Use of Platelet-Rich Plasma to Enhance Tendon Function and Cellularity

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Abstract

Clinical studies have shown inconsistent healing with subjective improvement after use of platelet-rich plasma (PRP) for tendinosis and partial tendon tears.

We conducted a study to assess changes after injecting PRP into an intact rabbit patellar tendon (PT) model. In the study group (n = 10), an incision was made over the PT and PRP was injected into multiple sites on the PT. The control group (n = 8) was injected with saline. PTs were harvested 7 and 28 days after injection.

Hematoxylin-eosin staining showed hypercellularity in the PRP group at 7 days, but the effect was not as marked at 28 days. At 7 days, polarized microscopy showed increased crimp density of collagen in the PRP group, compared with the control group, demonstrating up-regulation in collagen matrix. Cellular proliferation measured by tritiated thymidine was also significantly increased (P = .02) in the PRP group, compared with the control group, but the difference was not as significant at 28 days. At 7 and 28 days, there were no significant changes in basic fibroblast growth factor, insulin-like growth factor, vascular endothelial growth factor, or platelet-derived growth factor with 2B chains.

Injection of PRP into rabbit PT enhances collagen remodeling and hypercellularity with increased metabolic activity, which could have a positive effect on healing.

In recent years, use of platelet-rich plasma (PRP) has become a popular means for augmenting tendon healing. PRP is a plasma layer in which platelets are more concentrated after autologous blood is centrifuged. Degree of concentration depends on centrifugation technique, but the concentration of growth factors is associated with augmented healing in ligamentous, tendinous, and chondral injury models. Much of the research has been performed in animal models to try to assess the effects of applying PRP. In 2009, Lyras and colleagues[1] reported on changes that occur with excision of the central third of the patellar tendon (PT) and application of PRP to augment healing. Early tendon healing was improved in this rabbit model, though longer-term changes had a lesser effect. Aspenberg and Virchenko[2] also found short-term improvement in healing after transection of a rat Achilles tendon.

Clinical studies in humans have had inconsistent findings. In a prospective controlled study, Mishra and Pavelko[3] found that PRP was more efficacious than bupivacaine in managing chronic elbow tendinosis. Filardo and colleagues[4] demonstrated clinical effectiveness and good clinical improvement with use of PRP in a patellar tendinosis model. A prospective randomized clinical trial in which PRP was used to manage Achilles tendinosis showed no benefit over placebo.[5]

There is continuing debate regarding use and cost-effectiveness of PRP but also regarding mechanisms of action and whether presence of white blood cells (WBCs) in platelet concentrate is a benefit or detriment. To better understand this process, we extracted concentrated PRP and injected it into a normal PT in a rabbit model. This study allowed us to evaluate the histologic and biochemical effects of PRP on the PT. We evaluated cellular proliferation and molecular change in the regulation of platelet-derived growth factor with 2B chains (PDGF-BB), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), which could be important in healing after PRP injection.

Materials and Methods

Eighteen New Zealand White rabbits (age >4 years) were evaluated. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee, University of California, San Diego, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. We included 10 rabbits in the study group and 8 in the control group. All rabbits were anesthetized with a subcutaneous cocktail injection of ketamine 35 mg/kg for general anesthesia, xylazine 5 mg/kg for sedation, and buprenorphine 0.2 mg/kg for analgesia. In addition, the antibiotic enrofloxacin was administered to prevent infection.

PRP Collection

For each of the 10 PRP-injected rabbits, 8 to 10 mL of autologous blood was taken from an ear artery and placed in a centrifuge

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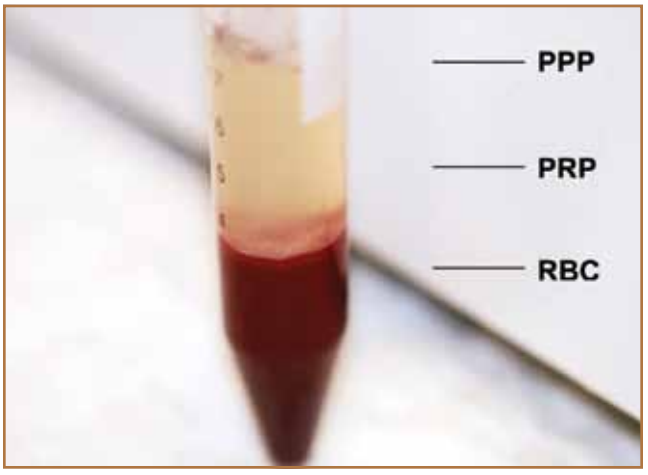

Figure 1. Platelet-rich plasma (PRP) layer between platelet-poor plasma (PPP) layer and red blood cell (RBC) layer after centrifugation.

Figure 2. Injection of platelet-rich plasma volume into distal patellar tendon. First injection was proximal to second injection being made here. As many as 8 injections were made in stepwise medial–lateral alternating fashion, 2 mm apart.

tube containing the anticoagulant acid citrate dextrose formula A. Of this blood, 0.5 mL was isolated for determination of whole blood platelet concentration. The rest was centrifuged at 2000 rpm for 15 minutes to separate the blood layers. A micropipette was used to decant the platelet-poor plasma layer from the PRP layer. This pipette was then used to collect the PRP layer, which consisted of the interphase zone between the plasma and the red blood cells (**Figure 1**). The platelets lay on top of the red blood cell layer, and this interphase was taken. Approximately 2 mL of PRP was obtained, of which 0.5 mL was isolated for determination of PRP platelet concentration. Blood and PRP were submitted to evaluate concentration of platelets and WBCs.

Surgical Procedure

For all 18 rabbits, isoflurane inhalation was used for general anesthesia. After the operative leg was prepared, a longitudinal incision was made over the medial PT, and the peritenon was identified and split longitudinally. The PT was identified from its origin at the patella to its insertion at the tibial tubercle and injected with 1.5 mL of PRP (study group) or saline (control group). An orthopedic surgeon (JL) used a 22-gauge needle to inject at 2-mm intervals, alternating between the medial and lateral aspects of the tendon in a stepwise fashion (**Figure 2**). About 8 injections were made, approximately 5 mm apart, to cover the tendon length. The peritenon was closed with running 4-0 Vicryl suture and the skin with inverted interrupted 4-0 Vicryl suture in the subcutaneous layer. The rabbit recovered in an incubator before being transferred to a cage in which its activity was unrestricted. There was no postoperative immobilization.

Tissue Harvest

Each rabbit was sacrificed after 7 or 28 days. A lethal dose of sodium pentobarbital was injected through a lateral ear vein after anesthesia. The PT was exposed in sterile fashion and dissected proximally above the patella and distally at the insertion of the tibial tubercle. The PT was then mounted on a tongue depressor and secured with staples without pinching the tissue. Once secure, the patella was removed and the distal half of the tendon separated. The proximal half, still connected to the depressor to prevent deformation, was placed in 10% neutral buffered formalin with 1% cetylpyridinium chloride until fixation was complete for histologic processing. The distal half was again divided into 2 sections. One section was placed in sterile culture media with 10% fetal bovine serum and 5 µCi/mL to measure cell proliferation. The other section was placed in RNA*later* (Qiagen, Valencia, California) for growth factor characterization. This procedure was repeated with the unoperated contralateral PTs for biochemical assessment.

Histologic Processing

The specimens were processed for paraffin embedding. Specimen sections were taken in the sagittal plane at 9 µm, and the slides were stained with hematoxylin-eosin (H&E). Each specimen was observed under light and polarized microscopy, and images of characteristic locations were obtained. Crimping patterns, visible with polarized light at 100× objective magnification, were recorded and analyzed with image analysis software (ImageJ version 1.43; US National Institutes of Health [NIH], Bethesda, Maryland).[6,7]

Biochemical assessment cell proliferation in normal and treated tendons was determined by quantifying the tritiated thymidine incorporated by tendon fibroblasts. After incubation at 37°C for 48 hours, the tendons were removed. They were washed overnight to remove unincorporated thymidine and then lyophilized. Aliquots were weighed, and radioactivity was determined with liquid scintillation spectrometry. Results were expressed as counts per minute per milligram dry tissue weight.[8]

Biochemistry

Gene expression was determined with reverse transcriptase-polymerase chain reaction (RT-PCR). Patellar tendons from

the PRP-treated and saline-treated groups were dissected after sacrifice (7 or 28 days after surgery, n = 4). A third group of normal control (n = 4) was also assessed to compare whether the growth factors from PRP and saline-treated were upregulated. All specimens were immediately placed into RNA*later* and stored at -80°C. The tendons were pulverized in liquid nitrogen, and the acid-guanidinium-thiocyanate-phenol extraction procedure was used to isolate total RNA.[9] Starting with 1 µg of total RNA, oligo $(dT)_{15}$ primers were used to synthesize first-strand cDNA. Based on published sequences,[10-15] PCR primer sets specific to selected coding regions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen I, bFGF, IGF, VEGF, and PDGF-BB were constructed as described in **Table I**. Amplifications were allowed to proceed through 34 cycles for all genes. NIH image analysis software (NIH Image, version 1.61; NIH, Bethesda, Maryland) was used to quantitatively scan RT-PCR profiles after agarose gel electrophoresis and ethidium bromide visualization. This software measures relative mean density over a fixed grayscale range after correction for background.

Statistical Assessment

All statistical values are presented as means and Standard Deviations (SDs). Population sizes were determined by power analysis (G*Power 3.1.3).[16] Assuming an effect size of 2.2 and power of 80%, a sample size of 5 rabbits per study group (PRP) and 4 rabbits per control group (saline) was established. Statistical differences were established by Student *t* test with significance level $\alpha = .05$.

Results

The effective concentrations of injected platelets by centrifugation were calculated by an independent laboratory using whole blood and PRP samples collected from each rabbit. Mean (SD) platelet concentration of the 7-day injections was 1.4 (0.3) times the whole blood counts. Mean (SD) concentration injected into the 2-day animals was 1.7 (0.7) times the whole blood counts. These concentrations, as well as WBC counts, are included in **Table II**.

Histology

Figure 3 illustrates 7-day results showing light and polarized H&E histologic sections at 100× magnification. The contralateral control slides (**Figures 3A, 3B**) show uniform collagen bundles with evenly distributed cells; the normal saline slides (**Figures 3C, 3D**) show disrupted collagen bundles and areas of concentrated cells with a disorganized crimping pattern (**arrows**); and the PRP slides (**Figures 3E, 3F**) show uniform collagen bundles with areas of concentrated cells (**arrows**) and a mostly uniform crimping pattern.

Figure 4 illustrates 28-day results showing light and polarized H&E histologic sections at 100× magnification. The contralateral control slides (**Figures 4A, 4B**) show uniform collagen bundles and a uniform crimping pattern with evenly distributed

Table I. Rabbit Primer Sequences (5'-3')

GAPDH	AGGTCATCCACGACCACTTC GTGAGTTTCCCGTTCAGCTC
VEGF	CCATGGCAGAAGAAGGAGAC CACACTCCAGGCTTTCATCA
IGF	CTCTGCTTGCTCACCTTCAC GCCTCCTCAGATCACAGCTC
PDGF-BB	CTACGGTGACCTGGTGGACT ACTCGTCCTTGCTCATGTCC
bFGF	TGCCCAAACTCCTCTACTGC TATAAAAGGCCGTCGGTGTC
Collagen I	TAAGAGCTCCAAGGCCAAGA GCCAGTTTCCTCATCCATGT

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor; IGF, insulinlike growth factor; PDGF-BB, platelet-derived growth factor with 2 B chains; bFGF, basic fibroblast growth factor.

Table II. Summary of Hematologic Analysis of Blood Concentrations (per mL) for Platelets and Red and White Blood Cells by Group

	7 Days			28 Days		
	WB	PRP	MC	WB	PRP	MC
Platelets, ×1000	252.25	365.25	1.44	212.25	323.25	1.71
Red blood cells, ×1,000,000	4.96	4.35	0.88	4.44	4.34	1.00
White blood cells, ×1000	3.20	9.56	2.99	3.66	10.73	2.95

Abbreviations: MC, mean concentration; PRP, platelet-rich plasma; WB, whole blood.

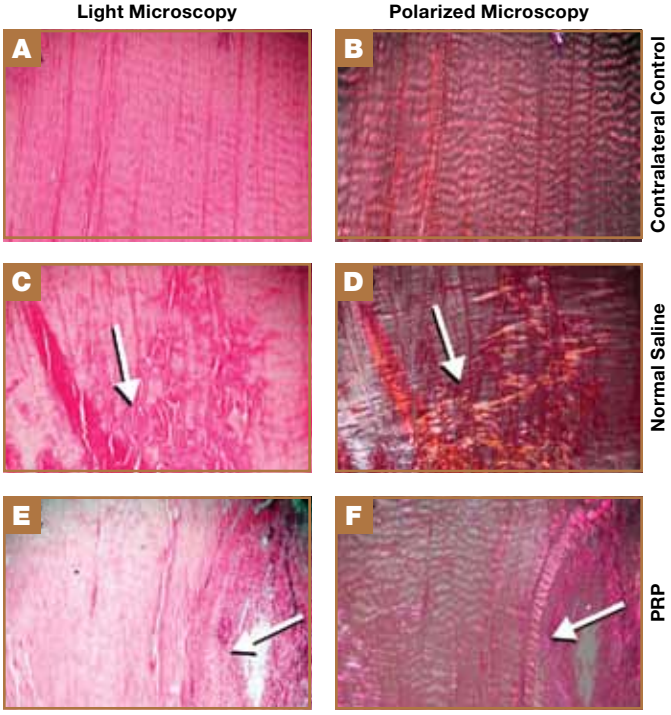

Figure 3. Light and polarized microscopy after 7 days (hematoxylin-eosin, original magnification ×100). PRP indicates platelet-rich plasma.

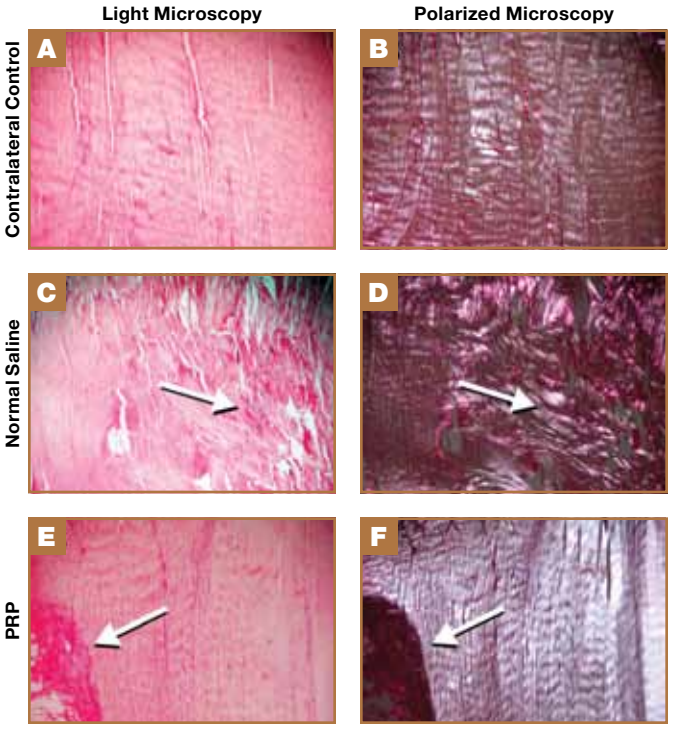

Figure 4. Light and polarized microscopy after 28 days (hematoxylin-eosin, original magnification ×100). PRP indicates platelet-rich plasma.

cells; the normal saline slides (**Figures 4C, 4D**) show disrupted collagen bundles and areas of concentrated cells with a disorganized crimping pattern (**arrows**); and the PRP slides (**Figures 4E, 4F**) show uniform collagen bundles with areas of concentrated cells (**arrows**) and a mostly uniform crimping pattern.

Assessment of polarized crimp densities is presented in **Figure 5**. Although there were no significant differences in the linear crimp density measures between PRP-injected repairs and contralateral controls at both 7 and 28 days, the saline-injected tendons tended to have reduced density, compared with the contralateral controls (P = .07 and .05 at 7 and 28 days, respectively). The normal saline-injected tendons had reduced crimp densities at 28 days, compared with the PRP-injected tendons (P = .07).

Biochemistry

DNA synthesis was determined by measuring the amount of ^{3}H-thymidine incorporated into the tendon cells. The results are shown in **Figure 6**. Seven days after injection, significantly more thymidine was incorporated into the PRP-treated tendons than into the saline-treated tendons—demonstrating a higher rate of DNA synthesis in the PRP-treated tendons at 7 days. However, there was no statistically significant difference 28 days after injection.

Semi-quantitative RT-PCR was used to determine mRNA expression of collagen I, bFGF, IGH, VEGF, and PDGF-BB in the tendons. The results are illustrated in **Figure 7**. There were no statistically significant differences for any of the

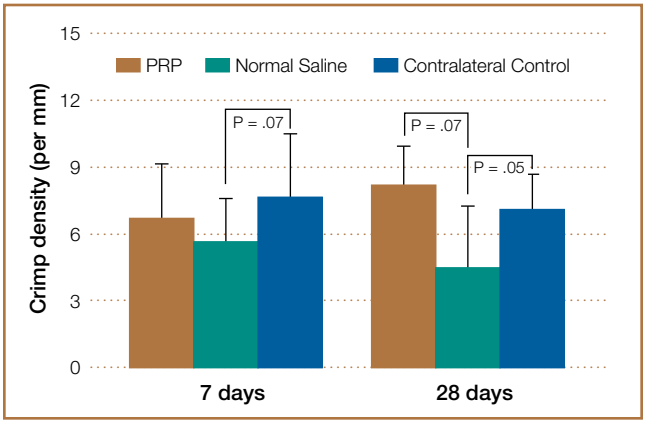

Figure 5. Assessment of crimp density for 7- and 28-day tendon repairs on platelet-rich plasma (PRP) and normal saline groups as well as grouped contralateral controls for both time points.

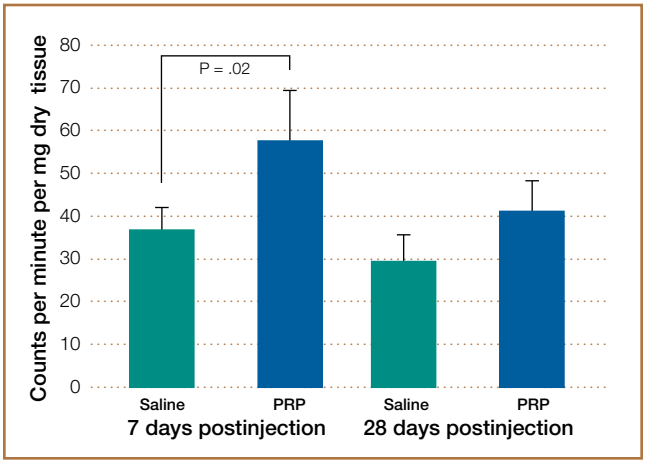

Figure 6. Tritiated thymidine incorporation in saline and platelet-rich plasma (PRP) treated tendon 7 and 28 days after injection.

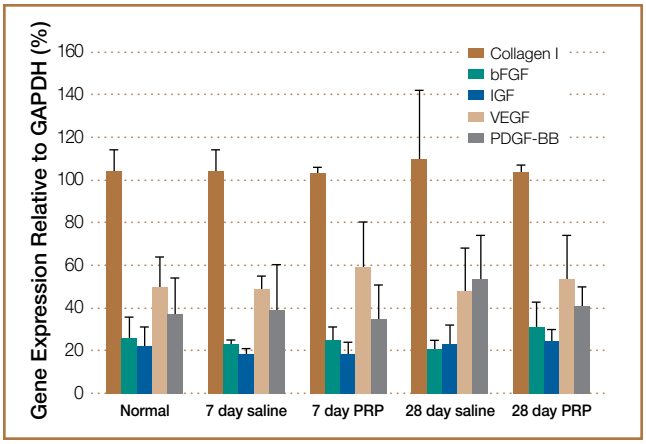

Figure 7. Reverse transcriptase–polymerase chain reaction results for growth factors 7 and 28 days after injection of platelet-rich plasma (PRP) and saline in patellar tendon. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; PDGF-BB, platelet-derived growth factor with 2B chains.

genes at either 7 or 28 days. However, there was a trend for increased bFGF expression in the PRP-treated tendons at 28 days, compared with the saline-treated tendons (P = .15).

Discussion

Use of PRP has been proposed as a new advancement in various aspects of orthopedic care. Musculoskeletal applications have been reported for augmentation in healing of bone, cartilage, tendon, and ligament. Many studies have shown some benefit from use of PRP, though others have noted no difference in benefit between PRP and placebo.[2,3,17-23]

Complicating the assessment of the efficacy of PRP is the lack of consistent data needed for an agreed-on standard for platelet concentration. Reported concentration varies from 1-3.2 times (GPS III system, Biomet Inc, Warsaw, Indiana) to 2-3.6 times (Magellan, Arteriocyte Medical Systems Inc, Hopkinton, Massachusetts) that in the peripheral blood.[24] In other preparations by SmartPREP (Harvest Technologies Corp, Plymouth, Massachusetts), Cascade (MTF Sports Medicine, A Division of the Musculoskeletal Transplant Foundation, Edison, New Jersey), and Autologous Condition Plasma (Webster Orthopedics, San Ramon, California), concentrations fall in between these rates. Whether to include WBCs in the preparation, or to exclude them, is also controversial. In addition, different studies use different concentrations with or without activation.

Other studies have demonstrated clinical improvement in function after PRP injection.[1,25-27] The present study evaluated the biochemical and histologic changes that occur with tendon reaction to PRP. Use of PRP (1.7 times platelet concentration in blood level) stimulated increases in cellular activity and matrix production in a rabbit PT. Thymidine uptake was increased, and the increased cellular activity was correlated with the histologic findings of increased cellular population of the tendon after PRP injection at both 7 and 28 days. These results demonstrated the effects of PRP on the cellular activity of tendons. The increase in cellular concentration may be due to migration of cells from the periphery of the tendon in the peritenon, stimulated by the growth factors in the PRP; cellular division does not appear to be a major factor, as there was no increase in cellular mitosis.

Collagen matrix production was also increased in the PRP-treated tendons, though not to a statistically significant degree (P = .07 at 7 days, P = .05 at 28 days). A rabbit model was used because rabbit tendons have demonstrated disorganized collagen bundle presentation with loss of normal crimping patterns in addition to the hypocellular findings.[28,29] The tendon structure was injured when the needle pierced the tendon; the reparative process was evaluated in both the PRP- and saline-injected PTs.

Changes in collagen matrix production were found with more organized collagen bundles at 7 days, and polarized microscopy showed an increase in collagen crimping at 7 days, demonstrating up-regulation in the collagen extracellular matrix.

The increase in cellular activity was statistically evident at 7 days, which may indicate that the stimulus provided by the active factors from the PRP is short-acting. This study was designed as a human tendinosis model; it used animals that have many of the characteristics of this disease. The growth factors that might stimulate a response have been postulated to include bFGF, IGF, VEGF, and PDGF-BB, as these have stimulatory effects on tendon structures.[30] It was therefore not surprising that these growth factors were not found at 7 and 28 days, as they provided an initial stimulatory effect. After that effect occurred, the growth factors were no longer present, as would be evidenced by the lack of a sustained increase in the stimulatory effect. Literature reviews[31,32] suggest that the increase in these factors may be short-lived and difficult to demonstrate. One study evaluated the effect of PRP on tendinopathies in 15 patients.[33] Interleukins, tumor necrosis factor α, VEGF, and chemokine (C-C motif) ligand 2 decreased from 30 minutes after injection to 3 hours after injection and returned to baseline after 24 hours. These changes correlated with cellular activity and collagen matrix production, which increased to a larger extent in the first 7 days than in the first 28 days. The beneficial effect of this stimulation was still evident, but the effect was beginning to be less marked the further removed from the time of PRP injection.

Our study results demonstrated an underlying stimulatory effect of PRP injected into a tendon structure. This effect occurred histologically with respect to collagen fiber and bundle formation and cellular findings. Other researchers have clinically evaluated the changes that occur in humans after PRP injection. Mishra and Pavelko[3] injected PRP into the lateral epicondyle of patients and noted decreased pain in this group, compared with a group injected with local anesthetic. Platelet concentration increased 539%. Peerbooms and colleagues[34] also showed benefit in using PRP to manage elbow lateral epicondylitis. Our results demonstrated the mechanism of action of PRP with increased collagen synthesis and cellular activity even with a lower concentration of platelets. Although investigators have evaluated different concentrations of platelets with results noted, the optimal level of platelet concentration is not known.

Another unknown is whether WBC activation improves PRP results or WBCs should be excluded.[25,35] Arguments have either supported or questioned the beneficial inflammatory effect of WBC activation. Lack of a clear understanding of the mechanism by which PRP works makes it difficult to ascertain its optimal composition.

There may also be differences in the effects of PRP injection on different tendons. Beneficial effects have been found on the common extensor tendon of the elbow but not on the Achilles tendon.[36-38] Therefore, different tendons with different stress characteristics and vascularity may have different responses to PRP.

The different growth factor dilution rates obtained with the various PRP delivery methods are thought to account for the differences in the stimulatory effect of PRP. Fibrin-rich matrix is thought to have a slower growth factor dilution rate, which may increase how long growth factors are provided to the surrounding tendon structure.[39] Research on this issue will help determine the type of PRP to inject to obtain optimal results in tendons.

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