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ORIGINAL ARTICLE

Comparison of the efficacy of intra-articular injections of hyaluronic acid and lactoferrin in mono-iodoacetate-induced temporomandibular joint osteoarthritis: A histomorphometric, immunohistochemistry, and micro-computed tomography analysis

Cansu Gül Koca, DDS, PhD[1], Bengisu Yıldırım, DDS, PhD[1], Özlem Özmen, MD[2], Muhammed Fatih Çiçek, DDS, PhD[1], Mehmet İğneci, DDS[1], Özge Kırarslan, DDS[1], Aras Erdil, DDS, PhD[1]

[1]Department of Oral and Maxillofacial Surgery, Uşak University Faculty of Dentistry, Uşak, Türkiye
[2]Department of Pathology, Burdur Mehmet Akif Ersoy University Faculty of Medicine, Burdur, Türkiye

Osteoarthritis (OA) is a chronic, degenerative joint disease with multifactorial etiologies affecting the articular cartilage and bone surfaces. Although the load-bearing joints such as the knee, hip, and spine in the body are most affected, it can also be observed in the joints such as the shoulder and temporomandibular joints (TMJ).[1] Osteoarthritis is characterized by pathological changes on the articular surfaces, including cartilage defibrillation, erosion of the joint surfaces, chondrocyte proliferation, subchondral sclerosis, disruption of

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Correspondence: Aras Erdil, DDS, PhD. Uşak Üniversitesi Diş Hekimliği Fakültesi, Ağız Diş ve Çene Cerrahisi Anabilim Dalı, 64200 Uşak, Türkiye.

E-mail: erdil.aras@gmail.com

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ABSTRACT

Objectives: This study aims to evaluate the efficacy of high-molecular-weight hyaluronic acid (HMWHA) and lactoferrin (LF) injections on temporomandibular joint (TMJ) cartilage and subchondral bone in mono-iodoacetate (MIA)-induced temporomandibular joint osteoarthritis model in rats.

Materials and methods: In this *in vivo* study, a total of 24 rats were divided into three groups as follows: saline group (Group 1), HMWHA group (Group 2), and LF group (Group 3) including eight rats in each group. The intra-articular injections were administered once a week for three weeks after osteoarthritis was induced. All animals were euthanized 28 days after induction of osteoarthritis, and TMJs were harvested for histomorphometric, immunohistochemical, and micro-computed tomography (CT) analysis.

Results: There was no significant difference between the HMWHA and LF groups in terms of the histomorphometric and immunohistochemical analysis results ($p>0.05$). According to the micro-CT analysis, the LF group had the highest mean bone volume fraction (74.9±0.5) and trabecular thickness (0.122±0.002), while the saline group had the lowest mean values (55.0±0.3 and 0.071±0.002, respectively) ($p<0.001$). There was no significant difference between the HMWHA and LF groups according to the micro-CT analysis ($p>0.05$). Both groups had better healing effects than the saline group in all analyses.

Conclusion: Lactoferrin has a healing effect at least as much as HMWHA in MIA-induced TMJ osteoarthritis. We suggest that LF may be evaluated in future clinical studies as a promising agent in the treatment of osteoarthritis.

Keywords: High-molecular-weight hyaluronic acid, lactoferrin, micro-computed tomography, matrix metalloproteinase-3, osteoarthritis, temporomandibular joint.

the cartilage matrix and suppression of the synthesis of its components.[2]

The data on the efficacy of pharmacological anti-inflammatory therapies or non-pharmacological methods such as occlusal splints for long-term pain management is inconclusive.[3] Intra-articular injections are relatively less invasive procedures, and materials such as hyaluronic acid (HA), platelet-rich plasma, and corticosteroids can be administered.[4] It has been reported that HA-based scaffolds and adipose tissue-derived stromal vascular fraction treatment increased hyaline cartilage formation and cartilage thickness in osteochondral defects in a rabbit model.[5]

Lactoferrin (LF) is an 80 kDa non-heme iron-binding glycoprotein that belongs to the transferrin family[6] and, recently, its proliferative and anti-apoptotic properties have been shown.[7,8] Chondrocyte apoptosis has been observed in the pathogenesis of OA. Osteoarthritic cartilage damage is always associated with increased apoptosis of the joint chondrocytes, and its suppression reduces the severity of OA.[9,10]

In the present study, we aimed to compare the effectiveness of LF and HA in a mono-iodoacetate (MIA)-induced osteoarthritic rat TMJ with a null hypothesis that LF and HMWHA could have an equal healing effect on the subchondral and articular structure of TMJ.

MATERIALS AND METHODS

This study included a total of 24 male 10-week-old Sprague-Dawley rats weighing 300 to 320 g with complete skeletal development. To avoid hormonal factors and the effects of these factors on bone and articular cartilage, male rats were preferred instead of female rats. The rats were randomly divided into three groups with eight rats in each group. All rats were housed in wire mesh bottom cages at 22±2°C with a daily 12-h light/12-h dark cycle. They were supplied with a regular diet and tap water throughout the experiment phase *ad libitum*.

Experimental procedures

General anesthesia was administered by intraperitoneal injections of 80 mg/kg of ketamine hydrochloride (Alfamine, Ege-Vet, Izmir, Türkiye) and 12 mg/kg of xylazine hydrochloride (Alfazyne, Ege-Vet, Izmir, Türkiye) into each experimental animal. The trichotomies were performed on the skin of the left TMJ regions and disinfected with a 10% povidone-iodine solution after general anesthesia. In all animals, a 50-μL quantity of 3 mg/mL sodium MIA (Sigma-Aldrich, Saint Louis, MO, USA) was injected into the left joints. After waiting four weeks for the formation of OA, the high-molecular-weight HA (HMWHA) (Hylan GF-20, Synvisc, Wyeth, Genzyme, Biosurgery Ridgefield, NJ, USA) and LF (Sigma-Aldrich Bovine Lactoferrin L-9507, Saint Louis, MO, USA) injections were administered once a week for three weeks. The groups were as follows:

Group 1 (sham injection): n=8, 50 μL saline administration

Group 2: n=8, 50 μL of 3 mg/mL Hylan GF-20 administration

Group 3: n=8, 50 μL of 100 μg/mL LF administration.

The intra-articular injections were administrated with a 29-gauge needle attached to a 1-mL plastic insulin syringe. The TMJ was palpated 5 mm anterior to the external auditory canal and 5 to 10 mm posterior to the lateral canthus of the left eye, while the mandible was manipulated.[11] When the mandibular condyle was identified, the needle was inserted from a posterosuperior direction with an angle of 30 to 40 degrees to the sagittal plane under the zygomatic arch. The needle was inserted 2 to 3 mm, until it came into contact with the posterolateral aspect of the mandibular condyle.[12] The content of the syringe was administered after negative aspiration. All injections were performed by a single surgeon using the same technique for both inducing OA and administering the experimental agents.

All animals were euthanized by anesthesia overdose 28 days after induction of OA in the TMJs. The TMJ capsule, articular disc, the mandibular condyle, and the retrodiscal tissues were harvested for micro-computed tomography (CT), histomorphometric, and immunohistochemical analyses.

Micro-CT analysis

All specimens were placed in the holders of the micro-CT (Scanco Medical μCT50, Switzerland) and scanned at 70 kVp energy, 114 μA intensity, 300 ms integration time, and 20 μm voxel size. A volume of interest (VOI: 0.4×0.4×0.6 mm) was selected in each sagittal position of the condyles. These values were determined for ideal visualization of the condylar surface and cancellous structure. All specimens were scanned with an 8 to 10-mm diameter, and the micro-CT Evaluation Program version 6.5 (Scanco Medical, Wangen-Brüttisellen, Switzerland) was utilized for analysis.

The morphological changes in the bone tissue were analyzed with a trabecular bone volume to total volume fraction (BV/TV). The parameters used to detect the alterations in the trabecular structure were as follows:

- The trabecular number (Tb.Nb), calculated as the average number of trabeculae per unit length;
- The trabecular thickness (Tb.Th), calculated as the mean thickness of trabeculae; and
- The trabecular separation (Tb.Sp), calculated as the mean distance between trabeculae.

Histomorphometric methods

Harvested joint samples were fixed in a 10% neutral formalin solution for histopathological and immunohistochemical examinations. After two days of fixation, samples were decalcified in ethylenediaminetetraacetic acid (EDTA) (0.1 M) solution for two weeks. Then, tissue samples were washed in running water for 8 h and routinely processed using automatic tissue processing equipment (Leica ASP300S, Leica Microsystem, Nussloch, Germany). The samples were embedded in paraffin wax, and all blocks were sectioned at three different levels by a fully automatic rotary microtome (Leica 2155, Leica Microsystem, Nussloch, Germany). Then, the sections were stained with hematoxylin and eosin (H&E) stain for histopathological examinations. Chondrocyte histology, articular cartilage tissue, and lesions of all sections at the osteochondral and subchondral bone tissue were examined. Articular cartilage was evaluated for thickness (regular, thick, thin), chondrocyte histology (normal, hypocellular, clustered), and subchondral bone architecture (regular, increase in trabecular bone). In addition, osteochondral junctions were evaluated according to their microscopic appearance as usual, invaginated, and weak junctions.[13] Histopathological evaluations were performed in a blinded manner by an experienced pathologist who was unaware of which groups the specimens pertained to.

Immunohistochemical examination

The streptavidin-biotin complex peroxidase method was used for immunohistochemical examination to coat sections taken from the TMJs on poly-L-lysine slides. Matrix metallopeptidase-3 (MMP-3) antibody (anti-MMP-3, ab52915; Abcam, Cambridge, UK) was used for this procedure at a 1/100 dilution. An UltraVision Detection System Anti-Polyvalent HRP kit (TP-060-HL) (Thermo Shandon Limited, Cheshire, UK) was used as the secondary kit, and 3,3´-diaminobenzidine (DAB) was used as the chromogen (Abcam, Cambridge, UK). Immunohistochemical analysis was performed according to the manufacturer's recommendations. Harris hematoxylin was used as a counterstain, and the slides were examined under a light microscope (Olympus CX41, Olympus Corp., Tokyo, Japan). Antibody dilution solution was used instead of primary antibodies for negative controls. Microphotography and morphometric analysis were performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corp., Tokyo, Japan). Immunohistochemical staining was scored semi-quantitatively between 0 and 3: 0= negative, 1= mild expression, 2= moderate expression, and 3= high expression. Statistical analysis was performed on the scores, and differences between groups were evaluated.

Statistical analysis

The study power analysis and sample size calculation were performed using the G*Power version 3.1 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).[14] The sample size was estimated using data from a previous study.[15] With an alpha value of 0.05 and statistical power of 80%, a minimum of 24 animals were required.

Statistical analysis was performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD) or number and frequency, where applicable. Considering the micro-CT and histomorphometric results, one-way analysis of variance (ANOVA) was used for normally distributed data, and Kruskal-Wallis and Mann-Whitney U tests were used for non-normally distributed data. The Duncan test evaluated differences between the groups obtained in the immunohistochemical analysis. A p value of <0.05 was considered statistically significant.

RESULTS

The condylar surface and trabecular structure analysis

Statistically significant differences were found among the groups for BV/TV ($p<0.001$). The LF group showed the highest mean BV/TV value (74.9±0.5), while the saline group showed the lowest (55.0±0.3) ($p<0.001$). There were also statistically significant differences among the groups for Tb.Th ($p=0.001$). The LF group had the highest mean Tb.Th (0.122±0.002), while the saline group had the lowest mean value (0.071±0.002). We observed statistically significant differences

TABLE I
Comparison of micro-CT parameters among groups

Micro-CT parameters	Study groups	n	Mean±SD	95% CI Lower bound	95% CI Upper bound	*p*
BV/TV (%)	Saline	7	55±0.3[a]	54.7	55.3	<0.001¶
	Lactoferrin	7	77.1±0.7[b]	76.4	77.8	
	HMWHA	7	74.9±0.5[c]	74.5	75.3	
Trabecular thickness	Saline	7	0.071±0.002[a]	0.069	0.073	0.001‡
	Lactoferrin	7	0.122±0.002[b]	0.120	0.125	
	HMWHA	7	0.118±0.001[c]	0.117	0.118	
Trabecular number	Saline	7	44.2±1.1[a]	43.2	45.2	Sal *vs.* LF <0.001†
	Lactoferrin	7	53.4±1.3[b,c]	52.2	54.6	Sal *vs.* HA <0.001†
	HMWHA	7	52.7±1.5[c]	51.2	54.1	LF *vs.* HA=0.918†
Trabecular separation	Saline	7	0.092±0.002[a]	0.090	0.094	Sal *vs.* LF <0.001†
	Lactoferrin	7	0.054±0.001[b,c]	0.052	0.055	Sal *vs.* HA <0.001†
	HMWHA	7	0.056±0.001[c]	0.055	0.057	LF *vs.* HA=0.78†

CT: Computed tomography; CI: Confidence interval; SD: Standard deviation; BV: Bone volume; TV: Total volume; HMWHA: High-molecular-weight hyaluronic acid; ¶ One-way ANOVA, post-hoc Bonferroni; ‡ Kruskal-Wallis; Different superscript letters represent significant differences among groups (p<0.05).

among the groups for the mean Tb.Nb (p<0.001). The Tb.Nb for the saline group was significantly lower than that for the other two groups (p<0.001), and no significant difference was found between the HMWHA and LF groups (p=0.918). Likewise, statistically significant differences were observed among the groups for Tb.Sp (p<0.001). The Tb.Sp for the saline group was significantly lower than that of the other two groups (p<0.001), and no significant difference was found between the HMWHA and LF groups (p=0.78) (Table I). The osteolytic degenerations in bone tissue are illustrated in Figure 1 and Figure 2.

Inflammatory articular cartilage alterations

Histopathological evaluation revealed inflammatory cell infiltration around the TMJs. The most severe inflammatory reactions were observed in the saline group, and HMWHA and LF treatments relatively caused reduced inflammatory reactions (Figure 3a-c). The articular cartilage tissues were markedly thinned in the saline group compared to the HMWHA and LF groups. The HMWHA and LF groups were similar to the regular appearance, and the hypertrophic joint thickness was observed more

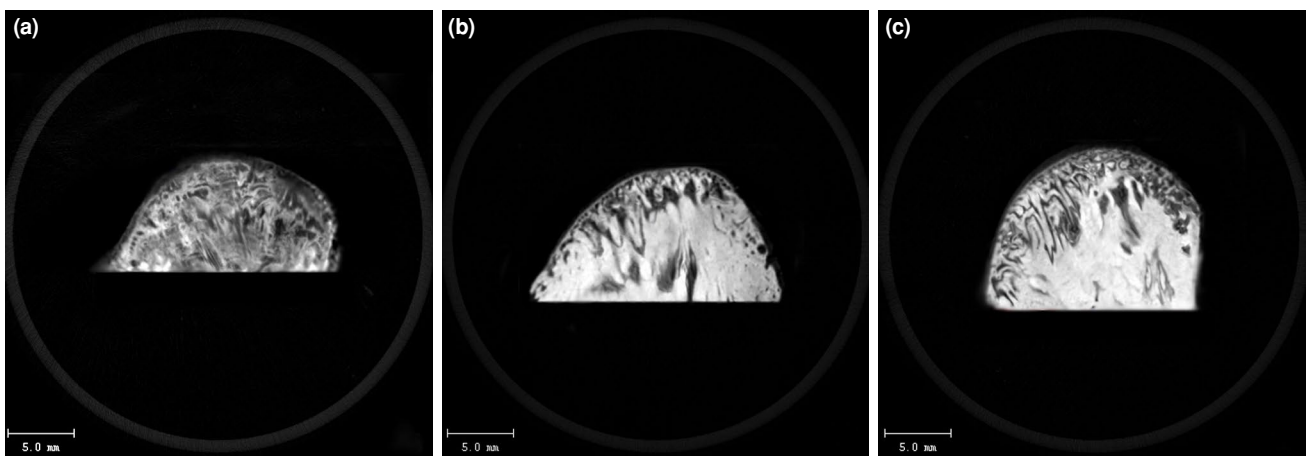


FIGURE 1. Representative micro-CT images from study groups. **(a)** HMWHA group, **(b)** Saline group, **(c)** LF group.
CT: Computed tomography; HMWHA: High-molecular-weight hyaluronic acid; LF: Lactoferrin.

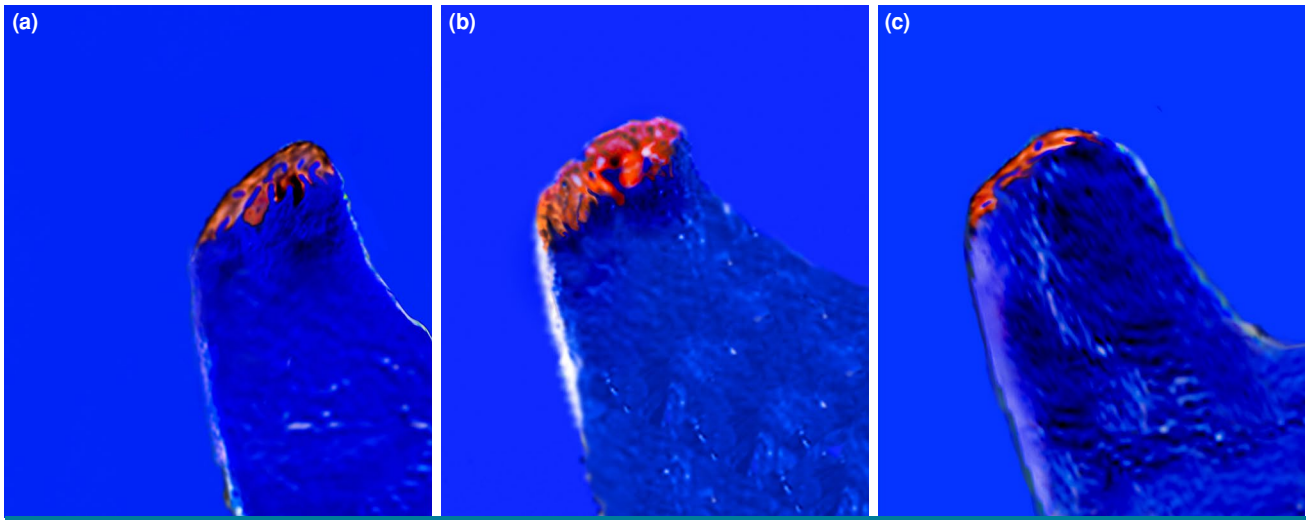


FIGURE 2. Micro-CT images showing the changes of the bone structure in each group. **(a)** (HMWHA group), **(b)** (Saline group) and **(c)** (LF group) images were obtained after image recording, and the natural bone structure is shown in blue and osteolytic bone changes are shown in red.
CT: Computed tomography; HMWHA: High-molecular-weight hyaluronic acid; LF: Lactoferrin.

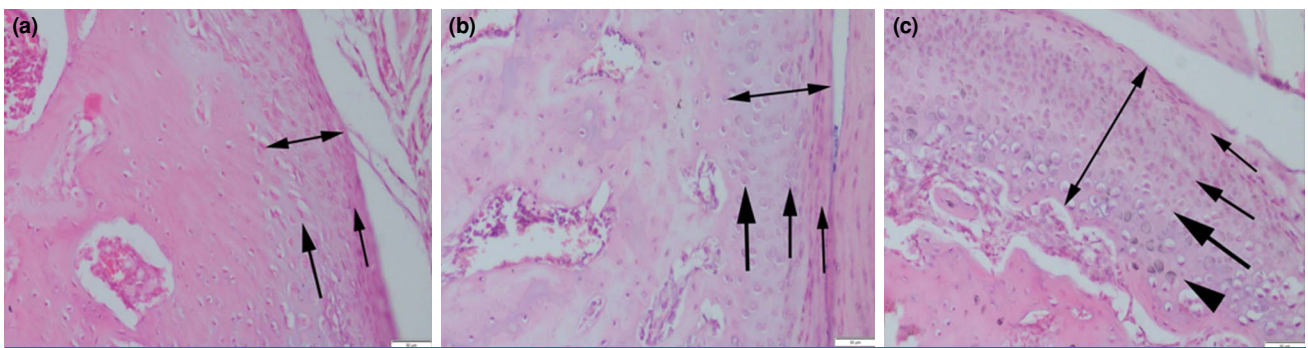


FIGURE 3. Microscopical appearance of the joint cartilage between the group, **(a)** decreased cartilage thickness (double headed arrow), decreases in articular (thin arrow) and proliferative (medium arrow) zones, lack of hypertrophic and transition zones in saline group; **(b)** relatively thickened joint cartilage in HMWHA group with slight increase in articular (thin arrow), proliferative (medium arrow) and transition (thick arrow) zones and without hypertrophic zone, **(c)** increase cartilage thickness and marked articular (thin arrow), proliferative (medium arrow), transition (thick arrow) and hypertrophic (arrow head) zones in LF group (H&E, Scale bars= 50 µm).
HMWHA: High-molecular-weight hyaluronic acid; LF: Lactoferrin.

often than the reduced joint thickness. While the highest hypocellularity was seen in the saline group, hypocellularity was slightly higher in the HMWHA group than in the LF group, but the numbers of clusters were equal. The rats in all groups exhibited invagination, and a weak junction was primarily seen in the saline group and the least in the LF group. The saline group noted relatively increased trabecular bone tissue, while the other groups exhibited a more normal appearance. The HMWHA and LF groups had better healing than the saline group (Figure 3a-c). The numerical distribution of histopathological findings of TMJs and statistical analysis are shown in Table II.

MMP-3 levels among groups

The immunoexpression of MMP-3 was increased in the saline group compared to the other groups. A marked decrease was observed in the LF group, while a mild increase was observed in the HMWHA group (Figure 4). Statistical analysis results of the immunohistochemical scores are shown in Table III.

TABLE II
The distributions of articular cartilage changes in all groups

Articular cartilage changes		Study groups		
		Saline (n)	HMWHA (n)	LF (n)
Cartilage	Normal	0	4	4
	Thinned	5	1	1
	Thickened	3	3	3
Osteochondral Junction	Normal	3	4	5
	Invagination	2	2	2
	Weak Junction	3	2	1
Subchondral bone	Normal	2	6	6
	Increase in trabecular bone	6	2	2
Chondrocytes	Normal	1	4	5
	Hypocellularity	4	2	1
	Cluster	3	2	2

HMWHA: High-molecular-weight hyaluronic acid; LF: Lactoferrin.

DISCUSSION

Regarding the current results, the intra-articular injections of HMWHA and LF in the MIA-induced TMJ OA model revealed statistically improved outcomes compared to the control group in histopathological, immunohistochemical, and micro-CT analyses. The null hypothesis of the study was accepted, as there was no significant difference between the outcomes of intra-articular HMWHA and LF injections, except for hypocellularity and MMP3 levels. Therefore, the LF injections were at least as effective as HMWHA injections on MIA-induced TMJ OA.

The effect of exogenous HMWHA on TMJ OA has been previously evaluated and the researchers argued that degenerative changes in the articular cartilage in the HMWHA group were significantly less than those in the control group.[13] Tolba et al.[16] and Lemos et al.[17] reported that HMWHA had a protective effect on cartilage by regulating endogenous HA, glycosaminoglycans, collagen synthesis, and preventing proteolytic enzymes, the inflammatory response, cartilage friction by providing lubrication. In the current study, articular cartilage was observed to be regular in four joints, and degenerative changes were observed in four joints (one thinning, three thickenings) in the HMWHA group. However, in the control group, in all samples, degenerative changes were observed (five thinning, three thickenings). The difference between these two groups was considered to be significant. The results of this study are consistent with the literature and indicate that HMWHA protects cartilage tissue. Another factor affecting articular cartilage degeneration is chondrocyte apoptosis.[13] Several studies have shown that one of the repair functions of HMWHA in TMJ OA is its chondroprotective effect.[16,18] Chondrocyte appearance is an early change in TMJ OA, which is vital, as hypocellularity is associated with chondrocyte apoptosis, and clustering is associated with the repair response.[13,19] In the current study, normal chondrocyte appearance in four joints, hypocellularity in two joints, and clustering in two joints were observed in the HMWHA group.

In contrast, chondrocyte appearance in one joint, hypocellularity in four joints, and clustering in

TABLE III
Comparison of MMP-3 levels among groups

Study groups	n	Mean±SD	*p*
Saline	8	2.3±0.5[a]	
HMWHA	8	1.3±0.9[b]	<0.001†
LF	8	1.4±0.5[b]	

MMP-3: Matrix metallopeptidase-3; SD: Standard deviation; LF: Lactoferrin; HMWHA: High-molecular-weight hyaluronic acid; † denotes the *p* value obtained by one-way ANOVA test. The Duncan test was performed to detect the inter-group differences. The mean ± standard deviation values carrying the different indices were significantly different.

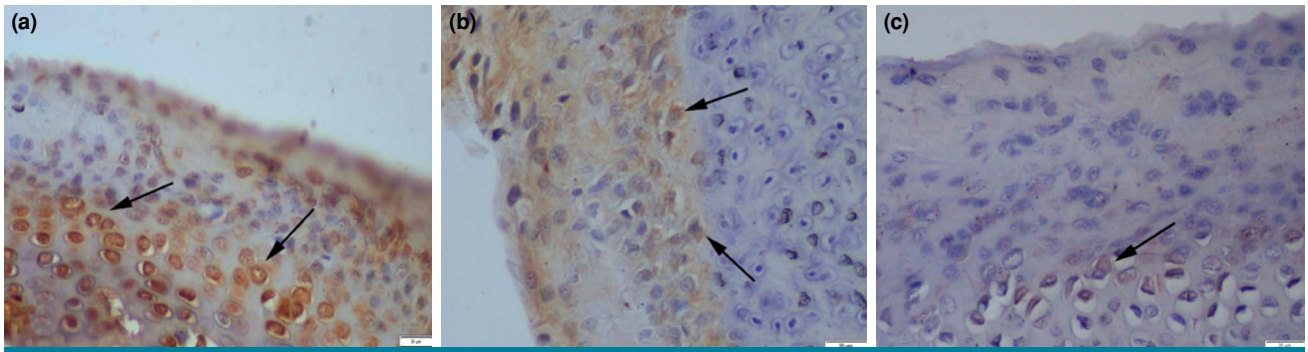


FIGURE 4. MMP-3 expressions in TMJ between the groups. **(a)** Marked increased in expression in saline group (arrows), **(b)** decreased expression (arrows) in HMWHA group, **(c)** slight expression in LF group (arrow), Streptavidin-biotin peroxidase method, Scale bars= 20 µm.
MMP-3: Matrix metallopeptidase-3; TMJ: Temporomandibular joints; HMWHA: High-molecular-weight hyaluronic acid; LF: Lactoferrin.

three joints were observed in the control group. As previously reported, the difference between the groups was not considered significant.[13] However, the authors argued that although there was no significant difference between the groups, the number of joints that appeared normal and clustered in the HMWHA group was higher than in the control group, inducing the repair response of HMWHA and could be effective in reducing the frequency of chondrocyte apoptosis. Another degenerative change observed during the early period of TMJ OA may occur in subchondral bone tissue. It has been reported that the decrease in prostaglandin (PG) levels and some alterations in the cartilage tissue increase the destructive factor of the force on the subchondral bone and affect the incidence of pathological changes.[13,20] In the literature, it has been reported that exogenous HMWHA positively affects subchondral bone tissue by concentrating in load-bearing areas during TMJ function, reducing the destructive effect of the incoming load with its lubricative characteristic, and increasing PG synthesis.[13] Also, in the current study, six normal joints and trabecular bone increases in two joints were observed in the HMWHA group. Two normal and trabecular bone increases in six joints were observed in the control group, and the difference between the groups was found to be significant. Furthermore, changes in the subchondral bone and articular cartilage structure may affect the osteochondral junction.[13] As a result of structural changes, the destructive effect of the force on the joint increases, which may cause some changes in the osteochondral junction.[13] In the current study, four joints were observed as regular, and two joints as invaginate and weak in the HMWHA group. Three joints were observed as regular, two as invaginate, and three as weak in the saline group. The difference between the groups was not considered significant, as previously indicated.[13] In addition, although the difference was not significant, the authors considered that the higher number of joints with normal osteochondral junctions in the HMWHA group might be due to the protective effect of HMWHA on articular cartilage and subchondral bone tissue.

There are studies, *albeit* limited, indicating the effects of LF on joints with OA. The anti-inflammatory effects were indicated with a significant decrease in interleukin (IL)-1 and IL-6 levels in the joints, cartilage, and synovial fluid with OA in the LF group.[21] Likewise, Xue et al.[22] induced OA in rat knees and reported that LF caused a marked decrease in IL-1 levels, which was achieved by the anti-inflammatory effect of LF. In this study, the typical structure of the articular cartilage in four joints, thickening in three joints and thinning in one joint in the LF group, and degenerative changes were observed in all the joints in the control group. In the literature, the protective effect of LF on the articular cartilage is significant.

The present study differs from other studies regarding the amount and frequency of LF administered and the joint for which OA was induced. Lactoferrin is a potent regulator of chondrocyte metabolism and eliminates some mediators that cause chondrocyte apoptosis.[21,22] In this study, five normal joints, hypocellularity in one joint, and clustering in two joints were observed in the LF group. The typical structure was observed in one joint, hypocellularity in four joints, and clustering in three in the control group. Although there was no significant difference between the groups, we believe that the low hypocellularity in the LF group

may be due to its protective effect on chondrocytes following the literature. Wang et al.[11] reported that osteoclastic activity triggered by vascular endothelial growth factor in TMJ OA might be among the factors causing degenerative changes in subchondral bone tissue. In this study, subchondral bone was regular in six joints, while trabeculation was increased in two joints. In the control group, subchondral bone was regular in two joints, while trabeculation was increased in six joints, and the difference between the groups was significant. We believe that the lower amount of degenerative changes observed in the articular cartilage and the possibility of suppression of osteoclastic activity with the anabolic effect may have led to fewer degenerative changes in the subchondral bone. In the LF group, the osteochondral junction was regular in five joints, invaginated in two joints, and weak in one joint, while it was regular in three joints, invagination in two joints, and weak in three joints in the control group. Although there was no significant difference between the groups, articular cartilage and subchondral bone tissue in the LF group were significantly healthier regarding the number of joints with normal osteochondral junctions.

The histomorphometric results of this study revealed non-significant differences between the LF and HMWHA groups in articular cartilage, chondrocyte appearance, and the subchondral bone or osteochondral junction. Also, both agents had a positive effect on OA. Since there is no similar study available in the literature, we speculate that these results were achieved with the specified effects on the joint for both agents. In addition, HA has the unique characteristic of providing lubrication on the TMJ and being the primary component of synovial fluid. Achieving successful results in joints with OA validates these characteristics of exogenous HA. A previous study reported that LF stimulated HA synthesis in dermal fibroblasts.[23] In this study, the possibility of exogenously administered LF affecting HA synthesis might be a factor leading to similar results for the two agents. Another factor may be that LF is a component of synovial fluid, such as HA, and its levels become elevated through exogenous administration. In the present study, LF deficiency was an essential factor in joints with rheumatoid arthritis, and treatments with exogenous LF were successful.

One of the primary changes seen in TMJ OA is a significant increase in MMP expression. In particular, MMP-3 is primarily produced by synovial cell lines and activates many MMP family members, playing a role in many degenerative functions, including articular cartilage destruction.[16] Tolba et al.[16] reported that HMWHA significantly decreased MMP-3 levels in the joint where TMJ OA was induced. A significant difference was observed in this study between the HMWHA group (1.25±0.88) and the control group (2.25±0.53). Also, Yan et al.[21] showed that LF significantly suppressed the expression of the cartilage-degrading enzymes MMP-1, MMP-3, and MMP-13 and exerted anti-inflammatory and anti-catabolic effects. In this study, the LF group (1.37±0.51) significantly suppressed MMP-3 expression, and this finding is consistent with the effect of LF on MMP-3, as reported in the literature.

Kim et al.[24] and Shi et al.[25] demonstrated that the decrease in BV/TV value directly affected the bone quality. In addition, the BV/TV value, not alone, but its conformity with other parameters, provided a complimentary analysis of the effect of OA on the TMJ microstructure (Tb.Th, Tb.Sp, Tb.Nb). Significant decreases in BV/TV, Tb.Th, Tb.Nb values and a significant increase in Tb.Sp values indicate bone loss and structural degeneration.

Additionally, a decline in Tb.Th values, while BV/TV and Tb.Nb values increased in the TMJ with OA indicate an endochondral maturation pathway, but not an increase in osteoblastic activity.[26] In the current study, except for Tb.Sp, all parameters were significantly higher in the LF and HMWHA groups than the saline group, indicating a more protective effect on the joint in terms of bone quality and loss. Also, the LF group had significantly higher BV/TV and Tb.Th values than the HMWHA group indicating the possible superior effect of LF on the repair response in the TMJ.

To the best of our knowledge, the current study is the first to compare the efficacy of intra-articular injection of LF with HMWHA in an MIA-induced TMJ-OA. The consistency between histomorphometric, immunohistochemical, and micro-CT findings of the study increases the authenticity of the healing effect observed in response to LF and HMWHA on TMJ OA. LF, which has been reported to have no toxic effects in studies conducted thus far and is already a part of synovial fluid and is a promising agent in treating TMJ OA.

Nevertheless, this study has certain limitations. First, the long-term effects of LF and HMWHA on TMJ OA were unable to be evaluated, as the study is not longitudinal in nature. Another limitation is that LF was used as a single dose with a uniform administration frequency and, therefore, no information concerning the ideal dose or frequency

of administration could be obtained. Also, there are some methodological limitations such as induction of OA. In the current study, TMJ OA was chemically induced. In the MIA-induced OA model, diffuse cell death and rapid destructive changes were observed, unlike spontaneous or posttraumatic OA.[28] As a result, although clinical features can be mimicked in rapidly progressive pathological changes such as in the current model, severe immediate histopathological changes observed in human OA were absent.[29] The absence of TMJ OA-related histopathological changes, such as disc perforation, subchondral bone exposure, and vertical splitting in the cartilage,[30] can be considered a disadvantage in this model. It should be kept in mind that this feature of the model may impact the effects of the treatment methods applied. In addition, the effects that may vary according to dose-dependent MIA administrations were not investigated in the current study. Furthermore, the animals used in the present study were 10-week-old male rats. However, whether age-related efficacy changes in MIA-induced models is an issue that needs further investigation.

In conclusion, our study results indicated that the administration of 50 µg of LF at 100 µg/mL and 150 µg of HMWHA once a week for three weeks in a MIA-induced rat TMJ OA model resulted in significant therapeutic effects, without any differences between the study groups. Based on these findings, we believe that LF may be as effective as HMWHA in the treatment of TMJ OA by determining the appropriate doses and frequency of administration.

Ethics Committee Approval: The study protocol was approved by the Afyon Kocatepe University, Animal Research and Ethics Committee (date: 03.06.2020, no: AKUHADYEK-221-20). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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