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Research Article

Efficacy of hyaluronic acid and conditioned serum in acute rotator cuff tear repair: A rat model study

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ABSTRACT

Objective: This study aimed to compare the effects of local hyaluronic acid (HA) and autologous conditioned serum (ACS) on the repair of acute rotator cuff (RC) tears and their impact on functional outcomes, specifically on running performance.

Methods: In this study, 25 male Wistar Albino rats, aged 16 weeks and weighing 350-400 g, were used. The rats were divided into 4 groups (n=6 per group). Group A was the sham group, and the 3 treatment groups were as follows: group B (primary repair), group C (primary repair+ACS), and group D (primary repair+HA). A standardized procedure was used to create an acute tear-and-repair model of the RC in each treatment group. The rats in group B received no injections. Group C received ACS 24, 48, and 72 h after surgery. Patients in group D received a single dose of HA at the operative site. After a 4-week follow-up period, the rats were subjected to an exercise protocol using a computer-monitored motorized treadmill. For each treadmill run, shocks were recorded as one penalty point using a computer (one point per shock). Subsequently, the rats were sacrificed and bone–tendon healing in each group was assessed histopathologically.

Results: Group A had the lowest penalty points in the final run and the highest penalty points. The best performance among the surgical groups was observed in group D. Groups C and D received fewer penalty points than group B. Group D received fewer penalty points than group C; however, there was no significant difference between them in pairwise comparisons (P=.132). When the statistical analysis of histological parameters was conducted, excluding the sham group, the least inflammation was observed in group D. Pairwise analysis between groups D and B revealed significantly fewer inflammatory cells in group D (P=.026). After pairwise analysis between groups, no significant differences were found in terms of fibroblastic proliferation, neovascularization, or fibrosis.

Conclusion: This study has shown that HA application in an acute RC tear repair model significantly reduced inflammation, accelerated tendon healing, and markedly improved running performance by reducing pain. Additionally, immunohistochemical evaluations revealed that following HA application, collagen fibers were reorganized, forming a regular and tight connective tissue structure.

Introduction

Rotator cuff (RC) tears cause pain and movement restriction in the shoulder joint. The incidence of full-layer RC tears is 6% under the age of 60 years, while the incidence is 30% above 60 years.[1,2] Full-layer RC tears in the young patient population generally develop due to acute trauma; it was found that those with surgical repair have reduced pain, return of strength, and high satisfaction.[3,4]

Hyaluronic acid (HA) is a high molecular weight polysaccharide found in abundant amounts in the extracellular matrix of synovial fluid and soft connective tissue.[5,6] Hyaluronic acid has various effects, such as protecting chondrocytes, providing lubrication, exhibiting anti-inflammatory properties, and preventing adhesion. In the subacromial region, it has been shown to prevent prostaglandin E2 production by inhibiting cyclooxygenase 2 (COX-2) through binding to the CD 44 receptors of fibroblasts.[7,8] It had positive effects on tendon healing by preventing inflammation.[9] In a study evaluating HA application in a rabbit model of complex RC tear, HA was shown to have positive effects on bone–tendon healing and biomechanical strength.[10]

Autologous conditioned serum (ACS) is an autologous blood product rich in endogenous interleukin (IL)-1 Ra used for treatment of joint osteoarthritis, spinal radiculopathy, tendon and muscle injuries.[11] Endogenous interleukin-1 Ra prevents inflammatory cytokine release by inhibition at the receptor level. Autologous conditioned serum was found to be rich in terms of anti-inflammatory cytokines like IL-4, IL-10, and IL-13.[12,13] In a study of a rat Achilles tendon repair model, ACS was shown to have positive effects biomechanically and histochemically on tendon healing.[14]

The aim of our study was to investigate the histochemical and functional effects of HA and ACS, administered locally after RC surgery, on supraspinatus tendon healing. There are no studies comparing the effects of HA and ACS in RC repair models. This gap

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in the literature has influenced the design of our study. Our hypothesis is that HA and ACS will provide better histological and functional outcomes than the primary repair group.

Material and methods

Bülent Ecevit University Animal Experiments Ethics Committee approval was obtained before the study on 06/10/2016 (2016-43-06/10). All procedures were conducted in accordance with the ARRIVE guidelines, ensuring transparency regarding the ethical standards followed during the study.

The study used 25 Wistar Albino rats with mean age 16 weeks and weights from 350 g to 400 g. The sample size was determined by power analysis. Twenty-four rats were randomly divided into 4 groups. Numbered by their tails, they were randomly selected into groups of 3 and kept in cages at 22°C under a 12-hour light–dark cycle, with 12 hours of light and 12 hours of darkness. They had free access to standard rat feed and water.

The groups were determined according to the randomly assigned numbers. Group A was the sham group (n:6), group B was the primary repair group (n:6), group C was the primary repair+ACS group (n:6), and group D was primary repair+HA group (n:6). The reason for not selecting the contralateral shoulder as the control group was to avoid potential tendinopathy that could develop due to overuse while the operated shoulder is healing.

Anesthesia was administered with ketamine (50 mg/kg Ketalar® Eczacıbaşı Istanbul, Türkiye) and xylazine (5 mg/kg Rompun® Bayer Istanbul, Türkiye). After sterile staining and covering, a longitudinal incision was made above the left scapulohumeral joint. The subacromial area was accessed between the fibers of the deltoid muscle. The supraspinatus tendon was cut at the insertion to the bone using a number 11 scalpel with the forearm in supination. With the aid of 0.5 mm wire, after opening a transverse hole in the humerus, 5.0 prolene was passed through the hole and anatomically sutured with the Mason Allen technique (Figure 1). Then the skin was primarily closed with 3.0 prolene and dressed with povidone iodine (Batticon®, Adeka, Türkiye). After surgery, all rats were randomly housed in cages. In the postoperative period, no immobilization method was applied to rat.

Figure 1. Primary repair of supraspinatus tear created with Masson Allen technique.

One rat was sacrificed and approximately 10 cc intracardiac blood was collected. The blood sample was placed in an Orthokine® (Orthogen AG, Düsseldorf, Germany) injector with 21 mm² surface area at 37°C. Samples were centrifuged at 3500 rpm for 10 minutes using a centrifuge (Hermle Labortechnik Z 206 A, Germany) (Figure 2). Following this, the concentrated serum was collected in 0.2-mL quantities and kept at –20°C. The samples were thawed and brought up to room temperature before injection, and they were injected into the surgery area of the RC at 170 μL using a 1-mL insulin injector at 24, 48, and 72 hours after the operation. This dose was calculated based on a previous study relative to the rats' body weight.[15] Group D received 0.1 mL/g Hyalone® (Fidia Fannaceutici S.p.A. Abano Terme (Padua)–Italy) administered to the repair region, as in previous studies.[16] No injection was administered to the primary repair group. A tunnel was drilled into the bone as a standard procedure.

After 4 weeks of monitoring, rats were brought to the computer-monitored motorized treadmill at Ankara University Department of Physiology. The 4 groups underwent an exercise protocol in a dark room, only lit by the computer screen. The exercise protocol was conducted on a 4-band treadmill with adjustable speed, slope, and stimulus. In the literature, treadmills are used for the functional evaluation of acute rat RC repair models.[17] Different exercise protocols can be employed.[18] The aim is to interpret tendon healing through running performance.

All rats were habituated to the treadmill for 5 minutes before every experiment. The system was designed to allow 4 rats to run on the treadmill at the same time. Each of the 4 separate treadmill bands had an electric stimulation unit at one end to encourage continuous running and at the other end to ensure temperature equilibrium (Figure 3). To keep the rats running, a low dose of electrical shock to the foot was used as deterrent. For each treadmill band, the shock was recorded as one punishment point by the computer (1 point per shock). When electrical stimulus was not effective, rats were poked with a small paintbrush and a punishment point was added for each poke. Assessment was performed after 5 days of exercise followed by a final run after 3 days of rest.[19] Before and after the final run, rats were weighed (Sartorius, Germany). The final run was completed in 0-30 minutes, 31-60 minutes, 61-90 minutes, and 91-110 minutes periods for each rat and each punishment point was recorded.

Rats were euthanized. The supraspinatus insertion was dissected using an 11 scalpel, including the bone, for histological examination and analysis. Specimens were delivered to histologists for investigation. The specimens were labeled with the group and the rat's number. After histological processing, the samples were embedded in

Figure 2. Incubation (A) and then centrifuge of blood (B) samples.

paraffin and sections with a thickness of 5 μm were made. These sections were stained with hematoxylin–eosin (HE) to identify the general features of tendon tissue and with the Masson trichrome and Sirus red stains to observe the morphology of the injured tissue in more detail (Figure 4). Photographs for all investigations and findings were taken using a Zeiss AX10 brand photo-microscope device.

Histopathological analyses were scored by 2 histologists who were blinded to the group, using the scoring system established by Shakouri et al[13] on HE-stained preparates. Scoring histopathologically rated distribution and density of inflammatory cells, neovascularization, fibroblastic proliferation, and fibrosis. Inflammatory cells, fibroblastic proliferation, and fibrosis were rated as none [0], mild [1], moderate [2], and pronounced [3]. For neovascularization, if the number of capillaries within a 0.45 micro diameter magnification area was 0-5 it was rated as mild [1], 5-10 capillaries was moderate [2] and more than 10 capillaries was pronounced [3]. Data underwent statistical analyses.

For immunohistochemical analyses, tissue samples were fixed in 10% neutral formalin and then blocked by undergoing routine tissue series procedures. Paraffin blocks prepared for all subjects were cut to 5 μm thickness using a Shandon Finesse 325 brand rotary microtome and tissue sections were placed on positively charged slides. With the aim of researching collagen-1 expression in these sections, the immunohistochemical avidin–biotin complex (ABC) method was used. All preparates were photographed and assessed with a Zeiss brand Axiolab A1 research microscope. Immunohistochemical analyses were not used in any statistical analysis or comparison.

Statistical analyses were performed using SPSS 19.0 (IBM SPSS Corp.; Armonk, NY, USA) program. Descriptive statistics are expressed as arithmetic mean ± SD and median (minimum-maximum). Comparison of groups was conducted the Kruskal–Wallis variance analysis. For 2-way comparison of subgroups in Kruskal–Wallis variance analysis, the Dunn test was performed. All assessments accepted $P < .05$ as significant.

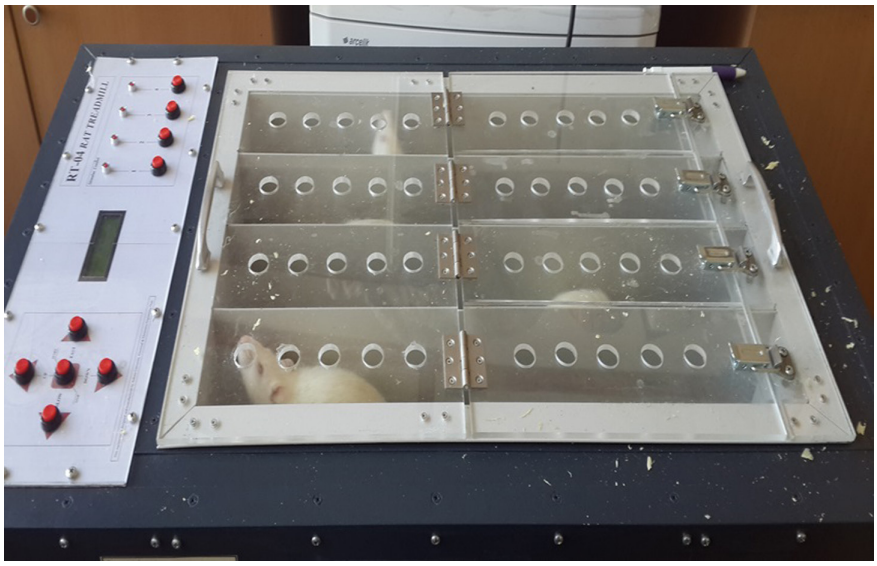

Figure 3. Four-line motorized treadmill with adjustable speed, slope, and stimulus.

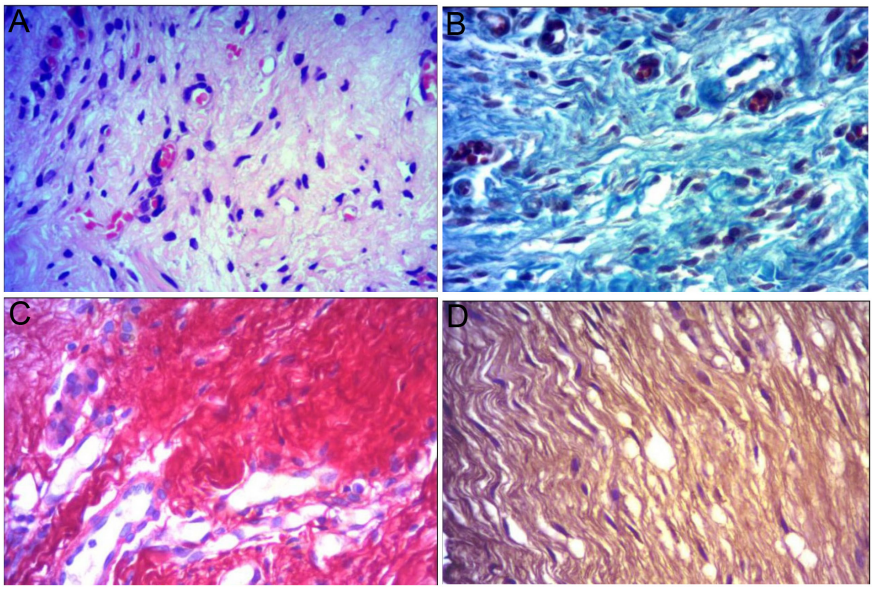

Figure 4. Includes the histological image results of group D with H–E (A), Masson trichrome (B), Sirius red (C), and immunohistochemistry collagen (D).

Bone–tendon healing plays a significant role in the repair of RC tears. The main problematic point in healing is the bone–tendon interface (BTI). In our study, we focused on RC tendon healing. In this respect, it has limitations in the evaluation of a comprehensive RC repair.

Results

Although reductions were observed in weight measurements before and after the final run in all groups, no significant differences were found. Group A had the lowest penalty points in the final run, while group B had the highest.The difference in run punishment points was significant ($P = .001$). The best run performance among surgical groups belonged to group D. Groups C and D received fewer penalty points than group B. Group D received fewer penalty points than group C; however, there was no significant difference in their pairwise comparisons ($P = .132$). While group B received more penalty points than group C, there was no significant difference in their pairwise comparisons (P: .132). A positive difference was found in pairwise comparisons between group D and group B (P: .004). Group D performed worse than Group A but had better punishment points than Group B (Table 1).

Histological analysis used the scoring system of Curtis et al.[20] When statistical analysis was performed by including the sham group, significant increases were identified in terms of histological scores between the groups. The group with the fewest inflammatory cells was group A. When group A was excluded, the group with the fewest inflammatory cells is group D. Group D had significantly fewer inflammatory cells compared to group B ($P = .026$), with no significant difference identified in 2-way analysis with the other groups (Table 2).

Neovascularization increased in all groups when group A was excluded. However, pairwise comparisons between groups did not reveal any significant differences. The least increase was observed in group D (Table 2).

Fibroblastic proliferation was found to be significantly lower in group A compared to the other groups ($P = .002$) in pairwise comparisons between groups. No significant statistical difference was found among the other groups (Table 2).

Fibrosis was found to be significantly lower in group A compared to the other groups ($P = .002$) in pairwise comparisons. No significant statistical difference was found among the other groups. Although it was least observed in group D after group A, there was no significant difference compared to the other groups (Table 2).

Discussion

The main finding of this study is that the application of HA in the acute RC tear repair model significantly reduces inflammation, accelerates tendon healing, and demonstrates the reorganization of collagen fibers into a regular and dense connective tissue structure. As functional evaluation, running performance was significantly improved in the HA-treated group. Neovascularization, fibroblastic proliferation, and fibrosis increased in all groups except the control group. However, no significant difference was observed when comparing the groups with each other.

For surgical treatment of RC tears to be successful, it is necessary to ensure tendon healing. To enhance tendon healing, bone marrow stem cells, growth factor, and a variety of supplements are used.[21,22]

Table 1. Punishment points as a result of final treadmill performance

	Group A control n=6	Group B primary repair n=6	Group C ACS n=6	Group D HA n=6	*P*
Punishment points (min-max)	5 (2-6)	13 (10-15)	10.5 (7-14)	8.5 (6-10)	.001

Group A, control; Group B, primary repair; Group C, primary repair+ACS; Group D, primary repair+HA. ACS, autologous conditioned serum; HA, hyaluronic acid.

Table 2. Histological analysis results for tendon repair tissue

Score (min-max)	Group A control n=6	Group B primary repair n=6	Group C ACS n=6	Group D HA n=6	*P*
Inflammation	**0** (0-0)	**3** (2-3)	**2.5** (2-3)	**2** (1-2)	**<.001**
Neovascularization	**0** (0-0)	**3** (2-3)	**2.5** (2-3)	**2** (1-3)	**.001**
Fibroblastic proliferation	**0** (0-0)	**2** (1-2)	**2** (1-3)	**2** (2-3)	**.001**
Fibrosis	**0** (0-0)	**2** (1-3)	**2** (1-3)	**1.5** (1-2)	**.002**

Group A, control; Group B, primary repair; Group C, primary repair+ACS; Group D, primary repair+HA. ACS, autologous conditioned serum; HA, hyaluronic acid.

The healing issues at the BTI have been a long-standing topic of research that remains unresolved. Healing at the BTI is mediated by mesenchymal and stromal stem cells. Hyaluronic acid enhances chondrogenesis at the BTI by activating mesenchymal stem cells in this region. Additionally, it promotes the differentiation of endogenous stem cells by stimulating the secretion of various cytokines and growth factors. It has been demonstrated that it increases biomechanical strength and enhances chondroid differentiation at the repair site.[10]

platelet rich plasma (PRP) functions as a concentrated source of platelets and contains a range of growth factors and cytokines essential for healing, thereby effectively enhancing bone and soft tissue recovery by accelerating processes such as cell proliferation and angiogenesis. Additionally, PRP has been shown to promote healing at the BTI. Although platelets release a variety of growth factors, transforming growth factor (TGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) are the most relevant in the injury healing cascade.[23] Mesenchymal stem cells migrate to the bone–tendon injury site approximately 5 days after the injury and differentiate into fibroblasts, chondrocytes, and osteoblasts. This process establishes the connection between the tendon and bone. In a study, it was demonstrated that a PRP injection on the seventh postoperative day maintained the optimal concentration of cytokines at the BTI for a longer duration, effectively promoting RC healing.[24]

In a study comparing hyaluronic acid and microfracture applications in a RC repair model, it was demonstrated that the fibrin clot formed after microfracture contains mesenchymal stem cells and growth factors and also enhances vascularization. The combined application of microfracture and HA was found to result in better healing of the BTI and an increase in biomechanical strength.[9]

We aimed to compare the efficacy of HA and ACS administration after acute RC tear. Our study is the first comparing the effects of HA and ACS on tendon healing. According to the histopathological results of our study, the group with HA administration had significantly less inflammation than the primary repair group. There was less inflammation than the group with ACS administration but this was not statistically significant. Neovascularization, fibroblastic proliferation, and fibrosis scores were significantly increased compared to the control group, though there was no superiority among the groups undergoing surgery. Running performance was better in the group with HA administration. The data indicate that HA accelerates tendon healing and remodeling, while also increasing running performance by reducing pain and inflammation (Table 3).

In a RC repair model, HA administration was found to accelerate tendon healing by reducing inflammation and it additionally increased both tendon healing and biomechanical strength when used with microfractures.[9] In our study, HA clearly reduced inflammation and accelerated tendon healing and we found the group administered HA had better running performance.

It was found that HA applied into the rat Achilles tendon caused acute inflammation but increased neovascularization.[25] In our study, HA administration increased neovascularization; however, it did not create a significant difference compared to the other groups.

Osti et al[26] found that HA reduced pain and increased functionality in for the treatment of RC tears. In our study, the results were similar. Rat running performance was better in the group administered HA, which we believe is linked to reduced pain. As a result, the rats were able to complete the runs with fewer punishment points.

Due to the anti-inflammatory cytokines and growth factors contained in ACS, it is used for treatment of muscle injury, low back and sciatica pain and especially for osteoarthritis. Beneficial effects have been supported by randomized controlled studies.[11] In an experimental rat Achilles tendon rupture model, when ACS and saline applications were compared, it was shown that ACS was beneficial for biomechanical and histochemical healing of Achilles tendon repair. Autologous conditioned serum was found to increase tendon strength and accelerate its remodeling.[14] In a study by Majewski et al,[15] it was reported that ACS increased type 1 collagen and decreased type 3 collagen. Thus, it was stated that ACS influences tendon strength by increasing collagen accumulation. However, it was found that the effects on tendon remodeling appeared at the eighth week, and no significant difference was observed in the earlier period. In our study, ACS was observed to have positive effects on run penalty points and tendon healing. However, its effects on run performance and tendon healing were not found to be superior to those of HA. We believe that comparing the results with studies that involve longer follow-up periods for tendon analyses would provide more accurate conclusions.

In an experimental study on rat Achilles tendon healing, ACS was found to provide better histological results than both the PRP and control groups. This aspect is similar to our study. Vascular endothelial growth factor, which plays an important role in early tendon healing by regulating angiogenesis, is present in PRP but not in ACS. Biomechanical results on the 30th day were found to be similar for both groups, and no superiority was demonstrated. This has been attributed to the incomplete process of collagen cross-linking and remodeling.[27]

Another experimental study investigating the effects of PRP and HA on rabbit RC injuries, no superiority was observed between the 2 groups in terms of VEGF and collagen parameters. However, in the group where PRP and HA were combined, an increase in VEGF expression, an improvement in biomechanical indices, and enhanced healing of the BTI were observed, effectively promoting RC healing.[28] Although this study design closely resembles ours, in our study,

Table 3. The analysis results of the pairwise comparisons of the groups

	Inflammation (*P*)	Neovascularization (*P*)	Fibroblastic proliferation (*P*)	Fibrosis (*P*)	Treadmill performance (*P*)
Group A-B	.002	.002	.002	.002	.002
Group A-C	.002	.002	.002	.002	.002
Group A-D	.002	.002	.002	.002	.004
Group B-C	.699	.699	.589	.699	.132
Group B-D	.026	.132	.132	.485	.004
Group C-D	.065	.240	.589	.240	.240

Group A, control; Group B, primary repair; Group C, primary repair+ACS; Group D, primary repair+HA. ACS, autologous conditioned serum; HA, hyaluronic acid.

combined use was not preferred among the groups. Future studies combining ACS and HA will provide significant contributions to the literature.

In our study, the treadmill method was utilized for functional evaluation based on running performance. Acute or chronic RC tear models are commonly preferred for assessment in the literature.[17] Post-repair mechanical stress has been reported to have positive effects on bone–tendon healing.[29] Treadmill was used to create mechanical stress. Moderate-intensity treadmill training can accelerate early-stage healing of the supraspinatus. It provides better BTI healing and enhanced biomechanical properties. This is explained by a significant increase in peripheral neuropeptides involved in tissue healing.[18] Additionally, it has been shown to suppress CX3CL1 release, inhibit local osteoclast differentiation, and promote BTI healing by reducing osteolysis.[30] In our study, the reduced inflammation in the group treated with HA has alleviated pain, thereby leading to an improvement in running performance.

In animal models, gait analysis, passive range of motion, open field tests, staircase tests, and treadmill endurance tests can be used to assess shoulder function following RC tears. While all methods have their advantages and disadvantages, there is no reliable scientific data proving which method is most accurately associated with human RC tears. A full-thickness RC tear may lead to changes in gait analysis, while a partial tear may not cause any significant changes. Passive range of motion may not clearly distinguish between tendon tears or repairs. Open field and staircase tests are more likely to be influenced by emotional responses. The treadmill endurance test may provide data that is not specific to a particular limb.[31] However, animal models continue to be frequently used for functional evaluation. In a study comparing the repair of rat RC tears using suture anchor and transosseous techniques, functional evaluation was performed using gait analysis.[32] In another study conducted on the rat Achilles tendon repair model, functional evaluation was performed using gait analysis, while no biomechanical assessment was carried out.[33] In this respect, it resembles our study.

Immunohistochemical staining is commonly used for evaluating bone–tendon healing. However, changes in the 3-dimensional structure of this region are as important as the differences in its protein composition. Micro-CT is used to evaluate the footprint of the RC repair site. Its resolution is low, and it is utilized to analyze bone volume differences following repair.[34] Three-dimensional images of the humeral head are reconstructed using micro-CT. Additionally, bone mineral density, bone volume/total volume ratio, trabecular number, trabecular thickness, and trabecular separation can be calculated. Another method used to assess the BTI is biomechanical analysis, which measures rupture load, ultimate tensile strength, stiffness, and distance of repaired enthesis on the supraspinatus. The BTI healing is evaluated by comparing these measurements with those of the intact side RCs.[35]

There are some limitations to our study. The first is the inclusion of the minimum number of subjects required for statistical validity. The second is that biomechanical analysis could not be performed. The third is that the supraspinatus repair model was induced after acute injury in rats; however, in humans RC tear may develop with a background of chronic tendinopathy. Also the rapid intrinsic healing in animal models may limit the applicability of these results to human clinical outcomes, where healing rates are slower. The fourth is the absence of a detailed evaluation of the BTI, which plays a crucial role in complete RC healing. Functional assessment can be strengthened by comparing treadmill testing with functional tests such as gait analysis, passive range of motion, open field tests, or staircase tests. Additionally, biomechanical analysis and micro-CT imaging can be used to evaluate BTI. Future studies should address this gap by incorporating bone–tendon healing assessments. It is important to consider this when evaluating the results.

Conclusion

The results of our study indicate that HA significantly accelerates healing in acute tendon tears, reduces inflammation, and markedly decreases pain, thereby improving running performance. Immunohistochemical evaluations have shown that following HA application, collagen fibers reorganize, forming a regular and tight connective tissue structure.

Ethics Committee Approval: This study was approved by the Ethics Committee of Bülent Ecevit University Animal Experiments Local Ethics Committee (Approval no.: 2016-43-06/10, Date: 06/10/2016).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author Contributions: Concept –Y.Ö.; Design – Y.Ö., E.Ş.; Supervision – E.Ş., M.F.A.; Resources – Y.Ö., A.S.; Materials – Y.Ö., İ.M.; Data Collection and/or Processing – Y.Ö., G.B.; Analysis and/or Interpretation – Y.Ö., A.S.; Literature Search – Y.Ö., İ.M.; Writing – Y.Ö., G.B.; Critical Review – Y.Ö., E.Ş.

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