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Research Article
Effects of Circadian Rhythm Hormones Melatonin and 5-Methoxytryptophol on COXs, Raf-1 and STAT3

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Abstract

Background and Objective: Circadian rhythm hormones melatonin (MEL) and 5-Methoxytryptophol (5-MTX) have antioxidant and anti-inflammatory effects on the biological systems. The aim of this study was the investigation of Cyclooxygenase-1 (COX-1), Cyclooxygenase-2 (COX-2), Rapid Accelerated Fibrosarcoma-1 (Raf-1) and Signal Transducer and Activator of Transcription 3 (STAT3) inflammatory markers in the temporomandibular joint arthritis (TMJA) model) and effects of pineal hormons melatonin (MEL) and 5-Methoxytryptophol (5-MTX) on these parametres. **Materials and Methods:** Wistar albino (200-250 g) 40 rats of both sexes were used in this study. The arthritis model was created by intraarticularly injecting zymosan dissolved in physiological saline solution (2 mg/40 µL) into the left TMJ'S, while the sham group was created by only injecting 40 µL physiological saline solution. Intraperitoneal applications of MEL (15 min before zymosan) and 5-MTX (30 min before zymosan) were conducted for therapy. Data were analyzed using one-way analysis of variance (ANOVA). **Results:** The animals were decapitated after 6 h. COX-1, COX-2, Raf-1 and STAT-3 levels were examined with the RT-PCR technique. Articular structural damage was assessed histologically. In arthritis group, the activity of COX-2, Raf-1 and STAT3 were increased, therapy applications led to decreased these parameters. However, a significant difference was not observed in COX-1. In the histological evaluation, obvious articular degeneration and disc congestion in the arthritis group regressed with therapy. **Conclusion:** In this study, COX-2, Raf-1 and STAT3 have been suppressed by the therapeutic effect of MEL and 5-MTX.

Key words: Temporomandibular joint, arthritis, melatonin, 5-Methoxytryptophol, COX, Raf-1, STAT3

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Arthritis is an generalize inflammatory disease and seen very common in the world. Although there are several types of arthritis, the most common types of related to TMJ are osteoarthritis and rheumatoid arthritis. In generalized osteoarthritis patients, the incidence of TMJ osteoarthritis is 40%, whereas in rheumatoid arthritis cases in general, the incidence of TMJ rheumatoid arthritis is 50%[1,2]. Temporomandibular joint arthritis (TMJA) is characterized by hyperplasia of fibroblasts and cell infiltrations in the synovial tissue. In the inflammation, the dominant macrophages, enzymes and proteases released from the fibroblasts, degrade the articular matrix and the cartilage. In accordance with authors previous studies and based on other studies, synoviocytes (= synovial fibroblasts = fibroblast-like cells) and released from IL-1β, IL-6, IL-10, TNF-α, TGF-β1 and IFN-γ inflammatory cytokines stimulation with Matrix Metalloproteinases (MMPs), Cyclooxygenase-2 (COX-2), Rapidly Accelerated Fibrosarcoma 1 (Raf-1) enzymes and Signal Transducer and Activator of Transcription 3 (STAT3) protein all play a major role in the cartilage and bone destruction in TMJA pathogenesis[3-5]. It has been previously shown that COX-2 is a key enzyme responsible for the development of inflammation in zymosan-induced arthritis[6]. In studies conducted on rats, COX-2 was found to be increased in the synovial tissue of the TMJA models, however, with the inhibition of COX-2, the inflammatory response was decreased and COX-1 levels remained the same, even though there was a change in the degree of inflammation[6,7]. Raf-1 is thought to be an undetermined reaction of synovium in arthritis against a variety of pathological stimuli[8]. In this context, in one study the downregulation of Raf-1 caused a decrease in the invasive potential of the synovites and is shown through rats with Sever Combined Immunodeficiency (SCID)[9]. STAT3 proteins also play an important role in inflammation. In the study, it is shown that during inflammation, the increase of STAT3 protein levels with cytokine levels increases the activation of proteolytic enzymes and thus accelerates the inflammation[10]. At the same time, in experimental studies on TMJA, the STAT3 protein was reported to be at high levels within synovial cells[11,12]. Melatonin (MEL) is a circadian rhythm hormone which is produced primarily by pinealocyte in the pineal gland also secondly release in the other tissues, such as the liver, kidney, etc. and plays a role in the circadian rhythm, regulation of sleep and other biological activities[13]. The MEL is known to have both antioxidant and tissue protective effects with anti-inflammatory activity. In recent studies on the synovial tissue of arthritis, particularly in the synovial boundary layer, COX-2 activation increased, whereas COX-1 levels remained unchanged. This increase was amended, even when MEL was administered in supra-pharmacological concentrations (1 mM)[7,14,15]. There have not been adequate studies on MEL apart from its activity with Raf-1 enzyme and STAT3 protein on different inflammation models. In particular, MEL has not been studied in arthritis as well as its effects on these two mediators. The 5-Methoxytryptophol (5-MTX) is another circadian rhythm hormone which is created in two different ways by the degradation of MEL and serotonin and contributes to neuroendocrine and physiological functions[16]. In particular, in the light-dark cycle, it shows adverse effects with MEL. Since the secretion of MEL is high in the dark[17], 5-MTX is secreted more in daylight[14]. However, due to the removal of free radicals and its antioxidant activity, it also shows similarities with MEL. In a study conducted by Satue *et al.*[18] in order to assess the effects of these two pineal indoles on bone marrow formation, it was revealed that 5-MTX has more protective effects with its antioxidant and anti-inflammatory effects compared with MEL. However, there is no literature on the activation of COX-1, COX-2, Raf-1 and STAT3 in arthritis by 5-MTX. Therefore, the present study is the first to examine the activity of both MEL and 5-MTX on the COXs, Raf-1 enzymes and STAT3 protein in TMJA. Therefore, the aim was to investigate the effects of MEL and 5-MTX on the joint tissue in the zymosan-induced TMJA model and whether their combined use would have a synergistic effect in therapeutic applications.

MATERIALS AND METHODS

The study was carried out in Marmara University, Istanbul, Turkey between August, 2015 and February, 2016.

Animals: The study comprised 5 groups with 8 rats per group.

Sham group: The rats were anesthetized with ketamine (100 mg kg^{-1}) than 40 µL of saline was administered intraarticularly into the left TMJ.

Temporomandibular joint arthritis (TMJA)-saline group: About 2 mg zymosan dissolved in 40 µL saline was

administered into the left TMJ with a 30 gauge 1 mL insulin syringe intraarticularly (ia)[19].

TMJA-5-Methoxytryptophol (5-MTX) group: Thirty minutes before the administration of zymosan (ia), 5 mg kg^{-1} 5-MTX was administered intraperitoneally (ip).

TMJA-Melatonin (MEL) group: Fifteen minutes before the administration of zymosan (i.a.), 10 mg kg^{-1} MEL was administered intraperitoneally (ip).

TMJA-[MEL+5-MTX] group: Thirty minutes before the administration of zymosan (i.a.), 5 mg kg^{-1} 5-MTX was administered intraperitoneally (ip), 15 min before 10 mg kg^{-1} MEL was administered intraperitoneally (ip).

Preparation of TMJ tissues: Six hours after the i.a. administrations, the rats were decapitated. COX-1, COX-2 , Raf-1 and STAT3 levels were all examined with the Real Time-Polymerase Chain Reaction (RT-PCR) technique to assess inflammatory enzymes and proteins in the articular tissue and articular structural damage was assessed by histologically[20].

Cyclooxygenase-1 and 2 (COX-1, COX-2), Rapidly Accelerated Fibrosarcoma-1 (Raf-1) and Signal Transducer and Activator of Transcription 3 (STAT3): Left TMJ tissues were homogenized with lysis buffer by using rotor-stator. Homogenates were used immediately after the homogenization.

RNA isolation and cDNA preparation: Total RNA was isolated using a Roche High Pure RNA Tissue Kit following the manufacturer's instructions. cDNA was transcribed with a Roche Transcriptor High Fidelity cDNA Synthesis Kit following the manufacturer's instructions. Recently isolated RNA (10 µL) was added to 2 µL hexamer primer. In total, 12 µL volume was incubated for 10 min at 65°C. Four microliters transcriptor high fidelity reverse transcriptase reaction buffer, 0.5 µL protector RNase inhibitor, 2 µL deoxynucleotide mix, 1 µL DTT, 1.1 µL transcriptor high fidelity reverse transcriptase were added, mixed thoroughly and incubated for 10 min, 60 min and 5 min, at 29, 48 and 85°C, respectively and then placed on ice. After this process, the obtained cDNA samples were kept at -80°C in a freezer.

Real time PCR reactions: Real-time PCR was performed based on the settings developed and validated previously through the use of QIAGEN QuantiTect SYBR Green PCR Kit. Ptgs1 (COX-1), Ptgs2 (COX-2), Raf-1 and STAT3 genes were analyzed via QIAGEN, QuantiTect Primer Assays, Rn_Ptgs1_1_SG (NM_017043, XM_006234063, Rn_Ptgs2_1_SG (NM_017232), Rn_Stat-3_1_SG (NM_012747, XM_006247257, XM_006247258, XM_006247259), Rn_Raf-1_1_SG (NM_012639, XM_008763217, XM_008763218), respectively, according to the manufacturer's instructions. Briefly, 4 µL of cDNA was used in a reaction volume of 16 µL. Ten microliters probe master, 2 µL primer probe and 4 µL dH_2O were added as well. Hypoxanthine guanine phosphoribosyl-transferase (HPRT) was used as a housekeeping gene and positive controls were added to the end of the rows for each gene. The RT-PCR reactions are specified as follows: Subsequent to 95°C 15 min initial denaturation, 40 cycles of [94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec]. The RNA concentration in each group was found to be between 13.2-21.7 ng μL^{-1} (average 16.5±2.71 ng μL^{-1}), with an A260/A280 ratio of 1.86±0.14. For each reverse transcription reaction, the RNA volume added was 10 µL, so the amount was at least 130 ng/reaction.

Analysis and quantification was performed using Roche Light Cycler 480 software. The expression fold change was calculated according to the $2^{\Delta\Delta CT}$ method.

Histological studies

Light microscopy: Tissues were washed with 10% formalin for at least 3 h or overnight in tap water and dehydrated with increasing alcohol concentrations then stored overnight in 60°C paraffin. The next day, the tissues were embedded in paraffin blocks:

- After the blocking process, which was performed with decreasing concentrations of alcohol and was left in distilled water
- The next step, treated with hematoxylin and eosin, followed by washing 2 times with toluene and the tissue was covered with entellan. Finally, the tissue was examined under a light microscope

Statistical analysis: In the present study, the levels of COX-1, COX-2, Raf-1 and STAT3 proteins measured in the TMJ tissues were compared by means of one-way analysis of variance (ANOVA). Tukey's test was used as a further analysis in binary comparisons. The p-values less than 0.05 were considered significant.

Table 1: In the temporomandibular joint arthritis (TMJA) model on rats, activities of Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 COX-2 of all groups

	TMJA				
Activities	Sham	Saline	5-MTX	MEL	5-MTX+MEL
COX-1 (expression fold change, $2^{\Delta\Delta CT}$)	0.85±0.09	1.05±0.12	0.86±0.13	0.97±0.17	0.93±0.14
COX-2 (expression fold change, $2^{\Delta\Delta CT}$)	0.95±0.24	6.52±0.45***	1.80±0.21^{+++}	2.01±0.27^{+++}	1.59±0.28^{+++}

***p<0.001, $^{+++}$p<0.001, MEL: Melatonin, 5-MTX: 5-Metoxytryptophol

RESULTS

Cyclooxygenase-2 (COX-2) levels were found to be significantly higher in the TMJA group in comparison with the sham group. In contrast, MEL, 5-MTX and MEL+5-MTX administered groups elevated levels dropped significantly according to the TMJA group and were close to the sham group values. No significant difference was observed between the groups when the COX-1 levels were analyzed (Table 1, p<0.05-0.001). Rapidly Accelerated Fibrosarcoma-1 (Raf-1) and Signal Transducer and Activator of Transcription 3 (STAT3) findings in the TMJA group, the Raf-1 (Fig. 1, p<0.05-0.001) and STAT-3 (Fig. 2, p<0.05-0.001) levels were significantly higher in comparison to the sham group. In contrast, the MEL, 5-MTX and MEL+5-MTX administered groups elevated levels dropped significantly according to the TMJA group. Only the MEL and 5-MTX treatment groups had Raf-1 and STAT3 levels higher than the sham group. Between the combined applications and single applications, only the MEL administered group was observed to have a significant reduction in Raf-1 levels, while the other parameters did not show any difference. Histologic findings for the sham group, proper morphology was observed of the TMJ condyle (Fig. 3a). In the TMJA group, disc congestion and articular degeneration was determined (Fig. 3b). In the 5-MTX group, a decrease in articular degeneration was determined (Fig. 3c). In the MEL group, similar to the 5-MTX administered group, degeneration was decreased (Fig. 3d). In the MEL+5-MTX group, an obvious decrease in the degeneration was observed (Fig. 3e).

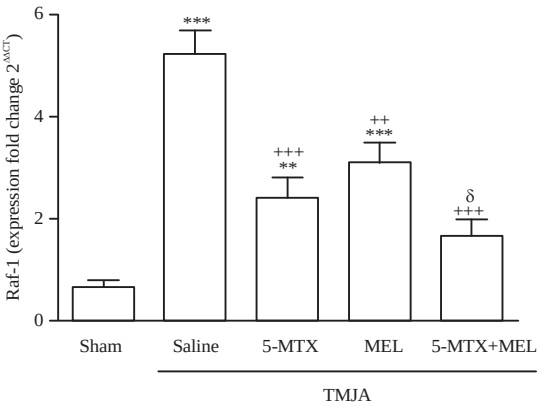

Fig. 1: Tissue belonging to all groups for rats in the model temporomandibular joint arthritis (TMJA) Rapidly Accelerated Fibrosarcoma-1 (Raf-1) levels. MEL: Melatonin, 5-MTX: 5-Methoxytryptophol

**p<0.01, ***p<0.001 according to the sham group, $^{++}$p<0.01, $^{+++}$According to p<0.001 according to TMJA group, $^{\delta}$p<0.05 comparisons to MEL group

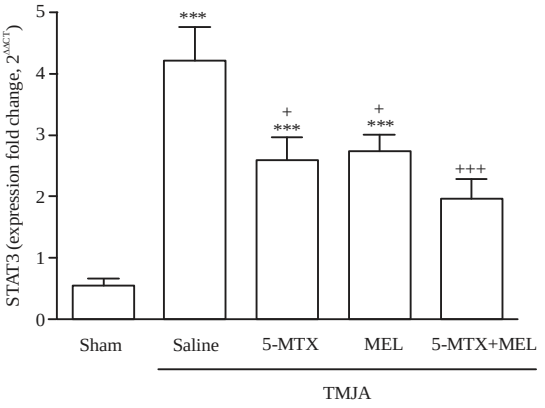

Fig. 2: Temporomandibular joint arthritis (TMJA) in rats tissue models for all groups Signal Transducer and Activator of Transcription 3 (STAT3) levels. MEL: Melatonin, 5-MTX: 5-Methoxytryptophol

***p<0.001 according to the sham group, $^{+}$p<0.05, $^{+++}$p<0.001 comparison made according to TMJA group

DISCUSSION

Inflammation associated with arthritis is a serious health problem that affects many people worldwide. Although many studies have been conducted on the subject and many applications have been administered in the treatment of synovial inflammations due to joint diseases, there is no

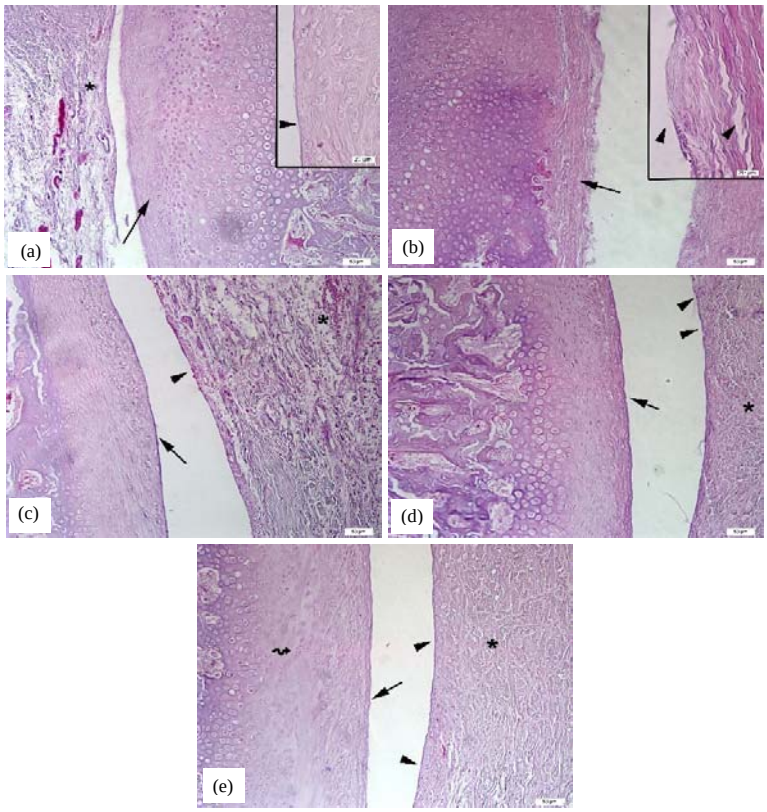

Fig. 3(a-e): (a) Sham group: Normal articular cartilage (arrow) and the disc (*), perichondrium (arrow head), (b) Temporomandibular joint arthritis (TMJA)+saline group: Destruction of cartilage (arrow), perichondrial degeneration present (arrow head) (c) TMJA+5-Methoxytryptophol (5-MTX) group: Cartilage (arrow), articular disc surface (arrow head) and connective tissue (*) decreased degeneration, hypertrophic chondrocytes present, (d) TMJA+Melatonin (MEL) group: Cartilage (arrow), articular disc surface (arrow head) and connective tissue (*) decreased degeneration, hypertrophic chondrocytes present and (e) TMJA-[MEL+5-MTX] group: Cartilage (arrow), articular disc surface (arrow head) and connective tissue (*) decreased degeneration, reduced hypertrophic chondrocytes present (curved arrow)

perfect cure. Understanding the parameters that cause arthritis and reducing them will be the priority of the treatment. Different mechanisms are suggested to explain TMJA characterized by pain, tenderness and limitations in jaw movement. In the formation of the derangement, mononuclear cell infiltrations, complement and coagulation systems, cytokines, arachidonic acid pathway (COX-2) and proteolytic enzymes such as Raf-1 and STAT3 proteins are thought to be involved[21]. These mediators often cause structural damage to all tissues, including the synovial space. According to this knowledge, TMJ arthritis was induced through zymosan, which is a polysaccharide synthesized from yeast cell walls and has been shown to promote the formation of the synovitis by causing an increase in cell migration in the vascular permeability with mononuclear cell infiltration[22]. In a particular zymosan inflammation model study, the effects of 0.25, 0.5, 1 and 2 mg doses applied at different times were evaluated[21]. In accordance with the literature[23] findings and the authors' previous study[2], the results showed that injecting 2 mg of zymosan causes intense inflammation after 6 h. Therefore, in this study, the extent of inflammation was assessed in the condylar tissue at this time point. Cyclooxygenase (COX) are responsible for cell inflammation[24]. There are two well-known types of its isoforms. Normally, COX-1 levels are high in cells under normal conditions and through which, secrete mediators play a role in platelet activation, vasoconstriction and smooth muscle cell proliferation. However, COX-2 enzymes are low under normal physiological conditions and play a role in vasodilation, inflammation, fever and pain through cytokines such as IL-1β and TNF-α, protease and endotoxins[22]. Thus, the importance of COX-2 activation in the growth of chondrocytes and osteoclastic bone resorption increase is emphasized[25,26]. Seki *et al.*[25] previously demonstrated in a clinical study that

COX-2 levels are higher than COX-1 in TMJA. In the TMJA group, COX-1 levels remained unchanged compared to the sham group, whereas in the TMJA group, COX-2 levels increased significantly in comparison with the sham group. Additionally, the results for the present study are in parallel with previous clinical and experimental studies, in which MEL was shown to have an anti-inflammatory effect by inhibiting the COX-2 activation in inflammatory diseases such as multiple sclerosis, ulcerative colitis, systemic lupus erythematosus and type 1 diabetes mellitus[27,28]. In the study conducted by Cutando *et al*.[29], it was found that, although MEL shows a strong affinity to both to COX-1 and COX-2, when applied to the alveolar socket locally during tooth extraction, the inflammation was only prevented by inhibiting the activation of COX-2. The results in this study are consistent with Cutando *et al*.[29]. Although it is claimed that MEL does not have an important physiological role in dentistry, it is recommended for protective use for oral bacterial and viral diseases in post-operative wound healing, the formation of bone tissue in oral surgery, in autoimmune diseases such as Sjogren's syndrome, in periodontal disease, aphthous ulceration, lichen planus, oral cancer and toxic dental materials[30]. To the authors knowledge, this is the first study showing that 5-MTX has a direct effect on COX activity. As it is a by-product of MEL disintegration, although its anti-inflammatory effect has been determined, its effects on COX have not been confirmed in the literature. Thus, the present study suggests that the exogenous application of these two hormones that are released according to the dark/light cycle may develop a new treatment protocol for their working mechanism in TMJ related research.

Raf-1 causes Mitogen-Activated Protein Kinase (MAPK) pathways, leading to the activation of proliferation, differentiation, the anti-apoptotic effect in tumor formation, angiogenesis and inflammation. Although MAPK pathways are known to cause inflammations, Raf-1 has a similar effect and the nuclear factor-κB (NF-κB) pathway plays an important role as well[31]. Even though the importance of Raf-1 in inflammations is known, to date, there have been no studies on the role of Raf-1 on TMJ arthritis. The authors' previous study[2] led to the measurement of Raf-1 enzymes that cause the degradation of NF-κB dependent cytokines in the synovial inflammation of the zymosan-induced TMJ. There has only been one study that has demonstrated the role of Raf-1 in arthritis molecular pathogenesis. Yeung *et al*.[32] showed that the inhibitor protein Raf-1 blocks the activation of inflammatory pathways such as MAPK and NF-κB. Lin *et al*.[33] also reported that the Raf-1 inhibitor GW 5074 substance prevents inflammation in lung epithelial cells. Similar to these studies, the present study also showed an increase in Raf-1 activation in the TMJA group. On the other hand, there have been an insufficient number of studies on the Raf-1 activation related to MEL or 5-MTX. Present study results suggest that MEL and 5-MTX therapies play a role in decreasing Raf-1 activation in the TMJA. Based on these findings, although MEL and 5-MTX treatment may be a remedy for arthritis, further studies are needed to explain the molecular mechanisms. The STAT proteins are defined as the regulation of gene transcription mediated by interferon (IFN) and they cause proliferation, migration and inflammation[10]. From the STAT subgroups, STAT3 protein in particular plays an important role in inflammation with the increase of inflammatory cytokines[34]. As evidence of this, information in a previous study showed a rise in the STAT3 protein levels during inflammation and that levels of cytokines such as TNF-α and IL-6 increase with the activation of MMP-2 and MMP-9 proteolytic enzymes and are indicated to accelerate the inflammation[35].

Yang *et al*.[36] study on 28 patients with arthritis revealed that in fibroblast like cell cultures, IL-34/STAT3/micro RNA 21 (miR-21) pathways are shown to play an important role in the pathogenesis of inflammation. Lee *et al*.[37] also suggested that in collagen-mediated arthritis, an increase of STAT3 protein in the synovial tissue model increases the osteoclastic activity. On the contrary, in a single study conducted in 1999 on the relationship between STAT3 protein and activation of the cytokines on TMJ arthritis in a disc culture from a bovine's TMJ, no correlation was found between STAT3 protein and cytokines, however, in later arthritis studies, this hypothesis was refuted[35]. In the present study, in the TMJA group, the STAT3 protein expression has increased in the TMJ tissues. Hence, these results are in concordance with the literature. In studies conducted to determine the protective effects of MEL, whether it be the liver tissue or the heart tissue, it was claimed that the regeneration was the result of the anti-apoptotic effect of the STAT3 protein, nonetheless, Min *et al*.[38] reached different results and stated that, in the inflammation model that they created through applying lipopolysaccharides to the microglia cells, STAT3 expression increased with cytokines and the damage was improved with MEL treatment. The present study is compatible with Min *et al*.[38] study. Until now, no studies have been conducted in regard to the effect of the agent 5-MTX's effects on the STAT3 protein. However, the results from the present study indicate that the suppression of STAT3 protein levels in the TMJA helps to explain the anti-inflammatory mechanism of this pineal hormone, which is released in light. Histological evaluation showed that the TMJA group had articular degeneration and congestion of the discs in condyle tissue, which is in line with a previous study that

demonstrated formation of structural damage in TMJ, the rise of COX-2 levels was accompanied with an increase of other inflammatory mediators[7]. Overall, MEL and 5-MTX application showed a decrease of articular degeneration in the condyle tissue, which is in agreement with the other biochemical parameters evaluated and with the previous findings in the literature.

CONCLUSION

To date, this study is the first to examine the exogenous application of the dark hormone MEL and the daylight hormone 5-MTX on arthritis. In general, this article focused on how the presence of COXs, STAT3 and Raf-1 in the arthritis and the positive role of MEL and 5-MTX affected the reduction of these mediators. In the literature, the search for alternative treatment principles still continues in a situation where such factors play a role. Naturally, applications for primarily grounded reasons should first take place. This is one of the aims of preventing the complications of inflammatory disease. There is little potential for side effects in treatment, the simultaneous application of darkness hormone melatonin and the daylight hormone 5-Methoxytryptophol will ensure that the results are more successful.

Thus, although these results suggested that the use of MEL and 5-MTX will improve the clinical applications, more extensive and comparative clinical and experimental studies are still needed before these agents can be used in clinics and the authors' believe that future studies will be able to provide further insights.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of dark hormone MEL and the daylight hormone 5-MTX on arthritis This study will help the researchers to uncover the critical area of arthritis that many researchers were not able to explore. Thus, a new theory on these pineal hormones combination may be arrived at.

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