ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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PROTECTIVE EFFECT OF THE RAMELTEON, A MELATONIN AGONIST, AGAINST METHOTREXATE-INDUCED BONE-TOXICITY

MELATONİN AGONİSTİ OLAN RAMELTEONUN METOTREKSAT KAYNAKLI KEMİK TOKSİSİTESİNE KARŞI KORUYUCU ETKİSİ

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Öz

Amaç

Metotreksat (MTX) büyüme durması ve kemik mineral yoğunluğunun azalması dahil olmak üzere kemik morbiditesine neden olan anti-metabolittir. Epifiz bezi tarafından üretilen melatonin, insan kemik hücrelerinde de birçok olumlu etkiye ve kemik üzerinde olumlu etkilere sahiptir. Ramelteon (RMT), seçici olmayan bir melatonin reseptör agonistidir. Bu çalışmada, bir melatonin agonisti olan ramelteonun MTX kaynaklı kemik toksisitesi üzerinde koruyucu bir etkisinin olup olmadığını araştırdık.

Gereç ve Yöntem

Sıçanlar Grup 1 kontrol grubu olmak üzere 4 gruba ayrıldı; Grup 2 MTX grubu (20 mg/kg); Grup 3 MTX+RMT (20 mg/kg + 10 mg/kg); Grup 4 RMT (10 mg/kg). Ratlara gruplara göre ikinci gün oral ramelteon ve intraperitoneal mtx uygulandı. 7 gün sonra uzun kemikler hematoksilen-eozin (HE) boyama ile histolojik olarak ve Catepsin K ve RUN X2 boyama ile immünohistokimyasal olarak değerlendirildi. İstatistiksel analiz için grupların immünhistokimyasal skorları gruplar arasında karşılaştırıldı, bu amaçla SPSS-22.00 paket programı ile Oneway ANOVA Duncan testi kullanıldı.

Bulgular

Kemik doku kesitlerinin H&E boyamasında kontrol grubu (grup I) ile deney grupları (grup II-grup III-grup IV) arasında anlamlı bir fark yoktu ($p>0.05$). CAT-K ve RUN-X immün boyamasında grupların hiçbirinde pozitif boyanma gözlenmedi ($p>0.05$).

Sonuç

Ramelteonun, sağlam kemik dokusu üzerinde yüksek doz metotreksatın neden olduğu kemik toksisitesinde, kemik döngüsünde, histopatolojik ve immünohistokimyasal olarak anabolik bir işlevi olmadığı gösterilmiştir.

Anahtar Kelimeler: Metotreksat; Ramelteon; Melatonin; Catepsin K, RUN X2

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Abstract

Objective
Methotrexate (MTX) used anti-metabolite, causes bone morbidity, including growth arrest and reduced bone mineral density. Melatonin, produced by the pineal gland, has also multiple positive effects in human bone cells, and positive effects on bone. Ramelteon (RMT) is a non-selective melatonin receptor agonist. In this study, we investigated whether ramelteon, a melatonin agonist, has a protective effect on MTX-induced bone toxicity.

Material and Methods
The rats divided into 4 groups, including Group 1 control group; Group 2 MTX group (20 mg/kg); Group 3 MTX+RMT (20 mg/kg + 10 mg/kg); Group 4 RMT (10 mg/kg). Oral ramelteon and intraperitoneal mtx were applied to the rats on the second day according to the groups. After 7 days, long bones were evaluated histologically with hematoxylin-eosin (HE) staining and immunohistochemically with Catepsin K and RUN X2 staining. For statistical analysis immunohistochemical scores of the groups were compared between the groups for this purpose, the Oneway ANOVA Duncan test was used by SPSS-22.00 package program.

Results
There was no significant difference between the control group (group I) and the experimental groups (group II-group III-group IV) in H&E staining of bone tissue sections ($p>0.05$). No positive staining was observed in any of the groups in CAT-K and RUN-X immunostaining ($p>0.05$).

Conclusion
It was showed that ramelteon has no anabolic function in bone turnover, histopathological and immunohistochemical, in bone toxicity induced by high-dose methotrexate on intact bone tissue.

Keywords: Methotrexate, Ramelteon, Melatonin, Catepsin K, RUN X2

Introduction

Methotrexate (MTX) is a cytotoxic drug commonly used at low dosage as a disease-modifying drug in rheumatologic diseases (1). MTX, used anti-metabolite, causes bone morbidity, including growth arrest and reduced bone mineral density (BMD). At chemotherapeutic doses, MTX inhibits RNA/DNA synthesis via the inhibition of dihydrofolate reductase. After MTX treatment, bone formation is attenuated (2), and bone synthesis (indicated by levels of circulating osteocalcin) and mineralization are depressed (3). It is metabolized in the liver. MTX and its metabolites are excreted by the kidney both by glomerular filtration and proximal tubular secretion. The plasma half-life is less than 10 hours, but may increase in renal failure (4).

Melatonin, produced by the pineal gland, has been shown to be an effective antioxidant and free radical scavenger, and has in vivo and in vitro activity because of its lipophylic property which can easily enter the nucleus and other organelles (5). Many in vitro studies have shown multiple positive effects of melatonin in human bone cells (6, 7), and positive effects of melatonin on bone (8, 9). Ramelteon is a non-selective melatonin receptor agonist. In the United States, it received Food and Drug Administration approval in 2005 for the treatment of insomnia (10). Ramelteon is a melatonin receptor agonist with both high affinity for melatonin MT1 and MT2 receptors and selectivity over the MT3 receptor. Ramelteon demonstrates full agonist activity in vitro in cells expressing human MT1 or MT2 receptors. Following oral administration of ramelteon, 84% is excreted in the urine and approximately 4% in the faeces. Elimination is essentially complete 96 hours post-dose (11).

RUNX2 is a multifunctional transcription factor that controls skeletal development by regulating the differentiation of chondrocytes and osteoblasts and the expression of many extracellular matrix protein genes during chondrocyte and osteoblast differentiation (12). During bone development, RUNX2 induces osteoblast differentiation and increases the number of immature osteoblasts, which form immature bone (13). Cathepsin K (Cat K), a member of cysteine proteases, is predominantly expressed in osteoclasts and plays crucial roles in degradation of bone matrix composited of hydroxyapatite and protein, especially type I collagen (14). Cat K with a relatively restricted expression pattern exhibits high activity against elastin and type I collagen and is obviously responsible for the relation of osteoclastic bone resorption (15).

The aim of this study was to investigate the protective effect of the ramelteon against methotrexate-induced bone-toxicity. We hypothesized that CAT-K activity

would increase, ramelteon would increase bone formation and repair, and RUN-X2 activity would increase in the group given methotrexate.

To our knowledge, this is the first study to investigate the effects ramelteon on methotrexate induced bone-toxicity.

Material and Methods

Ethical Approval

All experiments were conducted in accordance with the Ministry of Health of Turkey, the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (NIH) of the United States. The Experimental Animal Center and Animal Experiments Ethics Committee of Süleyman Demirel University approved all experimental procedures in this study (Date: 11/09/2020, Number: 06/14).

Animals and Surgical Procedures

32 male Wistar rats (20 to 24 weeks of age, weighting 200–300 g) were purchased from the Experimental Animal Center, Isparta, Turkey. Experimental animals divided into 4 groups (Table 1):

Control group; Oral gavage with 0.1 ml of saline for 7 days and also on the 2nd day intraperitoneal (i.p.) saline applied.

Methotrexat group; On the second day of the experiment, a single dose of 20 mg/kg MTX (i.p., Methotrexate 50 mg/ml flk, Kocak, Turkey) administered and saline administered by oral gavage for 7 days.

MTX + RMT 10mg group; On the second day of the experiment, a single dose of 20 mg/kg MTX (i.p., Methotrexate 50 mg/ml flk, Kocak, Turkey) administered and 0.1 ml 10 mg/kg RMT was given by oral gavage for 7 days.

RMT10 mg group; On the second day of the experiment, a single dose of i.p. 0.1 ml of saline was applied with oral gavage and 0.1 ml of 10 mg/kg RMT was given for 7 days.

At the end of seven days, anaesthesia was produced by means of a cocktail prepared by using xylazine hydrochloride (10mg/kg) and ketamine hydrochloride (90mg/kg) i.p. Then, all rats were sacrificed, bone tissue samples obtained were placed in 10% nitric acid solution for histochemical and immunohistochemical analyzes.

Histological Evaluation

Hard bone tissue samples in fixative were immersed in 10% nitric acid. The solution was changed daily, and the degree of tissue softening was determined with a needle. Decalcification was performed in the tissues for 2–10 days. Afterward, the tissues were washed overnight and were dehydrated in ethanol (50-60-70-80-90-100%), made transparent in xylol and embedded into paraffin. Samples were sectioned with a thickness of 3–4 mm by using a sliding microtome (Leica SM2000R, Germany) from the prepared paraffin blocks and stained by H–E than covered with entellan evaluated with photomicroscope according to scoring by Refaiy (16).

Immunohistochemical Evaluation

Two series of sections taken from all blocks drawn on poly-L-lysine coated slides were stained immunohistochemically for Catepsin K (Catalog no: sc-48353, Santa Cruz Biotechnology) and RUN X2 (Catalog no: sc-390351, Santa Cruz Biotechnology)

Table 1 32 male Wistar rats divided into four groups. The drug doses applied to the experimental group and the placebo doses applied to the control group are given in the table.

Days	Control (0.1ml)	MTX (20mg/kg)	MTX +RMT (20mg/kg + 10mg/kg)	RMT (10mg/kg)
First day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)
Second day	**Salin (O.G) + Salin (I.P)**	**Salin (O.G) + MTX (I.P)**	**RMT (O.G) + MTX (I.P)**	**RMT (O.G) + Salin (I.P)**
Third day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)
Fourth day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)
Fifth day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)
Sixth day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)
Seventh day	Salin (O.G)	Salin (O.G)	RMT (O.G)	RMT (O.G)

expression by streptavidin biotin technique according to manufacturer instruction. The sections were incubated with the primary antibodies for a period of 60 min, and immunohistochemistry was carried out using biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate. EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) (Abcam, Cambridge, UK) were used as secondary antibody. Diaminobenzidine (DAB) was used as the chromogen. For negative controls, antigen dilution solution was used instead of primary antibody. Tissue samples were analysed and evaluated and the receptor densities observed were identified using the semi-quantitative evaluation method

To evaluate the severity of the immunohistochemical reaction of cells with markers, semiquantitative analysis was performed using a grading score ranging from (0) to (3) as; follows:

(0-) = negative,
(1) = focal weak staining,
(2) = diffuse weak staining,
(3) = diffuse strong staining.

For evaluation, 10 different areas under 40X objective magnification in each section were examined. The results were saved and statistically analyzed.

Statistical Analysis

For statistical analysis, of spermatozoon counts and immunohistochemical scores of the groups were compared between the groups for this purpose, the Oneway ANOVA Duncan test was used by SPSS-22.00 package program. The level of significance was considered at $P < 0.05$.

Results

Histochemical Results

Structural changes in the bone tissue sections of the control and experimental groups were evaluated according to the grading of Refaiy et al. (1). There was no significant difference between the control group (group I) and the experimental groups (group II-group III-group IV) in H&E staining of bone tissue sections ($p>0.05$). Normal histological findings were observed in the bone tissues of both the control and experimental groups (Figure 1).

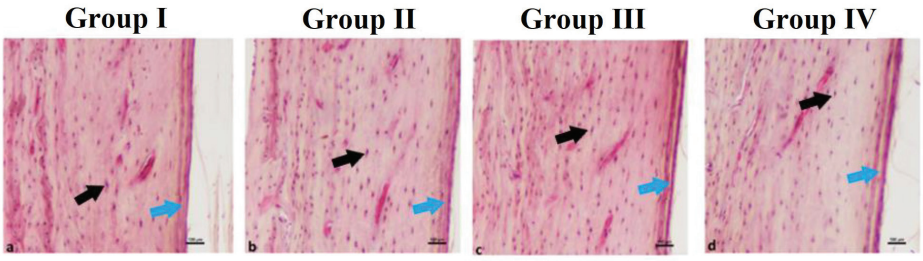

Figure 1

Histopathological findings in the bone tissue of the control and experimental groups. a: control group (group I); no histopathological findings were found, b: Mtx group, c: Mtx + Rmt group, d: Rmt group bone tissue samples, no difference was found between them and the control group. Black arrow: osteocytes in the lacunae, blue arrow: Periosteum. H-E x20, scale bar=100 µm.

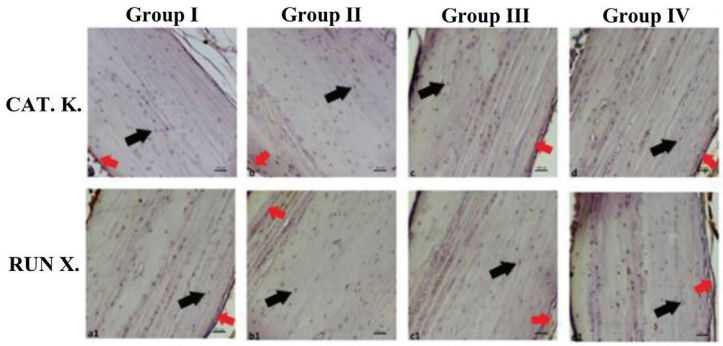

Figure 2

RUN X and CAT K immunostaining in bone tissue of control and experimental groups. a-a1: control group; b-b1: Mtx group, c-c1: Mtx + Rmt group, d-d1: Rmt group bone tissue samples. No positive staining was observed in any of the groups in immunostaining with CAT K and RUN X. Black arrow: osteocytes in the lacunae, red arrow: Periosteum. DABx20, scale bar=100 µm.

Immunohistochemical Results
There was no significant difference between the control group (group I) and the experimental groups (group II-group III-group IV) in Catepsin K and RUN X2 staining of bone tissue sections ($p>0.05$). No positive staining was observed in any of the groups in CAT-K and RUN-X immunostaining ($p>0.05$), (Figure 2).

Discussion

We investigated whether ramelteon, which is used in the treatment of insomnia and major depression in adults, has a toxic effect on long bones due to experimentally induced methotrexate in rats. Our histopathology results show that the application of MTX 20 mg/kg, combined with MTX 20 mg/kg and RMT 10 mg/kg, and RMT 10 mg/kg resulted in no significant difference between groups in H&E staining of bone tissue sections. The IHC staining results showed no positive staining was observed in any of the groups in immunostaining with CAT-K and RUN X, when compared.

Methotrexate is an antimetabolite that interferes with the metabolism of folic acid. Low-dose methotrexate is an effective first-line treatment for early rheumatoid arthritis and other inflammatory arthropathies, and as a maintenance therapy in Crohn disease, vasculitis and refractory atopic dermatitis (17). When looking at overall toxicity, pancytopenia, hepatotoxicity, pulmonary toxicity are major, painful stomatitis and gastrointestinal discomfort are the most common minor adverse effects (18). High-dose methotrexate (500 mg/m2<) is used to treat a number of adult and childhood cancers, neverteless can exert significant nephrotoxic effects in 2-12% of patients, including acute kidney injury (AKI) (19). Decreased bone formation and increased bone resorption are seen in children treated with high-dose MTX in combination with corticosteroids (20). Animal studies have provided our understanding of the mechanisms underlying chemotherapy-induced skeletal damage. In Wheeler et al's study comparing the long-term and short-term effects of methotrexate on bone volume in rats, the Cancellous bone volume was significantly lower in the MTX-treated group, the Cancellous mineralizing surface and longitudinal bone growth were always significantly depressed (21).

Melatonin, produced by the pineal gland, has been shown to be an effective antioxidant, and has in vivo and in vitro activity because of its lipophylic property which can easily enter the nucleus and other organelles (5). Also, in recently years, the in vitro osteoblastic differentiation and bone formation effect has been demonstrated (22-24). Melatonin at pharmacological doses (30 mg/kg) stimulates osteoblast differentiation and mineralization of matrix and it may regulate osteoclastic activity via superoksid dismutase in vitro. Therefore, the effect of melatonin in fracture healing depends in part on the free radical scavenging and osteoblastic- osteoclastic regulatory properties of melatonin (23). Ramelteon (RMT) is non-selective peripheral melatonin receptor (MT) agonist too. Kose D. et al has showed their application of RMT 3 mg/kg resulted in bone healing with increased new knitting bone tissue but in different RMT doses they reported that there was no difference between 6 mg/kg RMT dose and 3 mg/kg RMT dose on bone union (24).

RUNX2 is a multifunctional transcription factor that increases osteoblastic activity by regulating the expression of many extracellular matrix protein genes (13). During bone turnover, RUNX2 induces osteoblast differentiation and increases the number of immature osteoblasts (12). CAT-K is mostly produced by osteoclasts and exhibits high activity against type I collagen, the main collagen of the bone matrix, and is responsible for bone resorption with increased osteoclastic activity (15). In our study, we aimed to observe the increase in osteoclastic function of MTX with CAT-K in cortical and cancellous long bone localizations in rats which administered high-dose MTX, and the increase in osteoblastic activity and anabolic activity of RMT with RUNX2 against this catabolic activity. However, we did not observe any significant histopathological and immunohistochemical differences in both the cortical and spongiotic components of the long bones between the four groups, which can be explained by the small sample size and and the intact bone tissue without damage used in our study.

Conclusion

Our study showed that ramelteon has no anabolic function in bone turnover, histopathological and immunohistochemical, in bone toxicity induced by high-dose methotrexate on intact bone tissue.

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

Ethical Approval
All experiments were conducted in accordance with the Ministry of Health of Turkey, the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (NIH) of the United States. The Experimental Animal Center and Animal

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Authors Contributions
RD: Methodology, Validation, Supervision, Writing - original draft, Writing - review & editing
TB: Investigation, Visualization
DKD: Conceptualization, Ideas
ES: Resources, Data curation
DC: Investigation, Visualization

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