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Effects of human amniotic fluid and membrane in the treatment of Achilles tendon ruptures in locally corticosteroid-induced Achilles tendinosis: An experimental study on rats

İbrahim Çoban M.D.[a], İsmail Safa Satoğlu M.D.[a], Alper Gültekin M.D.[a], Burçin Tuna M.D.[b], Hasan Tatari M.D.[a,*], Mustafa Fidan Ph.D.[c]

[a] *Dokuz Eylül University, School of Medicine, Department of Orthopaedics and Traumatology, Balçova, İzmir, Turkey*
[b] *Dokuz Eylül University, School of Medicine, Department of Pathology, Balçova, İzmir, Turkey*
[c] *Dokuz Eylül University, School of Medicine, Department of Biophysics, Balçova, İzmir, Turkey*

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Abstract

Background: To determine the effects of human amniotic fluid and membrane in the treatment of Achilles tendon ruptures, 72 tendons of 36 Wistar rats were injected with betamethasone sodium phosphate.
Methods: By the end of fourth week, both tendons were tenotomized and repaired, then the samples were divided into three groups. The first group was left untreated after suturing. Human amniotic fluid was injected to the second and amniotic fluid and membrane were both administered to the third group. Twenty-four tendons were scored at the end of the first week, and 24 at the end of the second week histopathologically, and 24 biomechanically at the end of the third week.
Results: There was a significant statistical difference only between the histopathological results of Groups 2 and 3 at the first week.
Conclusions: Human amniotic membrane and fluid do not add anything to the healing process of Achilles tendon ruptures in the early phase.

Keywords: Achilles tendinosis; Human amniotic fluid; Human amniotic membrane; Achilles tendon rupture

1. Introduction

Treated either conservatively or surgically, healing in any tendon following a rupture exists with a scar tissue that is mechanically different from the original [1]. Besides this, a variable degree of adhesion takes place around the healing tissue that interferes with the outcome [2–6]. To overcome this problem, some biochemical agents [2,7–12] were discussed in the literature besides the other treatment modalities. One of these, hyaluronic acid, a natural constituent of the synovial fluid, was demonstrated to promote tendon healing and decrease adhesion formation [2,3,9,12–14] when placed between flexor tendons and their sheaths after tendon repair. Human amniotic fluid obtained by amniocentesis during the second trimester of gestation, which is known to include high molecular weight hyaluronic acid and hyaluronic acid stimulating activator, was also proved to be effective in preventing peritendinous adhesion formation without impairment of healing of flexor tendons of rabbits [4]. Moreover, human amniotic membrane has been demonstrated to be effective on the same entity [15].

In this setting, the purpose of this experimental study was to determine the histopathological and biomechanical effects of human amniotic fluid injection and combined application of human amniotic membrane wrapping and human amniotic fluid injection in the treatment of Achilles tendon ruptures by using the model of locally corticosteroid-induced tendinosis.

* Corresponding author at: Mithatpaşa Cad. No.: 794 D: 2, 35280 Köprü, İzmir, Turkey. Tel.: +90 232 2318010; fax: +90 232 2772277.
E-mail address: hasan.tatari@deu.edu.tr (H. Tatari).

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2. Material and methods

After the approval of the Clinical Research Local Ethics Committee and signing of informed consent forms, amniotic fluid was obtained from the amniocentesis of normal pregnant women who attended the Gynecology and Obstetrics Department and were proved to be seronegative in between the 16th and 24th weeks of their gestation. The fluid was kept at −20 °C and was used within a week.

After the same ethical procedure, the amniotic membrane was obtained from Caearean sections. The amniotic epithelium layer was dissected from the corion layer and washed with sterile saline solution to get rid of blood, embedded in 10% gentamicin sulphate solution at +4 °C and was used in 4 h.

After the approval of the Animal Research Local Ethics Committee, the experiment was carried on 72 Achilles tendons of 36 male Wistar rats weighing between 200 and 250 g. Both tendons were injected with 0.1 ml betamethasone sodium phosphate (Diprospan-Eczacıbaşı, Turkey) intratendinously by the senior author with a 22-gauge needle at 3-day intervals for 4 weeks. The reason of choosing this kind of degeneration model was the findings of the recent experimental studies indicating that tendon and paratenon degeneration can be obtained to some degree with local corticosteroid injections [16,17]. During this period, the rats were kept in individual cages and had freedom of movement and free access to food and water.

The second part of the experiment was started on the 30th day. The surgical procedure was performed under general anesthesia induced by intraperitoneal injection of 80 mg/kg ketamine (Ketalar-Eczacıbaşı, Turkey). After the skin over the tendon was shaved and disinfected with povidone–iodine, a 2-cm incision beginning from the insertion of the tendon to the calcaneus was made. The paratenon was incised carefully and the tendon was demonstrated. The tendon was cut horizontally at a distance of 1 cm from the calcaneal insertion. Then, the rats were divided into three randomized main groups:

Group 1 (24 tendons) (control group): The tendon was primarily repaired by modified Kessler suture with no. 5/0 PDS.

Group 2 (24 tendons): The tendon was primarily repaired by modified Kessler suture with no. 5/0 PDS and 0.3 ml amniotic fluid was injected under the paratenon after it was repaired.

Group 3 (24 tendons): The tendon was primarily repaired by modified Kessler suture with no. 5/0 PDS and amniotic membrane was wrapped around the repaired area of the tendon with no. 6/0 propylene and 0.3 ml amniotic fluid was injected under the paratenon.

Then, each main group was divided into three subgroups so as each subgroup included eight tendons of four rats. Therefore, while 24 tendons were evaluated biomechanically at the end of the third week, 24 were included into the early period (first week) histopathological examination, and the last 24 into the late period (second week) histopathological examination. The distribution of the tendons in the subgroups can be seen in Table 1.

Table 1
Distribution of the operated tendons

	1st week (histopathological)	2nd week (histopathological)	3rd week (biomechanical)
Group 1 (*n*)	8	8	8
Group 2 (*n*)	8	8	8
Group 3 (*n*)	8	8	8

At the end of the operation, the paratenon was repaired with 4/0 vycril, and the skin was closed with 4/0 propylene and the rats had freedom of movement and free access to food and water in their cages.

For prophylaxis, each rat was administered Cefazolin Sodium (50 mg/kg) intramuscularly for 48 h beginning an hour before the operation.

For histopathological analysis, the rats were sacrificed and the tendons and paratenons were excised from the distal insertion site to the musculotendinous junction proximally with maximum care taken not to damage the tissue and the samples were sent to the Pathology Department. They were fixed in 10% formalin for 48 h and embedded in paraffin after tissue processing. The paraffin blocks were cut into 5-μm sections and after staining with hematoxylin & eosin, the sections were evaluated under light microscope using Tang's scale [18] (Table 2).

For biomechanical analysis, the rats were sacrificed at the end of the third week and the tendons and paratenons were excised including the whole gastrosoleus muscle proximally and the calcaneus distally. The samples were protected in saline and the biomechanical tests were done at the same day in Biomechanics Laboratory with the help of two specially designed clamps (Fig. 1). The proximal clamp had a groove to hold the gastrosoleus muscle and was covered with emery not to allow it to come out. The distal clamp had a groove not to damage the calcaneus but to fix it properly. After both screws of both clamps were screwed, traction was applied to the tendon using Shimadzu test machine (AG-I, 10 kN, Japanese) under 5 mm/s velocity until the tendon ruptured.

Table 2
Tang's tendon healing scale

Excellent	Continuity of the tendon is well established; the epitenon is smooth
Good	The intratendinous collagenous bundles healed well; but the epitenon had been destroyed by adhesions
Fair	The intratendinous collagenous bundles are irregularly arranged and partly interrupted by adhesions
Poor	Disconnection of the repair site or repair site is connected to a large extent by granulation or adhesion tissues

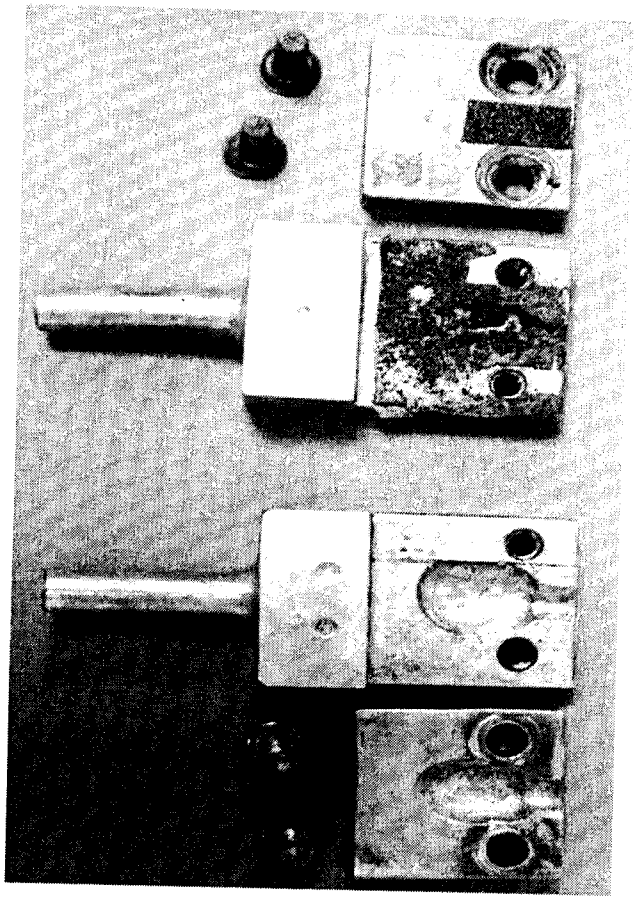

Fig. 1. Specially designed clamps used in the biomechanical tests.

2.1. Statistical analysis

Non-parametric Mann–Whitney *U*-test was used to detect any statistical difference between the histopathological scores of three groups and Wilcoxon test was used to determine any significant difference between the histopathological scores of the first and second weeks of each group. The statistical evaluation of the result of biomechanical study was made by Mann–Whitney *U*-test. SPSS 11.0 software was used for statistical analysis and p values less than 0.05 were considered significant.

3. Results

One of the rats was excluded from the experiment because of re-rupture of the tendon at the follow-up, but none of them suffered from infection at the operation site. All the other rats were in good condition in convenient room temperature.

The mean histopathological scores of the tendons in Groups 1, 2 and 3 and statistical results can be seen in Table 3. According to the statistical analysis evaluated by Wilcoxon test, there was only a significant difference between the scores of the first and second week of Group 1 in favor of the second week ($p = 0.046$) (Fig. 2). The scores of the second week in Group 2 (Fig. 3) were better than the first week, but there was no statistical significance like the correlation between the first and second weeks of Group 3 (Fig. 4) where the scores of the first week was better than the second week. No significant statistical difference could be demonstrated when the first week results of Groups 2 and 3 compared with Group 1 although the mean score of Group 3 was better than the others.

There was a significant difference between the first week scores of Groups 2 and 3 in favor of Group 3 ($p = 0.04$) (Table 4).

When the scores of the second week were compared, no statistical significance could be seen between any groups.

In Table 5, the results and the comparisons of the biomechanical tests done in all three groups can be demonstrated. No significant difference could be shown between the tensile forces of Groups 2 and 3 when compared with Group 1 just like the difference between the ones of Groups 2 and 3 (Table 6).

Table 3
The mean histopathological scores of the tendons in Groups 1, 2 and 3 and statistical results (Wilcoxon test)

Group	1st week (histopathological)	2nd week (histopathological)	p
Group 1	2.375 ± 0.517	2.875 ± 0.353	0.046*
Group 2	2.000 ± 0.755	2.875 ± 0.353	0.053
Group 3	2.857 ± 0.377	2.250 ± 0.886	0.059

* Statistically significant.

Table 4
Statistical significance of differences in group means (Mann–Whitney *U*-test)

Group comparison	p
1 vs 2 (1st week)	0.382
1 vs 3 (1st week)	0.121
2 vs 3 (1st week)	0.040*
1 vs 2 (2nd week)	1.000
1 vs 3 (2nd week)	0.195
2 vs 3 (2nd week)	0.195

* Statistically significant.

Table 5
The results of the biomechanical tests at the end of the third week

Groups	Mean tensile strength
Group 1	42.498 ± 17.80
Group 2	46.561 ± 7.492
Group 3	44.018 ± 9.648

Table 6
The comparisons of groups after the biomechanical tests

Group comparison	p
1 vs 2	0.574
1 vs 3	0.867
2 vs 3	0.694

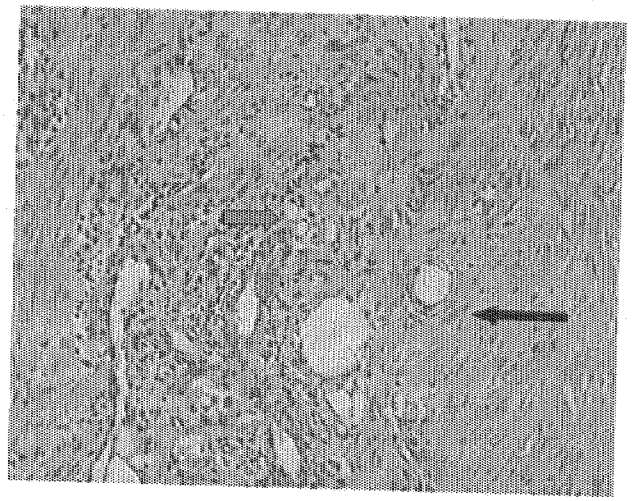

Fig. 2. Histopathological appearance of a specimen, belonging to the control group, that is poor according to Tang's scale. Red arrow shows the inflammation with giant cells where the black one shows the irregular collagen distribution and tenocytes. Hematoxylin & eosin, 20×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

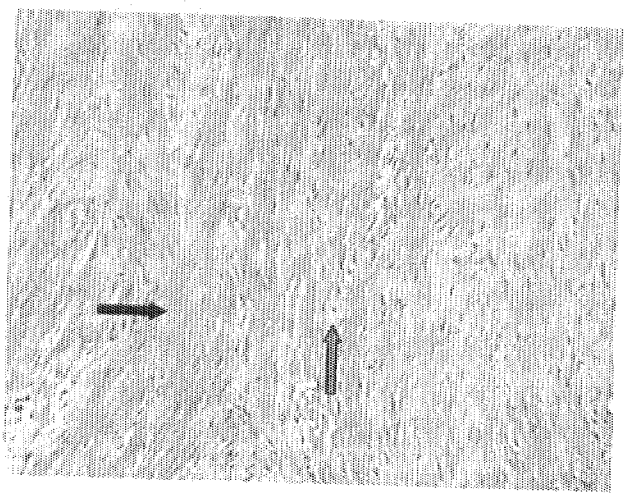

Fig. 4. Histopathological appearance of a specimen, belonging to the amniotic membrane and fluid group, that is good according to Tang's scale. Black arrow demonstrates the collagen distribution and the red one demonstrates the fibroblasts parallel to the collagen bundles. Hematoxylin & eosin, 20×.

4. Discussion

The studies on tendon healing in the literature are mostly focused on the effort to quicken healing and decrease adhesion formation. Platelet concentrate injections have improved Achilles tendon healing after a segment removal [19] and insulin-like growth factor I has decreased time to functional recovery after transection of the tendon [20] in rat models.

Heparin was another biochemical agent that was shown to increase DNA concentration and vascularization in the injured tendon [1].

There is a great interest in amniotic fluid since fetal wound healing found to be rapid without scarring, fibrosis and inflammation in a process that is different from adult wound healing [21,22]. Longaker et al. have established that wound fluid from the fetus has contained high levels of hyaluronic-acid-stimulating activity in contrast to adult wound fluid and [21] and in another study, they have demonstrated that regeneration ability of fetal tissues was due to the stimulation of the mesenchimal cells by the high concentration of hyaluronic acid in the amniotic fluid [23]. After these studies, many others on tendon healing related with hyaluronic acid have been published.

Human amniotic membrane was also found to be effective on the healing of peripheral nerves [24] and on the development of chondral tissue in perichondral flaps [25].

Based on this knowledge, at the beginning of the present study, it was thought that amniotic fluid would be effective on the healing of degenerative tendon rupture because of the rich content of growth and trophic factors in addition to hyaluronic acid [23].

Contrary to the literature, no significant difference could be found in the histopathological results of the group in that amniotic fluid was used in the present study. When we researched the literature, we saw that the tendons which were demonstrated to heal better with hyaluronic acid were the ones those had synovial sheaths [2–4,12,13]. For hyaluronic acid is one of the more important constituents of the synovial

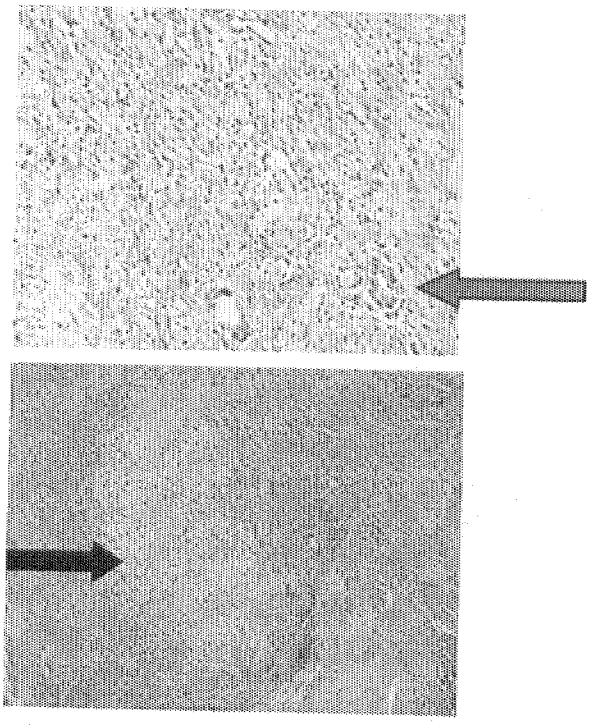

Fig. 3. Histopathological appearances of a specimen, belonging to the amniotic fluid group, that is fair according to Tang's scale. Black arrow shows the granulation tissue and the red one shows inflammatory cell infiltration. Hematoxylin & eosin, 20×.

fluid, only the studies done on the tendons which have synovial sheaths can be compared to the previous ones. The rat's Achilles tendon does not have a synovial sheath [26]; and this can be the only reason of reaching no significant statistical result in the present study. This was also confirmed in another animal study in terms of adhesion formation in a rat Achilles tendon model [6].

The preferred species for the experiments has been rabbit, chicken and rat in the previous studies. Especially, rabbits [4,6,13,14] and chickens [15] have been used in the experimental studies to observe the effects of amniotic fluid and hyaluronic acid on the flexor tendons because they had synovial membranes. Even though they did not have synovial membranes, we preferred rats with the literature support [15–17], because they were easily available in our laboratory.

Another contradictory side of the present study is the number of amniotic fluid application. If hyaluronic acid treatment in tendon degeneration is compared with viscosupplementation in osteoarthritis treatment, it can be supposed that more than one injection can be more effective on the results. We used amiotic fluid once at the operation time as Özgenel et al. [4,27] defined in their model despite the fact that hyaluronic acid was eliminated in a week when applied onto the wound [28] and only one dose did not have any effect on the healing [6]. For the presence of hyaluronic acid on the wound for a long time would be more effective on healing [2,4,14], we think that more than one injection would be more effective.

In the present study, Tang's scale was used to evaluate the histopathological healing of the tendon as we found some previous publications which used that scale [4,27] and some others [6,14].

In biomechanical tests, we had some problems because of the anatomical shape of the tendons. We had to use the muscle part because of the shortness of the tendon. After we covered the proximal clamp with emery, we were able to hold the tendon securely without peeling off the apparatus.

However, there are some limitations in the present study. First of all, it was not possible for us to measure the dosage of hyaluronic acid and its stimulating factor in 0.3 ml amniotic fluid that we have injected in the tendon. We also could not have an opinion about if there are some other proteins in the amniotic fluid and membrane that can quicken healing and decrease adhesion formation in the tendon.

On the other hand, the present experiment deals only with the early phase of the tendon healing process.

5. Conclusion

In this experimental study, we could not reach any significant statistical result between the groups as we expected at the beginning. This can be due to inadequate number of the subjects.

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