Investigation of the biomechanical and histopathological effects of autologous conditioned serum on healing of Achilles tendon

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Abstract

Objective: The aim of this study was to evaluate the effects of autologous conditioned serum (ACS) on the healing of transected rat Achilles tendons via the assessment of biomechanical and histological parameters.

Methods: The study was conducted on 45 male Sprague–Dawley rats. Five rats were used as donors for ACS preparation. Animals were randomly assigned to the experimental or control group. In both groups, the Achilles tendon was cut transversely and then sutured. In the placebo control and ACS-treated groups, saline or ACS, respectively, was injected into the repair zone three times after surgery. Ten rats from each group (ACS group, n = 20; control group, n = 20) were euthanized at days 15 and 30 after surgery for histopathological (n = 5) and biomechanical (n = 5) testing. The histopathological findings were interpreted using the Bonar and Movin scales. Tendon remodelling was evaluated via the immunohistochemical staining of collagen type 3. Biomechanical effects were assessed by tensile testing.

Results: The Bonar and Movin scale scores were significantly better in the ACS-treated group on both day 15 (p = 0.003 and p = 0.003, respectively) and day 30 (p = 0.005 and p = 0.004, respectively). The immunohistochemical density of collagen type 3 was significantly lower in the ACS-treated group on day 30 (p = 0.018). The type 1/3 collagen ratios of the groups were similar on days 15 and 30, as determined by Sirius Red staining (p = 0.910 and p = 0.133, respectively). In the biomechanical assessment results, the ACS-treated group’s maximum load to failure values were significantly higher on day 15 (p = 0.049).

Conclusion: Injection of ACS had a positive effect on the histopathological healing of rat Achilles tendons on days 15 and 30 and on biomechanical healing on day 15. ACS treatment contributed to lowering the collagen type 3 density by day 30. According to our study, ACS may be favourable for the treatment of human Achilles tendon injuries and tendinopathies.

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Introduction

The Achilles tendon is the strongest, largest tendon in the body. However, it commonly ruptures in middle-aged men who exercise. The incidence of tendon rupture is estimated as 18/10 000. Along with surgical and conservative methods for Achilles tendon rupture treatment, novel treatment methods have been developed due to the establishment of new biological approaches. Numerous articles have shown that individual growth factors are useful for tendon healing in animal models. Such growth factors include vascular endothelial growth factor (VEGF), transforming...
growth factor (TGF) β-1, platelet-derived growth factor, insulin-like growth factor-1, basic fibroblast growth factor (FGF-2) and bone morphogenic proteins (BMP-12 and BMP-13).5–11

Due to mechanical stress in tendons, interleukin 1 (IL-1) expression upregulates and stimulates the release of cytokines, which play a role in inflammation. This pathway is a potential contributor to existing inflammation in tendinopathy, and the inhibition of such a pathway may be useful for tendinopathy treatment.12 IL-1RA is a natural competitive inhibitor of IL-1; by inhibiting the signal pathway, it prevents the inflammatory cascade.

Autologous conditioned serum (ACS), which is used for the treatment of osteoarthritis and similar inflammatory diseases, is an injectable agent rich in endogenous IL-1RA. Meijer et al reported that contact between the blood and small glass spheres allowed a rapid, strong increase in the synthesis of many anti-inflammatory cytokines, including IL-1RA.13 ACS is also rich in anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13; furthermore, its tumour necrosis factor (TNF)-α, FGF-2, VEGF and hepatocyte growth factor (HGF) values are high.14,15

The aim of the present experimental study is to examine whether local ACS treatment implemented after tendon surgery would be useful for healing over a 4-week period. Our hypothesis is that ACS administration will have positive immunohistochemical, histopathological and biomechanical effects on the healing of Achilles tendons.

**Material and methods**

Local ethics committee approval for animal experimentation was obtained on 08.06.2015 (no. 2015/26). Forty-five 12-month-old adult male Sprague–Dawley rats with a mean body weight of 400–450 g, including five rats as ACS donors, were used in the study. The animals were kept five rats to a cage at a temperature of 22°C±1°C under a 12-h: 12-h light–dark cycle. They were fed ad libitum with standard rat feed and had free access to water.

Forty rats were divided into two groups, with group 1 as the control group (n = 20) and group 2 as the ACS group (n = 20). Prophylactic gentamycin (8 mg/kg) was administered to the rats 30 min prior to the surgical procedure. The surgery was initiated with administration of inhaled anaesthesia, which started with 4% isoflurane (Forane) as an induction dose and continued with 2% as a maintenance dose. The posterior side of the right cruris was shaved, iodine was applied under aseptic surgical conditions and the area was covered using a sterile green cover; a standard posterior longitudinal incision of 2 cm was applied, and the Achilles tendon was revealed (Fig. 1). A complete transverse incision was performed using a no. 11 scalpel (Plusmed, Turkey) at 4–5 mm on the proximal side of the Achilles tendon–calcaneus junction. The end of the Achilles tendon was non-traumatically resutured using the modified Kessler method PDO II 4/0 (BOZ, Turkey). The incision site was sutured with four 3/0 propylene (Dogsan, Turkey) sutures placed at equal distances by under sterile conditions, and dressing was applied with povidone iodine (BatticonR, Adeka, Turkey; Fig. 2). No immobilisation method was applied to the rats during the postoperative period.

The five rats included in the donor group were decapitated after the collection of 5–6 cc of blood under anaesthesia. The blood samples collected from the donor group were transferred into special Orthokine (Orthogen AG, Düsseldorf, Germany) injectors containing glass spheres, with a surface area of 21 mm, under a temperature of 37°C. The samples were centrifuged using a centrifugation device (Megafuge, Kendro, Germany) at a rate of 3500 rpm for 10 min; following this, concentrated serum was collected in 0.2-mL quantities and kept at −20°C. The samples were

![Fig. 1. Exploration of the Achilles tendon.](image1)

![Fig. 2. Postoperative Achilles tendon with sutures at equal distances.](image2)

![Fig. 3. Autologous conditioned serum (ACS) administration after surgery.](image3)
**Fig. 4.** Histological effect of autologous conditioned serum (ACS) (a) Control group, day 15 (haematoxylin and eosin [H&E], 100 × magnification). The nuclei in tenocytes are round and slightly enlarged, while the cytoplasm is less detectable. More than two capillary clusters are observed in each of the 10 large magnification areas. Significant dissociation and total loss of alignment are observed in the collagen fibres. (b) Autologous conditioned serum (ACS) group, day 15 (alcian blue staining, 100 × magnification). Abundant mucin presence is noted. (c) ACS group, day 15 (trichrome staining, 100 × magnification). Marginal loss of collagen fibre bundles and fibre dissociation are observed. (d) ACS group, day 30 (Sirius Red staining, 200 × magnification). Type 1/3 collagen staining pattern (type 1 collagen fibres are thicker and red; type 3 collagen is thinner, opaque and green). (e) Control group, day 30 (200 × magnification). The type 3 collagen staining pattern is observed via immunohistochemical staining.
Tendon tissue samples were also removed. For the biomechanical analyses, the left Achilles tendon to prevent the biomechanical measurements from leaving the plantaris tendon in place during the removal of the calcaneus was removed with the femur condyle. Care was taken to ensure that the histopathological preparation processes, the materials were used for evaluation.

Samples were obtained from the tendon repair area for histopathological evaluation. Tendon tissue samples were fixed using a 10% formaldehyde solution and kept in 5% formic acid. After the histopathological preparation processes, the materials were embedded in paraffin blocks and sliced. The sections were stained with haematoxylin and eosin (H&E), Masson’s trichrome and alcian blue (pH 2.5; Fig. 4a–c). Each variable was scored between 0 and 3, as follows: 0, normal; 1, slightly abnormal; 2, abnormal; and 3, markedly abnormal. The total semi-quantitative histological score varied between 0 (normal tendon) and 24 (severest abnormality).17

Table 1
Statistical analysis of the groups according to Bonar scoring.

| Histopathology Bonar scale | Control group | ACS group |
|----------------------------|---------------|-----------|
| Mean ± SD                  | Median (IQR)  | Mean ± SD | Median (IQR)   | p         |
| Day 15                     | 11.0 ± 0.0    | 11 (11–11)| 10.0 ± 0.0    | 10 (10–10) | 0.003⁴     |
| Day 30                     | 7.0 ± 0.0     | 7 (7–7)   | 5.6 ± 0.5     | 6 (5–6)    | 0.005⁴     |
| p                          | 0.003         | 0.005     | 0.003         | 0.005      |

a p<0.05.
The Biomechanical results revealed a significantly higher $F_{\text{max}}$ value on day 15 in the ACS group ($p = 0.049$). $F_{\text{max}}$ values on day 30 were similar between groups ($p = 0.127$; Table 5).

### Discussion

Problematic treatment complications are common after Achilles tendon ruptures. Since endogenous repair is not flawless, some symptoms may persist and result in delayed return to exercise. Hence, there is increasing interest in novel treatment methods, including biological approaches, to treat these injuries. ACS treatment has been found to be useful in tendinopathies. In our study, which considered the effects of ACS treatment after Achilles tendon rupture repair, the results demonstrated that ACS was effective in histopathological healing at day 15, and this effect was shown to continue at day 30. However, while the biomechanical effect was significant at day 15, no significance could be shown at day 30. Moreover, while no significant immunohistochemical difference in the collagen 3 ratio was shown at day 15, the ratio was significantly lower at day 30. With such results, using the ACS treatment, we could show a significant effect of the high collagen 1 ratio on tendon stiffness and strength at day 30 at the earliest. ACS treatment increased the expression of collagen 1; however, a quantitatively significant result could be obtained at day 30.

Our findings are in agreement with Majewski et al’s study, which involved biomechanical, histological and immunohistochemical evaluations of the outcomes of ACS treatment administered after Achilles tendon surgery. In their study, which was performed on 80 rats, useful effects of ACS administration on the collagen composition, histological appearance and mechanical strength of tendon regeneration were shown. Majewski et al reported an increase in type 1 collagen and decrease in type 3 collagen in the ACS treatment group. They also found that ACS enhanced the expression of collagen mRNA and collagen deposition, which affect tendon resistance and collagen fibre maturation. Furthermore, they reported that significant effects on tendon remodelling appeared in week 8, although there was no significant difference at week 4. We could not see a significant difference between the ACS and control groups using Sirius Red staining in the present study. Since our study period was 4 weeks long, we think that significant results would be obtained for a longer period. We obtained faster and more significant results using H&E staining. Moreover, we demonstrated significantly better Bonar and Movin scores in the ACS group than in the control group at both days 15 and 30.
Although no immunohistochemical difference in collagen 3 density was observed on day 15, we detected significantly lower levels of collagen 3 on day 30. We think that ACS is effective in bringing about collagen remodelling, but the remodelling outcomes, which present in the late term, are significant at week 4 and later.

Like our findings showing a positive effect of ACS on tendon rupture repair, beneficial effects of ACS have been shown for the treatment of muscle injuries, tendinopathy and osteoarthritis in animal and human studies. The immunohistochemical results we obtained are comparable to the results of one study considering the effect of single-dose ACS on tendon healing in racehorses with tendinopathy. The immunohistochemical analysis of type 1 and type 3 collagen quantities demonstrated an earlier, more permanent healing in tendinopathy of the superficial digital flexor tendon with a single dose of intralesional ACS injection in comparison to placebo control group. Transient flattened morphology of the nuclei of tenocytes in tendons treated with ACS and increase in expression of type 1 collagen are indicators of reduced proliferation and increased differentiation in this cell type. Furthermore, the healing effect of ACS treatment in the experimental group compared with the control group was explained by the early reduction of limping.

The results of the study should be viewed in the context of its limitations. The first limitation is that, due to the experimental design, a minimum number of subjects, sufficient for statistical validity was included in the present study. A second limitation is the lack of clinical and functional results. Finally, the Achilles rupture was created deliberately in the present study, which did not allow demonstration of the presumptive degeneration that occurs before rupture.

Conclusion

Our study findings showed that postoperative administration of ACS is biomechanically and histochemically beneficial for the healing of transected rat Achilles tendons. The biomechanical effects of ACS may enhance tendon strength recovery. The histopathological and immunohistochemical effects may accelerate tendon remodelling. Further experimental and clinical studies for determining dose and period should be conducted. Figs. 1–5.

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