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The Effect of Autologous Platelet Rich Plasma in the Treatment of Achilles Tendon Ruptures: An Experimental Study on Rabbits

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Background: Achilles tendon ruptures are characterized by a long recovery period, high re-rupture rate and late return to work. To overcome these difficulties and augment tendon repair, many agents have been used.
Aims: To determine the effect of autologous platelet rich plasma (PRP) in the treatment of Achilles tendon ruptures in rabbits.
Study Design: Animal experimentation.
Methods: The study included 14 New Zealand albino rabbits that were divided randomly into 2 groups, A and B, each containing seven rabbits. On day zero, all 28 Achilles tendons were tenotomized and repaired. In group A, the tendons were injected with PRP post-surgery, whereas those in group B were left untreated. On day 28, the right tendons in both groups were examined histopathologically via both light and electron microscopy, and the left tendons were subjected to biomechanical testing.
Results: The histological and biomechanical findings in both light and electron microscopy in group A were better than those in group B, but the difference was not significant. According to Tang's scale, the mean value in Group A was 3.57, while it was 3.0 in Group B. The mean value of Group A for the length of collagen bands was 48.09 nm while the mean value of Group B was 46.58 nm (p=0.406). In biomechanical tests, although stiffness values were higher in group A, the difference between groups was not significant. In addition, maximum load values did not differ between groups A and B.
Conclusion: PRP had no effect on the healing process 28 days post-Achilles tendon rupture.
Keywords: Achilles tendon, electron microscopy, platelet-rich plasma, tendon healing, tendon rupture

Achilles tendon ruptures are characterized by a long recovery period, high re-rupture rate and late return to work (1-3). Treated either conservatively or surgically, some patients may present with re-ruptures during the late phases of healing, generally between the 4th and 12th weeks (1,4), especially when the tissue quality is poor. As such, some biochemical agents including hyaluronic acid (5), calcitonin (6) and butyric acid (7), and various biomaterials, such as amniotic membrane and fluid (8), porcine intestinal submucosa (9,10), porcine dermal patch (11-13) and human dermal tissue matrix (14) have been used to augment tendon repair.

Researchers have recently exhibited interest in growth factors that were shown to have positive effects on tendon healing (15-19) via acting on target cells present at the site of injury (1). Many studies have reported that autologous platelet-rich-plasma (PRP), derived from a patient's venous blood, includes

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a mixture of growth factors (20-22) and can be used to treat tendon injuries as well as other sports-related disorders, and enhance cellular response to injury (20-22) and angiogenesis (23), despite the lack of evidence-based findings. However, the number of animal studies (1,24-28) that have reported both histopathological and biomechanical findings post-administration of PRP to the Achilles tendon and the number of human trials (3,29-32) are insufficient.

As such, the present experimental study aimed to determine the histopathological and biomechanical effects of autologous PRP on the treatment of Achilles tendon rupture.

MATERIALS AND METHODS

The study protocol was approved by the Local Animal Research Ethics Committee. Following sample dimension analysis, the experiment was performed using 28 Achilles tendons from 14 adult male New Zealand albino rabbits at the Local Research Center. The mean weight of the rabbits was 3520 (range, 2800 to 4200) grams.

PRP preparation, pilot study

To prepare PRP, a pilot study was performed using two rabbits and the method described by Yokota (33). Blood (10 cc) was obtained from the ear vein of each rabbit; however, because the optimum platelet concentration could not be achieved, the samples were centrifuged for a second time at 2500 rpm for five minutes, as described by Efeoglu et al. (34). The upper limit of the automatic hematology apparatus used to determine the platelet count was 1×10^6 mL; therefore, PRP was diluted 4-fold to measure the platelet count. When the rabbit platelet concentration increased 3.5-fold, it was selected for use in the study.

Blood collection and PRP preparation

Blood samples were obtained from the ear vein of all 14 rabbits after the left ear of each was shaved and cleaned with xylol. Blood (10 cc) was collected into EDTA Vacutainer tubes and 0.5 cc of blood was collected into pediatric EDTA Vacutainer tubes via a 24G catheter. Blood in the pediatric tubes was used to measure the platelet counts, whereas the larger tubes, following 5-minute centrifugation, were sent via cold chain to the hematology laboratory to prepare PRP.

Blood samples were first centrifuged at 1500 rpm for ten minutes, and the plasma at the upper level was removed with the buffy coat and separated from the red blood cells. Then, the sample was centrifuged again at 2000 rpm for ten minutes to separate the platelet-poor part in the upper level. The remaining sample was centrifuged again at 2500 rpm for ten minutes and the lower third (approximately 1.5 cc) was considered to be PRP. After adding 0.5 mL of calcium chloride ($CaCl_2$) to PRP for activation, 200 µL was removed from each PRP sample and the platelet count was measured after 4-fold dilution. PRP was then transported to the surgical suite via cold chain.

The platelet count in the blood samples and in PRP were measured and evaluated statistically via the non-parametric Mann-Whitney U test (Table 1) and the difference between two groups, regarding the beginning and injection-time values separately, was not significant ($p>0.05$). In both groups, the platelet count increased about 3.5-fold.

Operative procedure

On day zero, the operative procedure was performed under general anesthesia induced via IM injection of ketamine 40 mg/kg (Ketalar, Eczacıbaşı; İstanbul, Turkey) ten minutes after premedication with 10 mg/kg IM xylazine HCL (Rompun, Bayer; İstanbul, Turkey). The skin over both Achilles tendons was shaved and disinfected with povidone-iodine (Betadine, Drogsan; Ankara, Turkey), followed by a 2 cm incision beginning from the distal insertion of the tendon. Following incision of the paratenon longitudinally, the tendon was cut horizontally 1.5 cm from the calcaneal insertion. Immediately thereafter, all tendons were primarily repaired via modified Kessler sutures using no. 4/0 PDS.

The rabbits were then randomly divided into two groups. In group A (7 rabbits, 14 tendons), after suturing the paratenon

TABLE 1. Platelet counts in both groups in the collected blood samples and PRP

	A	B	p value
Platelet count at the beginning (number/µL)	394±71	413±94	0.623
Platelet count in PRP (number/µL)	1352±562	1586±423	0.465

PRP: platelet rich plasma

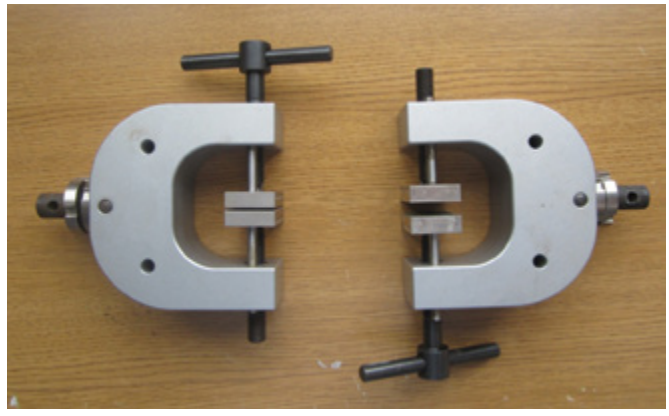

FIG. 1. Specially designed clamps for biomechanical testing

with 4/0 vicryl, PRP was injected onto the repaired tendon, under the paratenon, and the skin was closed using no. 4/0 propylene. In Group B (7 rabbits, 14 tendons), the paratenon and skin were closed, without injecting PRP onto the repaired tendons. Post-surgery, the rabbits were housed in individual cages (66 cm × 72 cm × 47 cm), were free to move, and had access to food and water. Antibiotic prophylaxis was not used. Wound care was performed at 2-day intervals using povidone-iodine (Betadine, Drogsan; Ankara, Turkey).

The right leg of each rabbit in both groups was evaluated histopathologically, whereas each left leg was evaluated biomechanically.

On day 28, the rabbits in both groups were sacrificed under general anesthesia induced via IM injection of 10 mg/kg xylazine HCL (Rompun, Bayer; İstanbul, Turkey) and 40 mg/kg IM ketamine (Ketalar, Eczacıbaşı; İstanbul, Turkey), followed by intracardiac infusion of 5 cc of 7.5% KCL (Basel Kimya; İstanbul, Turkey) via an intravenous catheter needle (Medflon Eastern Medikit Ltd.; Delhi, India).

After sacrifice, the incisions were re-opened, and the tendons and paratenon were excised including the calcaneus distally and the musculotendinous junction proximally, avoiding damage to the healing tissue.

The right Achilles tendons from both groups were split longitudinally into two equal pieces; one was sent to the pathology department in 10% formalin for light microscopic examination and one was sent to the histology and embryology department in glutaraldehyde for electron microscopic examination. The left tendons from both groups were fixed in 10% formalin and subjected to biomechanical testing.

Histopathologic evaluation

Light microscopy

The samples were embedded in paraffin post-tissue processing and the paraffin blocks were cut into serial 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) and were evaluated under a light microscope by a blinded pathologist, according to Tang's tendon healing scale (35, Table 2). For each sample, four sections were examined and the mean value was calculated.

Transmission electron microscopy

Samples were immediately fixed in 2% paraformaldehyde plus glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), and then stored at 4°C for 24 hours. The samples were post-fixed with osmium tetroxide in 0.1 M sodium phosphate buffer for 90 minutes at room temperature, and were subsequently contrasted with 0.5% uranyl acetate in 70% acetone and 1% phosphotungstic acid overnight at 4°C. The tissues were processed using routine procedures and embedded in araldite. After semi-thin sections were taken with an ultratome (Leica Microsystems; Wetzlar, Germany), ultrathin (<0.1 μm) sections were mounted on grids and analyzed using a transmission electron microscope (TEM, Carl Zeiss Libra 120 EFTEM; Stuttgart, Germany).

TABLE 2. Tang tendon healing scale

Tendon healing scale	
Excellent	Continuity of 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

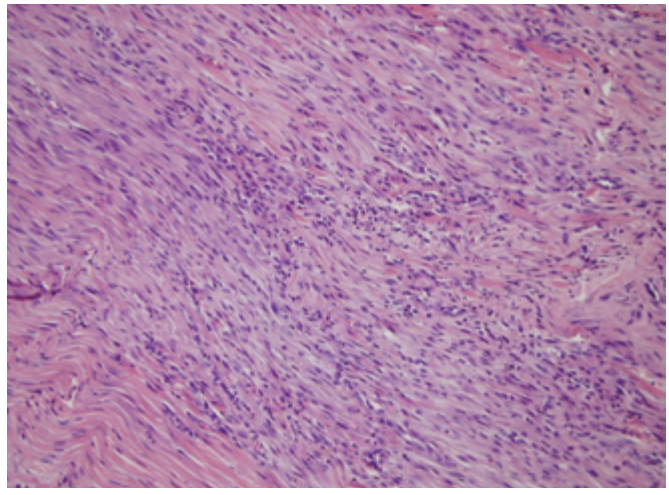

FIG. 2. Group A. Fibrosis, foreign body multinuclear giant cells, lymphocyte and polymorph nuclear leucocytes (PNLs) containing inflammation, H&E, X200 (Tang Phase 3)

Histomorphometry

Digital image analysis software (UTHSCSA ImageTool v.3.0; San Antonio, TX, USA) was used for image processing. The diameter of each collagen band was measured from transverse sections. Morphometric measurements were compared between the two groups.

Biomechanical testing

The Biomechanics Laboratory of the institute was used for biomechanical tests and the tendons were subjected to tensile testing in an axial tensile testing machine (AG-I 10 Kn; Shimadzu, Japan) using specially designed clamps (Figure 1).

TABLE 3. Histological scores of the tendons according to Tang's scale

Groups	Tendon number	Tang's scale
Group A	1	4
	2	4
	3	3
	4	4
	5	4
	6	3
	7	3
Group B	8	4
	9	2
	10	4
	11	2
	12	3
	13	3
	14	3

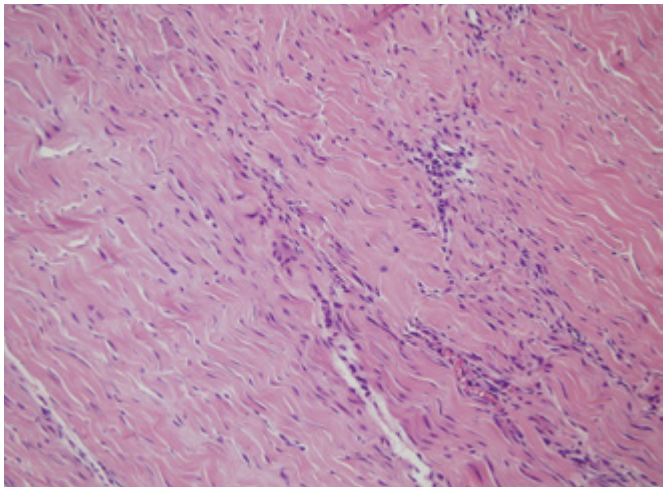

FIG. 3. Group B. Fibrosis, foreign body multinuclear giant cells, lymphocyte and polymorph nuclear leucocytes (PNLs) containing inflammation, H&E, X200 (Tang Phase 3)

After performing preload at 1 N, in order to not cause a rupture in the healing or adjacent tissue, each tendon underwent tensile testing with a loading speed of 10 mm/min until failure.

The process was continued until the tendon-tendon complex broke down at any point. The maximum tensile strength and stiffness values were recorded.

Statistical analysis

Data were analyzed using SPSS v.11.0 for Windows (SPSS Inc. Released 2003. SPSS for Windows, Version 11.0; Chicago, IL, USA). The non-parametric Mann-Whitney U test was used to detect any statistically significant differences in electron microscopy, histomorphometry, and biomechanical test findings between the two groups, and the chi-square test was used to detect any statistically significant differences in light microscopy findings. The level of statistical significance was set at $p<0.05$.

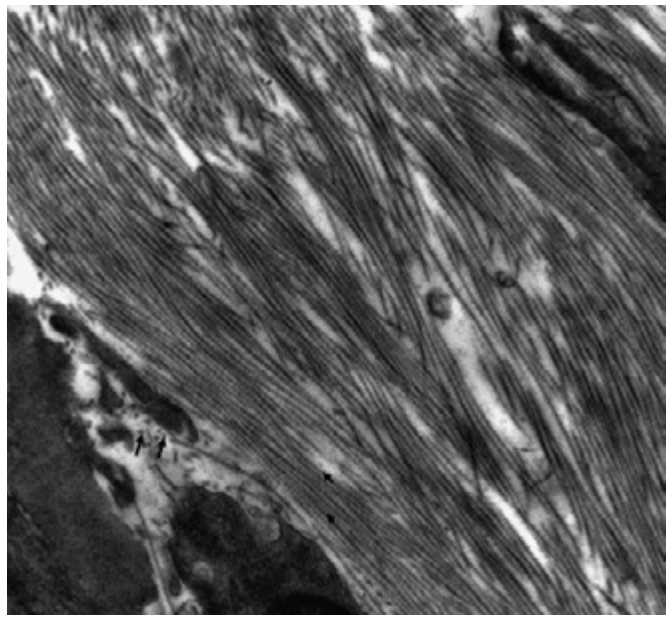

FIG. 4. Microphotograph of a sample from group A shows the tendon cell and transverse (arrows)/longitudinal (arrowheads) collagen bundles (*: nucleus) X5000.

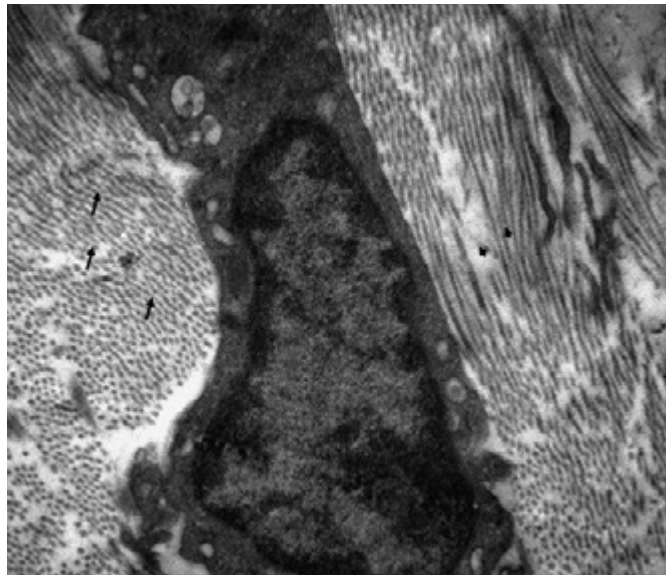

FIG. 5. Microphotograph of a sample from group B shows the tendon cell and transverse (arrows)/longitudinal (arrowheads) collagen bundles (*: nucleus) X5000.

RESULTS

All rabbits were kept comfortable, at a convenient room temperature, and no infection was observed at the operation site.

Light microscopy results

The histological scores of the tendons based on Tang's scale are shown in Table 3. Although group A scores were better

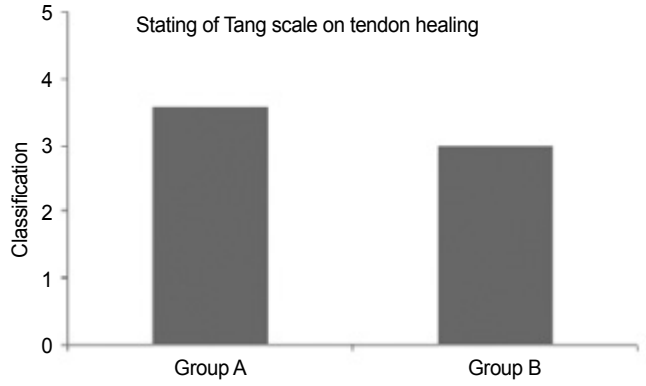

FIG. 6. Comparison of the groups according to Tang's scale

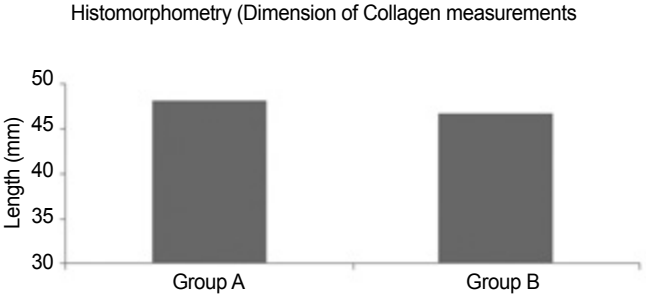

FIG. 7. Diameters of transverse sections of collagen fibers

(Mean value in Group A: 3.7, Group B: 3.0), there was no significant difference between the two groups. A comparison of Tang's scale scores between the two groups is shown in Figure 6. The histological appearance of a sample from each group is shown in Figures 2 and 3.

Electron microscopy results

Fine structure findings

Both groups were evaluated in terms of fine structure based on microphotographs. The nucleus and organelles of the tendon cells were determined to be structurally normal; the difference between groups was not significant. The mean value of Group A among the length of collagen bands was 48.09 nm while the mean value of Group B was 46.58 nm (p=0.406) (Figure 7). Microphotographs from each group are shown in Figures 4 and 5.

Biomechanical findings

Although stiffness values were higher in group A, the difference between groups was not significant (Figure 8). In addition, maximum load values did not differ between groups A and B (Figure 9).

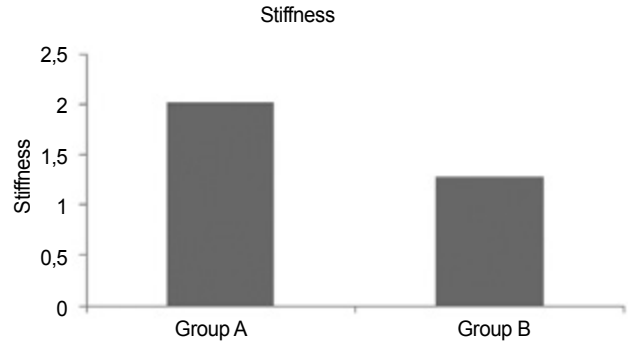

FIG. 8. Comparison of stiffness measurements between the groups

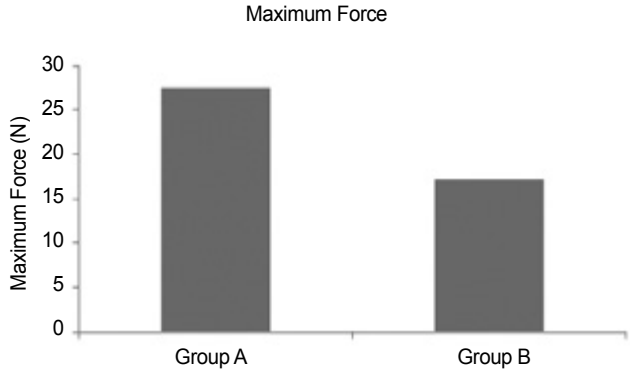

FIG. 9. Comparison of maximum load measurements between the groups

DISCUSSION

The present experimental study aimed to determine both the histopathological and biomechanical aspects of the effect of PRP on Achilles tendon healing post rupture. To the best of our knowledge, PRP (a biomaterial with potentially beneficial effects on the healing of almost every tissue) has been previously studied in terms of the histopathology and biomechanics of Achilles tendon ruptures (25-28), but the present study is the first to use autologous PRP and electron microscopic evaluation.

Kajikawa and Aspenberg (21,25) used donor rats and sacrificed them after collecting their blood and injecting the derived PRP into other rats. Rabbits were used in the present study because we wanted to use autologous blood and PRP. We think that rabbits are more suitable than rats for such research. To the best of our knowledge, the present experimental study is the first to examine tendon healing and PRP obtained via autologous blood.

The literature does include some clinical and experimental studies on the effect of PRP on tendon healing (1-3,21-32). As PRP contains growth factors, it is rational to include an examination of research on growth factors in the present discussion.

Jelinsky et al. (19) reported that rhBMP12 and rhBMP13 accelerated the speed of Achilles tendon repair in a rat model, as reported in other BMP studies (15,16,36). Zhang et al. (17) reported the promising effect of vascular endothelial growth factor on rat Achilles tendon repair. Despite these positive findings, Konerding et al. (2) studied the effect of short-term application of a combination of growth factors on tendon healing in cases of conservative and operative treatment, and reported that the use of growth factor did not result in a significant biomechanical or histological improvement in collagen type ratios.

Nonetheless, we think that growth factors have a positive effect on tendon healing, as mentioned above, but they are expensive and difficult to obtain, and their production requires advanced laboratory conditions and technology, which is why their use in routine clinical treatment remains limited. Additionally, the use of PRP might be considered a more rational approach than use of individual or combinations of growth factors, as it contains all growth factors.

Most experimental studies on PRP and tendons report positive findings. Aspenberg and Virchenko (25) reported the positive effect of platelet concentrate on Achilles tendon healing in rats based on biomechanical testing, as confirmed via histological scoring. They observed improvements in maximum stress as much as 21 d and 28 d post-injury. Their findings were confirmed by Anitua et al. (37), who studied tendon cell cultures. Kajikawa et al. (21) used PRP during the early phase of patellar tendon healing in rats and reported that locally injected PRP was a useful activator of circulation-derived cells for enhancement of the initial tendon healing process.

According to Lyras et al. (23), PRP stimulates vascularization and tissue organization in rabbit Achilles tendons. Kaux et al. (27) histologically and biomechanically examined repaired rat tendons, and concluded that mechanical resistance was significantly higher in the tendons in the PRP group on d 30 post-injury.

Clinical studies on PRP and tendons are fewer in number than experimental studies and the findings are inconsistent. Sanchez et al. (1) used PRP fibrin matrices in 12 athletes with Achilles tendon rupture in a case-control study. They reported that the athletes treated with PRP had better range of motion and returned to sport training sooner than those in the control group. Unfortunately, Schepull et al. (3) conducted a randomized clinical trial and concluded that PRP was not useful in the treatment of Achilles tendon ruptures, as there was no difference in the heel raise index and Achilles Tendon Total Rupture Score between those treated with PRP and those that were not. Moreover, they reported that PRP had a negative effect on tendon healing. In some other recent retrospective and prospective clinical studies, the same results were obtained, showing that adding PRP to the treatment regimen was not clinically and functionally superior (31,32).

In contrast to earlier experimental studies, there was no significant difference in the histopathological and biomechanical evaluation findings between the two groups in the present study, which may have been because of the small number of animals studied. More significant findings might be obtainable using a larger study group, but ethical considerations limited the number of rabbits in each group to seven, which is considered a limitation of the study. The result of this experimental study cannot influence the use of PRP in daily practice, mostly because of the different results of the material when compared to the clinical studies on human beings.

According to Lui et al. (38), cell number decreases to restore the normal cell-to-matrix ratio at the late stage of tendon healing in rats. The researchers reported that this finding was due to apoptosis during the late phase of tendon healing, with a maximum value 28 days post-injury. This finding was confirmed by other studies (39,40), suggesting that tendon strength decreases in most cases after the 28th day post-injury, which is why the tendons were examined 28 days post-injury in the present study.

Sasaki et al. (41) evaluated the Achilles tendon in rats via electron microscopy and reported that it was useful for visualizing the 3-dimensional network of collagen fibers, especially during the first 28 days post-injury, which is another reason why tendons in the present study were evaluated 28 days post-injury. We think that electron microscopy is the best tool for the evaluation of tendon structure, in terms of morphometric measurement and objective findings, as reported by Hazard et al. (42).

In conclusion, although our results showed some differences between the PRP-injected and control groups, these differences were not statistically significant. Future studies with sample sizes adequate to look for significant differences should be considered. However, it can be stated that PRP did not significantly affect the healing process in Achilles tendon ruptures in a rabbit model 28 days post-injury.

Ethics Committee Approval: Ethics committee approval was received for this study from the Local Animal Research Ethics Committee.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.T.; Design - B.Ş., S.G.; Supervision - H.T.; Resource - B.Ç., E.K.; Materials - S.G.; Data Collection &/or Processing - B.Ş., M.A.Ö.; Analysis &/or Interpretation - A.B., S.Ö., N.Ş.; Literature Search - B.Ş., H.T.; Writing - B.Ş., H.T.; Critical Reviews - H.T., H.Ö.

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