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Protective Effects of Ramelteon on Acute Lung Injury in Endotoxin-Induced Sepsis in Rats

Sıçanlarda Endotoksin Kaynaklı Sepsiste Akut Akciğer Hasarı Üzerine Ramelteon'un Koruyucu Etkileri

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Abstract

Introduction: Sepsis is a life-threatening excessive systemic inflammatory reaction syndrome to infection that usually occurs in patients with bacteremia. The respiratory system is one of the structures most affected by acute organ damage. Melatonin plays an important role in regulating various physiological functions of the body, including antioxidant and anti-inflammatory. Ramelteon (RAME) is the first melatonin receptor agonist confirmed for clinical use. The goal of this study is to determine the effects of RAME on endotoxin-induced septic lung injury in rats.

Materials and Methods: Thirty-two female rats were separated randomly into four groups (n=8). Group healthy received intraperitoneal normal saline, group sepsis received intraperitoneally 10 mg/kg lipopolysaccharide (LPS), group sepsis+RAME2 received 10 mg/kg LPS plus 2mg/kg RAME, and group sepsis+RAME4 received 10 mg/kg LPS plus 4mg/kg RAME. RAME was administered by oral gavage 1 hour before LPS administration. The lung tissues were collected 12 hours after LPS administration and investigated molecularly (qRT-PCR analyses of Tumor Necrosis Factor-α, nuclear factor kappa-β, and interleukin 1-beta mRNA expression) and histopathologically (staining with Harris Hematoxylin and Eosin Y).

Results: TNF-α, NF-κβ, and IL-1β levels significantly decreased dose-dependent in the septic rats following RAME administration. RAME administration ameliorated histopathological injury in lung tissues due to sepsis.

Conclusion: RAME ameliorated the inflammatory response in endotoxin-induced sepsis. These findings suggest that RAME can be a promising agent by contributing to alternative preventive treatment methods for sepsis with its anti-inflammatory effect.

Keywords: : Inflammation; lipopolysaccharide; melatonin; ramelteon; sepsis.

Özet

Amaç: Sepsis, genellikle bakteriyemili hastalarda ortaya çıkan, enfeksiyona karşı yaşamı tehdit eden aşırı sistemik inflamatuar reaksiyon sendromudur. Solunum sistemi akut organ hasarından en çok etkilenen yapılardan biridir. Melatonin, antioksidan ve antiinflamatuar dahil olmak üzere vücudun çeşitli fizyolojik işlevlerinin düzenlenmesinde önemli bir rol oynar. Ramelteon (RAME), klinik kullanım için onaylanan ilk melatonin reseptörü agonistidir. Bu çalışmanın amacı sıçanlarda endotoksin kaynaklı septik akciğer hasarı üzerine RAME'nin etkilerini belirlemektir.

Gereç ve Yöntem: Otuz iki dişi sıçan rastgele dört gruba ayrıldı (n=8). Sağlıklı grup intraperitoneal normal salin, grup sepsis intraperitoneal 10 mg/kg lipopolisakkarit (LPS), grup sepsis + RAME2 10 mg/kg LPS ile birlikte 2mg/kg RAME ve grup sepsis+RAME4 10 mg/kg LPS ile birlikte 4mg/kg RAME aldı kg. RAME, LPS uygulamasından 1 saat önce oral sonda ile uygulandı. LPS uygulamasından 12 saat sonra akciğer dokuları toplandı ve moleküler (tümör nekroz faktörü-α, nükleer faktör kappa-β ve interlökin 1-beta) ve histopatolojik (Harris Hematoxylin ve Eosin Y ile boyama) olarak incelendi.

Bulgular: TNF-α, NF-κβ ve IL-1β seviyeleri, RAME uygulamasını takiben septik sıçanlarda doza bağlı olarak önemli ölçüde azaldı. RAME uygulaması, akciğer dokularında sepsise bağlı histopatolojik hasarı düzeltti.

Sonuç: RAME endotoksin kaynaklı sepsiste inflamatuar yanıtı iyileştirdi. Bu bulgular, RAME'nin antiinflamatuar etkisi ile sepsis için alternatif koruyucu tedavi yöntemlerine katkıda bulunarak umut verici bir ajan olabileceğini düşündürmektedir.

Anahtar Kelimeler: Enflamasyon; lipopolisakkarit; melatonin; ramelteon; sepsis.

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Introduction

Sepsis is a life-threatening excessive systemic inflammatory reaction syndrome to infection that usually occurred in patients with bacteremia (1). The septic response is due to excessive secretion of inflammatory cytokines including tumor necrosis factor-α (TNF-α), nuclear factor kappa-β (NF-κβ), and increased free radicals (2). Overexpression of inflammatory cytokines causes sepsis-related damages, such as septic shock and multiple organ failure/dysfunction including injury in endothelial and epithelial cells of lung. (3). Melatonin, an endogenous molecule, plays a significant role in the organizing of various physiological functions including anti-inflammatory (4). However, melatonin cannot be used for a long time, exogenous melatonin causes undesirable adverse effects of hormonal stress on the chronological rhythm (5). Ramelteon (RAME) is the first peripheral melatonin receptor agonist confirmed for clinical use in humans for the treatment of insomnia (6). It affects both melatonin-1 and melatonin-2 receptors with high affinity (7). There are also studies showing that RAME administration reduces markers related to inflammation (8). However, the effect of RAME, peripheral melatonin receptor agonists that do not affect the central nervous system, against endotoxin-induced septic lung injuries is unknown. Based on all this information, this study aimed to determine the protective effects of RAME on endotoxin-induced septic lung injury in rats with molecular and histopathological analyses.

Materials and Methods

Animals: Thirty-two female rats (Sprague Dawley, 10 - 12 weeks, 240 - 260 g) were used in the experiment (n=8) and rats were provided by the Atatürk University Medical Experimental Research Center. The care and use of the rats were confirmed by the Atatürk University Institutional Animal Care and Use Committee.

Chemicals: LPS (E. coli O55: B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RAME (Rozerem© 8 mg tab) was purchased from Abdi Ibrahim, Turkey. Ketamine (Ketalar) was obtained from the Pfizer Drug Company, in Turkey. Xylazine (Basilazin 2%) was obtained from BioTek, Turkey. All other chemicals were obtained from Sigma-Aldrich Company (Germany).

Experimental design: Thirty-two rats were randomly separated into 4 equal groups (n=8). To investigate the effect of the RAME, two different doses of RAME were applied with oral gavage to the septic rats (Table 1).

Table 1: Experimental groups and design to investigate the effects of RAME in the lung tissue on endotoxin-induced sepsis in rats

Group No	Group Name (n=8 for each)	Applied
1	Healthy	Sham operated control
2	Sepsis	Sepsis control group, LPS was administrated intraperitoneally with a dose of 10 mg/kg
3	Sepsis+RAME 2	2 mg/kg Ramelteon was administrated orally 1 hour before LPS administration.
4	Sepsis+RAME 4	4 mg/kg Ramelteon was administrated orally 1 hour before LPS administration.

Sepsis:10 mg/kg LPS (Lipopolysaccharide), RAME 2:2mg/kg Ramelteon; RAME 4:4mg/kg Ramelteon

Endotoxin-induced sepsis and treatments: The endotoxin-induced sepsis model was created by a single intraperitoneally injection of 10 mg/kg LPS to the rats as noted in previous studies [9]. RAME at doses of 2 and 4 mg/kg rats (9) were applied by oral gavage 1 hour before the LPS injection. The rats had free access to water but not food for 12 hours after the LPS injection. All rats were euthanized with the application of an anesthetic consisting of a ketamine-xylazine combination 12 hours after LPS injection (11). Lung tissues of all rats were gathered and stored under suitable terms for analysis.

Molecular analyses: Relative quantification of gene expression (real-time reverse transcriptase-polymerase chain reaction) The relative TNF-α, NF-κβ, and interleukin 1 beta (IL-1β) mRNA expression analyses were performed with StepOnePlus Real-Time PCR System technology (Applied Biosystems, USA) using cDNA

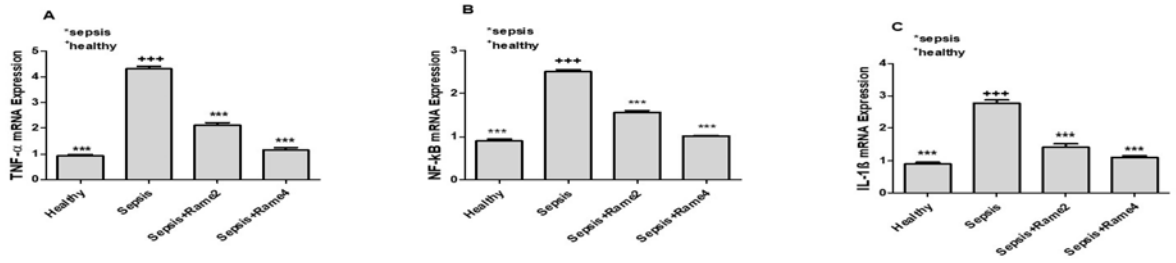

Figure 1. A-C: The molecular results of the effects of RAME on acute lung injury in endotoxin-induced sepsis. **A:** TNF-α mRNA expression levels; **B:** NF-ϰB mRNA expression level; **C:** IL-1β mRNA expression level. Sepsis (10 mg/kg LPS), Sepsis+RAME2 (2mg/kg Ramelteon), and Sepsis+RAME4 (4mg/kg Ramelteon). The expression of mRNAs was detected using quantitative Real-Time PCR analysis. β-actin was used as the reference gene. Results are expressed as relative fold compared with healthy animals. Each bar is expressed as mean value ± SD. Significant differences were detected between all groups, compared to sepsis groups (*p<0.05, ** p<0.01, *** p<0.001) and compared to healthy groups ($^{+}$ p<0.05, $^{++}$ p<0.01, $^{+++}$ p<0.001) by one-way analysis of variance test and Tukey tests. SD: standard deviation.

Table 2: Means±standard deviations of the molecular results of the effects of RAME in the lung tissue on endotoxin-induced sepsis in rats

Compound	Healthy	Sepsis	Sepsis+Rame2	Sepsis+Rame4
IL1-B	0.90±0.04	2.77±0.08	1.42±0.08	1.10±0.04
NF- ϰβ	0.90±0.04	2.50±0.04	1.55±0.04	1.02±0.01
TNF-α	0.93±0.03	4.31±0.08	2.11±0.07	1.16±0.08

IL-1β: interleukin 1 beta; nuclear factor kappa-β (NF-ϰβ); **TNF-α:** tumor necrosis factor-α. **Sepsis:**10 mg/kg LPS (Lipopolysaccharide), **RAME 2:** 2mg/kg Ramelteon; **RAME 4:** 4mg/kg Ramelteon

synthesized from rat RNA as noted in previous studies (5). All probes and primers were purchased as TaqMan Gene Expression Assays. The expression data for β –actin (Applied Biosystems, USA) in each lung tissue was used as the housekeeping gene, and the primers and probes for the β -actin were designed by Primer Design. Ct values were automatically transformed into delta delta Ct ($2^{-\Delta\Delta Ct}$).

Histopathological analyses light microscopy: Preparation of solutions, dehydration and clearing procedures of tissue samples, preparation of sections and staining with Harris Hematoxylin and Eosin Y were carried out in line with previous studies for histopathological evaluation (5). The lung tissues collected from rats for histopathological investigation were fixed in 10% neutral formalin for 48 to 72 hours. Then routine operations are performed. Serial sections of 3-µm thickness were cut and stained with hematoxylin and eosin before being examined under a light microscope (Olympus BX51, Japan). Edema area, alveolar wall thickness, and inflammatory cell infiltration in lung tissues were investigated by light microscope. At least 5 areas in every lung tissue slide at ×40 magnification were evaluated and appointed to define the violence of the alterations using scores on a scale. (Grade 0: - (0% absent), grade 1: + (0-33% mild), grade 2: ++ (33-66% moderate), grade 3: +++ (66-100% severe)) (12).

Ethical consent: Ethics Committee Approval was obtained with the decision dated 17.09.2020 and protocol number: 42190979-000-E.2000225791 of Atatürk University Animal Experiments Local Ethics Committee.

Statistical analysis: Data are expressed as means±standard deviation (SD). Statistical analyzes were performed with One-way ANOVA and Duncan's multiple comparison tests using the IBM SPSS 25.0 package program. *p* value less than 0.05 was considered statistically significant.

Results

Molecular results: To evaluate whether 2 and 4 mg/kg RAME alleviated endotoxin-induced sepsis, TNF-α, NF-ϰβ, and IL-1β mRNA expression levels in the lung tissue of rats were analyzed. TNF-α, NF-ϰβ, and IL-1β mRNA expression levels increased in the lung tissue of the sepsis group. RAME application significantly decreased TNF-α (p<0.05) and NF-ϰβ (p<0.05) and IL-1β (p<0.05) mRNA expression levels induced by sepsis, dose-dependent manner, compared to the sepsis group. (Figure 1 A-C / Table Figure. 1 A-C / Table 2)

Table 3: Histopathological scoring results of the effects of ramelteon on acute lung injury in the sepsis

Groups	Edema Area	Alveolar Wall Thickness	Inflammatory Cell Infiltration
Healthy	-	+	-
Sepsis	+++	+++	+++
Sepsis + RAME 2	++	+	+
Sepsis + RAME 4	++	+	+

Sepsis:10 mg/kg LPS (Lipopolysaccharide), **RAME 2:** 2mg/kg Ramelteon; **RAME 4:** 4mg/kg Ramelteon
Grade 0: - (0% absent), **Grade 1:** + (0-33% mild), **Grade 2:** ++ (33-66% moderate), **Grade 3:** +++ (66-100% severe).

Histopathological Results: The histopathologic features of the RAME effects on the e lung injury in sepsis were shown in Figure 2, and the histopathologic scores were given in Table 3. No pathological marks were observed in the lung tissues of the healthy group (Figure 2-A). Signs of severe sepsis were observed in the lung tissues of the sepsis groups. A vasodilation in alveolar capillaries and a raise in alveolar wall thickness were observed. Leukocyte infiltration was determined in the whole lung parenchyma, although more severe around the pulmonary artery and terminal bronchioles. Edematous areas were observed in the periphery of the pulmonary artery. In some parts of the section, it was observed that the integrity of the alveolar wall was lost and the lung tissue was damaged (Figure 2-B). It was observed that the histopathological damage caused by sepsis improved in RAME-administered groups depending on the dose. Although there was no reduction in periarterioema findings, a decrease in leukocyte infiltration was observed in both bronchiole-related areas and interalveolar septum in the lung tissues of the 2 and 4 mg/kg RAME administered groups, compared to the sepsis group. An increase in alveolar volume was observed in the lung tissues of both treatment groups, consistent with signs of improvement. (Figure 2-C, D).

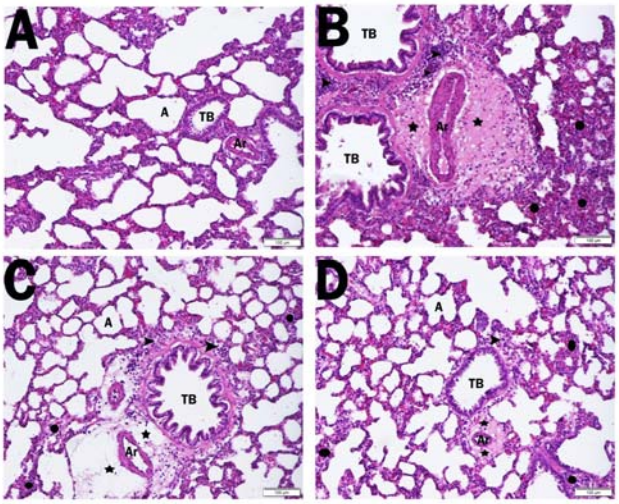

Figure 2. A-D: Hematoxylin-eosin staining findings of the effects of RAME on acute lung injury in endotoxin-induced sepsis. **A:** Healthy; **B:** Sepsis (10 mg/kg LPS), **C:** Sepsis+RAME2 (2mg/kg Ramelteon), and **D:** Sepsis+RAME4 (4mg/kg Ramelteon) LPS: Lipopolysaccharide TB: Terminal Bronchiole, A: Alveole, Ar: Arteriole, Star: Edematous Areas, Arrow Heads: Leukocyte Infiltration Areas, Points: Alveolar septum thickening)

Discussion

Sepsis is systemic inflammation caused by infection and is characterized by persistent fever, leading to microcirculation damage, organ damage including damage to the lungs, and multi-organ failure (13). As at present, there is no potent drug available to treat lung inflammation, which has a high mortality rate, the effects of RAME against acute lung injury in endotoxin-induced sepsis model were investigated molecularly and histopathologically. The primary mechanism causing the inflammatory reaction to sepsis includes the LPS defined through pattern identification receptors, followed by the organized expression of proinflammatory cytokines such as TNF, and IL-1β (14). TNF-α has a leading role in the emergence and progress of systemic inflammation. It can stimulate the production of other inflammatory markers, including IL-1β, and aggravate the damage of tissues and organs (15). Excessive production of IL-1β can be a mark of the beginning of inflammation such as sepsis (16). NF-κβ, the predominant transcription factor, induces inflammatory marker proteins, including chemokines, cytokines, and inducible enzymes (17). Therefore, suppression of markers, such as TNF-α, NF-κβ, and IL-1β is highly significant in the treatment of sepsis. In light of this information, in this study, the mRNA expression of TNF-α, IL-1β, and NF-κβ in lung tissues after LPS and treatment with RAME were investigated to appraise the potential medicinal importance of RAME in sepsis. TNF-α, NF-κβ and IL-1β mRNA expressions raised in the sepsis group. These results are compatible with those of previous studies (18,19), which also defined endotoxin-

induced raises in cytokine levels. The increase in TNF-α, NF-κβ, and IL-1β mRNA expression in septic rats reduced compatible with the RAME dose. This influence of RAME may be owing to anti-inflammatory effects connected to melatonin agonism (20). It is known that the anti-inflammatory effects of melatonin intercede with the NF-κβ signaling pathway, and the molecular efficiency of melatonin is owing to the prevention of NF-κβ (21). TNF-α inhibition followed by NF-κβ inhibition is also expected. Previous studies have shown that TNF-α affects the JAK-STAT pathway in cells (22). Also, activation of melatonin receptors inhibits JAK2/STAT3 signal pathway (23), causing chemokine and cytokine secretion [(24). Also, Celinski et al. (25) reported that activation of melatonin receptors reduced the levels of pro-inflammatory cytokines such as TNF-α. Proofs that RAME exerts an anti-inflammatory effect by depressing proinflammatory cytokines has been previously shown in acute ocular inflammation (26), methotrexate-induced cerebral toxicity (27) in rats, and isoflurane-stimulated cytotoxicity in the brain in the human model (28). Moreover, it was previously confirmed that RAME played a protective role by inhibiting TNF-α, NF-κβ, and IL-1β in a rat model of endotoxin-induced neuroinflammation (29). Consistent with the previous studies, our result suggests that RAME reduced organ dysfunction by preventing the raise in cytokines due to sepsis. In systemic inflammatory illnesses including sepsis, one of the most affected members of the body is the lung (30). Earlier studies indicated that sepsis created histopathological injury in the lung tissue (19). In the present study, the lung tissues of rats after LPS and treatment with RAME were investigated histopathologically to evaluate the potential medicinal importance of RAME in sepsis. Widespread histopathological alterations including common inflammatory cell infiltration were observed in the lung tissues of the LPS group. RAME corrected histopathologic injury in the lung tissue due to sepsis. Our histopathological results are consistent with both our molecular findings and the previous studies. In conclusion, it was determined in the present study that RAME ameliorated the alteration in anti-inflammatory parameters stimulated by sepsis. RAME improved histopathological injury of lung tissues caused by endotoxin-induced sepsis. RAME could be a promising agent by contributing to alternative preventive treatment methods for sepsis with its anti-inflammatory effect.

Ethical Consent: Ethics Committee Approval was obtained with the decision dated 17.09.2020 and protocol number: 42190979-000-E.2000225791 of Atatürk University Animal Experiments Local Ethics Committee.

Conflict of Interest: The authors declare that they have no conflict of interest of financial or personal nature.

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Figures: The authors declare that the figures used belong to this work.

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