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Original Article

Intra-articular hyaluronate, tenoxicam and vitamin E in a rat model of osteoarthritis: evaluation and comparison of chondroprotective efficacy

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Abstract: Objective: The aim of this experimental study was to evaluate and compare the chondroprotective efficacy of intra-articular hyaluronic acid, tenoxicam and vitamin E in osteoarthritis. Methods: An osteoarthritis model was created by anterior cruciate ligament transection and medial menisectomy in knees of 28 rats. The rats were randomized into four groups; first group served as a control group and received intra-articular injections of saline solution, intra-articular HA, intra-articular tenoxicam and intra-articular Vit E were applied to the treatment groups. First intra-articular injections were applied at second week postoperatively and repeated once a week for 5 weeks. At 8th week after the operation groups were compared based on the histologic scores of cartilage degeneration by Mankin Histological Grading Scale. Results: Total cartilage degeneration score was significantly increased in the control group (P=0.004). Total Mankin scores of HA, tenoxicam and Vit E groups were significantly lower than the control group (P=0.004, P=0.016, P=0.012 respectively). There was no statistically siginificant difference between the treatment groups in terms of total Mankin scores (P>0.05). Conclusion: Intra-articular application of HA, tenoxicam and Vit E are chondroprotective in early osteoarthritis model in rats. Chondroprotective activity of tenoxicam and Vit E are comparable with the beneficial effects of HA on articular cartilage.

Keywords: hyaluronic acid, tenoxicam, vitamin E, osteoarthritis, chondroprotective activity

Introduction

Osteoarthritis (OA) is the most prevalent form of arthritis and one of the leading causes of chronic disability which is becoming more and more pronounced as the population ages. Various genetic, biologic and mechanical factors contribute to disease process in OA. A large cascade of events lead to breakdown and degeneration of cartilage in a progressive manner ultimately resulting in the damage of all the structures of the joint. Compounds that are intended to prevent, retard, stabilize or reverse the development of histopathological and eventual mophological changes caused by OA are under investigation worldwide by many researchers. The ideal treatment of OA should focus on prevention of articular cartilage damage and many compounds are under investigation for this purpose [1]. Hyaluronic acid (HA) which is a type of glucosaminoglycan and a major ingredient of synovial fluid is one of the most investigated molecules in the treatment of OA. Viscosupplementation with intaarticular (HA) has positive effects in pain relief and joint function improvement in the knee OA, relatively high cost is the only major disadvantage. Experimental and in vitro studies displayed chondroprotective efficacy of HA besides its favorable clinical effects [2-4]. Several physiological effects probably contribute to the mechanisms by which HA exert its clinical and chondroprotective effects; it enhances proteoglycan synthesis, reduces the production and activity of proinflammatory mediators and matrix metalloproteinases (MMP) and alters the behavior of

Table 1. Total Mankin Scores of treatment and control groups and comparison of groups in terms of total Mankin scores

		[1]Control	[2]HA	[3]Tenoxicam	[4]Vit E
Total Mankin Score	*Min-Max (Median)*	5-13 (9)	0-6 (2)	1-7 (5)	1-8 (3)
	mean±SD	8.14±2.73	2.28±1.98	4.57±1.90	3.86±2.41
	[a]P	0.004**			
	Paired comparisons; [b]P	[1-2]P: 0.004**		[1-4]P: 0.012*	[2-4]P: 0.172
		[1-3]P: 0.016*		[2-3]P: 0.053	[3-4]P: 0.402

[a]Kruskal Wallis Test; [b]Mann Whitney U Test; *P<0.05, **P<0.01.

Table 2. Structure scores of treatment and control groups and comparison of groups in terms of structure scores

		[1]Control	[2]HA	[3]Tenoxicam	[4]Vit E
		n (%)	n (%)	n (%)	n (%)
Structure Score	0	0 (0%)	2 (28%, 6)	1 (14%, 3)	1 (14%, 3)
	1	0 (0%)	3 (42%, 9)	2 (28%, 6)	2 (28%, 6)
	2	3 (42%, 9)	2 (28%, 6)	3 (42%, 9)	2 (28%, 6)
	3	1 (14%, 3)	0 (0%)	1 (14%, 3)	2 (28%, 6)
	4	2 (28%, 6)	0 (0%)	0 (0%)	0 (0%)
	5	1 (14%, 3)	0 (0%)	0 (0%)	0 (0%)
	Min-Max (Median)	2-5 (3)	0-2 (1)	0-3 (2)	0-3 (2)
	mean±SD	3.14±1.21	1.00±0.82	1.57±0.98	1.71±1.11
	[a]P		*0.019**		
	Paired comparisons; [b]P	*[1-2]P: 0.005***	*[1-4]P: 0.056*		*[2-4]P: 0.206*
		*[1-3]P: 0.028**	*[2-3]P: 0.253*		*[3-4]P: 0.790*

[a]Kruskal Wallis Test; [b]Mann Whitney U Test; *P<0.05, **P<0.01.

Table 3. Cells scores of treatment and control groups and comparison of groups in terms of cells scores

		[1]Control	[2]HA	[3]Tenoxicam	[4]Vit E
		n (%)	n (%)	n (%)	n (%)
Cells score	0	0 (0%)	5 (71%, 4)	0 (0%)	3 (42%, 9)
	1	3 (42%, 9)	1 (14%, 3)	5 (71%, 4)	3 (42%, 9)
	2	3 (42%, 9)	1 (14%, 3)	2 (28%, 6)	0 (0%)
	3	1 (14%, 3)	0 (0%)	0 (0%)	1 (14%, 3)
	Min-Max (Median)	1-3 (2)	0-2 (0)	1-2 (1)	0-3 (1)
	meant±SD	1.71±0.76	0.43±0.79	1.29±0.49	0.86±1.07
	[a]P		*0.026**		
	Paired comparisons; [b]P	*[1-2]P: 0.013**	*[1-4]P: 0.070*		*[2-4]P: 0.351*
		[1-3]P: 0.244	*[2-3]P: 0.028**		*[3-4]P: 0.154*

[a]Kruskal Wallis Test; [b]Mann Whitney U Test; *P<0.05.

immune cells [3]. HA has significant effects on inflammatory mediators including cytokines, proteases and prostaglandins. HA also has antioxidant effects in various systems providing protection of articular chondrocytes against the damage induced by oxygen-derived free radicals [4-6]. Tenoxicam is a nonsteroidal anti-inflammatory drug (NSAID) and analgesic of the oxicam class, is closely related to piroxicam and has a long half life which enables it to be administered in knee and shoulder joint for pain management [7, 8]. Vitamin E (Vit E) is a

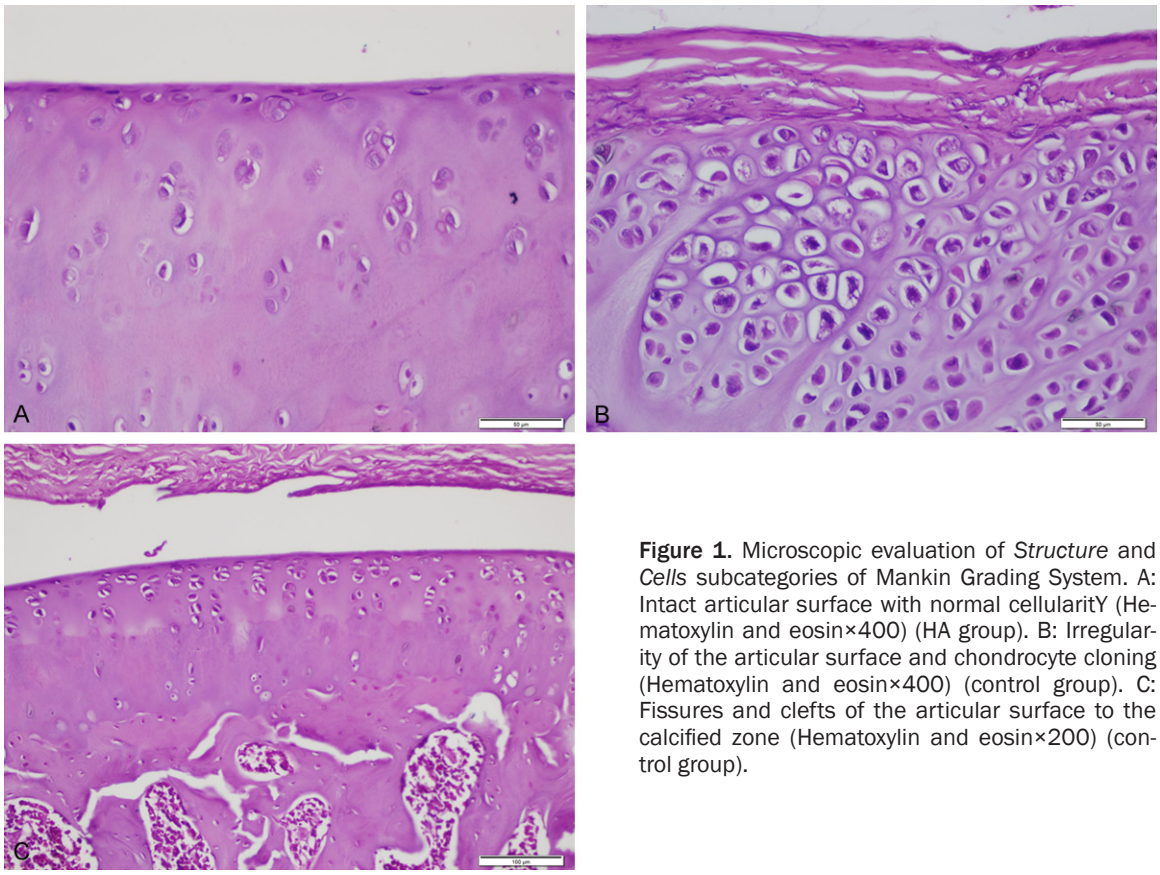

Figure 1. Microscopic evaluation of *Structure* and *Cells* subcategories of Mankin Grading System. A: Intact articular surface with normal cellularitY (Hematoxylin and eosin×400) (HA group). B: Irregularity of the articular surface and chondrocyte cloning (Hematoxylin and eosin×400) (control group). C: Fissures and clefts of the articular surface to the calcified zone (Hematoxylin and eosin×200) (control group).

fat soluble vitamin with strong antioxidant functions. It has multiple natural and synthetic forms but alpha tocoferol is the one with greatest biologic activity [9]. The purpose of this study was to evaluate the chondroprotective efficacy of intra-articular tenoxicam and Vit E in order to exhibit new and cost effective alternatives for viscosupplementation. The effects of intra-articularly administered HA, tenoxicam and Vit E on the prevention of cartilage damage in experimentally induced knee OA was assessed in this study.

Materials and methods

Animal model and surgery

Twenty eight Sprague-Dawley male rats weighing 350-400 gr were used. Rats were housed in metal cages where they were allowed free acsess to solid diet and tap water. All rats were anesthetized by intramuscular injection of 10 mg/kg xylazine (Rompun, Bayer, Turkey) and 50 mg/kg ketamin (Ketalar, Pfizer). The right knees of the rats were prepared for the operative procedure. After shaving the knee joint, the skin was disinfected and a parapatellar skin incision was made on the medial side of the joint. Then a modified prepatellar arthrotomy was made with complete exposure of anterior cruciate ligament (ACL) and medial meniscus. ACL was resected and medial meniscus was completely removed. A positive anterior drawer test ensured complete transection of the ligament. The medial retinaculum was repaired and the skin was closed with sutures. The animals were allowed free cage activity postoperatively. Principles of laboratory animal care were followed during the whole study and all operative procedures and interventions were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the local Animal Care Committee.

Intra-articular injections

After the operations rats were randomly allocated into four groups each consisting of seven rats. First group served as a control group and received intra-articular injections of 0.2 ml sterile saline solution. Second group received 0.2

Table 4. Safranin-O staining scores of experimental and control groups and comparison of groups in terms of Safranin-O staining scores

		[1]Control	[2]HA	[3]Tenoxicam	[4]Vit E
		n (%)	n (%)	n (%)	n (%)
Safranin-O Staining Score	0	0 (0%)	2 (28%, 6)	1 (14%, 3)	1 (14%, 3)
	1	0 (0%)	4 (57%, 1)	1 (14%, 3)	3 (42%, 9)
	2	2 (28%, 6)	1 (14%, 3)	5 (71%, 4)	3 (42%, 9)
	3	3 (42%, 9)	0 (0%)	0 (0%)	0 (0%)
	4	2 (28%, 6)	0 (0%)	0 (0%)	0 (0%)
	Min-Max (Median)	2-4 (3)	0-2 (1)	0-2 (2)	0-2 (1)
	mean±SD	3.00±0.82	0.86±0.69	1.57±0.79	1.29±0.76
	[a]p		*0.002***		
	Paired comparisons [b]P	[1-2]P: *0.002***	[1-4]P: *0.005***		[2-4]P: *0.266*
		[1-3]P: *0.007***	[2-3]P: *0.075*		[3-4]P: *0.389*

[a]Kruskal Wallis Test; [b]Mann Whitney U Test; *P<0.05, **P<0.01.

ml intra-articular injections of HA (25 mg/2.5 ml, Adant, Meiji Seika Kaisha, Japan); a viscous solution consisting of a fraction of purified natural sodium hyaluronate in buffered physiological sodium chloride with a molecular weight of 0.6-1.2 million Da. Third group received 0.2 ml intra-articular injections of tenoxicam (20 mg/2 ml, Oksamen, Mustafa Nevzat, Turkey) and fourth group received 0.2 ml intra-articular injections of Vit E (300 mg/2 ml alpha tocoferol asetat, Evigen, Mefar, Turkey).

All injections were applied by starting 2 weeks postoperatively, once a week for a period of 5 weeks. The rats were sacrificed on the 8th week after the operation with high dose of intravenous thipentone sodium (Penthotal, Abbot) after intamuscular anestesia with xylocaine and ketamine.

Histopathological evaluation

Distal femur from the rat knee joints were fixed in 10% buffered formaline and decalcified in TDE solution (Sakura, Finatek Europe; BU, ML). Medial femoral condyles were sectioned and embedded in paraffin blocks. Tissue sections were stained with Hematoxylen-Eosin for cellular assessment. Safranin-O staining was used for detection of the proteoglycan content. Histological assessment was done according to the Mankin Histological-Histochemical Grading System, a 14-point histological grading devised by Mankin et al [10]. Mankin Grading System assigns seperate scores to structure, cell distrubition and density, Safranin-O staining and the integrity of tidemark. The scores in each of these subcategories were totaled for each sample: zero value represents normal cartilage while a high score represents damage to the cartilage.

Statistical analysis

NCSS (Number Cruncher Statistical System) 2007 & PASS (Power Analysis and Sample Size) 2008 Statistical Software (Utah, USA) program was used for the statistical analysis. Kruskal Wallis test was used for the intergroup comparisons of parameters without normal distribution and Mann Whitney U test was used for the determination of the group causing difference. Spearman's correlation analysis was used to assess the relationship between parameters. The results were evaluated in 95% confidence interval and at a significance level of P<0.05.

Results

No rats were lost during the experiment period and no infection was observed in the rat knees. Histological assessment was done according to the Mankin Grading System. Total Mankin score of the control group was between 5 and 13 with a mean of 8.14±2.73 and a median of 9. Total Mankin score of the HA group was between 0 and 6 with a mean of 2.28±1.98 and a median of 2. Total Mankin score of tenoxicam group was between 1 and 7 with a mean of 4.57±1.9 and a median of 5. Total Mankin score of the Vit E group was between 1 and 8 with a mean of 3.86±2.41 and a median of 3. Statistically significant difference was found in terms of total Mankin score between the groups (P=0.004).

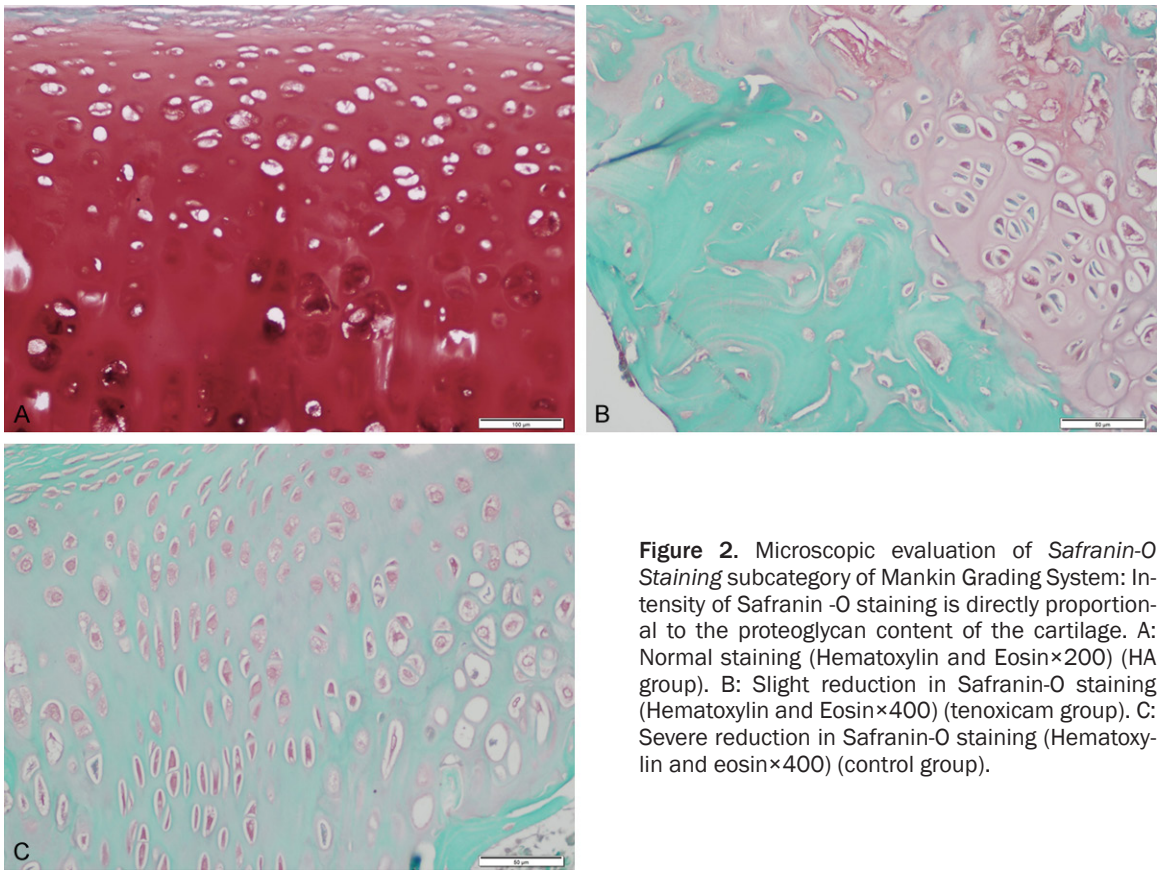

Figure 2. Microscopic evaluation of *Safranin-O Staining* subcategory of Mankin Grading System: Intensity of Safranin -O staining is directly proportional to the proteoglycan content of the cartilage. A: Normal staining (Hematoxylin and Eosin×200) (HA group). B: Slight reduction in Safranin-O staining (Hematoxylin and Eosin×400) (tenoxicam group). C: Severe reduction in Safranin-O staining (Hematoxylin and eosin×400) (control group).

Statistically significant difference was found in the comparison of HA, tenoxicam and Vit E groups (P=0.004; P=0.016; P=0.012 respectively) with the control group (**Table 1**).

First subcategory of Mankin Grading System is *Structure* which evaluates structure of articular cartilage by examining presence of surface irregularities and/or clefts in various zones of the cartilage. The cartilage structure scores of the control group were between 2 and 5 with a mean of 3.14±1.21 and a median of 3. The cartilage structure scores were between 0 and 2 with a mean of 1.00±0.82 and a median of 1 in the HA group. The cartilage structure scores of tenoxicam group were between 0 and 3 with a mean of 1.57±0.98 and a median of 2 and the cartilage structure scores were between 0 and 3 with a mean of 1.71±1.11 and a median of 2 in the Vit E group. Statistically significant difference was found between the control group and the treatment groups in terms of cartilage structure; the control group had higher scores (P=0.019) than the treatment groups (**Table 2**). When the study groups are compared, statistically significant difference was found in structure scores of HA and tenoxicam groups in comparison to the control group (P=0.005, P=0.028 respectively). Although statistically not significant (P=0.056) Vit E group showed a beneficial therapeutic trend in terms of cartilage structure in comparison to the control group. No significant difference was found between the treatment groups in terms of cartilage structure.

Second subcategory of the Mankin Grading System is *Cells* by which density and distrubition of chondrocytes is evaluated. Cellular abnormality scores were between 1 and 3 with a mean of 1.71±0.76 and a median of 2 in the control group. Cellular abnormality scores were between 0 and 2 with a mean of 0.43±0.79 and a median of 0 in the HA group. Cellular abnormality scores were between 1 and 2 with a mean of 1.29±0.49 and a median of 1 in the tenoxicam group. Cellular abnormality scores were between 0 and 3 with a mean of 0.86±1.07 and a median of 1 in the Vit E group. Statistically significant difference was found in terms of cellular abnormality scores between the groups (P=0.026). Cellular abnormality scores of HA

group were significantly lower than the control group (P=0.013). No statistically significant difference was found between tenoxicam and Vit E group in comparison to control group in terms of cellular abnormality (P=0.24, P=0.07 respectively). There was statistically significant difference between HA and tenoxicam groups in terms of cellular abnormality (P=0.028) (**Table 3**). Sections from the microscopic evaluation of *Structure* and *Cells* subcategories are seen in **Figure 1**.

Third subcategory of Mankin Grading System is *Safranin-O staining* by which the proteoglycan content of the cartilage is evaluated. Safranin-O scores were between 2 and 4 with a mean of 3.00±0.82 and a median of 3 in the control group. The Safranin-O scores were between 0 and 2 with a mean of 0.86±0.69 and a median of 1 in the HA group. The Safranin-O values were between 0 and 2 with a mean of 1.57±0.79 and a median of 2 in the tenoxicam group. The Safranin-O values were between 0 and 2 with a mean of 1.29±0.76 and a median of 1 in the Vit E group. Statistically significant difference was found between the groups in terms of Safranin-O staining (P=0.002). Total Safranin-O staining score of the control group was significantly higher than the HA, tenoxicam and Vit E groups (P=0.002, P=0.007, P=0.05, respectively). No ststistically significant difference was found between treatment groups in terms total Safranin-O staining scores (**Table 4**). Sections from the microscopic evaluation of *Safranin-O staining* are seen in **Figure 2**.

Fourth subcategory of Mankin Grading System is *Tidemark* by which integrity of cartilage tidemark is evaluated. The tidemark scores of the control group were between 0 and 1 with a mean of 0.29±0.49 and a median of 0. Tidemark was intact in all articular cartilage sections taken from HA and Vit E groups, hence tidemark scores of both groups were 0. Tidemark scores of tenoxicam group were between 0 and 1 with a mean of 0.14±0.38 and a median of 0. No statistically significant difference was found in terms of tidemark scores between the groups (P=0.266, P>0.05).

Discussion

The findings of our study revealed that intra-articular application of HA, tenoxicam and Vit E exerted chondroprotection individually in varying degrees in a rat model of OA. Studies have shown that although representing a traumatic form of OA, anterior cruciate ligament transection (ACLT) together with menisectomy models mimic pathogenesis of human OA in terms of cartilage degradation, subchondral bone sclerosis and osteophyte formation and provide useful experimental models for the evaluation of different molecules as potential disease modifying agents [11]. It has been shown that disease progression in ACLT joints is milder with less cartilage damage than that observed in the joints having ACLT together with meniscectomy with surface cartilage damage detected within 2 weeks post-surgery in the latter [12]. We applied intra-articular injections starting 2 weeks post-operatively in order to evaluate the chondroprotective efficacy of our study reagents during the ongoing osteoarthritic process. Consistent with the literature at 8th week postoperatively serious cartilage damage was detected in all rats in the control group with histological changes designating OA. Disruption in cartilage structure and chondrocytes, and proteoglycan loss displaying articular cartilage damage was prominant in the rats receiving intra-articular saline injections.

Viscosupplementation with HA is commonly used as a symptom modifying method in the management of knee OA and debate about its structure modifying activity is still ongoing. HA which is a major ingredient of synovial fluid is responsible for the viscoelastic properties by acting as a shock absorber during fast movements and as a lubricant during slow movements. Initial goal of intra-articular therapy with HA was to support and replace the synovial fluid that has lost its viscoelastic properties. Over time numerous in vivo and in vitro studies exhibited chondroprotective efficacy of HA in experimentally induced OA and cell cultures [2-6]. It coats the surface of the articular cartilage and shares space deeper in the cartilage among collagen fibrils and sulphated proteoglycans. In this respect HA protects the cartilage and blocks the loss of proteoglycans from the cartilage matrix into synovial space maintaining the normal cartilage matrix [4]. We observed similar results with the literature regarding the chondroprotective efficacy of inta-articular HA [3, 13, 14]. Highest chondroprotection was achieved with HA among the treatment groups in this study. HA was the most effective compound on preserving cartilage structure, chondrocytes and proteoglycan content. Our experi-

mental study once more displayed the chondroprotective efficacy of hyaluronate on a cellular level.

Besides the enhancement of proteoglycan synthesis, HA exerts anti-inflammatory effects by reducing the secretion of arachidonic acid from synovial fibroblasts, decreasing the production and activity of proinflammatory mediators and matrix MMPs [4-6]. It has been demonstrated that inflammation is involved in the pathogenesis of OA [15]. Mitsui et al. examined the effects of HA on the expression of mRNAs for proinflammatory cytokine production in IL-1-stimulated synovial fibroblasts derived from patients with rotator cuff disease [16]. They reported decreased expression of proinflammatory cytokine mRNAs and COX-2/PGE2 with the addition of HA in a dose dependant manner. Tanaka et al. investigated the anti-inflammatory effect of HA on human chondrocytes and reported supression of MMP-1 production by HA [17].

HA also has antioxidant effects [18, 19]. HA protects against the damage to articular chondrocytes by oxygen-derived free radicals, which are known to play a role in the pathogenesis of arthritic disorders [4]. Fukuda et al. demonstrated the inhibitory effect of HA on reactive oxygen species (ROS) in an experimental design of bovine articular chondrocyte culture [20]. Hyaluronan mediated protective effect against cell damage caused by hydroxyl radicals is dependent on hyaluronan molecular mass [21]. In an experimental study conducted by Miki et al. mechanical compression was applied to bovine cartilage mimicking the abnormal mechanical stress loaded on the articular cartilage leading to OA [22]. Inhibition of proteoglycan synthesis and enhancement of ROS was observed, externally added HA reversed the inhibition of proteoglycan synthesis and attenuated ROS synthesis supporting the antioxidant efficacy of HA.

We hypothesized that tenoxicam having anti-inflammatory effect and Vit E having both antioxidant and anti-inflammatory effects might have chondroprotective efficacy when applied intra-articularly. The mainstay of our hypothesis was the anti-inflammatory and anti-oxidant effects exerted by HA on the OA models. Tenoxicam is an NSAID commonly used for the symptomatic treatment of OA. The efficacy of NSAIDs is related to their concentration within the synovium; higher dosages of NSAIDs provide a greater improvement in the symptoms and lower concentrations of pro-inflammatory cytokines in the synovial fluid in patients with knee OA [23]. Tenoxicam can be administered inta-articularly [7, 8]. Intra-articular injection yields higher concentrations in the joint that can regulate the inflammatory process more effectively than systemic administration [24, 25]. Although efficacy of NSAIDS is well established in OA in terms of pain and functional parameters the literature about the possible chondroprotective efficacy of NSAIDs is lacking [26]. Previous reports has mentioned about detrimental effects of NSAIDs on articular cartilage [27] even at pharmacologic concentrations when given systemically by inhibiting glycosaminoglycan synthesis [28]. Although few in number some researchers displayed favorable effects of NSAID on osteoarthritic cartilage in animal OA models [29-31]. Recently Jiang et al. reported efficacy of intra-articular celecoxib in a rabbit model of OA in which delay in cartilage degeneration and impairment in the function of inflammatory mediators were exhibited by celecoxib application [32]. Our results about chondroprotective effect of intra-articular tenoxicam is also encouraging. Tenoxicam was effective in the preservation of cartilage structure and proteoglycan content in a similar way to HA. Although mean total cartilage degeneration score was highest in the tenoxicam group among the treatment groups, given the significant beneficial effects on cartilage structure and preservation of the proteoglycan content in the early stages of OA, tenoxicam deserves further investigations about its chondroprotective activity.

Vit E is a fat soluble vitamin with a highly flexible structure and strong antioxidant activity. It has several natural and synthetic forms but alpha tocopherol is the one with greatest biologic activity [9]. It has a major biological role in protecting cell membrane from oxidation by free radicals. Oxidative stress also plays a role in pathophysiology of OA as well as the proinflammatory factors that are locally increased during osteoarthritic process [33]. Sutipornpalangkul et al. reported significantly lower amounts of Vit E in the synovial fluid of osteoarthritic knees in a study comparing the levels of antioxidants in the osteoarthritic knees having severe cartilage damage with the knees having intact cartilage [34]. Placebo controlled clinical trials testing the efficacy of oral vitamin E in the treatment of OA are scarce and have contradictory findings [35, 36]. To our best knowledge

only report about intra-articular Vit E is by Kılıç et al. [37] investigating the effects of intra-articular injections of vit E in rabbits with experimentally induced hemarthrosis. Intra-articular Vit E resulted in preservation of joint surface displaying a potential chondroprotective effect. Consistant with the results of Kılıç et al. intra-articular Vit E exerted a chondroprotective effect in this experimental study of OA. Vit E was effective in the preservation of proteoglycan content in a similar way to HA. Vit E also exerted a beneficial trend in the preservation of articular structure although not reaching the significance level. Mean total cartilage degeneration score was of Vit E was in between the scores of HA and tenoxicam groups with the HA group having the least cartilage degeneration.

No detrimental effects on joint cartilage were detected by intra-articular application of tenoxicam or Vit E. Intra-articular HA may be combined with Vit E and/or tenoxicam to provide a better chondroprotective activity. Further studies are needed to display the possible combined effects of these compounds. One limitation of our study is the short follow-up time. This study will set an example for the future studies which can be designed in larger animal models and longer follow-up times with different dosages and combinations.

In conclusion, this study verified the chondroprotective efficacy of intra-articular HA and displayed beneficial effects of intra-articular tenoxicam and Vit E on cartilage preservation in early OA model. Whether combination of intra-articular HA with Vit E or tenoxicam results in enhanced chondroprotective activity or intra-articular Vit E and tenoxicam provides favorable clinical effects in knee OA warrants a human based study.

Disclosure of conflict of interest

None.

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