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BASIC SCIENCE

The effect of platelet-rich plasma gel in the early phase of patellar tendon healing

Dimitris N. Lyras · Konstantinos Kazakos · Dionysios Verettas · Sotirios Botaitis · George Agrogiannis · Anna Kokka · Michail Pitiakoudis · Athanasios Kotzakaris

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Abstract
Introduction The aim of this study is to assess if an application of platelet-rich plasma (PRP) gel would improve the mechanical properties of rabbit's patellar tendon after resecting its central portion.
Materials and methods Forty skeletally mature New Zealand White rabbits were used. Two groups ten rabbits each (PRP and control group) were used to evaluate mechanical properties and histology after 14 days and two groups ten rabbits each (PRP and control groups) were used to evaluate mechanical properties and histology after 28 days.
Results At 14 days, PRP group showed a 72.2% increase in force at failure, a 39.1% increase in ultimate stress, and a 53.1% increase in stiffness, as compared with controls. These changes were statistically significant ($P < 0.05$). At 28 days, there was no longer any significant difference between PRP and control groups ($P > 0.05$).
Discussion In our study, the mechanical properties of the regenerated tendon in the PRP group were significantly improved in relation to the control group. It appears that PRP has a strong effect in the early phase of tendon healing. This effect is probably due to the growth factors that are released from the platelets during activation.

Keywords Platelet rich plasma · Patellar tendon · Tendon healing · Rabbits · Biomechanics

D. N. Lyras (✉)
Democritus University of Thrace, Argyrokastrou 31B, Vrilissia, Athens, B.O. 15235, Greece
e-mail: dimitrislyras@yahoo.gr

K. Kazakos · D. Verettas
Democritus University of Thrace, Dragana, B.O. 68100, Alexandroupolis, Greece
e-mail: kazakosk@yahoo.gr

D. Verettas
e-mail: quack@otenet.gr

S. Botaitis · M. Pitiakoudis
Laboratory of Experimental Surgery, Democritus University of Thrace, Dragana, B.O. 68100, Alexandroupolis, Greece
e-mail: smpotait@med.duth.gr

M. Pitiakoudis
e-mail: mpitiak@med.duth.gr

G. Agrogiannis · A. Kokka
Department of Pathology, Kapodistrian University of Athens, Athens, Greece
e-mail: agrojohn@med.uoa.gr

A. Kokka
e-mail: anna.kokka@yahoo.gr

A. Kotzakaris
Ethniko Metsovio Polytechnio, Athens, Greece
e-mail: akotzaka@cpw.vionet.gr

Introduction

Platelet-rich plasma (PRP) is an autologous concentration of platelets in a small volume of plasma, and contains several growth factors including platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), fibroblastic growth factor, vascular endothelial growth factor (VEGF), insulin-like growth factor-1, and epidermal growth factor (EGF). PRP is a new application of tissue engineering and a developing area for clinicians and researchers. It has recently been investigated for the regeneration of bone [9, 12, 18], cartilage [2, 17], and ligaments [19], and for the treatment of open fractures [8].

Maffulli stated that the above growth factors could potentially be used therapeutically to accelerate the process

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of tendon regeneration. However, it is unlikely that a single growth factor could give a positive result, but the interaction of many factors present in the right concentration at the right time would be necessary [10]. Based on this statement we were encouraged to use PRP, a pool of growth factors, in order to enhance tendon healing.

The aim of this study was to assess if an application of PRP gel would improve the mechanical properties of rabbit's patellar tendon after resecting its central portion.

Materials and methods

The Ethics Committee in Thrace had approved this study (number T/4477), and institutional guidelines for the care and treatment of laboratory animals were adhered to.

Animals

Fifty-two skeletally mature New Zealand White rabbits, weighing an average of 4.2 (SD 0.41) kg each, were used for the study. Although there were both male and female rabbits, randomization was done without stratification for sex. The rabbits were housed one per cage with food and water available ad libitum. First, two groups ten rabbits each (PRP and control group) and two groups three rabbits each (PRP and control) were used to evaluate early repair biomechanically and histologically, respectively. They were killed after 14 days and the tendons harvested for mechanical and histological evaluation. Second, two groups (PRP and control group) ten rabbits each and two groups (PRP and control group) three rabbits each were killed after 28 days and tendons harvested for mechanical test and histological evaluation, respectively.

PRP preparation

Initially, we have used five rabbits as donors in order to measure some important parameters in blood and PRP. A standard hemocytometer was used to measure platelet counts and commercial enzyme-linked immunosorbent assay kits (R&D System, Inc., USA) were used to quantify the concentrations of PDGF-BB, TGF-β1, VEGF, and EGF according to the manufacturer's instructions.

Before performing the surgical procedure and immediately after general anesthesia, 8 ml of blood from an ear vein was collected in a tube. The blood was allowed to stand for 15 min, in order to reduce platelets activation during centrifugation. Once centrifugation was complete, the upper half was considered platelet-poor plasma and was removed by using sterile pipettes. The lower half, the PRP proper, was retrieved using a pipette by aspirating up to the interphase zone (consisting of blood cells) and was then placed into another glass tube. Two milliliters of PRP was collected for every 8 ml of blood. The PRP was applied in a gel form, manufactured by adding 0.5 ml of precoagulant solution in the tube with the liquid PRP and allowing approximately 15 min for the solution to become a gel.

Surgical procedure

The rabbits were anesthetized with an intramuscular injection of xylazine (Rompun® Injectable, Bayer) at a dosage of 5–7 mg/kg and 0.15 mg of atropine (DEMO S.A.). 10–15 min later, ketamine (Imalgene®, Rhone Merieux, France) at a dosage of 12–15 mg/kg was injected intramuscu1arly. During surgery, supplemental sedation was administered as required. Local anesthesia 1 ml of a 2% lidocaine–adrenaline solution (AstraZeneca, UK) was applied at regular intervals at the site of incisions.

The surgical procedure was performed according to the animal model described by Anaguchi et al. [3]. The skin of the right knee was shaved and the operation was performed under aseptic conditions. Following that, a longitudinal skin incision was made on the skin overlying the middle of the patellar tendon. The superficial surrounding fascia was cut longitudinally to expose the patellar tendon. Thereafter, the deep fascia overlying the tendon was opened and a full thickness, 3-mm-wide, and 10-mm-long tendon substance was excised from the central portion of the patellar tendon with a specially designed knife that had two stainless-steel surgical blades. The 3-mm width of the defect is approximately equal to one-third of the width of the tendon. The PRP gel was then applied and filled the tendon defect. The overlaying fascia was closed with a running suture of 4-0 nylon so that PRP gel applied into the resected portion would not flow out. Skin was closed with clips. The same procedure was performed in the opposite limb of the animals in PRP group and the same procedure was performed in both limbs in the control group, without the application of PRP into the patellar tendon defect. No immobilization was applied after surgery, and the rabbits were allowed unrestricted daily activities in their cages.

Harvesting

After 14 and 28 days the animals were killed with an overdose of intracardiac injection of 10% KCl solution under general anesthesia. Immediately after killing, each complex, consisting of patella, patellar tendon, and proximal tibia was dissectedfree from other tissues. For mechanical testing, each hind limb was wrapped in gauze moistened with physiologic saline solution, covered with an airtight plastic film, and then stored at −80°C until testing. Before mechanical testing, each limb was thawed overnight at 4°C.

Mechanical testing

Mechanical testing was performed at 14 and 28 days using a materials testing machine (Imperial 2500, MECMESIN, UK). The patella was fixed in a clamp and the tibia via a transversely inserted Kirchener-wire. Thus, both ends of the tendon were fixated via their bone attachments, so that tendon sliding could be avoided. The tendon was pulled at a constant speed of 1 mm/s until failure. Peak force, stiffness and energy uptake were recorded. Area and stress were calculated.

Histology

After the mechanical tests, the tendons of the 14 and 28 days group were prepared with routine methods for paraffin sections. The specimens were sectioned parallel to the longitudinal direction of the tendon.

From each tendon, six paraffin sections were stained with hematoxylin and eosin for the histological evaluation and two paraffin sections were stained with Masson's trichrome for the evaluation of collagen synthesis. All sections were analyzed by a single pathologist, who was blinded of the treatment groups.

Statistical analysis

All results are expressed as mean ± SD. Significant differences among groups were evaluated using the Mann–Whitney *U* test. A difference of $P < 0.05$ was considered to be statistically significant (SPSS 11.5.0).

Results

Platelet count and growth factor concentrations

Platelet concentration (mean ± SD) in whole blood, and PRP was $531 \pm 138 \times 10^3$ and $4{,}248 \pm 1{,}132 \times 10^3$, respectively. Furthermore, the concentration of TGF-b1 in whole blood and PRP was 52.14 ± 13.32 and 199.37 ± 36.62 ng/ml, respectively. PDGF-BB concentration was 3.1 ± 0.8 ng/ml in the whole blood and 22.32 ± 9.2 ng/ml in PRP. The concentration of VEGF in whole blood and PRP was 164.73 ± 98.72 and 957.46 ± 328.52 ng/ml, respectively. Finally, EGF concentration was 136.65 ± 86.53 ng/ml in the whole blood and 462.87 ± 271.69 ng/ml in PRP.

Histological analysis

At 14 days after injury, the gap was bridged by a synovium-like tissue in both groups and the fibroblasts were randomly oriented (Figs. 1, 2a, b). On the other hand, there was increased cellularity with changes in cell morphology, and the tenocytes became plump in PRP group. A mix of spindle-shaped fibroblastic cells and mononuclear cells was also present (Fig. 2b). The number of vessels was also increased in PRP group related to the control group (Fig. 2a, b).

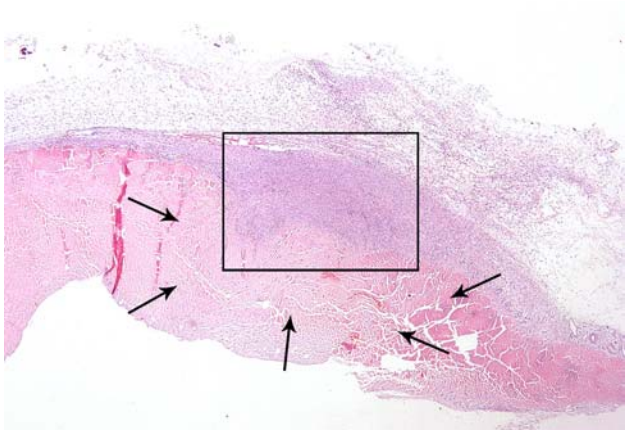

Fig. 1 Figure from a patellar tendon during the healing process (e–h, ×20 original magnification). *Rectangle* indicates the surrounding tissue which fills the wounded site and which is the area of interest. All *panels* from Fig. 2 represent details from this area. The *arrows* indicate the healthy tendon around the repairing site

At 28 days after injury, an almost healed tendon with some cellular activity was observed in the control group (Fig. 2c) and a completely healed tendon in PRP group (Fig. 2d). Furthermore, in PRP group the repair tissue which was bridging the gap became more fibrous with increased numbers of more distinct oriented fibroblasts which established a firmer lesion (Fig. 2d). The number of vessels decreased in both groups indicating the completion of healing process (Fig. 2c, d).

Finally, more collagen synthesis was observed in PRP group in the second, third, and fourth weeks in comparison with the controls. The greatest difference in collagen staining was demonstrated in fourth week (Fig. 3a, b).

Biomechanical analysis

At 14 days, PRP demonstrated a 72.2% increase in force at failure, a 39.1% increase in ultimate stress, and a 53.1% increase in stiffness, as compared with controls. These changes were statistically significant ($P < 0.05$; Table 1).

At 28 days, force at failure, stiffness, and energy had increased in the control group compared with 14 days, but the PRP group increased less, so that there was no longer any significant difference between PRP and control group ($P > 0.05$; Table 2).

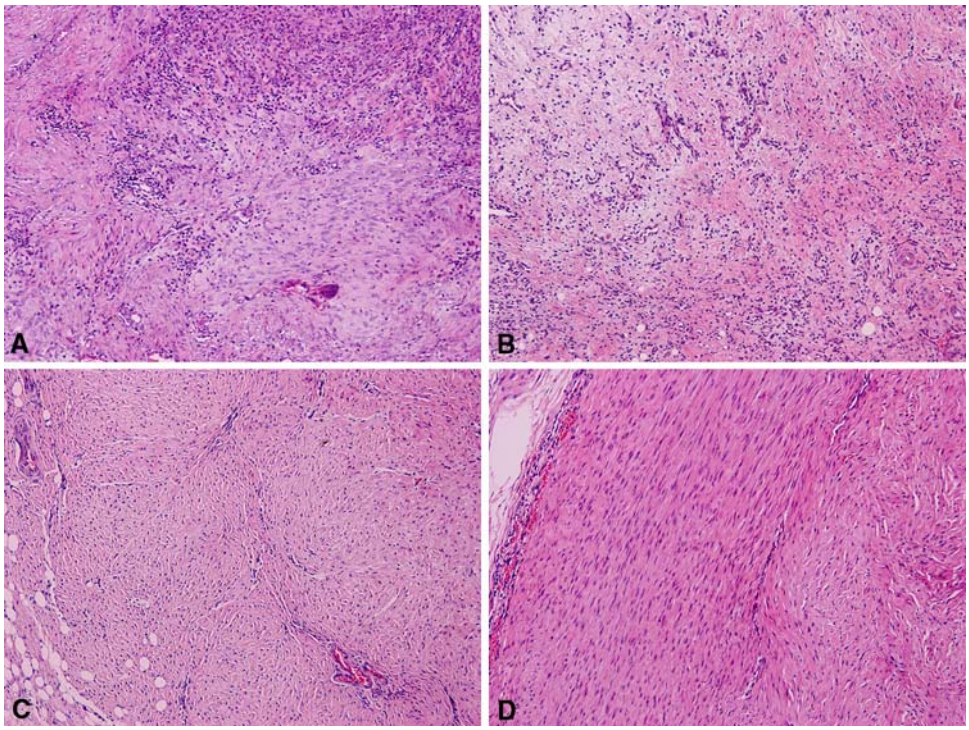

Fig. 2 Histology of patellar tendons in control group (**a**, **c**) and in PRP group (**b**, **d**) at 14 days (**a**, **b**) and 28 days (**c**, **d**) (all figures are under ×200 original magnification). At 14 days after injury, the gap was bridged by a synovium-like tissue in both groups and the fibroblasts were randomly oriented (Fig. 1a, b). There was increased cellularity with changes in cell morphology, and the tenocytes became plump in PRP group. A mix of spindle-shaped fibroblastic cells and mononuclear cells were also present (Fig. 1b). The number of vessels was increased in PRP group related to the control group (Fig. 1a, b). At 28 days, an almost healed tendon with some cellular activity was observed in the control group (**c**) and a completely healed tendon in PRP group (**d**). A more fibrous tissue was bridging the gap and an increased number of fibroblasts with a more distinct orientation were observed in PRP (Fig. 1d). The number of vessels decreased in both group indicating the completion of healing process (**c**, **d**)

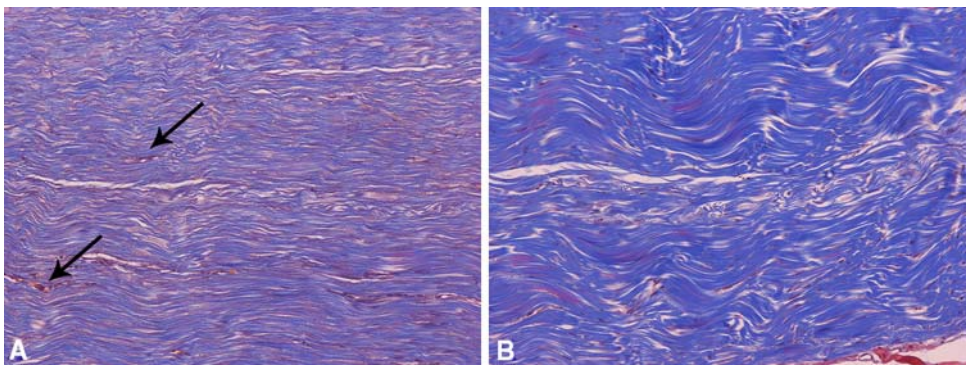

Fig. 3 Representative figures for mature collagene synthesis from control group (**a**) and PRP group (**b**), Masson's trichrome stain, original magnification ×200, at the fourth week of wound healing. *Blue* staining resembles mature collagen. Although both figures demonstrate a generally well-structured tendon, A has weaker blue staining than B, which means that collagen deposition is less in this case

Discussion

One of the early events of wound healing is angiogenesis, in which neovascularization prompts delivery of inflammatory cells and fibroblasts to the wound site. An injury such as a traumatic tendon rupture destroys the well-organized peri- and intratendinous network of blood vessels [1]. Wounding and inflammation provoke the release of growth factors and cytokines from platelets, polymorphonuclear leukocytes, macrophages, and other inflammatory cells [5]. These growth factors induce neovascularization and chemotaxis of fibroblasts and tenocytes and stimulate

Table 1 Effect of PR on mechanical properties of patellar tendon on day 14

Biomechanical data				
Treatment	*n*	Mean	SD	*P*
Force (N)				
PRP	10	136	5.4	<0.001
Control	10	79	3.4	
Stiffness (N/mm)				
PRP	10	49	11.8	0.005
Control	10	32	12	
Energy (N/mm)				
PRP	10	342	15.6	<0.001
Control	10	192	13.1	
Area (mm^2)				
PRP	10	58.2	15	0.735
Control	10	56	13.6	
Stress (MPa)				
PRP	10	3.2	0.5	<0.001
Control	10	2.3	0.3	

Table 2 Effects of PR on mechanical properties of patellar tendon on day 28

Biomechanical data				
Treatment	*n*	Mean	SD	*P*
Force (N)				
PRP	10	232	34.8	0.19
Control	10	212	33.1	
Stiffness (N/mm)				
PRP	10	86	11.2	0.4
Control	10	82	9.7	
Energy (N/mm)				
PRP	10	491	16.8	0.21
Control	10	482	14.3	
Area (mm^2)				
PRP	10	78	3.5	0.221
Control	10	76	3.5	
Stress (MPa)				
PRP	10	3.4	0.2	0.061
Control	10	3.2	0.2	

fibroblast and tenocyte proliferation and synthesis of collagen [14]. Considering that healing process results from the interaction of many factors, we have proposed the use of autologous platelet-rich plasma to simultaneously increase the concentration of several growth factors and subsequently to enhance tendon healing. For this purpose, we used a patellar tendon defect model in rabbits.

In our study, the mechanical properties of the regenerated tendon in the PRP group were significantly improved in relation to the control group. It appears that PRP has a strong effect in the early phase of tendon healing. This effect is probably due to the growth factors that are released from the platelets during activation. Moreover, the dose of PRP into the central defect of the patellar tendons could be increased, for example, by additional injections of PRP in a liquid form into the tendon mass. Thus, it might be possible to increase the effect in this rabbit model. The main limitation of the study is that there were no molecular-biological data to support our mechanical results. Specifically, we have not performed any assessment of the type of collagen matrix in the tendon. Immunostaining of type I and III collagens is essential to provide important information for the quality of the regenerated tendon. However, we believe the present study has contributed some new important information to the field of tendon regeneration.

The role of PRP in tendon and ligament repair has been studied in a few papers. Murray et al., in a canine and porcine anterior cruciate ligament reconstruction model [15, 16], and Aspenberg and Virchenko in a rat Achilles tendon rupture model showed the positive effect of PRP on ligament and tendon healing [4]. Although the influence of PRP on bone repair remains controversial [6, 7, 11] we believe that tendons and ligaments, which are poor vascularized fibrous tissues, are the field of choice for the PRP application. This is probably due to the strong effect of growth factors in angiogenesis and collagen synthesis [13, 20]. Further investigation, especially evaluation of angiogenesis and immunohistochemical detection of the main growth factors in the healed tendons, is necessary to evidence the role of PRP.

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