Joint Diseases and Related Surgery

Jt Dis Relat Surg
2023;34(1):158-165

ORIGINAL ARTICLE

In vivo study of the role of hyaluronic acid, N-acetyl cysteine, and deproteinized calf serum on injury-induced cartilage degeneration

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ABSTRACT

Objectives: The aim of this study was to compare the effects of hyaluronic acid (HA), N-acetyl cysteine (NAC), and deproteinized calf serum on cartilage healing after the creation of traumatic cartilage injury in a rat model.

Materials and methods: A total of 48 rats, each weighing an average of 350 g, were randomly separated into four groups of 12. An osteochondral defect was created, 2-mm-wide and 3-mm deep in each rat. Injections were made to the knees of the rats as saline solution in Group 1, deproteinized calf serum in Group 2, NAC in Group 3, and HA in Group 4. At the end of 12 weeks, all rats were sacrificed and tissues were evaluated histologically.

Results: The HA group had a better cell morphology, tissue morphology, surface architecture, and vascularity than the other groups ($p<0.001$). Matrix staining, chondrocyte clustering, and the assessment scores of the mid, deep, superficial zones, and overall were higher in the HA group than in the other groups ($p<0.001$). The NAC showed a better tissue morphology, cell morphology, and vascularity than the control group ($p=0.003$, $p<0.001$, and $p<0.001$, respectively).

Conclusion: Hyaluronic acid was the most effective agent in cartilage healing compared to NAC and deproteinized calf serum. In addition, the NAC was more effective compared to the control group.

Keywords: Actovegin, cartilage injury, hyaluronic acid, N-Acetyl cysteine, osteoarthritis.

Osteoarthritis (OA), which is the most common degenerative joint disease, is characterized by synovial inflammation, subchondral bone sclerosis, osteophyte formation, and, ultimately, narrowing of the joint space, resulting in pain and physical disability. Although aging, mechanical, catabolic, or genetic factors have been studied as etiological risk factors causing degeneration of articular cartilage, there are no clinical drugs which can effectively prevent or treat the progression of degeneration, yet.[1] However, three types of therapeutic agents that have been clinically proven to alleviate the clinical symptoms of OA are available in the current use. These are disease-modifying drugs such as hyaluronic acid (HA) and glucosamine, non-steroidal anti-inflammatory drugs (NSAIDs), and biological response-regulating drugs and steroids.[2]

In the pathogenesis of OA, there is believed to be an important role of proinflammatory cytokines such

Received: September 26, 2022
Accepted: November 14, 2022
Published online: December 27, 2022

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Citation: Özdemir M, Birinci B, Haberal B, Ok Atılgan A, Demirkale İ. *In vivo* study of the role of hyaluronic acid, N-acetyl cysteine, and deproteinized calf serum on injury-induced cartilage degeneration. Jt Dis Relat Surg 2023;34(1):158-165. doi: 10.52312/jdrs.2023.863

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as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-6, and matrix metalloproteinases (MMPs) responsible for cartilage matrix degradation and downregulation of chondrocyte extracellular matrix (ECM) molecules.[3-5] To date, 23 MMP proteins have been identified in humans, and increased levels of MMP have been shown to be associated with inflammation and degenerative diseases of the joints.[6] Several MMP inhibitors have been proposed for potential clinical use.[7] Similarly, it is also believed that inhibition of IL-6 overexpression in synovial fibroblasts (SFs) is necessary to prevent the progression of OA and to clarify the molecular mechanisms underlying IL-6 overexpression in SF.[8]

Experiments using animal models is a widely used method, often used to identify therapeutic drugs and biological markers as a primary and alternative tool for the study of human OA.[9] One such example of this modelling is the study by Kaneko et al.,[10] in which reactive oxygen species (ROS) and over-induced inflammatory cytokine expression (MMP-13) accumulated in chondrocytes as a result of mechanical stress were inhibited by the use of antioxidant N-acetyl cysteine (NAC). In another animal experiment, Şahin et al.[11] investigated the effectiveness of HA. In this model, they used HA-based acellular matrix scaffold to increase the adhesion, proliferation, and differentiation of stem cells to the defect area in the treatment of osteochondral defects. Another animal model investigated whether deproteinized calf serum, also known as Actovegin® (Takeda Pharmaceutical Company Ltd., Osaka, Japan), could reduce neural injury due to ischemia by benefiting from its positive effects such as improved oxygen utilization and uptake, improved cellular energy metabolism, increased glucose uptake, and decreased ROS production. It is a neuroprotective agent that inhibits the production and inhibition of apoptosis.[12]

Despite recent successful advances in knowledge about disease pathogenesis, treatment still remains challenging. Although many drugs such as strontium ranelate, intra-articular application of platelet-rich plasma, TNF-α blockers, hydroxychloroquine, glucosamine sulfate, chondroitin sulfate, and diacerein have been attempted, there is no preparation which can prevent the progression of OA and joint degeneration yet. In the present study, we hypothesized that NAC and Actovegin® would contribute positively to the amelioration of osteochondral injury, as well as or better than HA. We, therefore, aimed to examine and compare the effects of HA, NAC, and Actovegin® on healing after the creation of cylindrical osteochondral defects in the knee joint of rats.

MATERIALS AND METHODS

A total of 48 male Wistar rats, each weighing an average of 350 g, were randomly separated into four groups of 12. All the rats were kept in plastic cages at a mean temperature of 22° ± 2°C with a 12-h light-dark cycle, and free access to food and water.

An intraperitoneal anesthesia injection was made using 70 mg/kg of ketamine hydrochloride, and 7 mg/kg xylazine, and for postoperative pain control, 0.02 mg/kg of fentanyl was administered subcutaneously. Under anesthesia, arthrotomy was performed with a medial parapatellar approach to the knee joint. In the trochlea region of the femur, a 2-mm-wide and 3-mm-deep osteochondral defect was created in the right-side knee joint of each animal, using a 2-mm drill bit, as described by Meng et al. (Figure 1).[13] The knees of the rats in Group 1 (n=12) were injected with 1 mL saline alone. In Group 2 (n=12), 1 mL of Actovegin® was injected to the knees, in Group 3 (n=12), 1 mL of NAC

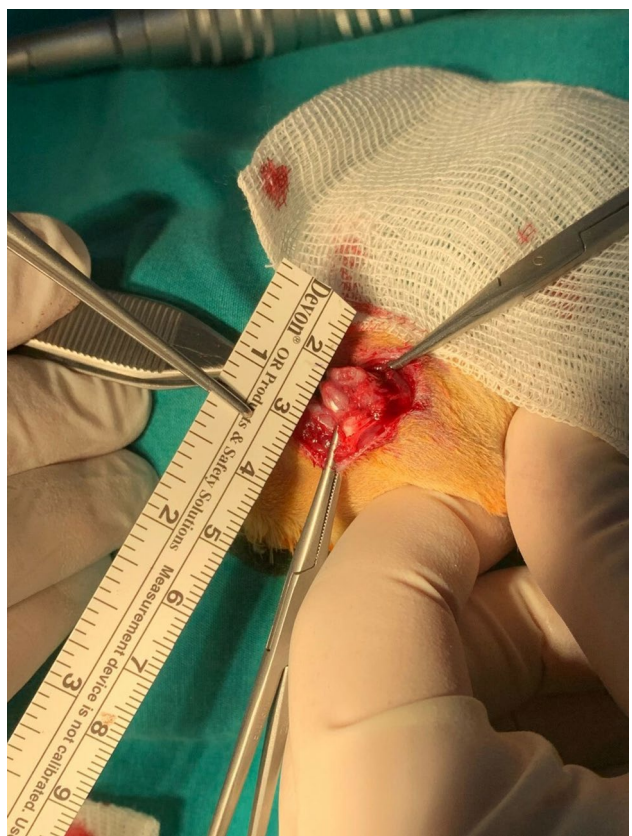


FIGURE 1. Cartilage defect in the femoral condyle of the knee joint.

(Asist.® 300 mg/3 mL 10%, Bilim Pharmaceuticals, Istanbul, Turkey), and in Group 4 (n=12), 1 mL of HA (Prostrolane®, Intraline Pharma Health, Istanbul, Türkiye). A single dose of each drug was administered to the rats, and no further treatment was applied until the day of sacrifice. All the rats were, then, kept in separate cages with no activity restriction. At the end of 12 weeks, all the rats were euthanized and histological examinations were performed.

Following fixation in buffered 10% neutral formaldehyde solution for 48 h, the tissues were decalcified at room temperature, sliced at 3-mm thickness, rinsed in buffer solution and, then, dehydrated in a graded ethanol series, and finally embedded in paraffin blocks. For every tissue sample, four sections of 4 to 5-μm thickness were cut with a standard microtome, then stained with hematoxylin and eosin (H&E), Masson trichrome (MT), and Safranin-O for the evaluation of cartilage regeneration. The tissue samples were examined under a light microscope (Model BX51; Olympus, Tokyo, Japan) by a pathologist blinded to the groups. Histological scoring was performed using the International Cartilage Repair Society (ICRS) scores II (Table I), with a continuous Visual Analog Scale (VAS) and 14 criteria to assess parameters related to chondrocyte phenotype and tissue structure. The VAS system scoring ranges from 0 to 100 (worst to ideal) marked on a 100-mm line by the assessor.[14]

TABLE I
The International Cartilage Repair Society Scores (ICRS II)

	Histological parameter	Score
1.	Tissue morphology (viewed under polarized light)	0%: Full-thickness collagen fibers 100%: Normal cartilage birefringence
2.	Matrix staining (metachromasia assessed by Safranin O)	0%: No staining 100%: Full metachromasia
3.	Cell morphology	0%: No round/oval cells 100%: Mostly round/oval cells
4.	Chondrocyte clustering (4 or more grouped cells)	0%: Present 100%: Absent
5.	Surface architecture	0%: Delamination, or major irregularity 100%: Smooth surface
6.	Basal integration	0%: No integration 100%: Complete integration
7.	Formation of a tidemark	0%: No calcification front 100%: Tidemark
8	Subchondral bone abnormalities/marrow fibrosis	0%: Abnormal 100%: Normal marrow
9	Inflammation	0%: Present 100%: Absent
10.	Abnormal calcification/ossification	0%: Present 100%: Absent
11.	Vascularization (within the repaired tissue)	0%: Present 100%: Absent
12.	Surface/superficial assessment	0%: Total loss or complete disruption 100%: Resembles intact articular cartilage
13.	Mid/deep zone assessment	0%: Fibrous tissue 100%: Normal hyaline cartilage
14.	Overall assessment	0%: Bad (fibrous tissue) 100%: Good (hyaline cartilage)

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). Conformity of the data to normal distribution was assessed using the Shapiro-Wilk test, and the Levene test was applied to evaluate homogeneity of variances. Continuous variables were expressed in median (25^{th}-75^{th} percentiles), while categorical variables were expressed in number and frequency. Whether the differences among the groups in each component of the ICRS (i.e., ICRS) score were statistically significant was examined using the Kruskal-Wallis test. Comparisons showing statistically significant p values in the Kruskal-Wallis test were applied with the Dunn Bonferroni multiple comparison test to identify from which group the difference originated. A p value of <0.05 was considered statistically significant.

RESULTS

The tissue morphology was statistically significantly better in the HA group than in NAC, Actovegin®, and the control groups (p<0.001). The NAC showed a better tissue morphology compared to the Actovegin® and control groups (Figure 2).

The matrix staining score was higher in the HA group compared to the NAC, Actovegin®, and control groups (p<0.001). The NAC group had a better matrix staining score than the Actovegin® and control groups (Figure 3).

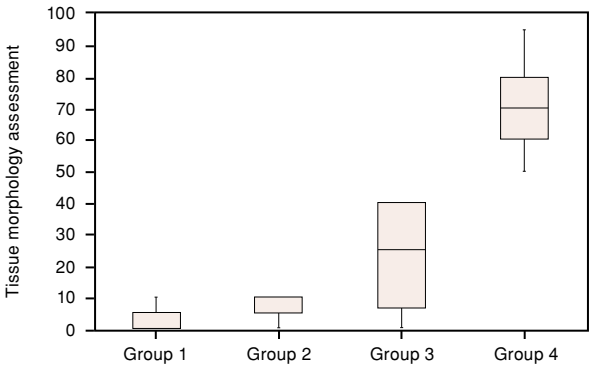


FIGURE 2. Comparisons of tissue morphology assessments of the groups.

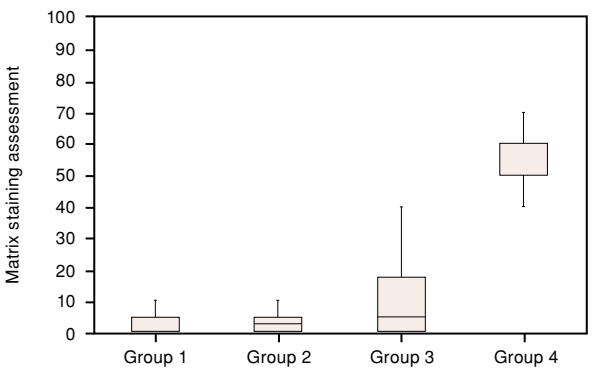


FIGURE 3. Comparisons of matrix staining assessments of the groups.

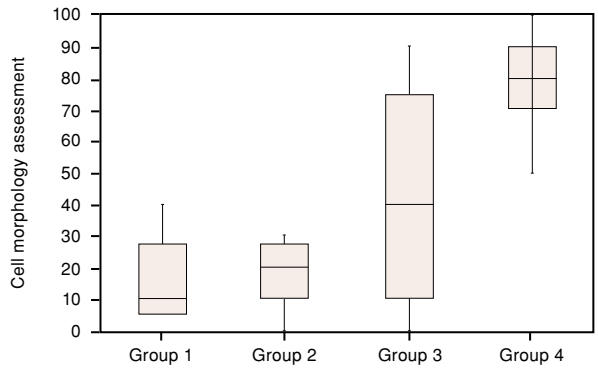


FIGURE 4. Comparisons of cell morphology assessments of the groups.

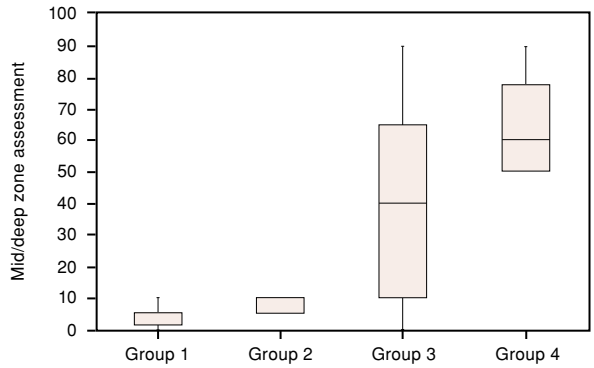


FIGURE 5. Comparisons of mid/deep zone assessments of the groups.

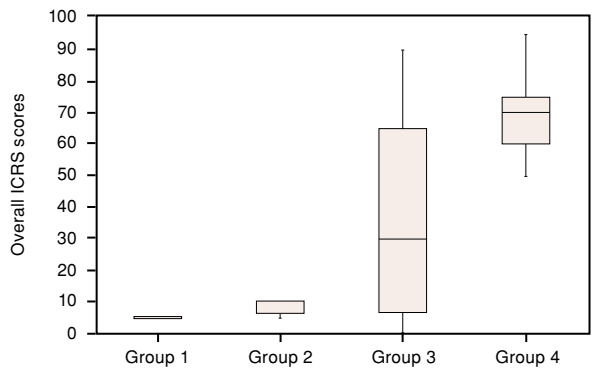


FIGURE 6. Comparisons of overall ICRS assessment of the groups.
ICRS: International Cartilage Repair Society.

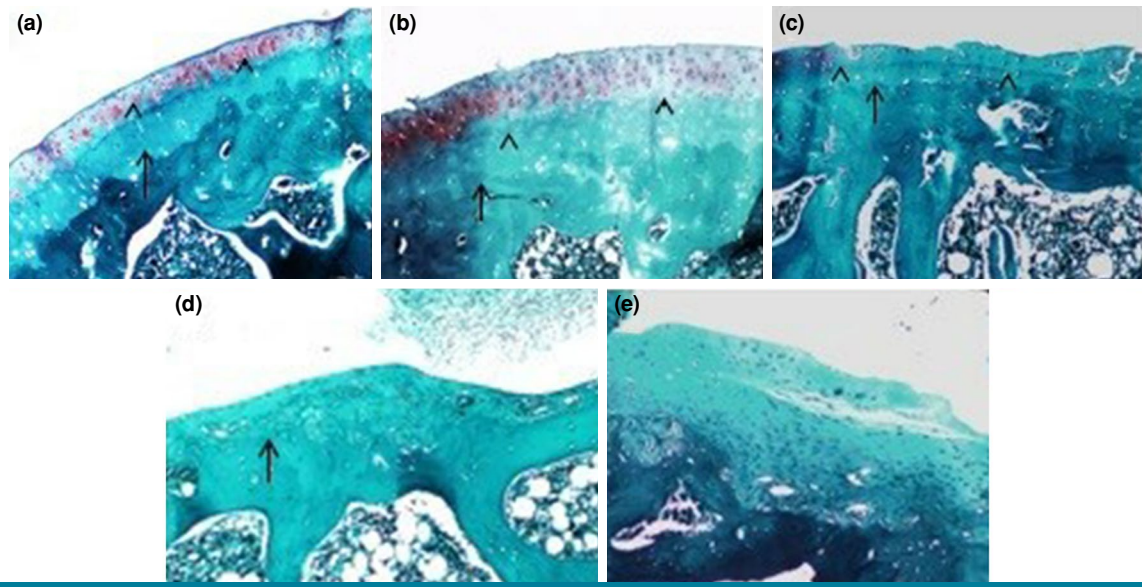


FIGURE 7. Hyaline cartilage formation, matrix production, baseline integration (arrow), and tidemark; (Safranin-O staining, x100 original magnification). **(a)** Cartilage formation, matrix staining, normal baseline integration (arrow), and tidemark (arrowhead) in normal cartilage. **(b)** Good hyaline cartilage formation and matrix production, nearly complete baseline integration and tidemark in the HA group; **(c)** Nearly good hyaline cartilage formation, matrix production, and nearly complete baseline integration and tidemark in the NAC group. **(d)** Poor hyaline cartilage formation and matrix production, focal baseline integration, and no tidemark in the Actovegin® group. **(e)** No hyaline cartilage formation or matrix production, no baseline integration, and no tidemark, only the presence of fibrous tissue in the control group.
HA: Hyaluronic acid; NAC: N-acetyl cysteine.

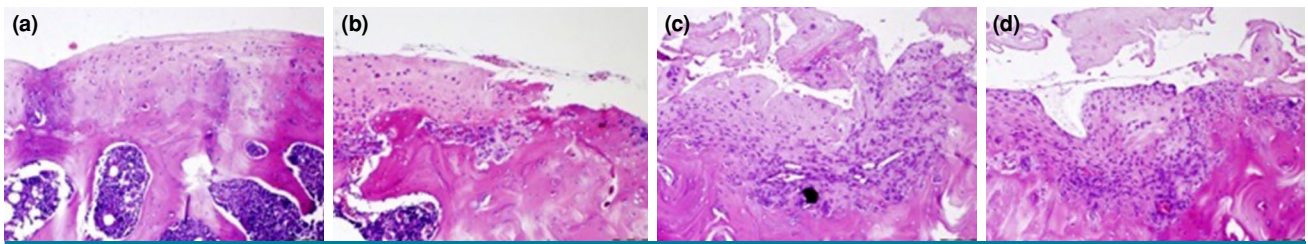


FIGURE 8. Decreased vascularization and inflammation in repair tissue; **(a)** (HA group), **(b)** (N-Acetyl cysteine group), **(c)** (Actovegin® group), and **(d)** (control group) (H&E, ×100 original magnification).
HA: Hyaluronic acid.

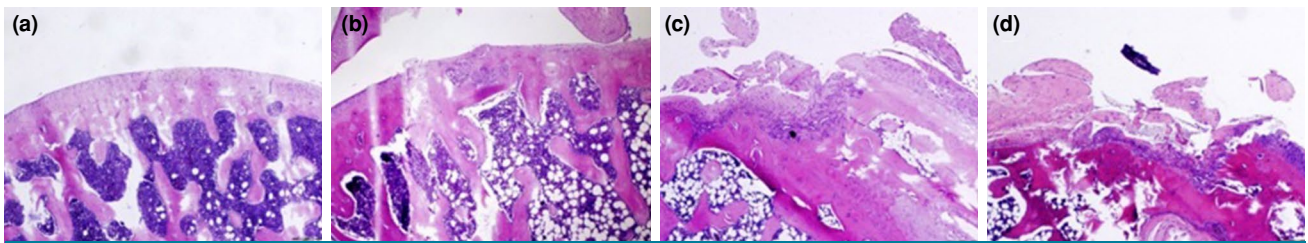


FIGURE 9. A smooth surface resembling intact cartilage seen in the HA group **(a)** and NAC group **(b)** and delamination, loosening, disruption, and surface irregularity in the Actovegin® group **(c)** and control group **(d)** (H&E, ×40 original magnification).
HA: Hyaluronic acid; NAC: N-acetyl cysteine.

The HA group had a significantly better cell morphology than the NAC, Actovegin®, and control groups ($p<0.001$). The NAC had a better cell morphology than the Actovegin® group and the control group (Figure 4).

The mid/deep zone assessment score was higher in the HA group than in the Actovegin® and control groups ($p<0.001$), and also higher in the NAC group than in the control group ($p=0.003$) (Figure 5).

TABLE II
The comparisons of the histological evaluations of the groups

	Group 1		Group 2		Group 3		Group 4		*p*†
	Median	25th-75th percentiles	Median	25th-75th percentiles	Median	25th-75th percentiles	Median	25th-75th percentiles	
Tissue morphology	5.0	0.0-5.0[a]	10.0	5.0-10.0[b]	25.0	6.3-40.0[c]	70.0	60.0-80.0[a,b,c]	**<0.001**
Matrix staining	0.0	0.0-5.0[a]	2.5	0.0-5.0[b]	5.0	0.0-17.5[c]	60.0	50.0-60.0[a,b,c]	**<0.001**
Cell morphology	10.0	5.0-27.5[a]	20.0	10.0-27.5[b]	40.0	10.0-75.0[c]	80.0	70.0-90.0[a,b,c]	**<0.001**
Chondrocyte clustering	100.0	20.0-100.0	0.0	0.0-100.0[b,d]	100.0	100.0-100.0[d]	100.0	100.0-100.0[b]	**<0.001**
Surface architecture	50.0	50.0-77.5[a]	85.0	72.5-90.0	80.0	70.0-90.0	90.0	80.0-93.8[a]	**0.004**
Basal integration	10.0	6.3-17.5[a]	10.0	10.0-10.0[b]	35.0	10.0-60.0	80.0	72.5-93.8[a,b]	**<0.001**
Formation of a tidemark	0.0	0.0-0.0[a]	0.0	0.0-5.0[b]	7.5	0.0-27.5	45.0	10.0-50.0[a,b]	**<0.001**
Subchondral bone abnormalities	0.0	0.0-50.0[a,e]	35.0	0.0-50.0[b]	70.0	50.0-90.0[e]	90.0	82.5-97.5[a,b]	**<0.001**
Inflammation	100.0	100.0-100.0[f]	55.0	10.0-100.0[b,d,f]	100.0	100.0-100.0[d]	100.0	100.0-100.0[b]	**0.014**
Abnormal calcification/ossification	100.0	100.0-100.0	100.0	100.0-100.0	100.0	100.0-100.0	100.0	100.0-100.0	N/A
Vascularization	10.0	0.0-47.5[a]	0.0	0.0-0.0[b,d]	70.0	50.0-90.0[d]	100.0	20.0-100.0[a,b]	**<0.001**
Surface/superficial assessment	0.0	0.0-5.0[a]	0.0	0.0-5.0[b]	40.0	5.0-65.0	80.0	72.5-80.0[a,b]	**<0.001**
Mid/deep zone assessment	5.0	1.3-5.0[a,e]	10.0	5.0-10.0[b]	40.0	10.0-65.0[e]	60.0	50.0-77.5[a,b]	**<0.001**
Overall assessment	5.0	5.0-5.0[a,e]	10.0	6.3-10.0[b]	30.0	6.3-65.0[e]	70.0	60.0-75.0[a,b]	**<0.001**

† Kruskal Wallis test; N/A: Not applicable; [a] Group 1 *vs.* Group 4 ($p<0.05$); [b] Group 2 *vs.* Group 4 ($p<0.05$); [c] Group 3 *vs.* Group 4 ($p<0.05$); [d] Group 2 *vs.* Group 3 ($p<0.05$); [e] Group 1 *vs.* Group 3 ($p<0.05$); [f] Group 1 *vs.* Group 2 ($p=0.032$).

The overall assessment score was significantly higher in the HA group compared to the Actovegin® and control groups ($p<0.001$), and also higher in the NAC group than in the control group ($p=0.027$) (Figure 6).

The chondrocyte clustering score was statistically higher in the HA and NAC groups, compared to the Actovegin® group ($p<0.001$ and $p=0.002$).

The HA group had a better surface architecture than the control group ($p=0.002$). The difference between the groups in respect of surface architecture scores was statistically significant ($p=0.004$).

The baseline integration and formation of tidemark scores of the HA group were statistically higher than in the Actovegin® and control groups ($p<0.001$ and $p<0.001$) (Figure 7).

Compared to the Actovegin® and control groups, the subchondral bone abnormalities score was significantly higher in the HA group ($p<0.001$ and $p=0.002$). The subchondral bone abnormalities score was higher in the NAC group than in the control group ($p=0.026$).

Vascularity was statistically significantly better in the HA group than in the Actovegin® and control groups ($p=0.023$ and $p<0.001$), and in the NAC group compared to the Actovegin® group ($p<0.001$) (Figure 8).

The inflammation score was higher in the HA group, NAC group, and the control group than in the Actovegin® group ($p=0.032$, $p=0.048$, and $p=0.032$, respectively).

The surface/superficial assessment score was statistically higher in the HA group than the Actovegin® and control groups ($p<0.001$ and $p<0.001$) (Figure 9). The comparisons of the histological evaluations of the groups are presented in Table II.

DISCUSSION

Our study results demonstrated that HA was more effective than NAC or Actovegin® in regaining normal cartilage features according to the ICRS II scores. Being a non-sulfated glycosaminoglycan with a low antigenic structure with excellent compatibility, HA is involved in the structure of cartilage in joints and in the composition of articular synovial fluid.[15] The American College of Rheumatology (ACR) and The European Alliance of Associations for Rheumatology (EULAR) guidelines for the management of patients with OA also recommend the use of HA.[16,17]

The NAC group showed more improvement than the control group. This result made NAC treatment researchable, applicable, and promising in cartilage

healing. Although NAC, which is a solid antioxidant, was seen to effectively slow the process of cartilage destruction, decrease synovial inflammation, and reduce inflammatory cytokines, and Actovegin® had a regulatory effect on oxidative metabolism and the redox-balance of cells to produce more oxidized substrates, neither of these were superior to HA.[18-20] Indeed, Actovegin® was not even superior to the control group.

Exogenous HA is absorbed by the synovial tissue 2 h after administration and completely disappears within four days. However, although it disappears in a short time, there are long-term curative effects, as it is able to scavenge reactive oxygen-derived free radicals, inhibit immune complex adhesion to polymorphonuclear cells, inhibit leukocyte and macrophage migration and aggregation, and regulate fibroblast proliferation.[21,22] This study clearly showed that cartilage tissue morphology, the cartilage surface/superficial and mid/deep zone assessment scores, baseline integration and formation of tidemark scores, and vascularity were superior in the HA group, which is consistent with previous findings in the literature.[21,22] According to the existing literature, our study still makes HA an effective and frequently used intra-articular agent. Moreover, it would continue to be used as a benchmark in future studies comparing cartilage healing.

Actovegin® is a biological drug that has been used for the treatment of muscle injuries for more than five decades. Several studies have shown that Actovegin® suppresses the inflammatory effects of oxidative stress on various human organ cells.[23] In an *in vivo* study by Søndergård et al.,[20] Actovegin® treatment resulted in a greater mitochondrial activity in injured cells of the cell wall compared to the control group. Actovegin® has also been shown to produce promising results in clinical applications. In a study of injured professional footballers, Lee et al.[24] reported that Actovegin® therapy reduced the return to play by eight days compared to physical therapy alone. However, in the current study, Actovegin® was not beneficial at any stage of cartilage healing, and no significant difference was found compared to the control group. There could be different reasons for this result. First, as there is still no consensus on the active content and therapeutic dose of Actovegin®, a sufficient level of active content in the joint may not have been provided. Another reason is that there may have been changes in Actovegin®, and this active substance has not yet been detected. Therefore, Actovegin® is not suitable for intra-articular use yet, and there is a need for further preclinical studies to more clearly define the active ingredients and dose.

Roman-Blas et al.[25] reported that NAC inhibited the synthesis of prostaglandin E2 (PGE-2), cyclooxygenase-2 (COX-2), and MMP-13, and the activation of NF-κB induced by IL-1β in synoviocytes in a chondrocyte culture. Based on these findings, it was concluded that NAC could be used in OA treatment. The current study showed that intra-articular administration of NAC accelerated cartilage healing compared to the control group, with an increase in tissue morphology score, cell morphology score, vascularization score, surface/mid and deep zone assessment scores, and particularly the overall assessment score according to the ICRS criteria. Although the histological results of NAC were worse than those of the group administered HA, the mechanism of action and the required intra-articular concentration are still unclear. It has been used clinically as an intra-articular injection, and its widespread use in the future is promising.[26] In a prospective clinical trial with 20 patients, both HA and NAC resulted in comparable reductions in anti-inflammatory cytokines and significant improvements in VAS, total Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores, pain, and functional scores. However, more preclinical studies are needed to establish the required dose and appropriate preparation.

Nonetheless, this study has some limitations, primarily that the optimal therapeutic dose of Actovegin® and NAC was not optimized, and further studies are needed on this subject. Another limitation is that, although the effects on acute cartilage damage were investigated, it should be kept in mind that OA is a chronic process. Finally, this is an experimental animal model study and, therefore, there is a clear need for further randomized-controlled human studies.

In conclusion, HA was the most effective agent in cartilage repair compared with to other groups in this study. In addition, NAC was found to be more effective compared to the control group. Contrary to expectations, Actovegin® was not histologically effective in cartilage healing. There is a need for further studies to investigate the specific mechanisms of the therapeutic effects of these active substances.

Ethics Committee Approval: This study was approved by Başkent University Animal Experimentation Ethics Committee (date: 06.05.2021, no: DA 21/05). National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978).

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

Author Contributions: Idea/concept: M.Ö.; Design: M.Ö., B.H.; Control/supervision: B.B., İ.D.; Data collection and/or processing: M.Ö., B.H., A.O.A.; Analysis and/or interpretation: M.Ö., B.H., B.B., İ.D. A.O.A.; Literature review: M.Ö., B.H., İ.D., A.O.A.; Writing the article: M.Ö., B.H., İ.D.; Critical review: İ.D.; References and fundings: M.Ö., B.H., B.B.; Materials: B.H., A.O.A.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding: The authors received no financial support for the research and/or authorship of this article.

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