Original Article

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Ramelteon Protects Intestinal Tissue Against Injury Caused by Methotrexate Via Showing Anti-apoptotic, Anti-inflammatory and Antioxidant Properties

Ramelteon, Anti-apoptotik, Anti-enflamatuvar ve Antioksidan Özellikler Göstererek Barsak Dokusunu Metotreksatın Neden Olduğu Hasara Karşı Korur

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ABSTRACT

Objective: Methotrexate (MTX), a drug used in the treatment of autoimmune diseases and cancers, is a folic acid antagonist, but it has toxic effects on the gastrointestinal system (GIS). In this study, we examined the anti-inflammatory, antioxidant and anti-apoptotic effects of Ramelteon (RAM), a melatonin receptor agonist, on the MTX-induced toxicity in the intestinal tissue of rats.

Methods: Thirty-two male Wistar albino rats were randomly divided into 4 groups; Control group, MTX group, MTX + RAM group, and RAM group. Single-dose 0.1 mL 20 mg/kg MTX, saline or 0.1 mL 10 mg/kg RAM orally was administered for 7 days. Animals were sacrificed at the end of 7 days after the last drug administration. Then, intestinal tissues were collected for biochemical, histopathological and immunohistochemical analyses.

Results: While normal histological findings and biochemical parameters were observed in the control and RAM groups, in the MTX group, mononuclear cell infiltrations, hemorrhagic areas, degenerations in the submucosa and Lieberkuhn crypts were observed in the intestinal sections. Caspase-3 (Cas-3) and tumor necrosis factor-alpha (TNF-α) expressions, total oxidant status (TOS) and oxidative stress index (OSI) increased and total antioxidant status (TAS) decreased in the MTX group. RAM treatment decreased Cas-3 and TNF-α expressions, TOS, OSI levels and increased TAS levels.

ÖZ

Amaç: Otoimmün hastalıklar ve kanserlerin tedavisinde kullanılan bir ilaç olan metotreksat (MTX), bir folik asit antagonistidir ancak gastrointestinal sistem (GİS) üzerinde toksik etkileri vardır. Bu çalışmada, bir melatonin reseptör agonisti olan Ramelteon'un (RAM) sıçanların barsak dokusunda MTX ile indüklenen toksisite üzerindeki anti-enflamatuvar, antioksidan ve anti-apoptotik etkilerini araştırdık.

Yöntemler: Otuz iki erkek Wistar albino rat rastgele 4 gruba ayrıldı: Kontrol grubu, MTX grubu, MTX + RAM grubu ve RAM grubu. Yedi gün boyunca tek doz 0,1 mL 20 mg/kg MTX, salin veya 0,1 mL 10 mg/kg RAM oral yoldan uygulandı. Hayvanlar, son ilaç uygulamasından sonra, yani 7. günün sonunda sakrifiye edildi. Daha sonra, barsak dokuları biyokimyasal, histopatolojik ve immünohistokimyasal analizler için toplandı.

Bulgular: Kontrol ve RAM gruplarında normal histolojik bulgular ve biyokimyasal parametreler gözlemlenirken, MTX grubunda mononükleer hücre infiltrasyonları, hemorajik alanlar, submukozada dejenerasyonlar ve barsak kesitlerinde Lieberkuhn kriptleri gözlendi. MTX grubunda kaspaz-3 (Cas-3) ve tümör nekroz faktör-alfa (TNF-α) ekspresyonları, total oksidan seviyesi (TOS) ve oksidatif stres indeksi (OSI) arttı ve total antioksidan seviyesi (TAS) azaldı. RAM tedavisi Cas-3 ve TNF-α düzeylerini, TOS, OSI seviyelerini azaltırken TAS seviyelerini arttırdı.

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Conclusion: In this study, RAM showed anti-apoptotic, antioxidant and anti-inflammatory effects on MTX-induced toxicity in intestinal tissue. Therefore, it was suggested that RAM might be used in MTX-like toxicities to alleviate the side effects on the GIS.

Keywords: Intestinal injury, inflammation, methotrexate, oxidative stress, ramelteon

Sonuç: Bu çalışmanın sonucunda RAM, barsak dokusunda MTX kaynaklı toksisite üzerinde anti-apoptotik, antioksidan ve anti-enflamatuvar etkiler gösterdi. Bu nedenle, GİS üzerindeki yan etkileri hafifletmek için MTX benzeri toksisitelerde RAM'nin kullanılabileceği öne sürülmüştür.

Anahtar Sözcükler: Barsak hasarı, enflamasyon, metotreksat, oksidatif stres, ramelteon

Introduction

Methotrexate (MTX), is a folic acid synthesis inhibitor that is widely used as a chemotherapeutic and immunomodulatory agent (1). It is used in the treatment of rheumatological diseases like arthritis as a Disease-Modifying Anti-Rheumatic Drug (DMARD) and several types of childhood and adult cancers such as osteosarcoma, non-Hodgkin lymphoma (2,3). However, it is a cytotoxic agent and can cause toxic effects on liver, kidney, bone marrow and gastrointestinal system (GIS) (4).

MTX, which inhibits DNA synthesis and cell proliferation by binding to dihydrofolate reductase, causes an increase in the production of reactive oxygen species (ROS) (6). This reduces the amount of antioxidants that protect against damage and causes an inadequate antioxidant response. It is known that ROS-induced oxidative damage may result from the inflammation process (7). Oxidative stress in the gastrointestinal tract may lead to the progression of inflammatory disorders. Disruption of the mucosal barrier in the gut may activate the innate immune system and lead to the expression of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) (8,9). This progression may result in the activation of apoptotic signaling pathways inside the cell depending on increased caspase-3 (Cas-3) levels (9,10).

The common side effects of MTX that limit its therapeutic usage on the GIS can be classified as diarrhea, nausea, vomiting, ulceration, enterocolitis, and mucositis (5). Therefore, elucidating the molecular mechanism of MTX is important for eliminating these side effects and improving the therapeutic efficacy of this agent.

Ramelteon (RAM) is a potent and selective melatonin receptor-1 and 2 (MT1 and MT2) agonist drug and is generally used in the treatment of insomnia (11,12). Studies revealed that this agent exhibited anti-inflammatory effects by reducing interleukin-6, TNF-α, interleukin-1beta, and transforming growth factor-β cytokine levels (13,14). Moreover, Kandezi et al. (15), demonstrated that RAM showed anti-apoptotic properties by inhibiting JNK/Bcl-2-Beclin-1 or JNK/Bcl-2/Bax signaling pathways and showed antioxidant effect via suppressing ROS production.

In this study, we aimed to investigate the underlying mechanisms of RAM's protective effects against MTX-induced intestinal toxicity through antioxidant, anti-inflammatory, and anti-apoptotic pathways.

Method

Animals

Adult male Wistar albino rats (n=32; 250-300 g) were purchased from the Animal Research Laboratory of Süleyman Demirel University. The animals were accommodated in standard laboratory conditions to acclimatize for at least seven days before experimentation. Then, they were group-housed under a 12:12-hour light: dark cycle with constant temperature (24±1 °C) and humidity (50±10%) with access to food and water ad libitum.

Experimental Procedures

All animal procedures were conducted following the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research at Süleyman Demirel University, Isparta (ethic no: 11.09.2020 06/15).

Thirty-two rats were randomly divided into 4 groups with 8 rats in each group: Control group: 0.1 mL of saline was administered by oral gavage (p.o.) for 7 days and intraperitoneal (i.p.) 0.1 ml of saline was administered on day 2. MTX group: 0.1 mL saline p.o. for 7 days and single dose 0.1 mL 20 mg/kg MTX i.p. (MTX 50 mg/mL vial, Koçak, Turkey) was administered on day 2 (16). MTX + RAM group: 0.1 mL 10 mg/kg RAM (Ramelda, Abdi İbrahim,Turkey) p.o. for 7 days and single dose 0.1 mL 20 mg/kg MTX i.p. was administered on day 2. RAM group: 0.1 mL 10 mg/kg RAM p.o. for 7 days and 0.1 mL of saline i.p. was applied on day 2 (17).

At the end of the 7 days after the last drug administration, animals were sacrificed under anesthesia with solution mixture containing ketamine (80-100 mg/kg) (Alfamin, Alfasan IBV) and xylazine bio 2% solution (8-10 mg/kg) (Bioveta, Czech Republic). Then, intestinal tissues were collected. One-half of the tissue was stored at (-20 °C) for biochemical measurements. The remaining part of the tissue was fixed in 10% buffered formaldehyde for histopathological and immunohistochemical analyzes. All these procedures are shown in Figure 1.

Histopathological Analysis

Intestinal tissues taken from rats were washed in water overnight, 10% neutral formaldehyde solution was used to fix them and embedded in paraffin. Samples from the prepared paraffin blocks were sectioned with a thickness of 3-4 mm with a sliding microtome (Leica SM2000R, Germany) and Hematoxylin-Eosin staining was performed, then covered with mounting medium. A

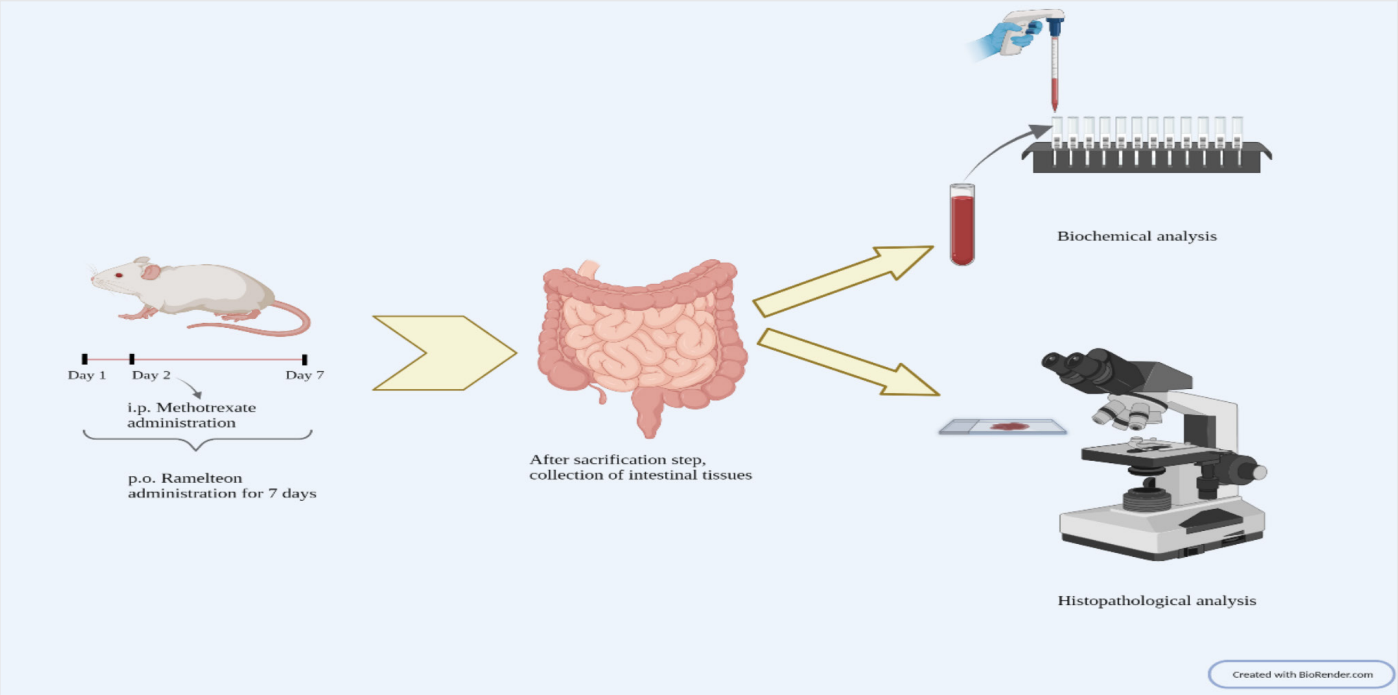

Figure 1. Graphical abstract for experimental procedures

semi-quantitative analysis of histopathological findings was then calculated to allow comparison between the groups. All groups were analyzed and evaluated with a photomicroscope according to scoring by Refaiy (18).

Immunohistochemical Analysis

Intestinal tissues with 3-4 µm thicknesses were fixed in 10% neutral formaldehyde solution and embedded in paraffin before histological methods were utilized. Tissues were stained with TNF-α (rabbit TNF-α antibody, Abcam, Cambridge, USA) and Cas-3 (rabbit Caspase-3 antibody, Abcam, Cambridge, USA) primary antibodies and were covered with mounting medium. Then, immunohistochemical methods were applied and results were evaluated by a semi-quantitative method (16).

Biochemical Analysis

Biochemical analyses included measurements of TAS, TOS, and OSI levels. Homogenization of intestinal tissue samples was carried out with the Ultra Turrax Janke & Kunkel T-25 homogenizer (IKA® Werke, Germany). By using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey), the total antioxidant status (TAS) and total oxidant status (TOS) were measured spectrophotometrically (Beckman Coulter AU5800, Beckman Coulter, USA), and according to results, OSI values were calculated using the formula OSI = TOS/TAS [Erel (19)]. In the TAS analysis which determined the antioxidative effect of the sample against the potent free radical reactions triggered by the produced hydroxyl radical; antioxidants were reduced in the sample from a dark blue-green colored 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulphonic acid; ABTS) radical to a colorless ABTS form. The changing of absorbance at 660nm was related with TAS level of the sample. The results are presented with millimolar Trolox equivalents per liter (mmol Trolox Eq/L) unit (19).

In the TOS analysis, oxidants found in the sample oxidized the ferrous ion-dianisidine complex to the ferric ion. The oxidation reactions were raised with glycerol molecules of the reaction medium. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The intensity of the color is related to TOS levels in the samples. TOS was measured spectrophotometrically at 530 nm by using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). Hydrogen peroxide (H_2O_2) was used in the calibration of this assay. Results were presented with micromolar H_2O_2 equivalents per liter (µmol H_2O_2 Eq/L) unit (20).

Statistical Analysis

Data were analyzed using Graphpad Prism software (Prism5, San Diego, California, US). One-way ANOVA followed by Bonferroni multiple comparison test was performed for analyzing statistical significance of differences between control and experimental groups. Differences were considered significant for $p<0.05$. All results were expressed as mean ± standard deviation.

Results

Total Oxidant Status (TOS), Total Antioxidant Status (TAS), and Oxidative Stress Index (OSI)

The TAS levels decreased significantly compared to the control group in the MTX administered group ($p<0.05$). In the MTX + RAM and RAM groups, TAS levels elevated compared to the MTX group ($p<0.01$ for both). TOS levels significantly elevated in the MTX group compared to the control group ($p<0.001$), decreased both in MTX + RAM and RAM groups

compared to the MTX group (p<0.001, p<0.05, respectively). OSI levels elevated in the MTX group compared to the control group (p<0.001). In MTX+RAM and RAM groups, OSI levels attenuated compared to the MTX group (p<0.01) (Figure 2).

Histopathological Findings

Significant difference was observed between the control group and the MTX, MTX + RAM groups in HE staining of intestinal tissue sections (p<0.05). Histopathological findings demonstrated mononuclear cell infiltration, hemorrhagic areas, degeneration in the submucosa and crypts of Lieberkuhn were detected in the MTX group and RAM treatment reversed these findings (p<0.05) (Figure 3, Table 1).

Immunohistochemical Findings

According to findings of immunohistochemical staining in intestinal tissue sections, TNF-α and Cas-3 expressions were found significantly higher in MTX, MTX + RAM groups compared to the control group (p<0.01, p<0.05, respectively, for both). TNF-α and Cas-3 expressions decreased significantly in the MTX + RAM group, compared to the MTX group (p<0.05, for both). No significant difference was examined between the control and RAM groups (Figure 4, Table 2).

Discussion

The MTX is already a widely used drug in the treatment of malignant and nonmalignant diseases. It is used in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (3). Although it is preferred as a chemotherapeutic agent, hepatic and renal toxicity that causes complications limit its usage. Due to the fact that it causes DNA damage, side effects are not limited to hepatic and renal toxicity, and it can also lead to harmful conditions in other tissues. MTX also affects human gut microbiota resulting in alteration of host immunity (21). These circumstances can cause gastrointestinal complications in patients. The findings from histopathological examinations might be consequences of microbiota changes depending on MTX administration. As a result of histopathological examination, mononuclear cell infiltration in intestinal tissue

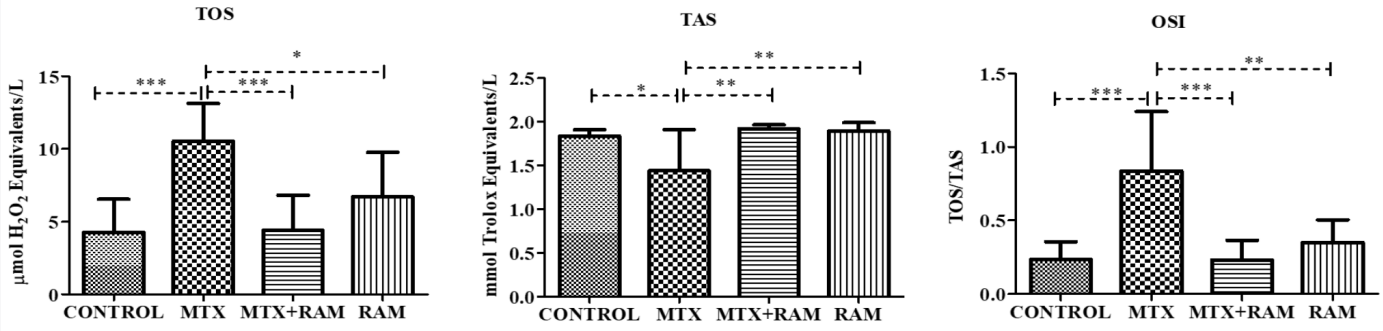

Figure 2. Values are represented as means ± SD. Comparison between groups and results of oxidative stress markers were assessed by one-way ANOVA test followed by post hoc Bonferroni multiple comparison test

MTX: Methotrexate, RAM: Ramelteon, TOS: Total oxidant status, TAS: Total antioxidant status; OSI: Oxidative stress index, SD: Standard deviation

***p<0.001 **p<0.01 * p<0.05

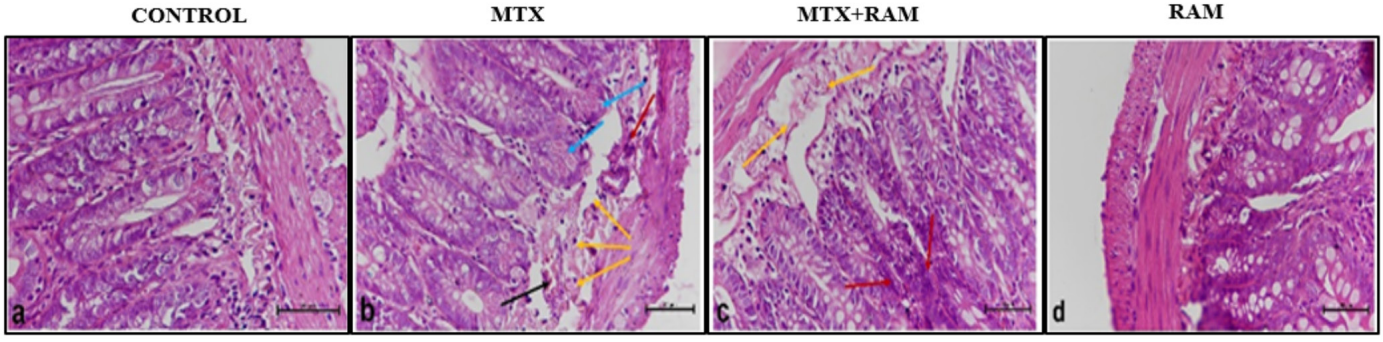

Figure 3. Histopathological findings in intestine tissues belonging to control and experimental groups: **a-** CONTROL group, no histopathological findings were found. **b-** MTX group, **c-** MTX + RAM group, **d-** RAM group. Red arrows; mononuclear cell infiltration, black arrows; hemorrhagic areas, yellow arrows; sinusoidal degeneration in the submucosa and blue arrows; degeneration in Lieberkuhn crypts, HE, x40

MTX: Methotrexate, RAM: Ramelteon

Table 1. Average score of histopathological findings between all groups

Groups	Mononuclear cell infiltrations	Hemorrhagic areas	Degenerations of submucosa	Degenerations of lieberkuhn crypts
Control	-	-/+	-	-/+
MTX	+++	++/+++	++	+++
MTX + RAM	+/++	++	-/+	++
RAM	-	-	-	-/+

(-), negative score: No structural changes; **(+)**, 1 positive score: Light structural changes; **(++)**, 2 positive score: Mild structural changes; **(+++)**, 3 positive score: Serious structural changes
MTX: Methotrexate, RAM: Ramelteon

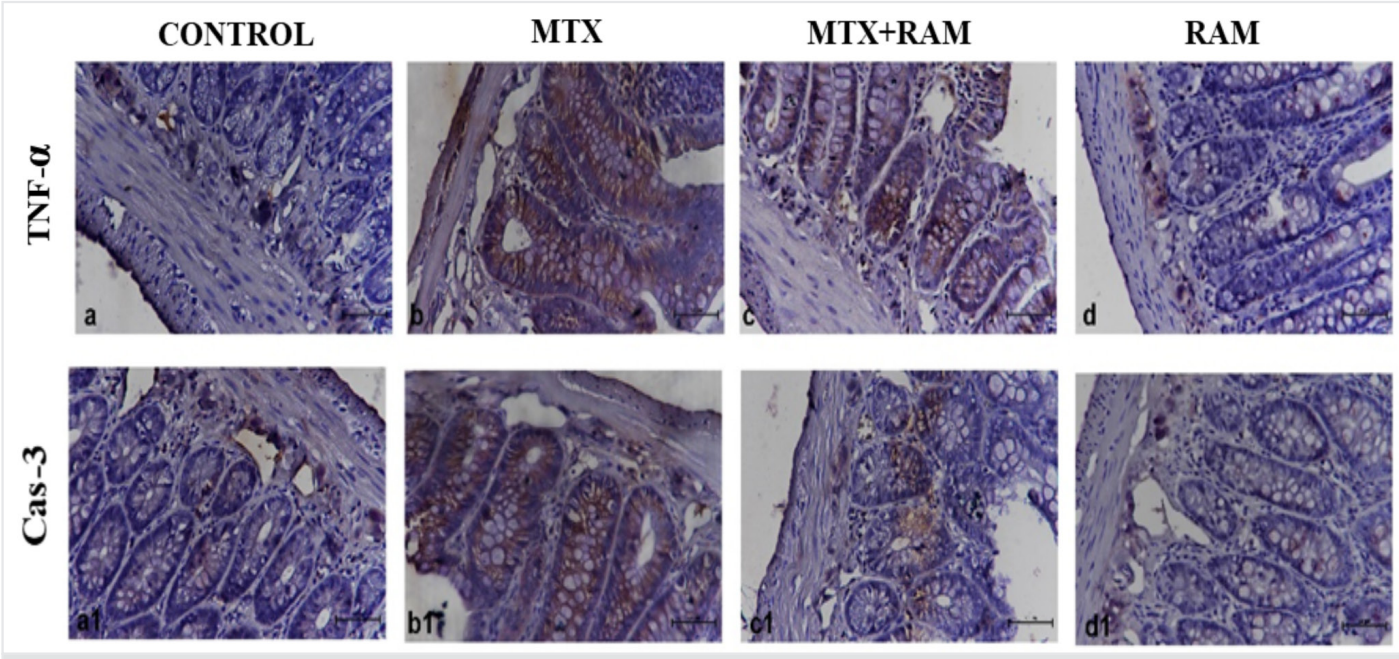

Figure 4. TNF-α, and Cas-3 immune staining in intestine tissues belonging to control and experimental groups: **a-a1**; CONTROL group, **b-b1**; MTX group, **c-c1**; MTX + RAM group, **d-d1**; RAM group. **a-a1**; CONTROL group, no positive staining, **b-b1**; MTX group, positive staining, **c-c1**; MTX + RAM group, mild positive staining, **d-d1**; RAM group, no positive staining, x40.

MTX: Methotrexate, RAM: Ramelteon, Cas-3: Caspase-3, TNF-α: Tumor necrosis factor-alpha

Table 2. TNF-α and cas-3 marking average degrees between all groups

	Control	MTX	MTX + RAM	RAM
TNF- α	0.19±0.463[a]	2.53±0.51[b]	1.65±0.453[c]	0.23±0.374[a]
Cas-3	0.15±0.354[a]	2.78±0.344[b]	1.78±0.364[c]	0.21±0.443[a]

Values are expressed as means ± SD. The comparison between groups and results are evaluated by one-way ANOVA. a, b, c; different characters indicate statistically significant differences in the same column, $p<0.05$, $p<0.01$, $p<0.001$.
TNF-α: Tumor necrosis factor-alpha, Cas-3: Caspase-3, MTX: Methotrexate, RAM: Ramelteon

was a clue for an inflammatory pathway that resulted in the occurrence of hemorrhagic areas, degenerations of the submucosa and formation of Lieberkuhn crypts.

Damaged intestinal tissue indicates that there may be blood flow from the hemorrhagic areas into the intestinal lumen. In addition, disruptions may occur in functions such as fluid absorption and mucus secretion due to degeneration in the submucosa layer and Lieberkuhn layers. As a result of all these, GIS complications such as diarrhea, blood in the urine or stools, vomiting, nausea and indigestion can be seen (22,23).

Moreover, MTX can lead to side effects due to both long and short-term usages. Myelosuppression, abnormalities of liver enzymes and toxicity in the male-reproductive system are possible adverse effects of long-term usage of MTX (24,25).

It is also known that MTX induces overexpression of ROS which initiates mucositis, followed by up-regulation of nuclear factor kappa B pathway-mediated pro-inflammatory cytokine production such as TNF-α which may stimulate apoptosis (26). Morsy et al. (27), studied the toxic effects of MTX on the intestine of rats and found that this agent increased oxidative and

nitrosative stress marker levels in the intestinal mucosa by causing up-regulation of nuclear factor kappa B, cyclooxygenase-2, and increasing TNF-α and Cas-3 levels. In this study, according to biochemical analyses and immunohistochemical examinations, increments in TNF-α, Cas-3 expressions and TOS and OSI levels in the MTX group confirmed this view.

The RAM is a widely used drug in the treatment of insomnia showing a high affinity for MT receptors; MT1 and MT2. Antioxidant effects of melatonin are thought to be the result of its radical-scavenging ability by the MT receptor (28). Moreover, Wang et al. (17), showed that RAM treatment improved dysfunction of brain endothelial, oxidative stress, and inflammation via activating nuclear factor erythroid 2-related factor 2 pathway in traumatic brain injury. In the isoflurane-induced in vitro cell culture model of brain microvascular endothelial cells, RAM downregulated p38 MAPK/NF-κB signaling pathway activation and exhibited an anti-inflammatory effect (29). In the present study, our results confirmed anti-inflammatory, antioxidant, and anti-apoptotic properties of RAM on short-term MTX-induced toxicity in intestinal tissue by diminishing TOS, OSI levels and TNF-α, Cas-3 expressions. In parallel to these results, it also decreased mononuclear cell infiltrations, hemorrhagic areas, degenerations in the submucosa and formation of Lieberkuhn crypts in the intestinal tissues. In line with these observations, to support conceivable molecular mechanisms of RAM on MTX-induced toxicity, additional studies are required.

Conclusion

The RAM, which was a melatonin receptor agonist, showed anti-apoptotic, antioxidant, and anti-inflammatory effects on MTX-induced toxicity in intestinal tissue. Although, MTX is a one of widely used chemotherapeutic agent in the treatment of many diseases, it has toxic effects on the tissues in dose dependent manner. This study showed that RAM could be a preferable drug candidate in MTX-like toxicities to alleviate the side effects on the GIS and might increase patient compliance during the treatment period. However, molecular studies are required to support histochemical, immunohistochemial and biochemical analyzes, therefore our studies will be continuing towards this goal.

Ethics

Ethics Committee Approval: All animal procedures were conducted following the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research at Süleyman Demirel University, Isparta (ethic no: 11.09.2020 06/15).

Informed Consent:

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: D.Ç., M.A.S, S.C., O.İ., E.K., Y.S.S., M.Ö., Design: D.Ç., M.A.S., O.İ., M.Ö.,

Data Collection or Processing: D.Ç., M.T., M.Y.A., Analysis or Interpretation: D.Ç., M.A.S., E.K., M.T., M.Y.A., Literature Search: D.Ç., M.A.S., S.C., O.İ., E.K., Y.S.S., Writing: D.Ç., M.A.S., S.C., O.İ., E.K., Y.S.S.

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References

1. Cronstein BN, Bertino JR. Methotrexate. Birkhäuser; 2012.
2. Editore S. Methotrexate in the treatment of rheumatic diseases. SICS Editore; 2014.
3. Jones V. Methotrexate: The Fast Action Drug Used to Treat Rheumatoid Arthritis, Severe Psoriasis and Breast Cancer. Independently Published; 2019.
4. Neradil J, Pavlasova G, Veselska R. New mechanisms for an old drug; DHFR- and non-DHFR-mediated effects of methotrexate in cancer cells. Klin Onkol 2012;25 (Suppl 2):2S87-92.
5. Freyer CW, Gier S, Moyer ME, et al. Leucovorin Rescue After Methotrexate Graft-Versus-Host Disease Prophylaxis Shortens the Duration of Mucositis, Time to Neutrophil Engraftment, and Hospital Length of Stay. Transplant Cell Ther 2021;27:431.e1-e431.e8.
6. Bedoui Y, Guillot X, Sélambarom J, Guiraud P, Giry C, Jaffar-Bandjee MC, et al. Methotrexate an Old Drug with New Tricks. Int J Mol Sci 2019;20:5023.
7. Zeeshan HM, Lee GH, Kim HR, Chae HJ. Endoplasmic Reticulum Stress and Associated ROS. Int J Mol Sci 2016;17:327.
8. Baer PC, Koch B, Geiger H. Kidney Inflammation, Injury and Regeneration. Int J Mol Sci 2020;21:1164.
9. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 2014;20:1126-67.
10. Cristiana F, Elena A. Reactive Oxygen Species (ROS) in Living Cells. BoD - Books on Demand; 2018.
11. Wang Q, Lu Q, Guo Q, Teng M, Gong Q, Li X, et al. Structural basis of the ligand binding and signaling mechanism of melatonin receptors. Nat Commun 2022;13:454.
12. Oishi A, Gbahou F, Jockers R. Melatonin receptors, brain functions, and therapies. Handb Clin Neurol 2021;179:345-56.
13. Yang S, Wang J, Wang D, Guo L, Yu D. Melatonin Receptor Agonist Ramelteon Suppresses LPS-Induced Neuroinflammation in Astrocytes. ACS Chem Neurosci 2021;12:1498-505.
14. Gu C, Wang F, Zhang YT, Wei SZ, Liu JY, Sun HY, et al. Microglial MT1 activation inhibits LPS-induced neuroinflammation via regulation of metabolic reprogramming. Aging Cell 2021;20:e13375.
15. Kandezi N, Majdi F, Davoudizadeh R, Motaghinejad M, Safari S. Preventive Properties of Ramelteon against Cocaine-Induced Autophagia and Apoptosis: A Hypothetic Role of TNF-α Receptor

Involvement and JNK/Bcl-2-Beclin1 or Bcl-2/Bax Signaling Pathway. Int J Prev Med 2020;11:36.

16. Asvadi I, Hajipour B, Asvadi A, Asl NA, Roshangar L, Khodadadi A. Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. Eur Rev Med Pharmacol Sci 2011;15:1003-9.
17. Wang J, Jiang C, Zhang K, Lan X, Chen X, Zang W, et al. Melatonin receptor activation provides cerebral protection after traumatic brain injury by mitigating oxidative stress and inflammation via the Nrf2 signaling pathway. Free Radic Biol Med 2019;131:345-55.
18. Refaiy A, Muhammad E, ElGanainy EO. Semiquantitative smoothelin expression in detection of muscle invasion in transurethral resection and cystectomy specimens in cases of urinary bladder carcinoma . African Journal of Urology 2011;6-10.
19. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004;37:277-85.
20. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38:1103-11.
21. Nayak RR, Alexander M, Deshpande I, Stapleton-Gray K, Rimal B, Patterson AD, et al. Methotrexate impacts conserved pathways in diverse human gut bacteria leading to decreased host immune activation. Cell Host Microbe 2021;29:362-77.e11.
22. Kuiken NSS, Rings EHHM, Tissing WJE. Risk analysis, diagnosis and management of gastrointestinal mucositis in pediatric cancer patients. Crit Rev Oncol Hematol 2015;94:87-97.
23. Nagakubo J, Tomimatsu T, Kitajima M, Takayama H, Aimi N, Horie T. Characteristics of transport of fluoresceinated methotrexate in rat small intestine. Life Sci 2001;69:739-47.
24. Haustein U-F, Rytter M. Methotrexate in psoriasis: 26 years' experience with low-dose long-term treatment . J Eur Acad Dermatol Venereol 2000;14:382-8.
25. Borovskaya TG, Shchemerova YA, Bokhan EA, Grigor'eva VA, Vychuzhanina AV, Poluektova ME, et al. Experimental Evaluation of Long-Term Toxic Effects of Methotrexate on Male Reproductive System. Bull Exp Biol Med 2021;171:37-40.
26. Keefe DMK. Gastrointestinal mucositis: a new biological model. Support Care Cancer 2004;12:6-9.
27. Morsy M, El-Sheikh A, Hamouda A. Protective mechanisms of thymoquinone on methotrexate-induced intestinal toxicity in rats. Pharmacogn Mag 2016;12(Suppl 1):S76-81.
28. Tomás-Zapico C, Coto-Montes A. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J Pineal Res 2005;39:99-104.
29. Wang T, Li Z, Xia S, et al. The Protective Effects of Ramelteon Against Isoflurane-Induced Insults and Inflammatory Response in Brain Microvascular Endothelial Cells. Neurotox Res 2021;39:67-86.