The Effect of Platelet-Rich Plasma on Muscle Contusion Healing in a Rat Model

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Background: Current therapy for muscle contusions is usually limited to nonsteroidal anti-inflammatory drugs and/or use of the RICE principle (rest, ice, compression, elevation); thus, other forms of treatment that can potentially accelerate the rate of healing are desirable.

Hypotheses: A local injection of platelet-rich plasma (PRP) would lead to accelerated healing rates compared with controls; also, delayed administration of PRP would lead to a blunted response compared with immediate treatment.

Study Design: Controlled laboratory study.

Methods: Forty-six male Lewis rats each underwent a single blunt, nonpenetrating impact to the gastrocnemius muscle via a drop-mass technique and subsequently received either a single injection of saline into the area of injury immediately after injury (controls, n = 11) or rat PRP (either immediately after injury [PRP day 0, n = 12], the first day after injury [PRP day 1, n = 12], or the third day after injury [PRP day 3, n = 11]). The primary outcome was maximal isometric torque strength of the injured muscle, which was assessed before injury as well as on postinjury days 1, 4, 7, 10, and 14. All animals were sacrificed on postinjury day 15. Histological and immunohistochemical analyses were performed on 6 specimens from each group after sacrifice.

Results: The mean platelet concentration in the PRP was 2.19×10^6 ($\pm 2.69 \times 10^5$)/μL. The mean white blood cell count in the PRP was 22.54×10^3/μL. Each group demonstrated statistically significant decreases in maximal isometric torque strength after injury when compared with preinjury levels, followed by significant increases back toward baseline values by postinjury day 14 (controls, 90.6% ± 7.90%; PRP day 0, 105.0% ± 7.60%; PRP day 1, 92.4% ± 7.60%; PRP day 3, 77.8% ± 7.90%) (P = .121). There were no statistically significant differences between the treatment and control groups at any of the time points. There were also no statistically significant differences between any of the groups in the percentage of centronucleated fibers (controls, 3.31% ± 5.10%; PRP day 0, 0.62% ± 1.59%; PRP day 1, 3.24% ± 5.77%; PRP day 3, 2.13% ± 3.26%) (P = .211) or the presence of inflammatory cells and macrophages.

Conclusion: In this rat contusion model, a local injection of PRP into the injured gastrocnemius muscle resulted in no significant differences in functional or histological outcomes, indicating no likely benefit to healing. Additionally, there was no significant difference between immediate or delayed administration of PRP.

Clinical Relevance: Before PRP can be recommended for the treatment of muscle contusion injuries, further translational and clinical investigations need to be performed.

Keywords: platelet-rich plasma; PRP; muscle; contusion; rat

Muscle contusions caused by blunt, nonperforating trauma and muscle strains are among the most common injuries in athletes.[10] Pain and restricted range of motion due to these injuries can lead to decreased performance and limited ability to play. Nonoperative management, including rest, ice, compression, elevation (RICE), is often considered the treatment of choice.[12] Early mobilization versus immobilization is somewhat controversial, and the benefit of other adjuvant therapies such as nonsteroidal anti-inflammatory drugs and corticosteroid injections is still debated.[5] Therefore, for severe contusions, more effective treatments are needed.

Platelet-rich plasma (PRP) is a locally administered agent that is being investigated for use in soft tissue healing. It is a concentrated solution of platelets derived from whole blood. Although the specific elements of PRP have yet to be completely defined, a concentration of approximately ≥1 million platelets/μL has been shown to be clinically useful.[16] Activation of the platelets, whether ex vivo (by thrombin and calcium) or in vivo by exposure to collagen,[7] leads to the local release of growth factors from the alpha and dense granules located in the platelet.

There are very few data on the effect of PRP on muscle injuries. A recent study utilizing a rat model found that PRP treatment resulted in a faster recovery time after

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repeated small muscle strain injuries (but not a single large strain injury).[9] However, currently, there are few published clinical or animal studies investigating the effects of PRP on healing of skeletal muscle contusions. In this study, we sought to employ a rat model of muscle contusion injuries to evaluate the effects of local PRP administration on muscle healing. We hypothesized that a local injection of PRP would lead to accelerated healing rates compared with controls. We also hypothesized that delayed administration of PRP would lead to a blunted response compared with immediate treatment.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee. A total of 46 male Lewis rats aged approximately 12 weeks each underwent a single blunt, nonpenetrating impact to the gastrocnemius muscle via a drop-mass technique. The rats were separated into 4 groups, with each rat receiving an injection into the injured gastrocnemius muscle in the following manner:

- Rats in group 1 (n = 11) received a single injection of 100 μL of saline within 2 hours of injury (controls).
- Rats in group 2 (n = 12) received a single injection of 100 μL of rat PRP within 2 hours of injury (PRP day 0).
- Rats in group 3 (n = 12) received a single injection of 100 μL of rat PRP on postinjury day 1 (PRP day 1).
- Rats in group 4 (n = 11) received a single injection of 100 μL of rat PRP on postinjury day 3 (PRP day 3).

The mean ± SD weight of the rats was 319 ± 15 g, with no significant difference between the groups (P = .249). After the injury, rats were allowed unlimited activity. The primary outcome measurement was maximal isometric torque strength of the injured muscle, which was performed before injury as well as on postinjury days 1, 4, 7, 10, and 14. All animals were sacrificed on postinjury day 15 (Figure 1).

Muscle Contusion Injury Model

The technique was adapted from that described by Crisco et al.[4] Briefly, the rats were anesthetized and placed in the prone position with the right hindlimb secured to a platform. The ankle was placed in the neutral position with the knee extended. Electrodes were placed subcutaneously on either side of the gastrocnemius muscle of the experimental leg. The muscle was then stimulated to tetanus for a period of 1 second throughout impaction to provide the most reliable/reproducible injury.[3] A mass weighing 500 g was dropped from a height of 33 cm onto an impactor shaped as a sphere on its bottom that directly contacted the skin over the rat gastrocnemius muscle, causing the injury (Figure 2). A pilot study demonstrated that this protocol results in a clinically relevant, reproducible injury (see the Appendix, available in the online version of this article at http://ajsm.sagepub.com/supplemental).

PRP Production

Seventeen male Lewis rats were used for the purpose of PRP production. We used Lewis rats because they are syngeneic (ie, inbred and genetically identical), and therefore, the PRP produced precluded any cross-reactivity or adverse immune reaction. Our laboratory developed a custom protocol for preparing rat PRP (because there were no commercial systems specific for animals) as follows. Whole blood was drawn from Lewis rats via an intracardiac puncture after euthanasia by CO_2 inhalation. The blood was pooled and collected in blood tubes containing the anticoagulant citrate phosphate dextrose. The whole blood was then centrifuged at 1000*g* for 15 minutes at 4°C, followed by a 5-minute rest period, followed by another cycle of centrifugation at 1000*g* for 15 minutes at 4°C. (This setting was determined to reliably produce rat PRP platelet concentrations of at least 4 times the whole blood levels, after a pilot experiment evaluating different centrifugation times and speeds [results unpublished]). The platelet-rich fraction of the supernatant was then isolated using a pipette technique and kept at room temperature.

All injections were performed within 2 hours of PRP preparation. In brief, 100 μL of the PRP solution was drawn into a 1-mL tuberculin syringe with a 27-gauge needle. The volume of injection was the same as that previously used in another study utilizing a rat muscle injury model.[9] The injection site was prepped in a sterile fashion, and the needle was inserted through the skin into the medial head of the gastrocnemius in the region of injury. Approximately half of the volume was injected into the medial gastrocnemius. Then, without completely withdrawing the needle out of the skin, it was redirected into the lateral head to allow for injection of the remaining volume. No additive was used to activate the PRP before injection as previous work has shown that PRP can be activated by exposure to collagen alone.[7]

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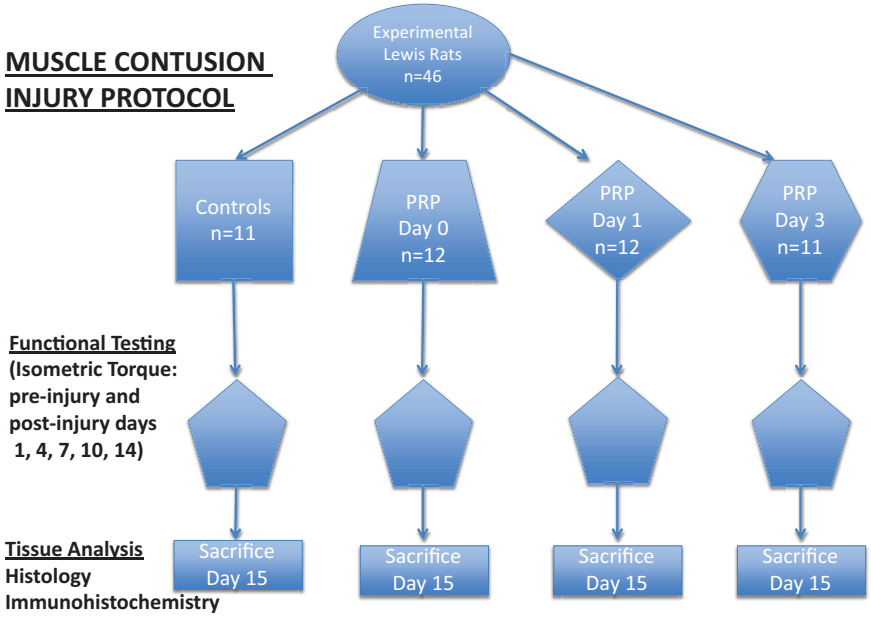

Figure 1. Flowchart demonstrating the study design.

Platelet Concentration and Growth Factor Analysis

The concentration of platelets in the PRP was determined using an automated cell counter. Aliquots from each preparation were analyzed for platelet cell count to ensure that the concentration was at least 4 times the whole blood levels. The concentrations of platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor–β (TGF-β) were determined by enzyme-linked immunosorbent assays (Quantikine Immunoassay Kits; R&D Systems) performed by Harvest Technologies on an aliquot of prepared PRP.

Biomechanical Testing (Maximal Isometric Torque Testing)

The primary outcome tested was maximal isometric torque strength of the gastrocnemius complex as a percentage of baseline (preinjury) values. For torque testing, the animal was first sedated with inhalation anesthesia and placed supine onto a custom platform. The foot was secured onto a footplate, and the tibia was stabilized with a Kirschner wire placed through the proximal metaphysis (Figure 3). A C-clamp was used to secure the thigh to a post with the knee at 90° of flexion. Muscle stimulation was performed using monopolar needle electrodes placed subcutaneously in the popliteal fossa/proximal calf. Contraction was induced by stimulation with a 15-mA constant current, and voltage was optimized for maximum contraction. All isometric contractions were performed with the foot orthogonal to the tibia (considered 0°). Maximal isometric torque testing was performed on the injured limb before injury (baseline value), 5 minutes after injury, and then 1, 4, 7, 10, and 14 days after injury.

Histological Analysis

Upon sacrifice, 6 rats from each group were chosen at random for tissue harvest and histological processing. Briefly, the injured gastrocnemius muscle was carefully dissected and placed in formalin for 72 hours. The tissue was then embedded in paraffin and sectioned in the axial plane in 5 μm–thick slices.

Evaluation of Muscle Regeneration

Tissue specimens were stained with hematoxylin and eosin to evaluate the general morphology and to determine the number of centronucleated muscle fibers. Centronucleated fibers are a marker of muscle regeneration.[15] Analysis of regenerating myofibers was performed by counting the number of fibers in 10 randomly selected microscope fields per sample. Samples were viewed under 100× magnification using a light microscope (Nikon Optiphot; Nikon Corp), and pictures were obtained with a digital camera (Nikon DMX1200; Nikon Corp) for recording purposes and later review.

Evaluation of Fibrosis

Masson trichrome staining (IMEB Inc) was used to evaluate the extent of fibrosis in the area of injury. After Masson trichrome staining, specimens were viewed at 40× magnification using a light microscope, and photomicrographs were obtained with a digital camera. Areas of fibrosis were carefully outlined and circumscribed using ImageJ software (National Institutes of Health). The ratio of the fibrotic area to the total cross-sectional area (also outlined using ImageJ software) was calculated to estimate the degree of fibrosis formation.

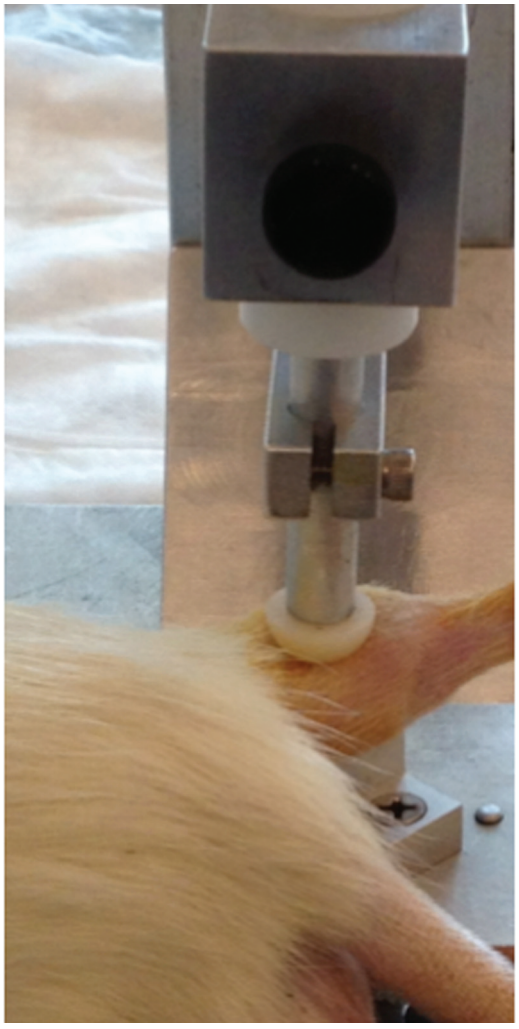

Figure 2. Injury model setup with the rat lying prone and impactor striking the gastrocnemius of the hindlimb.

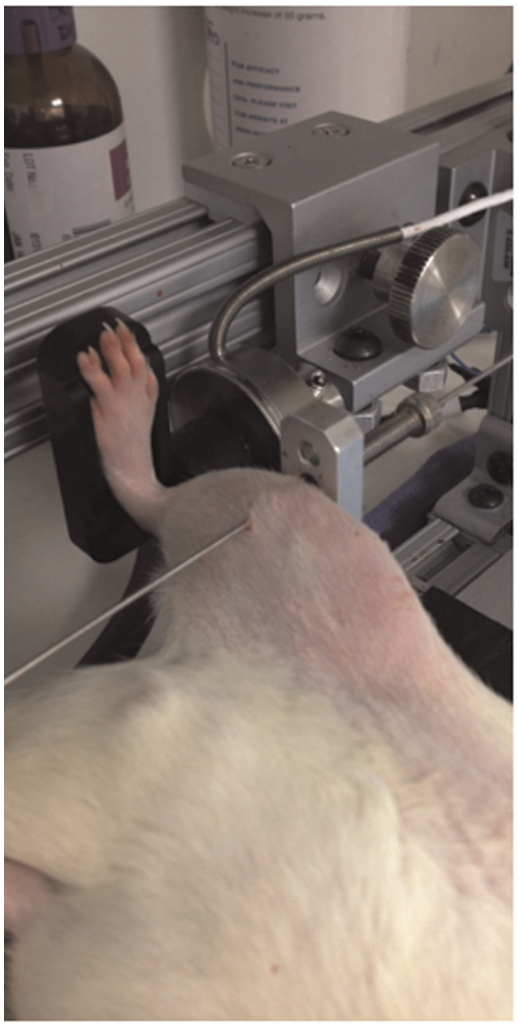

Figure 3. A rat being prepared for torque testing. The foot is placed on a foot holder attached to a torque sensor, and the tibia is secured with wire. Stimulation is performed with subcutaneous electrodes (not shown).

Immunohistochemical Analysis

Serial sections were treated with 3% H_2O_2 to quench endogenous peroxidase activity, and nonspecific antibody binding was blocked with serum-free protein block. Each primary antibody was applied to separate serial sections for 60 minutes at room temperature. Bound antibodies were visualized using a biotinylated link antibody and streptavidin–horseradish peroxidase system developed with 3,3′-diaminobenzidine (DAB, Dako Corp) as a substrate chromogen. We used the following antibodies to localize hematopoietic lineage cells: mouse anti-rat ED1 macrophage (ED1 antigen is a lysosomal glycoprotein expressed only by a subpopulation of macrophages and monocytes) and mouse anti-rat ED2 macrophage (ED2 antigen is a membrane glycoprotein found only on mature tissue macrophages) (Serotec Inc). The sections were counterstained with Mayer hematoxylin. Semiquantitative analysis was performed by reviewing 10 randomly selected fields per sample at 100× magnification using a light microscope and obtaining pictures with a digital camera. A grading system was used as follows: grade 0 (no positively staining cells), grade 1 (scant number of positively staining cells in the entire field), grade 2 (moderate number of positively staining cells in the entire field [ie, less than the majority of cells]), and grade 3 (large number of positively staining cells in the entire field [ie, the majority of cells]).

Statistical Analysis

A power analysis using pilot data was performed with the assistance of the institutional biostatistics department before commencing the study. The primary outcome measure used for the power analysis was isometric torque strength. This analysis determined that with α set at .05, β set at .20, and an effect size of 25%, 12 animals per group would be required.

Histological and immunohistochemical data were analyzed with a single-factor analysis of variance (ANOVA) using SigmaStat software (Systat Software Inc). Data from contractile testing were analyzed using a 2-way ANOVA with PRP injection date and days after injury and a cross-effect between the two as parameters. The variation between individual rats within each injection group was treated as a random effect. Tukey honest significant difference (HSD) post hoc tests were used to identify intraeffect significant differences. The 2-way ANOVA and post hoc tests were performed using JMP software (SAS Institute Inc), and statistical differences were considered at $\alpha = .05$.

RESULTS

Twelve rats underwent the procedure in the immediate treatment and PRP day 1 groups. Eleven rats underwent the procedure in the control and PRP day 3 groups because of anesthetic complications, resulting in 2 accidental death at the time of surgery.

Platelet and Growth Factor Concentrations

The mean platelet concentration in the PRP was 2.19×10^6 ($\pm 2.69 \times 10^5$)/μL. This was over 4 times greater than the mean whole blood platelet levels in a randomly selected sample of whole blood obtained before centrifugation. The mean white blood cell count in the PRP was 22.54×10^3/μL. The mean white blood cell count in rat whole blood was 5.21×10^3/μL.

In the aliquot of PRP tested for specific growth factors, the PDGF-AB, VEGF, and TGF-β concentrations were 330 pg/mL, 18 pg/mL, and 85 ng/mL, respectively. The PDGF-AB, VEGF, and TGF-β concentrations were 125 pg/mL, 10 pg/mL, and 32 ng/mL, respectively, in whole blood. Thus, PDGF-AB levels were 2.6 times greater in the PRP compared with whole blood levels from the same sample; VEGF levels were 1.8 times greater, and TGF-β levels were 2.7 times greater, respectively. (In that specific aliquot, the platelet concentration in the PRP was 1994×10^3/μL, whereas in whole blood, it was 464×10^3/μL, which is nearly 4.3 times less than that in PRP) (Table 1).

TABLE 1
Platelet and Growth Factor Concentrations in a Randomly Selected Aliquot of Whole Blood Used to Prepare PRP[a]

	Platelet Count, $\times 10^3$/μL	PDGF-AB, pg/mL	VEGF, pg/mL	TGF-β, ng/mL
PRP	1994	330	18	85
Whole blood	464	125	10	32
Factor difference	4.3	2.6	1.8	2.7

[a]PDGF-AB, platelet-derived growth factor–AB; PRP, platelet-rich plasma; TGF-β, transforming growth factor–β; VEGF, vascular endothelial growth factor.

Isometric Torque Testing

Each group demonstrated statistically significant decreases ($P < .05$) in maximal isometric torque strength after injury compared with preinjury levels, with the lowest values observed by approximately day 4 to 7 (Figure 4). This was followed by significant increases ($P < .05$) back toward baseline values by postinjury day 14. There were no statistically significant differences between the control and treatment groups at any of the time points ($P = .1208$). The only statistically significant differences were between testing days themselves ($P < .0001$). A Tukey HSD post hoc test showed that day 0 testing was significantly different from days 1, 4, 7, and 10 (but not day 14) ($P < .0001$). Day 14 was significantly different from days 4 and 7 ($P < .001$), day 10 ($P = .0007$), and day 1 ($P = .0334$). Day 7 was significantly different from days 1 and 10 ($P < .0001$), and day 4 was significantly different from day 10 ($P = .0003$) (Figure 4).

Histological Analysis

Histological analysis was performed on samples retrieved after sacrifice (postinjury day 15). The control group demonstrated the highest mean percentage of centronucleated fibers per visual field (3.31% ± 5.10%), and the PRP day 0 group demonstrated the lowest (0.62% ± 1.59%). The PRP day 1 group had a mean percentage of 3.24% ± 5.77%, and the PRP day 3 group had a mean percentage of 2.13% ± 3.26%. However, the difference in the percentage of centronucleated muscle fibers between treatment and control groups was not statistically significant ($P = .211$) (Figure 5).

The PRP day 1 group demonstrated the highest percentage of volume of fibrotic (scar) tissue (3.11% ± 3.65%), and the control group demonstrated the lowest (0.89% ± 1.95%). The PRP day 0 and PRP day 3 groups had a mean percentage of volume of 1.37% ± 2.44% and 2.78% ± 4.75%, respectively. However, the difference in the percentage of fibrotic (scar) tissue was not statistically different between any of the groups ($P = .158$) (Figure 6).

Immunohistochemical Analysis

The group treated with PRP immediately after injury demonstrated the lowest mean immunohistochemical grade (0.22 ± 0.41), whereas the group that received PRP on postinjury day 3 had the highest mean immunohistochemical grade (0.42 ± 0.61). The group treated with PRP on day 1 and the control group had a mean grade of 0.26 ± 0.74 and 0.34 ± 0.58, respectively. There were no statistically significant differences in the mean immunohistochemical grade between any of the groups ($P = .1158$) (Figure 7).

DISCUSSION

Platelet-rich plasma has received a tremendous amount of attention both in the medical literature as well as the lay

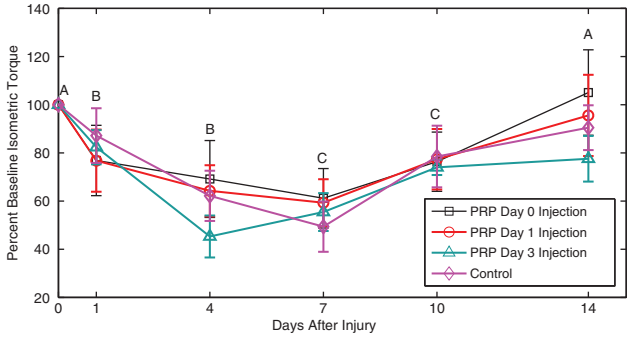

Figure 4. Mean maximal isometric torque strength by group over time. Corresponding letters indicate days that are not statistically different from one another.

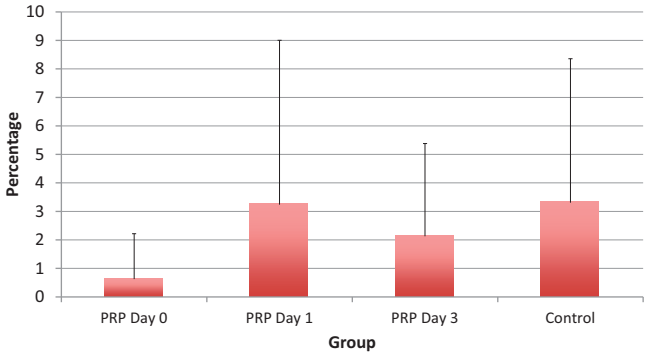

Figure 5. Percentage of centronucleated muscle fibers per group.

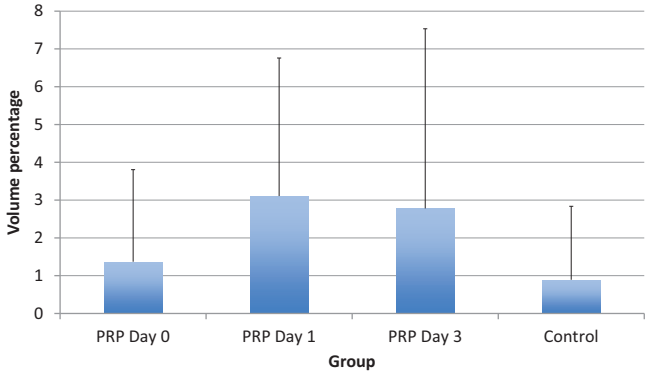

Figure 6. Percentage of volume of fibrotic tissue per group.

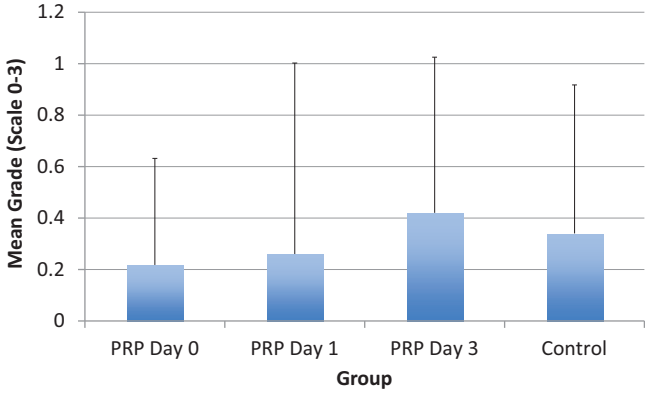

Figure 7. Mean immunohistochemical grade per group (antibody testing for ED1/ED2).

press over the last several years. Initial reports demonstrated positive findings, but these studies were limited primarily to small retrospective case series.[18,19,25] In a case-control study, Sanchez et al[25] reported that 6 athletes who underwent open Achilles tendon repair along with an injection of PRP recovered range of motion sooner and were able to return to running and training more quickly than 6 athletes who underwent open Achilles repair without a PRP injection. Mishra and Pavelko[19] reported significant pain relief in patients who received a single PRP injection for chronic elbow epicondylar pain at final follow-up.

More recently, clinical studies of higher level quality[6,8,21,24] have been published, but these have shown variable results. A recent randomized controlled trial demonstrated no effect on Achilles tendinitis treated with a PRP injection and eccentric exercise compared with controls[6]; however, a randomized controlled trial showed that PRP treatment of lateral epicondylitis had superior outcomes (pain scores and function) compared with steroid injections.[21] Some criticisms associated with these studies include the lack of isolated treatment and the use of a treatment (ie, steroid injection) that is not universally regarded as the gold standard. In addition, other studies, including a randomized controlled one from our institution, have shown no benefit in the treatment of rotator cuff injuries with platelet-rich therapies.[2,24,27]

Platelet-rich plasma has also been used empirically for the treatment of muscle injuries, although the literature on the effects of PRP on muscle healing is scant. Sanchez et al[25] presented a small retrospective series in which they reported positive findings, but they were limited in terms of the outcomes tested and the rigor of the study design. The authors reported performing ultrasound-guided injections of PRP into 22 injured muscles in 20 professional athletes. They noted full functional recovery in all patients in half the expected recovery time, without evidence of fibrosis and no reinjuries upon the resumption of normal activities. This study, however, is limited to an abstract that was presented at the 2nd World Congress on Regenerative Medicine in 2005 (Sanchez M, Anitua E, Andia I. "Application of Autologous Growth Factors on Skeletal Muscle Healing.") with no further follow-up. No other evidence level 4 or higher clinical study exists regarding PRP and muscle healing.

In the animal literature, Hammond et al[9] used a rat tibialis anterior strain model and found that PRP provided a positive benefit in terms of faster recovery for those rats that had multiple strain injuries but not in those in the single-strain

protocol group. The authors speculated that because recovery from the high-repetition protocol primarily relies on myogenesis, rather than sarcolemmal repair,[15] PRP may be more effective for injuries that rely on muscle regeneration rather than repair. Recent work by Terada et al[26] in mice also reported positive findings in those treated with a PRP injection and administration of losartan after a muscle contusion injury. More specifically, the authors reported improvement in muscle strength after PRP and losartan treatment as well as enhanced muscle regeneration and angiogenesis with decreased muscle fibrosis.[26]

Our study using a muscle contusion model showed no differences in terms of muscle contractile testing and histological outcomes as well as no evidence of increased inflammation by the time of sacrifice. The fact that our study showed no positive effect on healing after treatment with PRP may be because of a number of reasons. We utilized a rat model of muscle injuries limited to a single contusion event; as suggested by Hammond et al,[9] PRP may be of greater utility in multiple injury scenarios because these injuries may rely primarily on a muscle regeneration mechanism rather than sarcolemmal/muscle repair. Although it was originally thought that recovery of function after a skeletal muscle injury is predominately associated with the activation and proliferation of myogenic cells (ie, satellite cells),[1,14,23] recent work by Lovering et al[15] has shown that single-repetition muscle strain injuries result predominately in sarcolemmal (membrane) injuries, followed by early sarcolemmal repair (membrane resealing), rather than satellite cell activation and proliferation. Thus, the fact that we found no difference between the experimental and control groups may be because our injury model involved a single, large contusion event rather than multiple injuries.

Additionally, our PRP was prepared according to a protocol that we developed, and we did not rely on a commercial system. At the time this study was being conducted, there were no commercially available systems specifically for animal use. Indeed, several other groups had used noncommercial systems for producing rat PRP before this study.[11,13,20,22] These authors reported platelet concentrations in PRP of approximately 3 to 10 times that of whole blood. We settled on a protocol that resulted in platelet counts approximately 4 times greater than that in rat whole blood, as per the recommendation of Marx[16] with regard to human PRP. This resulted in specific growth factor concentrations that were similarly increased several-fold beyond whole blood values. Because the literature on PRP is still somewhat limited, there is yet no optimal PRP concentration, volume, or injection schedule that can be relied upon for ideal results. The PRP that we produced was collected from rats that were syngeneic (ie, inbred) so that should have precluded any risk of cross-reaction or enhanced immune reactivity (although other authors have used rat species that were not syngeneic without apparent adverse effects[9]). In addition, we also chose to utilize the PRP immediately after preparation rather than storing it for later use to minimize any effects of prolonged storage or freeze/thaw cycles as well as the fact that this is how it is typically prepared and applied clinically.

Our study has the following limitations. (1) The study may have been underpowered to detect small differences between groups; as mentioned in the Results section, 2 groups had animal numbers that were 1 less than the intended number. This was because of anesthetic complications that occurred at the time of surgery. Nevertheless, based on the post hoc statistical analyses, we do not believe that the addition of 1 animal in each of those 2 groups would have made a large difference in the outcomes. (2) The optimal concentration of platelets, timing of injection, and volume of solution to be injected have yet to be determined, especially in animal models. Additional research is needed to better define the effects of these variables. (3) The PRP that we generated had a relatively high concentration of white blood cells. Although the exact role of leukocytes in PRP is unclear, there is some evidence that increased levels can lead to elevated inflammatory cytokine production[17] and therefore, perhaps, a more catabolic rather than anabolic effect. (4) Only a single dose of PRP was used (ie, no repeated PRP injections), although no research to date has shown definitive superiority with multiple injections over single injection protocols. (5) The potential benefits of PRP for acute muscle healing may be limited; it may be that chronic injuries/lesions respond better than acute ones. (6) We did not quantify rat activity after injury, which may present a confounding factor. (7) Finally, the variability noted in the histological and immunohistochemical analyses may be caused by slight changes in the plane of sectioning of tissue samples.

In summary, PRP is being used to treat numerous soft tissue injuries (including muscle contusions), although clinical data are limited. In this rat contusion model, a local injection of PRP into the injured gastrocnemius muscle resulted in no significant differences in functional outcome at various postinjury time points, indicating no likely benefit to healing. Additionally, there was no significant difference between immediate or delayed administration of PRP. Further translational and clinical investigations need to be performed before PRP can be recommended for the treatment of muscle contusion injuries.

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