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Original Article

Effects of melatonin and 5-methoxytryptophol on synovial inflammation in the zymosan-induced rheumatoid arthritis in rats

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Abstract: The aim of this study is the investigation of synoviocytes, cytokines and metalloproteinases in the synovial inflammation model which is created by zymosan application to temporomandibular joint (TMJ) and effects of pineal hormones melatonin (MEL) and 5-methoxytryptophol (5-MTX) on these parameters. 200-250 g Wistar albino rats of both sexes were used for modeling arthritis in this study. Arthritis model was created by intraarticularly (i.a.) injecting 2 mg zymosan in 40 ml saline into the left temporamandibular joint of the rats while the sham group was created by only injecting 40 ml saline solution (intraarticulary). MEL and 5-MTX administration was made intraperitoneal before zymosan injection. 6 hours after zymosan or saline administration of MEL and 5-MTX the synovial fluid was collected from the animals and the synovial membrane was collected for histological assessment. The administration of zymosan increased the release of IL-1β and TNFα and the activity of metalloproteinases (MMM-9) and metalloproteinases-2 (MMP-2) and with this administration values got closer to the sham group. The histological evaluation showed significant increase in the intensity of synoviocytes that arose in the inflammation was found to subside. In conclusion, MEL and 5-MTX reducing inflammation in arthritis suggests these agents might constitute a new therapeutic principle clinically.

Keywords: Zymosan, synovial membrane, melatonin, 5-methoxytriptophol

Introduction

Temporomandibular joint (TMJ) disorder is a condition that results in a high degree of pain in speech, eating and other daily activities [1]. The most common of these disorders which is rheumatoid arthritis (RA) is musculo-skeletal discomfort which affects a large portion of the population and can be seen at any age [2, 3].

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease and the condition is characterized by particularly inflammatory cell infiltration of the synovial membrane [4]. Excessive release of inflammatory mediators that are produced by inflammatory cells, contributes to joint destruction. Mediators such as tumor necrosis factor-alpha (TNF-α) and interlokin1-beta (IL-1β) are associated with the pathogenesis of arthritis and causes an increase in the secretion of proteolytic enzymes such as matrix synovial metalopreoteinase-2 (MMP-2) and metalopreoteinase-9 (MMP-9) in stromal cells [5, 6]. These enzymes degrade specific peptide bonds of the extracellular matrix proteins and help expression of inflammatory cells. Zymosan which we used in our study to create the arthritis is a polysaccharide that is capable of severe and erosive synovitis that can be produced from yeast cell walls. The intraarticular injection of this agent has been shown to cause inflammation by enhancing the activation of cytokines such as TNF-α and IL-1β and proteolytic enzymes [7].

Melatonin (N-acetyl-5-methoxytryptamine) (MEL), the disintegration product of serotonin,

mainly released from the pineal gland especially in the dark, plays a role in many physiological processes such as circadian rhythm, immune function and sexual behavior [8]. In earlier studies antioxidant and anti-inflammatory effects of these agents has been shown [9, 10]. It creates anti-inflammatory effect by inhibiting the adhesion molecules stimulated by NF-κβ, reducing the migration of leukocytes from endothelial cells and thus preventing the PMN leukocytes from aggregating in the inflamed area [11, 12]. Consequently, it is stated that MEL is reported to inhibit tissue damage by inhibiting the generation of proteolytic enzymes and cytokines that are caused by the release of both direct and indirect free radicals during inflammatory events [13].

5-Methoxytryptophol (5-MTX), another pineal indole created by the decomposition of serotonin, plays a role in the regulation of many biological activities such as pro or anti-gonadotropic effects of biological rhythms and their reproductive behavior [14]. Features demonstrate an adverse effect on the light dark cycle with MEL. Since the secretion of MEL is high in the dark, while 5 MTX is secreted in light [15]. Despite a lack of studies with 5-MTX, antioxidant and immunomodulatory effects were observed [16, 17]. Studies in recent years focused on the evaluation of superiority or differences of the effects of MEL and 5-MTX [18, 19]. The recent study, assess the effects of these two pineal indoles on bone marrow formation revealed 5-MTX has more protective effects with its antioxidant and anti-inflammatory effects compared with MEL [20].

In this study we aimed to compare the effects of MEL and 5-MTX and to examine whether there is a synergistic effect of the combined used of MEL and 5-MTX on synovial inflammation model that created with zymosan application.

Materials and methods

200-250 g Wistar albino rats of both sexes were used. Animals were acclimated in the laboratory conditions two weeks prior to the experiment (20°C ± 2, 12 h light/12 hours dark). [This study has been done with the approval of Medipol University Animal Ethics Committee (Ref No. 38328770-46)].

Groups and experimental protocol

Our study comprised of 5 groups with 8 rats per group. The synovial inflammation rat model was set up as previously described [1].

Sham group: The rats in this group were administered saline intraarticularly (i.a.) to the TMJ under ketamine anesthesia (100 mg/kg, 40 ml).

Synovial inflammation (SI) group: 2 mg zymosan 40 ml was dissolved in saline and administered intraarticularly (i.a.) into the TMJ.

SI + melatonin group: 10 mg/kg intraperitoneally MEL injection 15 minutes before zymosan administration.

SI + 5-methoxytryptophol group: 30 minutes before injection of zymosan (i.a.), 5 mg/kg 5-methoxytryptophol intraperitoneally (i.p.) was injected.

SI + 5-methoxytryptophol + melatonin group: 5 mg/kg 5-MTX intraperitoneally 30 minutes before zymosan and 10 mg/kg melatonin intraperitoneally (i.p.) 15 minutes before zymosan were injected.

Synovial Fluid Collection, done on anesthetized rats by zymosan injection (i.a). 0.05 ml of EDTA in neutral buffered PBS used to wash TMJ cavity. Washing done twice with pumping and aspirating technique and then synovial fluid was collected and the rats were decapitated under anesthesia after 6 hours [1]. In synovial fluid, tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β) levels and matrix metalloproteinase (MMP-2 and MMP-9) activities were measured [21].

Histological studies

Light microscopy: Synovial membrane was excised after sacrificing animals. Then specimens were fixed in 10% neutral buffered formalin for 24 h, followed by demineralization in 10% EDTA, embedded in paraffin, and 5-6 μm sectioned along the synovial membrane. Synovial membrane sections were evaluated under light microscopy at 400×. For the specimens processed for routine hematoxylin-eosin (H&E) staining, histological analysis considered to the synoviocytes influx in the synovial membrane [1].

Table 1. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) values of all groups of synovial fluid in synovial inflammation (SI) model on rats created by zymosan

	SHAM	SI	SI-MEL	SI-5-MTX	SI-MEL + 5-MTX
TNF-α (pg/ml)	152 ± 11	254 ± 17***	179 ± 15^{+}	171 ± 11^{++}	150 ± 11^{++}
IL-1β (pg/ml)	51.8 ± 9.9	136.8 ± 14.8***	68.2 ± 9.1^{++}	70.3 ± 11.5^{++}	55.6 ± 9.6^{+++}

***P<0.001 comparisons according to the control group, $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 comparisons according to SI group.

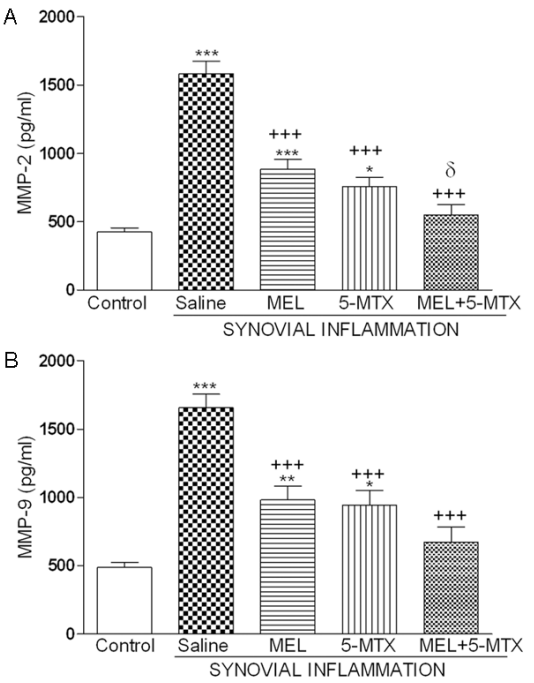

Figure 1. Activities of matrix metalopreoteinases (MMP-2, MMP-9) belonging to the synovial fluids of all groups in the (SI) model which was created by synovial inflammation with zymosan administration on rats. MEL: Melatonin, 5-MTX: 5-methoxytryptophol. **P<0.01, ***P<0.001 according to the control group; $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 comparisons according to SI group; P<0.05 comparisons according to MEL group.

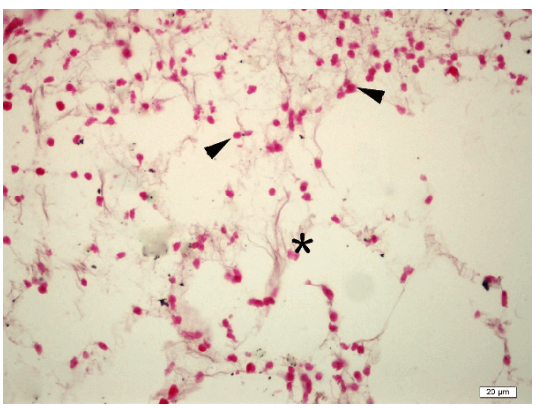

Figure 2. Sham group: The synoviocytes (arrowheads) observed with the spread morphology in the synovial membrane, synovia (*). Image magnification X400.

Examinations of Synovial Fluid

TNF-α, IL-1β, MMP-2, MMP-9 analyses: In synovial fluid the analysis and measurements of TNF-α (ELISA, BioSource Europe S.A. Catalog No. KRC 3014 Nivelles, Belgium) IL-1β (ELISA BioSource Catalog No. KRC0011, Nivelles, Belgium) MMP-2 (ELISA, Catalog No. Abnova KA0393, Taoyuan, Taiwan), MMP-9 (ELISA, Catalog No. Abnova KA0398, Taoyuan, Taiwan) were done considering appropriate criteria according to the instructions.

Statistical methods

One-way analysis of variance (ANOVA) based on statistical evaluation and as advanced analysis Tukey's test was performed. Where *P* values were less than 0.05, were considered significant.

Results

In SI group, TNF-α, IL-1β levels belonging to the synovial fluid were significantly higher than the control group (**Table 1**). In contrast to SI group increase in TNF-α, IL-1β levels in melatonin (MEL), 5-methoxytryptophol (5-MTX) and MEL+ 5-MTX administrated groups was significantly lower. In only MEL and only 5-MTX administered groups, TNF-α and IL-1β levels remained higher than the control group.

Upon the assessment of the synovial fluid's MMP-2 and MMP-9 activities, SI administered group was found to be significantly higher compared to the control group. In contrast, melatonin (MEL), 5-methoxytryptophol (5-MTX) administered groups has significantly dropped and was closer to control group values. Between the combined application and single applications, only MEL administered group showed significant reduction in their MMP-2 levels and other parameters did not show any variance (**Figure 1**).

The histological examination of the tissue made synovial tissue synoviocytes in sham group,

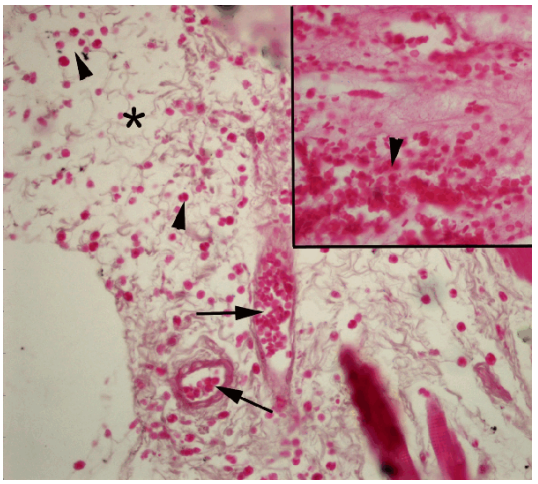

Figure 3. Saline administered synovial inflammation (SI) group: Intense congestion of the capillaries (arrows) and synoviocytes (arrowheads), intense synovia (*). Image magnification X400.

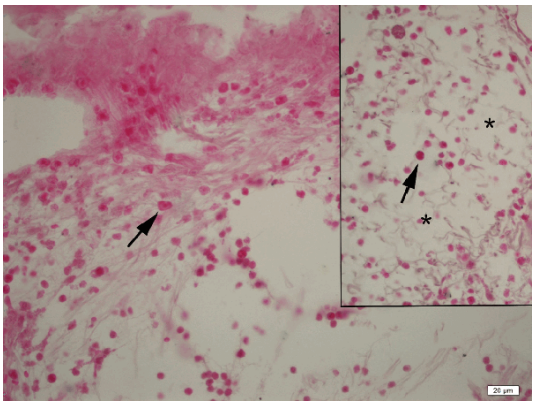

Figure 4. SI + 5-methoxytryptophol (5-MTX) group: Capillary congestion and synoviocytes intensity (arrowheads) moderate regression. Image magnification X400.

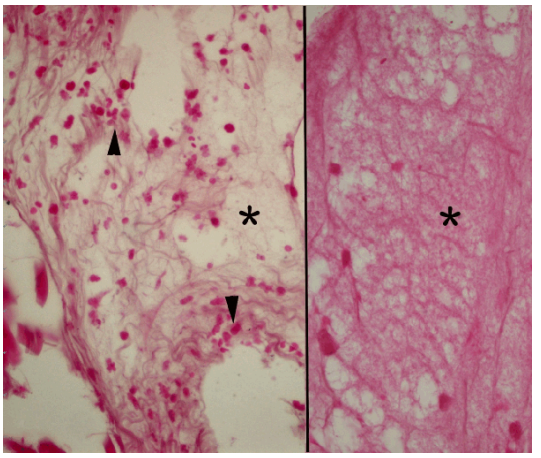

Figure 5. SI + melatonin (MEL) group: Capillary congestion and fairly significant regression in synoviocytes intensity (arrowheads), synovial cell density (*). Image magnification X400.

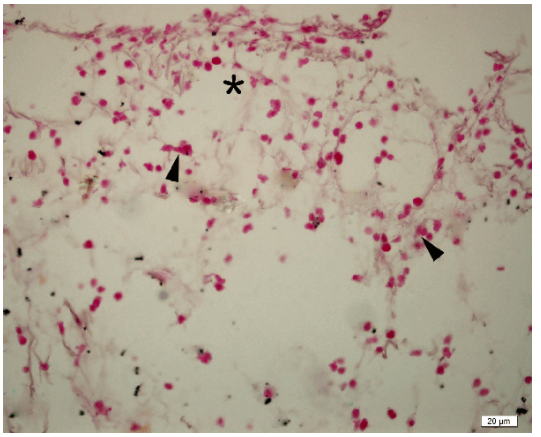

Figure 6. SI + 5-MTX + MEL: Synovium (*) significantly reduced synoviocytes intensity (arrowheads). Image magnification X400.

exhibits a smooth appearance (**Figure 2**) while SI group synoviocytes showed damage characterized by an increase in synoviocytes density and capillary congestion (**Figure 3**). SI + 5-MTX group showed the congestion decreased while cell density remain decreased (**Figure 4**). In SI + MEL group decreased congestion and a significant decrease in cell density was observed (**Figure 5**). SI + MEL + MTX group showed apparent reduction in density of synoviocytes (**Figure 6**).

Discussion

Inflammation associated with arthritis is a serious health problem that affects many people worldwide. In particular, it does not have a perfect cure despite many studies done on the subject and many applications administered in the treatment of synovial inflammation due to temporomandibular joint diseases [22].

Different mechanisms are suggested to explain temporomandibular joint disorders characterized by pain, tenderness and limitation of the jaw movement. Many mediators such as proteolytic enzymes, synoviocytes, monocytes/macrophages, platelets, complement and coagulation systems, cytokines are thought to be involved in the formation of injury [23]. These mediators are collected mainly in the synovial area and cause structural damage on the membrane.

In our study, zymosan which is a polysaccharide synthesized from yeast cell walls 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 [24-26]. Especially in zymosan inflammation models 0.25, 0.5, 1 or 2 mg doses tried and the effects in different times were evaluated [1]. Both literature findings and our preliminary study shows the 2 mg intervals zymosan injection causes intense inflammation after 6 hours.

Cytokines, secreted by immune cells, play a major role in hematopoiesis, cell division and differentiation control, tissue repair, and inflammation. Synovial inflammation leads to release of the pro-inflammatory mediators such as TNF-α, IL-1β from synovial cells and an increase of the biosynthesis of adhesion molecules that mediate leukocyte-endothelial cell adhesion [27]. In a recent study, it is stated that synoviocytes infiltration in the synovial fluid TNF-α and IL-1β levels are increased [28]. A clinic study of 31 arthritis patients suggests increased cytokines, TNF-α and IL-1β over inflammation by activating the collagen synthesis in fibroblast type 1 by activating metalloproteases [29]. In accordance with these literatures our study showed in zymosan caused inflammation also there was an increase in cytokines, TNF-α and IL-1β in synovial fluid. As is known, increase in pro-inflammatory cytokines indicate an inflammatory response by the organism in which inflammation is important starting causing tissue damage [30]. The positive effects of therapeutic agents on tissue parameters of our study given the levels of cytokines in synovial fluid of these agents in the synovial inflammation is significantly suppressed by supporting the important role of these cytokines. Therefore, the agents used in synovial inflammation treatment having inhibitory properties on cytokines expression will increase the success of treatment. The present study showed that MEL prevents the inflammation caused by IL-1β on mesenchymal stem cells and contributes to bone regeneration [23]. In the arthritis model in which the antioxidant and anti-inflammatory effect of MEL is studied has shown that these agents decrease levels of TNF-α and IL-1β [12].

The study has shown that antioxidant effects of 5-MTX which is another pineal indole, IL-6 is reduced which is a pro-inflammatory cytokines and increased IL-2 levels in the serum which is an inflammatory cytokines with an immune-modulator effect despite the studies about inflammations show a lack [31]. However, there aren't enough studies in the literature on the effects of 5-MTX on TNF-α and IL-1β. When all these studies are considered our findings conform to the literature, showing that MEL and 5-MTX's inhibitory effects on pro-inflammatory cytokines helps the tissue protective effects.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases, consists of 5 subgroups namely; collagenase, stromelysin, gelatinase, membrane-type, and others and be expressed in epithelial, mesenchymal, and haematopoietic cells [32]. In experimental and clinical studies, inflammation and gelatinase subfamily consists of connective tissue diseases in MMP-2 (gelatinase A) and MMP-9 (gelatinase B) shows the important role played [33]. The present study showed the increased activation of this proteolytic enzyme in experimental inflammation both created with zymosan and macrophage migration inhibitory factor to investigate the activity of MMP-2 [34]. On the other hand, the study suggest that the expression of MMP-9 increased in synoviocytes, macrophages and master cells in the experimental peritonitis model created with zymosan [35]. Therefore, these MMPs show activity by destroying the extracellular matrix proteins by breaking down specific peptide bonds in various cell and tissues and play a role in pathological conditions such as rheumatoid arthritis, tumor invasion, apoptosis and angiogenesis [36]. Our results from the study are consistent with the literature. Zymosan application resulted in synovial fluid MMP-2 and MMP-9 activation increasing significantly compared with the control group and with MEL and 5-MTX administration improved significantly. MEL's effects on expressions of MMP-2 and MMP-9 has been studied in, several models of inflammation such as spinal cord injury [37], colitis [38], cerebral ischemia [39]. Antioxidant and anti-inflammatory properties of MEL in these models was shown that the protective effect is created by reducing the expression of MMP-9 and MMP-2. However, effects of both MEL and 5-MTX's on MMP-2 and MMP-9 activation has not been studied in any arthritis model. But so far effects of 5-MTX on proteolytic

enzymes has not been studied before, it is a first with our research.

In conclusion: In our experimental study, pro-inflammatory cytokines and proteolytic enzymes increased and also that change observed in biochemical parameters accompanied by increase in synoviocytes and so the advert of tissue damage was observed. Pineal hormones melatonin and 5-methoxytryptophol, which we used for treatment has shown to significantly reduce this damage. To our knowledge this is the first study investigating the anti-inflammation effects of two pineal hormones in synovial inflammation and these two pineal hormones are normally responsible for the regulation of the circadian rhythm, effects are considered; 5-MTX that is released in brightness and MEL that is released in darkness. On the other hand this study being the first to research the effects of 5-MTX on cytokines levels and proteolytic enzymes is of importance in terms of contributing to the literature. Despite the fact more extensive and comparative clinical and experimental studies are needed for these agents to be used in the clinics our results suggest that MEL and 5-MTX's plays an important role on synovial inflammation repair thus we believe that further studies will shed light on later.

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Disclosure of conflict of interest

None.

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