EXPERIMENTAL STUDY

Acta Orthop Traumatol Turc 2016;50(3):366–372
doi: 10.3944/AOTT.2015.15.0271

Impact of platelet-rich plasma injection timing on healing of Achilles tendon injury in a rat model

Esra ÇİRCİ[1], Yunus Emre AKMAN[2], Erhan ŞÜKÜR[1], Erol Rüştü BOZKURT[3], Tolga TÜZÜNER[1], Yusuf ÖZTÜRKMEN[1]

[1]*İstanbul Training and Research Hospital, Department of Orthopaedics and Traumatology, İstanbul, Turkey*
[2]*Baltalimanı Bone Diseases Training and Research Hospital, Department of Orthopaedics and Traumatology, İstanbul, Turkey*
[3]*İstanbul Training and Research Hospital, Department of Pathology, İstanbul, Turkey*

Objective: The aim of this study was to evaluate the impact of the timing of platelet-rich plasma (PRP) application on the healing of Achilles tendon injury in a rat model.

Methods: Fifty-four female Sprague-Dawley rats were divided into 4 groups: PRP preparation group (n=6); Achilles tendon tear and 150 μL of PRP received on the day following the injury (PRP day 0, group 1, n=16); Achilles tendon tear and 150 μL of PRP received on the third day following injury (day 3 , group 2, n=16); Achilles tendon tear and 150 μL of saline received on the day following injury (PRP day 0, group 3, n=16). Rats were sacrificed at 3 weeks. Consequently, biomechanical and histologic analyses were performed.

Results: According to histological evaluation, inflammation, fibroblast density, epitenon thickness, and collagen fiber were significantly higher in group 1 than in group 2 ($p<0.05$). Biomechanical testing results of group 1 and group 2 were inferior to the control group, while the differences were not significant ($p>0.05$).

Conclusion: Based on histological criteria, results of the present study suggest that immediate injection of PRP for tendon injury improves tendon healing in rats. Although the use of PRP is well recognized in orthopedic surgery, we aimed to highlight the importance of immediate application of PRP for acute tendon injury.

Keywords: Achilles tendon; impact of injection timing; platelet-rich plasma; rat; tendon healing.

Tendon injury is a very common and restricting condition that results in impairment of quality of life. In most circumstances, it can affect personal and professional activities.[1,2]

Tendon tissue mainly consists of type I collagen fibers, elastic fibers, tenocytes, and water. Histopathological evaluation demonstrates disorganized and weakened collagen fibers, mucoid or lipoid degeneration, and neo-angiogenesis at the initial phase of tendinosis and throughout the healing process.[3,4]

Several methods to increase autologous healing potential have comprised the use of growth factors to improve cell proliferation, chemotaxis, and angiogenesis, as well as to influence cell differentiation during tendon healing.[5,6]

Correspondence: Esra Çirci, MD. İstanbul Eğitim ve Araştırma Hastanesi, Ortopedi ve Travmatoloji Kliniği, İstanbul, Turkey.
Tel: +90 212 – 360 50 60 e-mail: esracirci@hotmail.com

Submitted: June 08, 2015 **Accepted:** September 29, 2015

Available online at
www.aott.org.tr
doi: 10.3944/AOTT.2015.15.0271
QR (Quick Response) Code

Platelet-rich plasma (PRP) is obtained by centrifuging whole blood, which, when completed, produces a cellular constitute of platelet-enriched plasma. PRP includes numerous growth factors, including insulin-like growth factors 1 and 2, transforming growth factor, vascular endothelial growth factor, fibroblast growth factor, and hepatocyte growth factor. These growth factors are able to directly adhere to the exterior of the cell membrane to activate healing. They stimulate cellular signaling that provokes angiogenesis, cell proliferation, cell differentiation, and matrix formation the healing process.[7]

Although PRP is proposed as a material to facilitate tendon healing,[6] the value of the application is not clearly defined. The application of PRP is well defined in various pathologies, especially in musculoskeletal injuries.[8] However, the impact of PRP application timing following injury has not yet been evaluated.

The aim of this study was to evaluate the impact of the application timing of PRP on the healing of Achilles tendon injury, as demonstrated in a rat model.

Materials and methods

This study was approved by the University Ethical Committee for Experimental Research on Animals and supported by our Hospital Research Fund. Fifty-four adult female Sprague-Dawley rats with a mean age of 12 months and mean weight of 293±37 g were included in this study. Rats were acclimatized to caged laboratory conditions and were allowed to feed on standard diet and water according to ad libitum protocol. Room temperature and humidity were maintained at 20–24 °C and 50–60%, respectively. The light cycle was fixed at 12 hours.

All rats were randomly divided into 4 groups.

In the PRP preparation group (n=6), 6 rats were used in order to prepare PRP.

In group 1 (n=16), right Achilles tendon tears were surgically created, and the rats received a single injection of 150 μL PRP on the day following the injury (PRP day 0).

In group 2 (n=16), right Achilles tendon tears were surgically created, and the rats received a single injection of 150 μL PRP on the third day following the injury (PRP day 3).

In group 3 (n=16), right Achilles tendon tears were surgically created, and received single injection of 150 μL of saline on the day following injury (sham group).

Rats were sacrificed at 3 weeks after surgery. Consequently, biomechanical evaluation and histologic analyses were performed. The study design is shown in Figure 1.

The rats were anesthetized with a combined intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Rats were shaved and aseptically prepared. For surgical exposure of the Achilles tendon, a 1-cm longitudinal midline incision in the skin overlying the tendon and central to the calcaneus insertion was made. Full-thickness tendon cuts were performed transversely 3 mm above the calcaneal insertion in each rats' right Achilles tendon (Figure 2). Free ends were not sutured. The skin was closed with absorbable 3–0 Monocryl sutures (Ethicon, Cornelia, GA, USA).

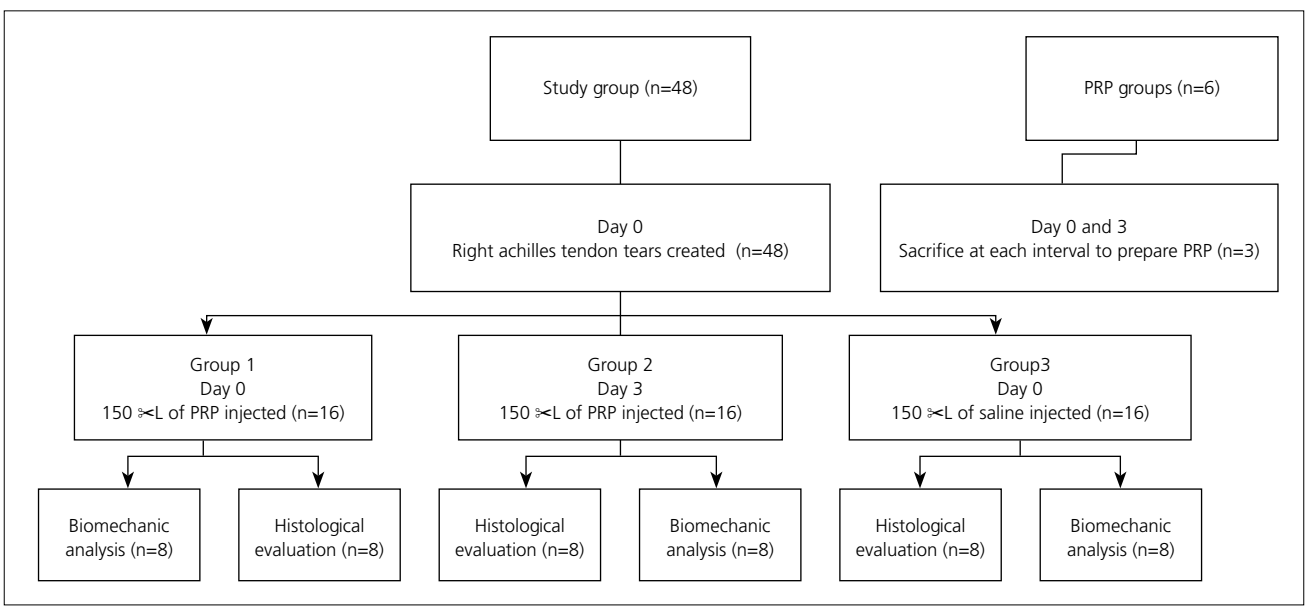

Fig. 1. Study design.

The rats were sacrificed with an intra-peritoneal injection of 200 mg/kg thiopental at 3 weeks after surgery. Each tendon was carefully dissected, and muscle was removed at the musculotendinous junction, leaving the tendinous part and calcaneus intact.

Six rats were sacrificed in order to produce PRP. Three rats were sacrificed in each study day (day 0 and day 3). Whole blood was drawn from rats via an intracardiac puncture after anesthesia. The blood was collected in tubes containing citrate phosphate dextrose. The whole blood was then centrifuged at 1800 rpm for 8 minutes.

All injections were performed within 1 hour of PRP preparation. One hundred fifty μL of PRP solution was drawn into an insulin syringe with a 27-gauge needle. Fufa et al. showed that PRP can be activated by exposure to collagen alone; for this reason, no additive was used for activation prior to injection.[9] The needle was inserted through the skin into the tendon in the region of the cut under sterile conditions. The same amount of sterile saline injection instead of PRP was applied to the sham group (group 3).

Platelet concentration of PRP was determined using an automated cell counter. Aliquots from each section preparation were examined for platelet cell count to confirm that the concentration was at least 3 times higher than the whole blood values.

Tissue samples were fixed in 10% neutral buffered formalin and dehydrated with alcohol. The fixed tissue was managed, inserted in paraffin, and sectioned at 3 μm. Consequently, tissue sections were stained with hematoxylin-eosin and Masson's Trichrome according to standard protocols for assessment of inflammation, fibroblast density, vascularity, epitenon thickness, collagen fiber orientation, chondroid, and osseous metaplasia.

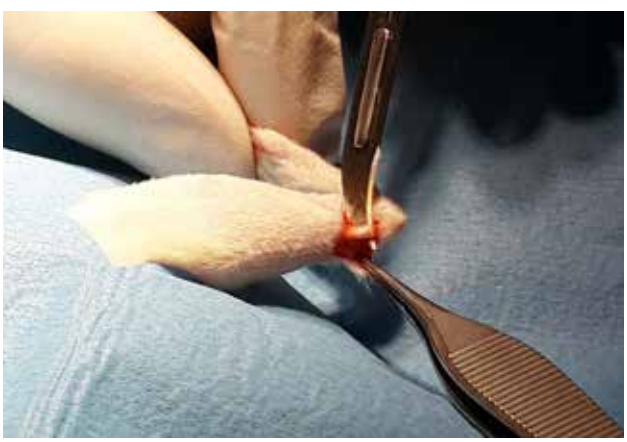

Fig. 2. Full-thickness tendon cuts of the right Achilles tendon. [Color figures can be viewed in the online issue, which is available at www.aott.org.tr]

Inflammation, vascularity, fibroblast density, epitenon thickness, and collagen fiber orientation were graded according to a 4-point grading system suggested by Movin et al.[10] Each histological variable is scored between 0 and 3, with 0 being normal, 1 slightly abnormal (mild), 2 abnormal (moderate), and 3 markedly abnormal (severe). The presence or absence of chondroid and osseous metaplasia was noted.

For each specimen, 2 slides were prepared. Slides were examined by the same pathologist under light microscope (Olympus BX50, Olympus Corporation, Tokyo, Japan) and were read blindly.

Specimens were kept at -20 °C until analysis. Achilles tendons' tensile strength was analyzed using MTS testing machine (MTS Acumen 3000, MTS Systems Corporation, Eden Prairie, MN, USA). Throughout the assessment, room temperature and humidity were controlled at 20±1 °C and 40%, respectively. The fresh frozen specimens were thawed at room temperature. The proximal and distal ends of the Achilles tendon were fixed between 2 pieces of sandpaper and clamped vertically in a custom-made clamp. The system was loaded to 250 N, with a displacement rate of 5 mm/min. The ultimate loads (load at failure) were determined.

Data were evaluated using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). A power analysis using pilot data was performed before beginning the study. This analysis was performed with 95% confidence interval and power of 80% (8 rats were used in each group).

Descriptive statistics were calculated, including frequency, mean and standard deviation, and minimum and maximum values. Chi-square test was used to ascertain the association among groups in terms of histological category. Kruskal-Wallis test was applied for biomechanical analysis. Significance level was set at 0.05.

Results

The tendons were observed to be intact with thickness gross appearance in all rats. No macroscopic difference was noted. Division of the histological grades of the groups is presented in Table 1. Mean histologic sum grades are given in Table 2.

Within each category, chi-square test demonstrated the association between the groups. According to statistical evaluation, inflammation, vascularity, and epitenon thickness were different among group 1, group 2, and group 3 ($p<0.05$). However, fibroblast density, collagen fiber orientation, chondroid, and osseous metaplasia were not different among the groups ($p>0.05$).

Table 1. Distribution of histological grades of the groups.

Histological parameter	PRP-received group–Day 0 (Group 1; n=8)	PRP-received group–Day 3 (Group 2; n=8)	Sham group (Group 2; n=8)
Inflammation grade*			
0	–	3	–
1	4	5	7
2	3	–	1
3	1	–	–
Vascularity grade*			
0	–	–	–
1	–	8	6
2	8	–	2
3	–	–	–
Fibroblast density grade*			
0	–	2	–
1	3	4	1
2	3	2	6
3	2	–	1
Epitenon thickness grade*			
0	–	3	–
1	3	5	7
2	5	–	1
3	–	–	–
Collagen fiber orientation grade*			
0	–	–	–
1	4	8	5
2	4	–	3
3	–	–	–
Chondroid metaplasia*	–	–	2
Osseous metaplasia*	–	2	2

*Values are given in numbers.

Table 2. Summary of mean histological sum grades of the groups.

Histological parameter	PRP-received group–Day 0 (Group 1; n=8)	PRP-received group–Day 3 (Group 2; n=8)	Sham group (Group 2; n=8)	p
Inflammation*	1.6±0.7	0.6±0.5	1.1±0.4	<0.05
Vascularity*	2.0±0.0	1.0±0.0	1.3±0.3	<0.05
Fibroblast density*	1.9±0.8	1.0±0.8	2.0±0.5	>0.05
Epitenon thickness*	1.6±0.5	0.6±0.5	1.1±0.4	<0.05
Collagen fiber*	1.5±0.5	1.0±0.0	1.4±0.5	>0.05
Total tendon histologic grade*	8.6±0.2	4.0±0.2	7.0±0.3	

*Values are given as mean grade±standard deviation.

Inflammation, vascularity, fibroblast density, epitenon thickness, and collagen fiber orientation were found to be different in the comparison of group 1 and group 2 ($p<0.05$). The measurements of these parameters were higher in group 1 than in group 2.

Only vascularity was higher in group 1 (Figure 3) than in group 3 ($p<0.05$). There were no differences in the other parameters between group 1 and group 3 ($p>0.05$).

All histological results were higher in group 3 than in group 2. Fibroblast density was higher in group 3 than in group 2 ($p<0.05$). There were no differences in the other parameters between group 3 and group 2 ($p>0.05$).

All specimens were evaluated successfully throughout biomechanical testing. Tendon failure was observed in the mid-zone of the tendon in all groups (Figure 4).

Biomechanical testing results of group 1 and group 2

Table 3. Results of biomechanical evaluation of the groups.

Biomechanical evaluation	PRP-received group–Day 0 (Group 1; n=8)	PRP-received group–Day 3 (Group 2; n=8)	Sham group (Group 2; n=8)
Load to failure (in newtons)*	53.6±4.0 (49–60)	55.1±4.9 (48–63)	62.9±9.5 (50–78)

*Ultimate load±standard deviation (minimum–maximum).

were inferior to the control group. According to Kruskal-Wallis test, significant difference was not found among the groups ($p>0.05$). Biomechanical evaluation results are reviewed in Table 3.

Discussion

This study demonstrated that immediate injection of PRP in a ruptured tendon provided faster healing when compared with the delayed injection and control groups. There was no macroscopic difference among the groups. Microscopic evaluation of the immediate PRP-injected group revealed more inflammatory cell migration, more vascularity, higher fibroblast density, thicker epitenon, and more collagen fibers compared with late-stage PRP-treated group. Even these extracellular matrix elements , which ensure the healing process in the site of the lesion, demonstrate superior results to the untreated control group than the late-stage PRP-treated group. We emphasize the superior outcome of PRP application in the acute phase in an injured tendon.

In a study by Parafioriti et al. performing PRP injection and evaluating the results by immunostaining and real time PCR, it was demonstrated that PRP improved tendon remodeling in the first week after surgery in rat Achilles tendon. However, after the second, fourth, and sixth postoperative weeks, tendons in the PRP-treated group had no difference when compared with the control group.[6] Lyras et al. concluded that PRP enhances and accelerates via overexpression of IGF-1 in the early phase of the tendon healing process.[11] Application of PRP at an early stage of the tendon disease was considered to be effective.

Although the literature is lacking in clinical prospective randomized studies investigating the usefulness of PRP management modality, Dallaudière et al. suggest that intratendinous injection of PRP improves tendon healing in rats according to clinical, ultrasound imaging, and histological data.[1] Sánchez et al. claimed that surgically repaired Achilles tendon tears treated with the application of platelet-rich fibrin matrix may present enhanced healing of the tendons and functional recovery

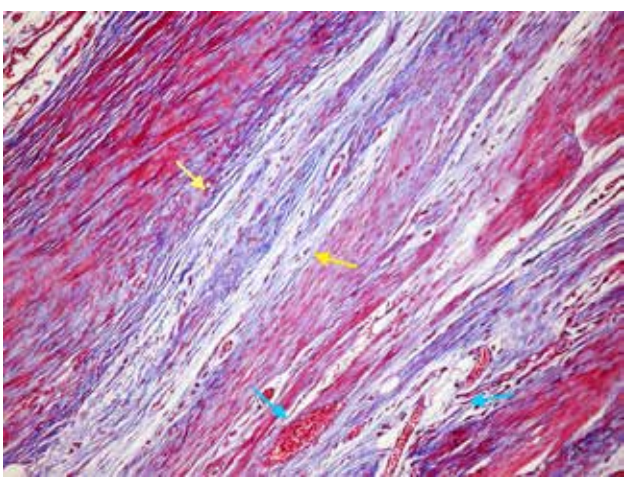

Fig. 3. Microscopic view of group 1. Disorganized collagen fiber orientation (yellow arrows) with increased number of capillaries (blue arrows) is seen (Masson's Trichrome x 20). [Color figures can be viewed in the online issue, which is available at www.aott.org.tr]

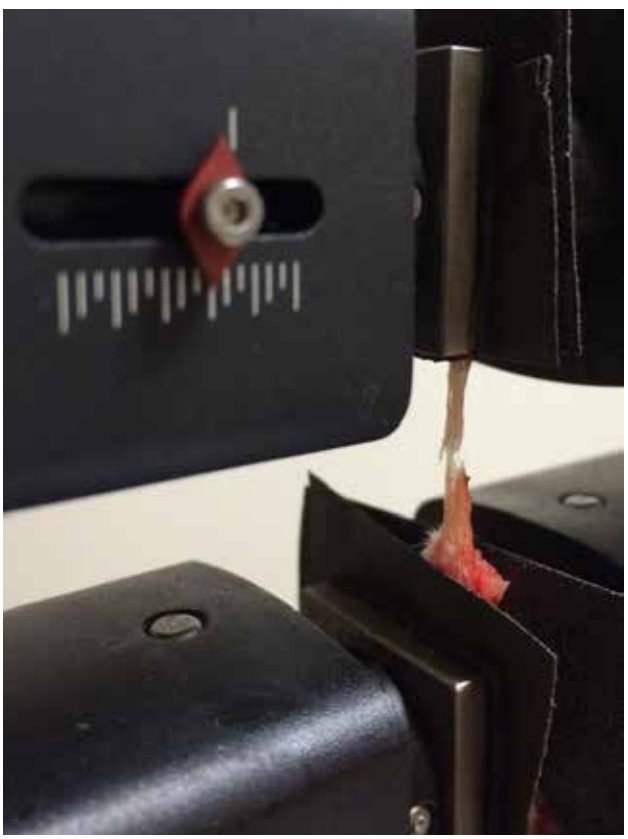

Fig. 4. Biomechanical analysis of the Achilles tendon; tendon visibly ruptured at the mid-zone. [Color figures can be viewed in the online issue, which is available at www.aott.org.tr]

in athletes.[12] On the contrary, De Carli et al. suggested that PRP for the surgical treatment of Achilles tendon rupture does not provide better clinical and functional outcomes.[13] Schepull et al. suggested that PRP is not useful for the treatment of acute human Achilles tendon ruptures regarding mechanical properties and functional outcomes.[14]

Researchers presented that PRP could provoke fibroblast proliferation and lead to an increase in their number. Fibroblasts migrate to the injured region and ensure the secretion of collagen type III, which is further replaced by collagen type I.[15,16] In the present study, better collagen organization and fibroblast density was observed in the early-phase PRP-injected group than late-phase PRP-injected group. However, our tensile data on tendons showed that PRP injection did not significantly enhance the tensile strength of the Achilles tendons at 3 weeks postoperatively. Solchaga et al. claimed that PRP did not improve the mechanical properties in a rat Achilles tendon,[17] whereas Kaux et al. suggested that ultimate Achilles tendon strength is significantly higher in PRP-treated groups. These results were attributed to higher expression of type I collagen.[18] Similarly, other researchers presented that PRP injection caused an enhancement of the mechanical properties.[19–22]

It is well known that the low healing capability of tendons is accompanied with the decline of the blood supply. Tendon healing requires vascularity, a process that is provided by growth factors[23] which can be transferred via PRP. Anitua et al. suggested that injection of pre-clotted plasma within sheep Achilles tendon could increase the cellularity and promote neovascularization.[24] de Mos et al. showed that platelet-rich clots enhanced the cell number in human tenocyte cultures.[25] Lyras et al. reported remarkable increase in angiogenesis when the rats received PRP in the early phase of tendon healing. Furthermore, they suggested that PRP may shorten the duration of healing processes.[22] These results demonstrate that vascularity is significantly better organized in the immediately PRP-injected group than in the control and late PRP-injected groups. The results of the present study showed immediate injection of PRP after tendon injury could provide better neo-vascularization. Delos et al. concluded no functional or histological differences between immediate and delayed application of PRP in muscle healing.[26]

PRP has a high intensity of white blood cells. As the precise function of leukocytes in PRP is not clear, there is little knowledge if an increased level can lead to increased inflammatory cytokine production. Thus, this process may be more of a catabolic rather than anabolic effect,[27] which can be more clearly seen in chronic tendon injuries. Our histological data in the control group showed better scores than the delayed injection group. This result may be attributed to the white blood cells, though further experimental analyses are needed to evaluate the effects of white blood cells in PRP.

There are some limitations of the present study. Evaluations were performed only in the third week following the injury, and immunohistochemical analysis and electron microscopic evaluation were not performed. Additionally, only a single dose of PRP was applied.

In conclusion, based on histological results, the present study demonstrated that the injection of PRP in the early phase of tendon injury improves tendon healing in rats. Although the use of PRP is well recognized in orthopedic surgery, we aimed to highlight the importance of immediate application of PRP for acute tendon injury.

Conflics of Interest: No conflicts declared.

References

1. Dallaudière B, Lempicki M, Pesquer L, Louedec L, Preux PM, Meyer P, et al. Efficacy of intra-tendinous injection of platelet-rich plasma in treating tendinosis: comprehensive assessment of a rat model. Eur Radiol 2013;23:2830–7.
2. Paavola M, Kannus P, Järvinen TA, Khan K, Józsa L, Järvinen M. Achilles tendinopathy. J Bone Joint Surg Am 2002;84-A:2062–76.
3. Virchenko O, Fahlgren A, Rundgren M, Aspenberg P. Early Achilles tendon healing in sheep. Arch Orthop Trauma Surg 2008;128:1001–6.
4. de Vos RJ, Weir A, Tol JL, Verhaar JA, Weinans H, van Schie HT. No effects of PRP on ultrasonographic tendon structure and neovascularisation in chronic midportion Achilles tendinopathy. Br J Sports Med 2011;45:387–92.
5. Maffulli N, Longo UG, Maffulli GD, Khanna A, Denaro V. Achilles tendon ruptures in elite athletes. Foot Ankle Int 2011;32:9–15.
6. Parafioriti A, Armiraglio E, Del Bianco S, Tibalt E, Oliva F, Berardi AC. Single injection of platelet-rich plasma in a rat Achilles tendon tear model. Muscles Ligaments Tendons J 2011;1:41–7.
7. Modarressi A. Platlet Rich Plasma (PRP) Improves Fat Grafting Outcomes. World J Plast Surg 2013;2:6–13.
8. Alsousou J, Thompson M, Hulley P, Noble A, Willett K. The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature. J Bone Joint Surg Br 2009;91:987–96.
9. Fufa D, Shealy B, Jacobson M, Kevy S, Murray MM. Activation of platelet-rich plasma using soluble type I collagen. J Oral Maxillofac Surg 2008;66:684–90.
10. Movin T, Gad A, Reinholt FP, Rolf C. Tendon pathology

in long-standing achillodynia. Biopsy findings in 40 patients. Acta Orthop Scand 1997;68:170–5.
11. Lyras DN, Kazakos K, Agrogiannis G, Verettas D, Kokka A, Kiziridis G, et al. Experimental study of tendon healing early phase: is IGF-1 expression influenced by platelet rich plasma gel? Orthop Traumatol Surg Res 2010;96:381–7.
12. Sánchez M, Anitua E, Azofra J, Andía I, Padilla S, Mujika I. Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. Am J Sports Med 2007;35:245–51.
13. De Carli A, Lanzetti RM, Ciompi A, Lupariello D, Vadalà A, Argento G, et al. Can platelet-rich plasma have a role in Achilles tendon surgical repair? Knee Surg Sports Traumatol Arthrosc 2015.
14. Schepull T, Kvist J, Norrman H, Trinks M, Berlin G, Aspenberg P. Autologous platelets have no effect on the healing of human achilles tendon ruptures: a randomized single-blind study. Am J Sports Med 2011;39:38–47.
15. Graziani F, Cei S, Ducci F, Giuca MR, Donos N, Gabriele M. In vitro effects of different concentration of PRP on primary bone and gingival cell lines. Preliminary results. Minerva Stomatol. 2005;54:15–22.
16. Rajabi H, Sheikhani Shahin H, Norouzian M, Mehrabani D, Dehghani Nazhvani S. The Healing Effects of Aquatic Activities and Allogenic Injection of Platelet-Rich Plasma (PRP) on Injuries of Achilles Tendon in Experimental Rat. World J Plast Surg 2015;4:66–73.
17. Solchaga LA, Bendele A, Shah V, Snel LB, Kestler HK, Dines JS, et al. Comparison of the effect of intra-tendon applications of recombinant human platelet-derived growth factor-BB, platelet-rich plasma, steroids in a rat achilles tendon collagenase model. J Orthop Res 2014;32:145–50.
18. Kaux JF, Drion PV, Colige A, Pascon F, Libertiaux V, Hoffmann A, et al. Effects of platelet-rich plasma (PRP) on the healing of Achilles tendons of rats. Wound Repair Regen 2012;20:748–56.
19. Aspenberg P, Virchenko O. Platelet concentrate injection improves Achilles tendon repair in rats. Acta Orthop Scand 2004;75:93–9.
20. Murray MM, Spindler KP, Abreu E, Muller JA, Nedder A, Kelly M, et al. Collagen-platelet rich plasma hydrogel enhances primary repair of the porcine anterior cruciate ligament. J Orthop Res 2007;25:81–91.
21. Murray MM, Spindler KP, Devin C, Snyder BS, Muller J, Takahashi M, et al. Use of a collagen-platelet rich plasma scaffold to stimulate healing of a central defect in the canine ACL. J Orthop Res 2006;24:820–30.
22. Lyras DN, Kazakos K, Verettas D, Botaitis S, Agrogiannis G, Kokka A, et al. The effect of platelet-rich plasma gel in the early phase of patellar tendon healing. Arch Orthop Trauma Surg 2009;129:1577–82.
23. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
24. Anitua E, Sanchez M, Nurden AT, Zalduendo M, de la Fuente M, Orive G, et al. Autologous fibrin matrices: a potential source of biological mediators that modulate tendon cell activities. J Biomed Mater Res A 2006;77:285–93.
25. de Mos M, van der Windt AE, Jahr H, van Schie HT, Weinans H, Verhaar JA, et al. Can platelet-rich plasma enhance tendon repair? A cell culture study. Am J Sports Med 2008;36:1171–8.
26. Delos D, Leineweber MJ, Chaudhury S, Alzoobaee S, Gao Y, Rodeo SA. The effect of platelet-rich plasma on muscle contusion healing in a rat model. Am J Sports Med 2014;42:2067–74.
27. McCarrel TM, Minas T, Fortier LA. Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy. J Bone Joint Surg Am 2012;94:143(1–8).