown in Table 2.

In control group, normal architecture was seen in hippocampal and cortical areas (Figure 1A-B). In LPS treated group, several apoptotic bodies and inflammatory cell infiltrations were observed in both hippocampus and cortex (Figure 1C-D). RML treatment added to LPS regressed the hippocampal and cortical histopathological findings compared to LPS only group (Figure 1E-F). Histological appearances of hippocampal and cortical areas belonging to RML group were in normal architecture, similar to controls (Figure 1G-H).

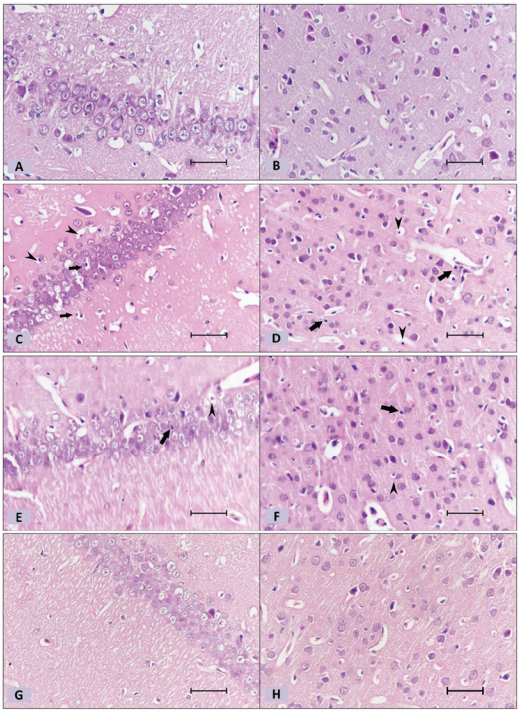

Figure 1:
Normal histological appearance of hippocampal (A) and cortical (B) tissues belonging to control group. Histopathological appearance of hippocampal (C) and cortical (D) tissues belonging to LPS group. Inflammatory cell infiltration (arrows) and apoptotic bodies (arrowhead). Histopathological appearance of hippocampal (E) and cortical (F) tissues belonging to LPS+RML group. Inflammatory cell infiltration (arrows) and apoptotic bodies (arrowhead). Normal histological appearance of hippocampal (G) and cortical (H) tissues belonging to RML LPS: Lipopolysaccharide; RML: Ramelteon; HE: Hematoxylin-Eosin, 40x, Bars = 50 µm

Table 1 Hematological results of the study groups

Group Parameter	Control		LPS		LPS + RML		RML	
	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
RBC	8.49 ± 0.67		8.28 ± 0.42		8.03 ± 0.39		8.17 ± 0.30	
Hb	14.5 ± 1.63		13.35 ± 2.34		14.50 ± 0.66		14.00 ± 0.52	
NEU	33.43 ± 4.35		85.20 ± 2.20[a]	a<0.001	85.24 ± 2.27[b]	b<0.001	55.5 ± 5.96[a, b]	a<0.001 b<0.001
WBC	3.33 ± 0.76		4.91 ± 0.69[a]	a:0.044	4.11 ± 1.04		6.12 ± 1.39[a, c]	a:0.002 c:0.005
PLT	561.25 ± 88.46		178.16 ± 84.07[a]	a<0.001	357.50 ± 96.00[a, b]	a:0.003 b:0.003	625.16 ± 91.08[b, c]	b<0.001 c<0.001
LYMP	63.40 ± 3.43		13.10 ± 2.03[a]	a<0.001	14.48 ± 3.74[a]	a:0.002	43.88 ± 5.85[a, b, c]	a<0.001 b<0.001 c<0.001
NLR	0.52 ± 0.08		6.68 ± 1.36[a]	a<0.001	6.64 ± 1.30[a, b, c]	a<0.001 b<0.001 c<0.001	1.30 ± 0.35[b]	b<0.001

Values were presentred as means + SD. LPS: Lipopolysaccharide, RML: Ramelteon, RBC: Red blood cell, Hb: Hemoglobin, NEU: Neutrophil, WBC: White blood cell, PLT: Platelet, LYMP: Lymphocyte, NLR: Neutrophil Lymphocyte Ratio. a: p<0.05 compared to control, b:p<0.05 compared to LPS, c:p<0.05 compared to LPS+RML

Table 2 Histopathological scores of cortical and hippocampal areas belonging to the study groups

Group Parameter		Control	LPS	LPS + RML	RML
Findings in cortical areas	Inflammatory cell infiltration	-	+++	+	-
	Apoptotic bodies	-	+++	+	-
Findings in hippocampal areas	Inflammatory cell infiltration	-	++	-	-
	Apoptotic bodies	-	++	+	-

LPS: Lipopolysaccharide, RML: Ramelteon, (-): no pathological finding, (+): low level pathological finding, (++): moderate level pathological finding, (+++): severe level pathological finding,

Discussion

One of the most important consequences of sepsis, known as the excessive inflammatory response developed against to a microbial infection, is multi-organ damage and the CNS is one of the organs affected in this pathological process (37, 38). This excessive inflammatory response observed in peripheral tissues manifests itself as NI in the CNS which constitutes the basic mechanism of the destruction that develops in neuronal tissues (39).

In experimental sepsis studies, bacterial LPS used to mimic microorganism attack actually does not create a complete picture of sepsis, but it is quite successful in forming NI underlying the pathogenesis of sepsis. In this study, we created a LPS-mediated NI model and evaluated the effects of RML, which is a melatonin agonist, especially in the hippocampal tissue.

The finding of severe inflammatory cell infiltrations, seen in both hippocampal and cortical tissues in the LPS group are consistent with previous studies evaluating LPS-mediated NI and it should first be interpreted that the experimental model was set up successfully (5, 40, 41)

Exaggerated inflammation is the main mechanism in LPS-mediated neural injury and it has led to the hypothesis that some molecules with anti-inflammatory properties may be protective against this damage. In vivo and in vitro studies confirm this hypothesis (5, 42-44). RML, which was used as a treatment molecule in our study, decreased the amount of inflammatory cells that increased by LPS in both hippocampal and cortical areas. Although the anti-inflammatory effects of RML in many organs or tissues have been demonstrated in both clinical and experimental studies, our study is the first to show the effect of this molecule on hippocampal NI (45-48).

The hippocampal tissue is important as it is one of the main site where NI occurs. (49). It has been shown that developing NI causes a decrease in intracellular pH in CA1 neurons in the hippocampus (50). Whether this change in pH is really a part of the damage mechanism and the effects of RML on this variable, especially in chronic use, should be evaluated in further studies.

Blood parameters change in septic conditions as well as in many diseases. In particular, WBC and neutrophil counts are important in terms of reflecting bacterial inflammation (51, 52). In our study, the significant increases of WBC and neutrophils counts in the LPS group is important in terms of being consistent with the literature and confirming the inflammatory model (53, 54). On the other hand, the significant decreases in these values in the RML group reveal the anti-inflammatory effect of the treatment molecule. Low LYMP numbers in addition to high neutrophil count observed in septic conditions were also shown in our study A non-significant elevation in LYMP count by RML may be related to a single dose treatment and should be evaluated in a long-term therapy, as well. NLR, which is currently used for many medical conditions, is calculated by dividing the neutrophil count by the LYMP count. This value, which was shown to increase in septic conditions in clinical and experimental studies, increased significantly in the LPS group in our study (55, 56). Changes in NLR are important in terms of rapid and practical evaluation of both the inflammation and the anti-inflammatory effect of the treatment. In experimental sepsis studies, LPS administration has also been shown to reduce the number of PLT (57). In consistent with this knowledge, in our study, the number of PLT decreased significantly in the LPS treated group compared to the control group and increased significantly with RML treatment. This effect of RML on PLT count may be protective against thrombocytopenia-related complications, especially in

septic patients who have hemorrhagic diathesis or are taking drugs that predispose to bleeding.

It is known that increased reactive oxygen species (ROS) and inflammation trigger apoptosis in septic conditions. LPS similarly induces apoptosis (58, 59). In our study, apoptotic bodies were observed at a significant rate in the cortical and hippocampal tissues of the experimental groups in which LPS was applied, supporting the literature findings. RML treatment regressed these changes. Besides the prominent anti-inflammatory properties of RML, its antiapoptotic effect, parallel to our results, has been demonstrated in traumatic lung injury and cocaine-induced NI (60). On the other hand, there is also a study showing that chronic RLM treatment does not cause a change in apoptotic indices and cognitive functions in the mouse Alzheimer model (61). So the effect and possible mechanisms of RML on apoptosis should be evaluated with study designs that include different doses and treatment durations of RML.

One of the limitations of our study is that LPS-related inflammation, apoptosis, and the protective effect of RML on these processes were demonstrated only by hematological and histopathological analyses. Further studies are needed to reveal the anti-inflammatory and antiapoptotic effects of RML with quantitative methods and to evaluate the possible mechanisms of action. In addition, in this study model, which was planned to mimic acute inflammation, a single dose of RML was used and only the structural changes in the hippocampal and cortical areas were examined. Considering the role of the hippocampus on learning and memory behavior, the impact of chronic RML use on NI-related learning-memory impairment should be evaluated with behavioral tests and further molecular studies.

In conclusion, RML can be a useful treatment option in the ongoing therapy of patients with NI due to various central causes, such as sepsis, aneurysm, or tumor in intensive care or clinical conditions, in terms of both regressing inflammation and treating sleep disorder secondary to the disease. In addition, RML can be a treatment alternative for many NI based neurodegenerative diseases. A single dose of RML administered in this study did not reveal a negative result, at least in terms of histology. Besides, the fact that this molecule is already a licensed product for another indication may make RML safer as a new treatment candidate in a new indication. However, there is a need for more comprehensive experimental studies and randomized controlled clinical studies on this subject.

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This article is a publication of a master's thesis.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
All experiments were performed under the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research of Suleyman Demirel University, Isparta (Approval No: 08.07.2020/04-04). During the test protocol, all precautions regarding animal welfare were taken.

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Availability of Data and Materials
Data are available on request due to privacy or other restrictions.

Authors Contributions
M.K: Conceptualization; Data curation; Investigation; Methodology; Visualization; Writing-original draft.

M.S: Conceptualization; Data curation; Investigation; Methodology; Visualization; Writing-original draft. Project administration; Writing-review & editing.

H.A: Investigation; Methodology; Data curation; Writing-original draft.

K.G: Formal analysis; Writing-original draft.

I.I: Formal analysis; Writing-review & editing.

Editorial
Although MS and KG, two of the authors of the article, are editorial board members of the journal, they have not taken part in any stage of the publication processes of this article.

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