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Effect of a single versus serial platelet-rich plasma injection on the healing of acute patellar tendon defect: an experimental study

Mohammad Taher Ghaderi[1], Omid Reza Momenzadeh[2], Fereidoon Mojtahed Jaberi[2], Negar Azarpira[3], Milad Bahari[1] and Mehdi Mohammadpour[1,4*]

Abstract

Background There is no consensus on the frequency and timing of platelet-rich plasma (PRP) injection in tendon healing. We aimed to evaluate the effectiveness of single versus multiple PRP injections in the healing of patellar tendon defects in the experimental model, through histological and biomechanical investigation.

Methods Forty-four male skeletally mature Dutch rabbits were randomly divided into the five study groups (A, B,C, D,E). After creating a longitudinal acute patellar tendon defect on both knees (One-third the width of the patella tendon), the right legs of the rabbits were used as the intervention group and the left legs as the control groups. Animals in groups A, B, and C were euthanized on days 7, 14, and 28, respectively, after the first PRP injection. Animals in group D received the second PRP injection on day 10 and was euthanized on day 14. Animals in group D received the second and third PRP injections on days 10 and 20, respectively, and were euthanized on day 28. The outcomes were evaluated histologically (modification of Movin's Grading) and biomechanically.

Results The inflammatory condition was exaggerated in groups D and E. Load at failure was higher in the non-injected side of groups D and E, while there was no significant difference between the right and left legs of the three groups A, B and C. In other word, groups with a single PRP injection were more resistant to the increasing load compared to the groups with multiple PRP injections.

Conclusions PRP improves tendon healing if injected early after injury, while its injection after the initial phase of injury hampers tendon healing. In addition, a single PRP injection seems to be more effective than multiple PRP injection. Therefore, in cases where PRP injection is indicated for tendon repair, such as acute tendon injury, we recommend using a single PRP injection during tendon repair surgery.

Keywords Platelet-rich plasma, Tendon defect, Patellar tendon, Rabbit model, Knee

*Correspondence:
Mehdi Mohammadpour
mehdi.668491@gmail.com
[1]Department of Orthopedics, Bone and Joint Reconstruction Research Center, School of Medicine, Shafa Yahyaian Educational and Medical Center, Iran University of Medical Sciences, Shafa Orthopedic Hospital, Baharestan Square, Mojahedin-e-Islam St., Tehran 1157637131, Iran
[2]Department of Orthopedics, Bone and Joint Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4]Clinical Research Development Unit, Taleghani Educational Hospital, Abadan University of Medical Sciences, Abadan, Iran

Background

Tendons are anatomical structures transmitting the mechanical force of the muscle to the corresponding bone, thereby ensuring smooth joint movements. [1] Tendon injuries are regarded as common health problems, mainly caused by age-related degeneration or overuse. Healing of tendon injury occurs slowly and barely recovers its original integrity and mechanical strength [2].

According to the available evidence, certain tendons, such as the patellar tendon, are more prone to injury [3]. If left untreated, patellar tendon defects result in disabling anterior knee pain and impaired routine activities, including walking [4]. Nonoperative therapies are the mainstream treatment for acute tendon injury and include different modalities such as anti-inflammatory medications, exercise therapy, extracorporeal shockwave therapy, and corticosteroid injections. Surgical treatment is selected for about 10% of patients who do not respond to nonoperative treatment [5]. However, there is no set of standard time and type of treatment for patellar tendon injury, and a considerable number of patients, particularly athletes, do not return to their pre-treatment level of activities. Anterior knee pain might remain, even 15 years after diagnosis [6]. Therefore, the development of more effective therapeutic strategies to improve the outcome of acute tendon injury is of significant clinical importance.

Platelet-rich plasma (PRP) is referred to as the concentrated autologous platelets in a small volume of plasma and contains significant amounts of various growth factors, including platelet-derived growth factor, insulin-like growth factor-1, epidermal growth factor, transforming growth factor, vascular endothelial growth factor, and etc. These growth factors are important components of tissue regeneration, angiogenesis, and restoration of biomechanical properties [7]. For these properties, PRP has been used for a variety of therapeutic purposes, including the regeneration of tissues with low healing potential, such as cartilage [8].

The safety and efficacy of PRP in the treatment of acute tendon injury have been reported in several studies [9–11]. However, there is no consensus on the effect size of this treatment, as well as on the number of injections required to achieve the best possible outcomes [12]. In this study, we aimed to compare the efficacy of a single versus serial PRP injection in the healing of acute tendon injury in the experimental model. We hypothesized that repeated PRP injections will improve histological and biomechanical quality and speed of tendon repair by increasing local growth factors and reducing local inflammation. The results of this study could help orthopedic surgeons to decide whether or not to use PRP in acute tendon injury, and whether to use single-dose PRP injection or repeated PRP injections.

Materials & methods

Study design

This study was approved by the ethics committee of the Shiraz University of Medical Sciences with the code IR.SUMS.REC.1389.2129. Welfare of the animal used in this research was respected according to the Declaration of Helsinki. All sections of the present study complied with the "ARRIVE guidelines" for reporting in vivo experiments in animal research. Completed "ARRIVE guidelines" checklist was included in Supplementary 1. In this study, Dutch rabbits were obtained from the Animal Center of Shiraz University of Medical Sciences.

In an experimental study, 44 skeletally mature male Dutch rabbits with an average weight of 2–2/5 kg were randomly divided into five study groups (A, B, C, D and E). First, all rabbits were numbered. Rats were randomized into 5 groups using Random allocation software [13] by an epidemiologist. Animals were kept in 12 h–12 h light-dark cycle condition at a constant temperature of 21 °C, in separate cages without activity restrictions. The first three groups (A, B and C) included eight rabbits each, while the next two groups (D and E) each consisted of 10 rabbits. All the rabbits underwent surgery to make a longitudinal acute tendon injury on both knees. The right legs of the rabbits were used to evaluate the effect of PRP injection on the healing of patellar tendon defect, while the left leg received no injection and was used as the control. Animals in groups A, B, and C were euthanized on days 7, 14, and 28, respectively, after the first PRP injection. Animals in group D received the second PRP injection on day 10 and was euthanized on day 14. Animals in group D received the second and third PRP injections on days 10 and 20, respectively, and were euthanized on day 28. After euthanizing, patellar tendons were harvested along with the patella and proximal tibia of both knees and transferred into 0.09 normal saline (Samen Pharmaceutical Company Products, Mashhad, Iran). Half of the extracted tissues were fixed in formalin 10% and sent for histologic examination, and the other half was used for biomechanical investigation. All surgical procedures were done under sterile conditions and by a single orthopedic surgeon.

PRP preparation

After general anesthesia, 8 ml peripheral blood was withdrawn from the rabbit's great aural artery and collected in a sterile tube containing 4 mL of platelet-rich plasma (PRP)anticoagulant.

citrate dextrose solution (ACD-A). Then the tube underwent two rounds of centrifugation. The first centrifugation was at the rate of 4500 rpm to separate red

blood cells. After that, the upper half of the serum layer, which included platelet-poor plasma, was removed, and the lower half, which included the "buffy coat", was collected in another sterile tube. Then, the extracted buffy coat was centrifuged at the rate of 3500 rpm for 10 min. The upper half of the centrifuged mixture was removed. The lower half, which included concentrated platelets, was mixed with 0.5 cc Pt. reagent for 15 min as the procoagulant solution to make a gel. For each rabbit, 1-1.5 cc PRP was prepared. At the end of the PRP preparation procedure, the platelet count of the obtained PRP was measured with a CBC measurement machine (SYSMEX ™ Sysmex America, Inc.) after one-tenth dilution. The platelet count was also checked by pathologist under the optical microscope(light microscope (Nikon, Tokyo, Japan) after smear preparation. The man platelet counts of the PRPs were 1,570,000 per microliter. All PRP preparation steps were performed by an orthopedist and a pathologist. PPR was prepared fresh and injected (utilized in 20˚C to 22˚C).

Surgical procedure

Animals were anesthetized with an intramuscular injection of appropriate doses of ketamine (25.00 mg kg-1; Alfasan, Woerden, The Netherlands and xylazine (5.00 mg kg-1; Alfasan, Woerden, The Netherlands Preoperative infection prophylaxis was done with ampicillin (25 mg/kg) 30 min before the surgery. After shaving the skin hair, the operation was started under sterile conditions. At the palpation of the acute tendon injury, in 70 degrees of knee flexion, the skin was incised 2–3 cm longitudinally (With No. 15 blade) after measurement with a sterile ruler. Paratenon was incised in line with skin incision and left unsevered for later repair. The central 3rd of the patellar tendon, in width, was removed along with its length from the patella to tibial tuberosity in its full thickness. An orthopedic specialist performed all surgeries. The middle 1/3 of the tendon was marked with a colored marker whose tip was 1 mm thick. Two lines were drawn at the central part of the tendon using this marker. A scalpel was used to remove the portion of the tendon between these two lines, which was 1–2 mm in distance [14–16]. So that a defect of 2–3 mm, appropriate to tendon size, was created. Then, prepared PRP gel was applied to the acute tendon injury of the right leg, and the paratenon was closed in layer with simple interrupted sutures (4−0 Vicryl; polyglactin; Ethicon, Johnson & Johnson) so that no leakage of gel could occur. After checking the patella tracking and ensuring complete closure of the paratenon and no PRP exit, the skin was closed in layer by cross mattress using 3/0 monofilament nylon suture (4/0 monofilament nylon (Supa Medical Devices Co., Tehran, Iran). The. No dressing was applied, and no immobilization device was implemented. The same procedure was done on the left side except for the application of PRP gel. Fentanyl skin patch was used to reduce rabbits' pain(12 mg/h). No activity restriction was planned after the operation. The rabbits were visited daily by an animal caretaker for food and water and twice weekly by an orthopedic surgeon for checking their wound and any potential problem. During the experiment, eight rabbits died of gastroenteritis (2 in group A, 1 in group B, 2 in group C, 2 in group D, and one in group E). The possible cause of death was diagnosed gastrointestinal stasis syndrome caused by changing the rabbits' feed to commercial rabbit pellets (Urom-Dordaneh. Feed Mill, Urmia, Iran). No other suspicious cause was observed.

We substituted the lost ones with another eight rabbits that underwent an operation with a similar procedure. The second and third injections were performed with the same procedure.

Sample harvesting

Rabbits were euthanized with intravenous injectionof thiopental sodium overdose (50.00 mg kg-1, Sandoz, GmbH, Kundl, Austria). For histologic examinations, samples were harvested with dissection of the middle 3rd of the tendon from patella proximally and tibial tuberosity distally. Then it was freed from surrounding tissues and immediately transferred into the 10% formalin. For biomechanical analysis, samples were dissected immediately ten block proximally from the quadriceps tendon and distally 5 cm to tibial tuberosity from the tibia. After freeing from surrounding tissue, the samples were wrapped in gauze moisturized with saline 0.09% (Samen Pharmaceutical Company Products, Mashhad, Iran) for biomechanical study. The samples related to the left leg were marked with a (2−0 Vicryl; polyglactin; Ethicon, Johnson & Johnson) suture. Eight weeks after the surgery, rabbits were put down by administering 50.00 mg kg-1 of thiopental sodium (Sandoz, GmbH, Kundl, Austria) through intravenous injection. Tendons from both hind limbs were harvested for biomechanical tests using a random selection of eight rabbits from each group. Sterile gauze soaked in normal saline was used to wrap all the samples. These samples were frozen at -20 °C to prepare for the tensile test.

Histology examination

After embedding the tissues in paraffin blocks, tissue sections were prepared transversely on the axis of the tendon by a pathologist. Six paraffin sections were prepared for each sample and mounted on slides. Three slides were stained with hematoxylin-eosin (H&E) and three with Masson trichrome. H&E slides were used for checking cellularity, fibroblast proliferation; tenocyte proliferation; vascularity, and the healing process in general. H&E

Table 1 Numerical scaling of histological parameters a modification of Movin's grading [36]

Grade	Cellularity	Vascularity	Collagen synthesis	Healing
1	4–7 inflammatory cell	1–2 vessels per site	Disorganized (< 10% parallel to each other) collagen fiber with the presence of fat cell	Aggregation of inflammatory cells without collagen synthesis (< 25% similarity to natural tendon healing)
2	7–10 inflammatory cells	3–4 vessels per site	25–50% of collagen fibers are parallel to each other	25–50% similarity to natural tendon healing
3	10–13 inflammatory cells	5–6 vessels per site	50% of collagen fibers are parallel to each other	50–75% similarity to natural tendon healing
4	13–15 inflammatory cells	More than seven vessels per site	Organized, parallel collagen fibers	> 75% similarity to natural tendon healing

Fig. 1 Biomechanical testing of tendon failure at constant load

slides were also used to check the inflammatory profiles of the specimens. The Masson trichrome slides were used to check the amount of collagen synthesis and collagen orientation. All histologic studies were done by one expert pathologist who wasn't aware of the specimens' group distribution. Internal consistency between two pathologists was confirmed with Cronbach's alpha coefficient equal to 0.92. Evaluation of histopathological variables was done using Bonar and Movin scores [17–19], whose validity and reliability have been confirmed for evaluation of tendon tissue. Numerical scaling was used for easy comparison of the histologic variables (Table 1).

Biomechanical test

All biomechanical tests were performed by one researcher at room temperature within one hour of harvest. The machine "Hounsfield, HTE-5000 N, H50KS, made in England" located at ISIRI (the Institute of Standard and Industrial Research of Iran, Fars Branch) was used for biomechanical analysis. First, using a 1.6 mm K-wire, a transverse hole was created in the proximal tibia, just posterior to the tibial tuberosity. At the proximal part of the sample, an ethibond (Multifilament Polyester Synthetic Nonabsorbable coated Braided Surgical Suture 5 (LINX-BOND) suture was used in the Krakow technique. Krakow suture was then fixed to the upper clamp of the machine, while K-wire was fixed to the inferior clamp. Constant loud was applied to the tendon at a speed of 1 mm/second. Peak load to failure was recorded (Fig. 1).

Sample size collection

Determining the sample size of animal studies is often based on "resource equation method" [20]. Based on this method, the sample size required for this study was estimated to be 30 rabbits. However, based on the opinion of the methodologist, G* Power software [21]was used to ensure that the sample size was sufficient to conduct this experimental study. The appropriate sample size for this animal study based on the effect size estimate of 0.81 for the difference in the effect of local administration of PRP on the angiogenesis of acute tendon injury in New Zealand white rabbits compared to the control group, based on the study of Mansour et al., [22] with an alpha of 5% and a power of 80%, the minimum number 8 rabbits were estimated for each group(total of 40 rabbits) by an epidemiologist.

Statistical analysis

SPSS for Windows, version 16 (SPSS Inc., Chicago, Ill., USA) for statistical analysis of data. The Shapiro-Wilk test was used to test the normality of variables distribution. A comparison of mean values between the right and left legs were made using a Wilcoxon Rank Sum Test. The Kruskal-Wallis test was used to compare variables between different groups. A P-value 0.05 was considered significant.

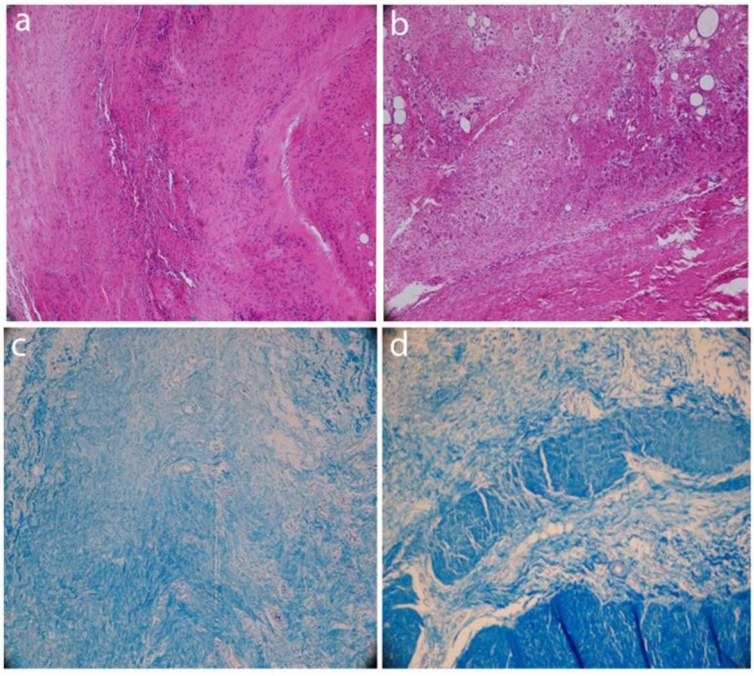

Fig. 2 Histologic slides of the 1st group (**a** & **b**): Less inflammation and cellular proliferation were seen in the control group (**a**); Severe chronic inflammation, giant cell reaction, and microvascular proliferation in the PRP group (**b**). The gap was bridged in both groups by randomly oriented fibroblasts (H & E × 200). Masson trichrome staining of the 1st group (**c** & **d**): Comparison for collagen synthesis between the control group (**c**) and PRP group (**d**). Darker blue staining in PRP injected site indicates more collagen synthesis, although disoriented fibers are obvious in both slides (Masson trichrome × 200)

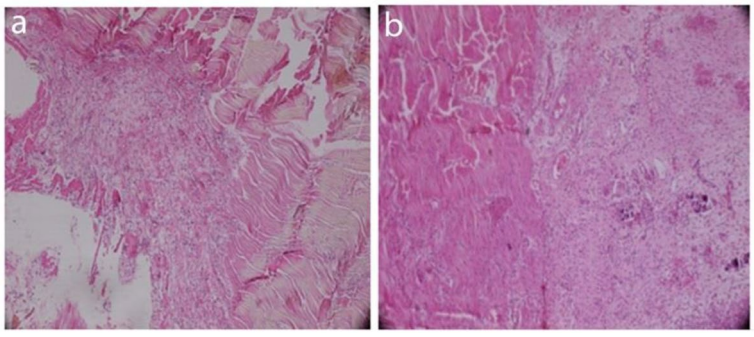

Fig. 3 Histologic slides of the 2nd week: (**a**) control group; (**b**) one PRP injection; At 14 days after injury, cellularity diminished in both groups. A mixed population of spindle-shaped fibroblast and mononuclear cells were also present. The number of vessels was increased in the PRP group related to the control group (H & E × 200)

Results

Histological analysis (within-group comparison)

Grossly, it was clear that the amount of regenerated tissue was increasing with time, and we could clearly define the site of the defect by palpation of a concavity at 7th and 14th days (group A, B, D), but it was obscured at 28th day (group C and E). Microscopically, although we did not do immunohistochemistry to define cell type, there were some cells in plump in favor of tenocytes. However, we could not differentiate it from myofibroblast.

In group A, the amount of cellularity, fibroblast and tenocyte proliferation, and vascularity were higher with PRP injection, and this difference was statistically significant ($P<0.05$). Collagen synthesis and orientation were the same between the right and left legs. The healing process was better in the right leg but without statistical significance (Fig. 2).

In group B, cellularity and vascular proliferation were better in PRP injected side with statistical significance ($P<0.05$). The amount of fibroblast was the same. Tenocyte proliferation, collagen synthesis, orientation, and healing process were also better with PRP injection but with no statistical significance (Fig. 3).

In group C, most of the parameters became closer to each other in injected and non-injected sides, and most differences were not statistically significant($P>0.05$). The

only significant difference was in the tenocyte proliferation, which was significantly more in the intervention leg ($P<0.05$). The amount of cellularity and collagen synthesis was slightly better in PRP injected group, and the orientation and healing process were reversed.

In group D, cellularity and tenocyte proliferation were better with two doses of PRP injection and with statistical significance ($P<0.05$). Vascularity was also better with two injections, although not significantly. Also, collagen synthesis, orientation, and the healing process increased, with only collagen synthesis showing a statistically significant difference ($P<0.05$).

In group E, the amount of cellularity and tenocyte proliferation significantly increased with three doses of PRP injection comparing the non-injected side. The vascular formation also increased in the PRP injected group, but without a statistically significant difference ($P>0.05$). Fibroblast proliferation was more on the control side but without significance ($P>0.05$). The amount of tenocyte proliferation, collagen synthesis and orientation, and the healing process were significantly better on the control side ($P<0.05$).

Detailed grading of the histologic parameters of each group is demonstrated in (Tables 2 and 3; Fig. 4).

Histological analysis (between-group comparison)

Comparison of histological parameters after 7 and 14 days of a single PRP injection (group A and B, respectively) showed a decreased amount of cellularity and vascular formation and increased rate of healing and collagen orientation with time.

Further analysis included comparing the histologic parameters between the groups who were euthanized on day 14 (group B and D) and the groups who were euthanized on day 28 (group C and E) (Fig. 5).

On the 14th day, cellular and fibroblast proliferation and vascular formation had higher levels in the rabbits receiving two PRP injections (group D) compared to those who only received one PRP injection (group B), with two later parameters showing a statistically significant difference ($P<0.05$). But the amount of collagen orientation and synthesis, and healing process were lower in group D, with only the former reaching a statistically significant ($P<0.05$) (Fig. 6).

On the 28th day, cellular and fibroblast proliferation and vascular formation had higher levels in the group receiving three PRP injections (group E) compared to the group receiving only one PRP injection (group C), but differences were not significant. Collagen orientation and synthesis and also healing process all were lower in group E than group C, with a significant statistical difference ($P<0.05$) (Table 4; Fig. 7).

Table 2 The detailed histological parameters in the injected and non-injected legs of each study group

Group	Right/left	Cellularity grade				Fibroblast proliferation grade				Vascularity grade				Collagen orientation grade				Collagen synthesis grade				Healing grade			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
A	Right	0	0	0	4	0	4	0	0	0	0	1	3	3	1	0	0	3	1	0	0	2	2	0	0
	Left	0	3	0	1	3	1	0	0	0	4	0	0	3	1	0	0	3	1	0	0	4	0	0	0
B	Right	0	0	2	2	0	4	0	0	0	0	0	4	0	2	2	0	1	3	0	0	0	4	0	0
	Left	0	3	1	0	0	4	0	0	0	3	1	0	0	4	0	0	2	2	0	0	2	2	0	0
C	Right	0	2	2	0	0	1	3	0	0	0	4	0	0	0	2	2	0	0	2	2	0	0	2	2
	Left	0	3	1	0	0	1	2	1	0	0	4	0	0	0	1	3	0	0	2	2	0	0	1	3
D	Right	0	1	0	4	0	5	0	0	0	0	1	4	3	2	0	0	4	1	0	0	4	1	0	0
	Left	0	3	2	0	0	2	3	0	0	0	2	3	0	4	1	0	2	3	0	0	2	3	0	0
E	Right	0	0	3	2	0	3	2	0	0	0	1	4	0	4	1	0	0	3	2	0	0	4	1	0
	Left	0	2	3	0	0	1	**2**	2	0	0	3	2	0	0	2	3	0	0	2	3	0	0	1	4

Table 3 The difference of histological parameters between right and left legs in different groups

Group characteristics			*P*- values						
Group	**Harvesting day**	**No of injections**	**cellularity Rt. / Lt.**	**Fibroblast Rt. / Lt.**	**Tenocyte Rt. / Lt.**	**Vascularity Rt. / Lt.**	**Collagen orientation Rt. /LT.**	**Collagen synth. Rt. /Lt.**	**Healing Rt. / Lt.**
A	7	One	0.046*	0.083	0.059	0.059	1.000	1.000	0.157
B	14	One	0.059	1.000	0.157	0.059	0.157	0.317	0.157
C	28	One	0.564	0.317	0.059	1.000	0.317	0.564	0.564
D	14	Two	0.098	0.083	0.025*	0.564	0.046*	0.317	0.317
E	28	Three	0.046*	0.157	0.059	0.157	0.038*	0.063	0.038*

RT: right; LT: left; No: number

*$P<0.05$ is considered significant

Inflammatory profile

Mononuclear cellswere present in all the specimens with PRP and without PRP injection. Polymorphonuclear (PMN) cells, plasma cells, and giant cells were present only on PRP injected side. PMN cells were present in 2 specimens on the 14th day of the first group and one specimen on the 14th day of the 2nd group. Plasma cell was seen in 2 specimens on the 14th and 28th day in group A and one specimen in group E on the 28th day. Also, the giant cell was present in PRP injected specimens in all of the right legs on the 7th day, two specimens on the 14th day in groups A & D, and one specimen on the 28th day in each group. Also, we saw some fat cells in all specimens with PRP injection; these cells were more prominent in samples of 2 and 3 doses of PRP injection in comparison to 1 dose.

Biomechanical analysis (within-group comparison)

In the first three groups, with only one dose of PRP injection on the first day, the difference between load at failure of right and left legs was not significant statistically ($P=0.58$, 0.46, and 0.46 respectively), although in the right leg (PRP injected side) it was slightly higher. In group D, load at failure was higher on the non-injected side; however, the difference was not statistically significant. In group E, load at failure was significantly higher in the non-injected side, both clinically and statistically ($p<0.05$) (Table 5).

Biomechanical analysis (between-group comparison)

After 14 days, the effect of one or two doses of PRP injection on the load at failure (groups B and D respectively) was not significantly different ($P=0.12$), although it was better in group B with only one injection. After 28 days, the effect of one or three doses of PRP injection on the load at failure (groups C and E respectively) was significantly different ($P<0.05$), so that one injection group had a higher load at failure than the three injection group.

Discussion

In this study, we evaluated the effect of the frequency of PRP injection on acute tendon injury healing. We observed better histological healing in early phases (7 and 14 days) with one dose of PRP injection on the first day of injury, but these effects were less prominent on day 28. The biomechanical property improved over time in groups receiving one dose of PRP. However, in the group receiving two and three PRP injections, collagen orientation, the healing process, and biomechanical parameters were all less improved compared to the control group, despite better cellular properties and vascularity. Indeed, the application of more PRP injections exaggerated the inflammatory reaction and cellular proliferation, thereby hindering the progress of acute tendon injury healing proves.

The effect of PRP injection on patellar tendon healing has been investigated in some earlier studies. Lyras et al. studied the impact of PRP gel on the mechanical properties of rabbit's patellar tendon following the resection of its central portion. Forty skeletally mature rabbits were randomly assigned to the PRP and control group. Half of the rabbits in each group were evaluated for histology and biomechanical properties on day 14, and the other half were investigated on day 28. At 14 days, force at failure, ultimate stress, and stiffness were all significantly increased in the PRP group compared to the control group. However, at 28 days, no significant difference was observed between the two groups. They concluded that PRP injection has a strong effect in the early phase of tendon healing [23] Similarly, the impact of PRP injection on the tendon healing properties was more prominent in the early phase of the present study.

In their other study, Lyras et al. evaluated the impact of local administration of PRP on the angiogenesis of patellar tendon defect in 48 New Zealand white Rabbits in comparison with the control group. The Rabbits were euthanized one, two, three, and four weeks after the intervention.

According to the histological examination, the healing process was superior in the PRP group compared with

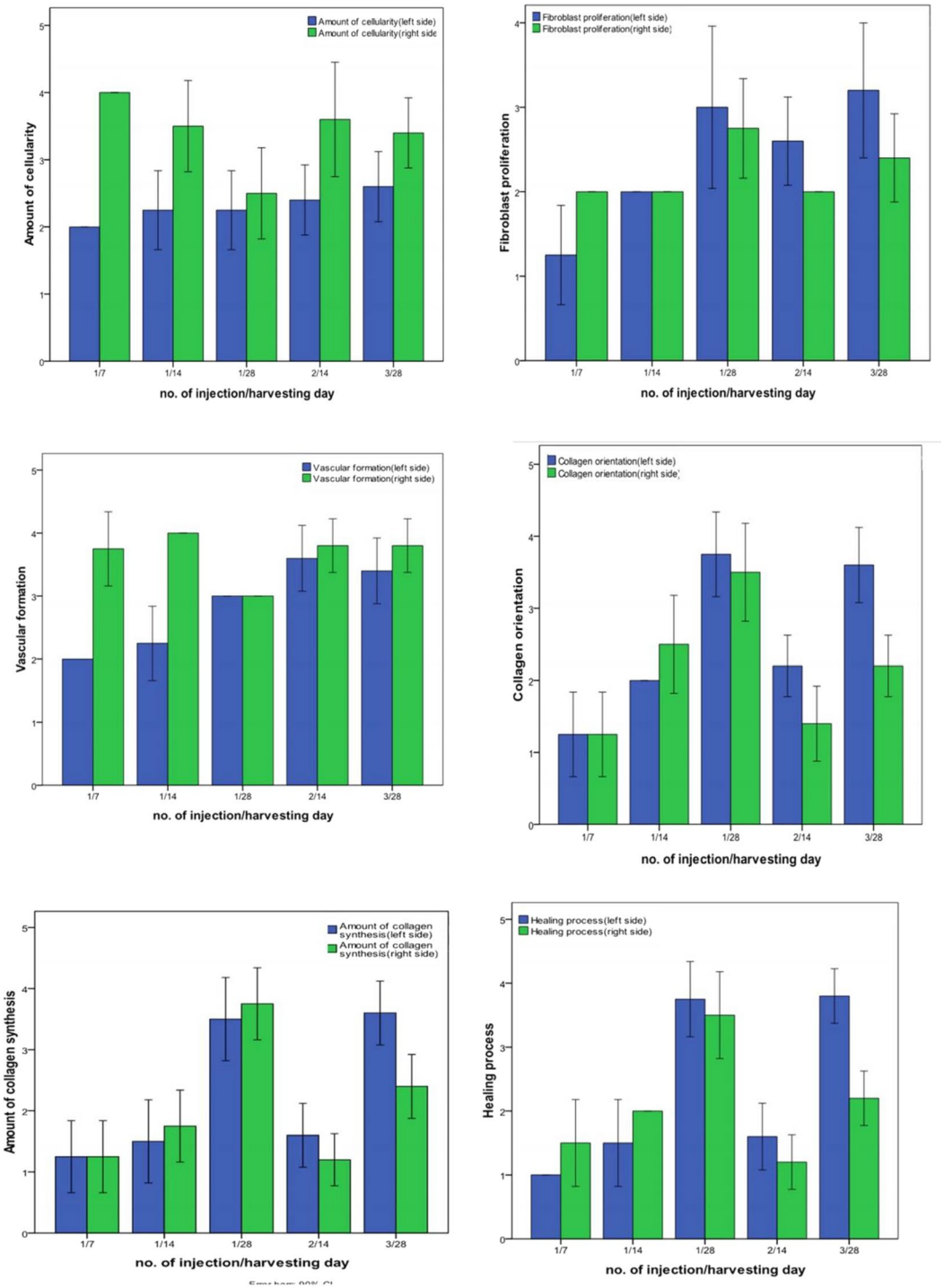

Fig. 4 Comparison of histologic parameters between the right and left legs in each group

the control group. The formed tissue in the PRP group was more mature and denser with less elastic fibers, particularly in the third week. In the PRP group, angiogenesis was significantly higher in the first and second weeks and significantly lower in the third and fourth weeks. Again, these results support a strong effect of PRP in the early phase of patellar tendon healing, like ours.

De Almeida et al., in a randomized controlled trial, evaluated the effect of PRP injection on patellar tendon healing. Twenty-seven patients were randomly divided to the PRP injection ($n=12$) or no PRP injection ($n=15$) in the patellar tendon harvest site during the reconstruction of the anterior cruciate ligament. Patellar tendon healing (gap area) was evaluated after six months using

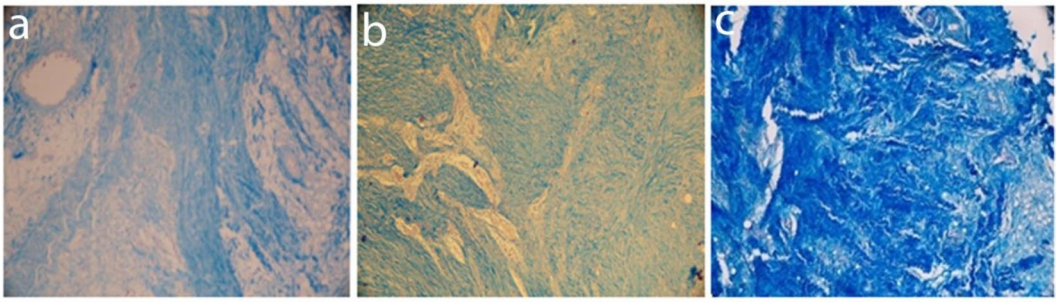

Fig. 5 ; Histologic slides of the 2nd week (2nd & 4th group): Comparison for collagen synthesis between control (**a**), one PRP injection (**b**), and two PRP injections (**c**). Darker blue staining in PRP injected site indicates more collagen synthesis, although with disoriented collagen fibers in both slides (Masson & Trichorom staining × 200)

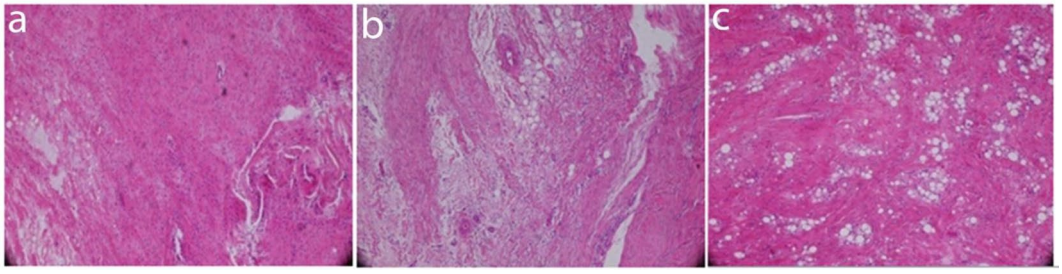

Fig. 6 Histology slides of the 4th week (3rd & 5th group): Histology of patellar tendons in the control group (**a**), one PRP injection (**b**), and two PRP injections (**c**). At 28 days, the tendon with some cellular activity was observed in both PRP groups. The control group showed more healing in relation to the treated groups. The fibroblasts were more prominent and oriented in the control group and one PRP injection compared to rabbits in group 5. In the treatment groups, the fat cells were present in favor of the disordered healing process, especially in group 5. The number of vessels decreased in both groups indicating the completion of the healing process (H & E × 200)

Table 4 Comparison of histological parameters between the different study groups

	***P*-value**					
Harvesting day	**D. cellularity**	**D. fibroblast**	**D. vascularity**	**D. collagen orientation**	**D. collagen synth**	**D. healing**
14th day (groups B &D)	0.945	0.068	0.014	0.007	0.238	0.127
28th day (groups C&E)	0.287	0.388	0.267	0.014	0.046	0.032

P < 0.05 is considered significant

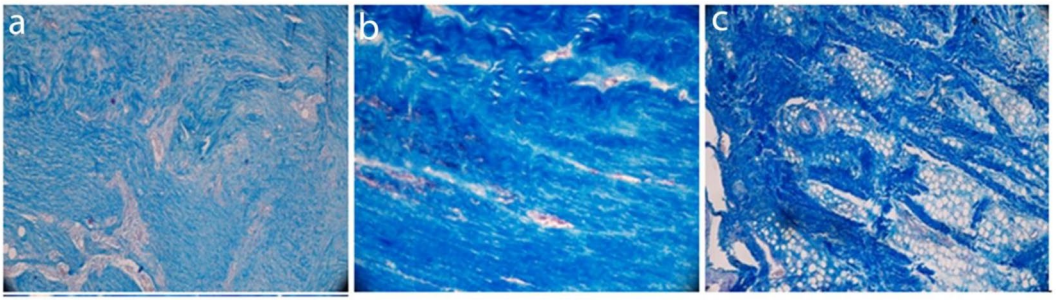

Fig. 7 Histologic slides of the 4th week (3rd & 5th group): Comparison for mature collagen synthesis between the control group (**a**), one PRP injection (**b**), and two PRP injections (**c**). Blue staining resembles mature collagen deposition and demonstrates a generally well-structured tendon. Weaker blue staining in the treated group is associated with less collagen deposition than the control group and one PRP injection. In addition, collagen fibers are more oriented in the control group and one PRP injection group (Masson trichrome × 200)

Table 5 The difference of load at failure between right and left legs in each group

Group	No. of PRP injection	Mean difference (Newton)	90% confidence interval		*P*-value
			Upper	Lower	
A	one	6.25	33.76	-11.69	0.581
B	one	21.82	67.85	-24.2	0.465
C	one	19.6	93.64	-54.44	0.465
D	two	-17.54	10.71	-45.79	0.225
E	three	64.4	-7.86	-12.93	0.o8o

$P<0.05$ is considered significant

MRI, which showed a significantly smaller gap in the PRP group compared to the control group (4.9 vs. 9.4 mm^2). The visual analog scale was significantly lower in the PRP group (3.8 vs. 5.1). Isokinetic testing and limb function were not significantly different between the PRP and control group [24] We evaluated the efficacy of PRP injection in the experimental model. However, similar to the study of Almeida et al., we observed beneficial effects of one PRP injection on the day of injury.

Contrary to our results, in a randomized controlled clinical trial, DJ Keene et al., [25] reported that PRP infusion was not superior to placebo in improving objective muscle tendon function, patient-reported function, or quality of life after acute Achilles tendon rupture. In a clinical trial study, LA Rossi et al., [26] in a 2-year follow-up examining autologous PRP therapy compared to a control group in seventy-five patients with acute muscle injuries, did not report a significant superiority of autologous PRP treatment over placebo for tendon healing and re-tear rate. In another randomized controlled clinical trial, W Dai et al., [27] showed no significant difference in improvement of mechanical, functional parameters and tendon healing for treatment of tendinopathy with platelet-rich plasma infusion compared to placebo. AP Boesen et al., [28] in a clinical trial study, did not report a significant superiority for the use of PRP in non-surgically treated ATRs. This heterogeneity in the results can be justified due to the difference in studies design (experimental versus RCT), adherence to instructions after treatment, time from injury to treatment, and time to evaluate outcomes. In the experimental studies, the studied samples are patients with tendon injuries, that the mechanism of injury and also following the principles of post-mortem in them may be different compared to experimental studies which are carried out in controlled conditions.

The efficacy of PRP injection on patellar tendon healing has also been investigated in some other studies [29–32]. However, there is no clear consensus on the optimal timing and frequency of PRP injection in patellar tendon healing. Dallaudière et al., evaluated the efficacy of a second intratendinous PRP injection in 24 patients with tendon tear or tendinosis and the incomplete response of the first injection. Six weeks after the second injection, the size of the residual lesion under ultrasound imaging was not significantly different from that of six weeks after the first injection. Clinical pain and function of the patients were not significantly different between the first and second PRP injections. They concluded that a second intratendinous PRP injection does not accelerate the tendon healing, nor does it improve clinical pain and functional outcomes following the incomplete efficiency of the first PRP injection [33] Consistent with the study of Dallaudière et al., in the current study, the second and third PRP injections reinforced the inflammatory reaction, thereby postponing the tendon healing stages.

The healing process follows in a highly orchestrated fashion that includes the inflammatory phase, cellular and matrix proliferation phase, and final phase of maturation and remodeling in which growth factors and fibronectin stimulate fibroblasts proliferation, migration, and synthesis of the extracellular matrix. This process is tightly regulated to preserve the balance between degradation and synthesis, and any intervention in this process might result in unwanted outcomes [34–37]. The results of the present study reveal that PRP injection in the early phase of injury has beneficial effects on the healing process.

However, PRP injection after the initial phase not only does not have a positive effect on the tendon recovery, it may also delay the recovery process. These results suggest that a single dose of PRP injection results in better histologic tendon healing and biomechanical properties than two and three PRP injections.

The present study was not without limitations. A small number of rabbits could be regarded as the main limitation of this study. This limitation led to an insignificant statistical association between several variables, while they seemed significant clinically. We used rabbits with both sexes, which could be regarded as a confounding factor. Another pitfall was to obtain PRP with the traditional method of centrifugation while using a manufactured device made for this purpose could provide more reliable results. In addition, we did not evaluate the type of regenerated collagen immunohistochemically. This limitation also led to the inability to differentiate between tenocytes and myofibroblasts.

Conclusion

PRP injection enhances the microscopic quality of the early stage of tendon repair tissue, significantly affecting its biomechanical and histological quality. However, administering PRP after the initial injury stage impedes tendon healing histologically and biomechanically, likely due to an inappropriate injection timing that disrupts the natural healing process. While our study revealed a

significant difference in biomechanical and histological outcomes between single and serial PRP injections, further research is needed to investigate the impact of serial PRP injections on inflammatory cytokine levels, tendon repair tissue quality, and microscopic healing components of tendon tissue. Clinically, our study suggests that using PRP to enhance the regenerative tendon tissue quality during the early phase of tendon repair shows promise. Still, multiple PRP injections do not provide an advantage over single injections when evaluating the biomechanical and histological appearance of the tendon tissue. Furthermore, a single PRP injection was more effective than multiple PRP injections. Therefore, in cases where PRP injection is recommended for tendon repair, such as in patellar tendon defects, we advise a single PRP injection during tendon repair surgery. Follow-up studies over the long term in humans will help clarify the role of single versus serial PRP injections in managing the acute phase of tendon injury.

Abbreviations

PRP	Platelet-rich plasma
ACD-A	Anticoagulant citrate dextrose solution
PMN	Polymorphonuclear

Supplementary Information

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Supplementary Material 1

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Author contributions
M M and M T G designed and supervised the study. M M and M B performed the methodology. O R M, F M J, N A and M M prepared figures and data analysis. M T G and M M wrote the first versions of the manuscript with contributions from all authors. All authors assisted with editing the manuscript and approved the final version.

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Data availability
The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The current study was performed in compliance with the Guidelines of the Iranian regulations for imported animals. All experimental protocols and procedures were conducted by the ethical committee guidelines of the Iran University of Medical Sciences with the code IR.IUMS.FMD.REC.1399.719. All sections of the present study complied with the "ARRIVE guidelines" for reporting in vivo experiments in animal research. Dutch rabbits were obtained from the Animal Center of Shiraz University of Medical Sciences.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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