Winner of the Cabaud Award

Use of Platelet-Rich Plasma Immediately After an Injury Did Not Improve Ligament Healing, and Increasing Platelet Concentrations Was Detrimental in an In Vivo Animal Model

Robert F. LaPrade,*†‡ MD, PhD, Laurie R. Goodrich,§ DVM, PhD, Jennifer Phillips,§ MS, Grant J. Dornan,† MS, Travis Lee Turnbull,† PhD, Michael L. Hawes,‖ DVM, Kimi D. Dahl,† MS, Ashley N. Coggins,† BS, John Kisiday,§ PhD, David Frisbie,§ DVM, PhD, and Jorge Chahla,† MD, PhD

Investigation performed at the Department of BioMedical Engineering, Steadman Philippon Research Institute, Vail, Colorado, USA, and Orthopaedic Research Center and Laboratory Animal Resource Center, Colorado State University, Fort Collins, Colorado, USA

Background: Limited information in basic science and clinical trials exists to determine if ligament healing may be accelerated with the use of biological adjuvants, such as platelet-rich plasma (PRP). However, there has been widespread acceptance of PRP for use in clinical practice, despite an inadequate understanding of its biological mechanism of action.

Purpose: To determine whether a single dose of PRP could accelerate ligament healing and correspondingly improve histological characteristics and biomechanical properties when injected immediately postoperatively into the injured medial collateral ligament (MCL) of New Zealand White rabbits.

Study Design: Controlled laboratory study.

Methods: Eighty skeletally mature New Zealand White rabbits (160 knees) were used. The MCL was torn midbody to simulate a grade 3 tear. After an acute injury of the MCL, the administration of autologous PRP at 3 different platelet concentrations (0 million/uL, platelet-poor plasma [PPP]; 0.6 million/uL, 2 times the baseline [2× PRP]; and 1.2 million/uL, 4 times the baseline [4× PRP]) was performed and compared with a saline injection control in the contralateral knee. Histological analysis and a biomechanical endpoint characterization were utilized to assess ligamentous healing and compare it to a sham surgery group.

Results: The PPP (P = .001) and 4× PRP (P = .002) groups had a significantly lower collagen subscore than the sham surgery group. No other differences were observed among the treatment groups, including the vascularity subscore and overall ligament tissue maturity index score. Compared with saline-injected contralateral knees, the maximum load for PPP and 2× PRP was not significantly different (P = .788 and .325, respectively). The maximum load and stiffness for knees treated with 4× PRP were significantly less than for the saline-treated contralateral knees (P = .006 and .001, respectively).

Conclusion: One single dose of PPP or 2× PRP at the time of injury did not improve ligament healing. In addition, 4× PRP negatively affected ligament strength and histological characteristics at 6 weeks after the injury.

Clinical Relevance: The current practice of treating knee ligament injuries with PRP may not improve healing at low doses of PRP. The decreased mechanical properties and histological appearance of the torn MCL suggest that high doses of PRP decrease the quality of repair tissue. Further in vivo studies are necessary to determine the dosing and timing of PRP administration after a ligament injury before the widespread use of PRP to treat ligament injuries is recommended.

Keywords: medial collateral ligament; ligament; platelet-rich plasma; rabbit; biologics; knee

The American Journal of Sports Medicine
1–11
DOI: 10.1177/0363546517741135

It has been reported that the next most significant treatment advancement in orthopaedic sports medicine since the use of the arthroscope will be biologics to augment and potentially accelerate the healing of injured tissue.[23]

Platelet-rich plasma (PRP) has been identified as a biological treatment that may be effective in healing musculoskeletal tissue because of its inherently high concentrations of beneficial growth factors.[6-8,11,18,30] These growth factors are derived from the alpha granules of platelets and are released in response to many stimuli.[21,22] When platelets are activated, they release several growth factors that have been demonstrated to improve tissue healing, especially during the inflammatory phase of healing.

In recent years, PRP has emerged as an accessible and United States Food and Drug Administration–approved source of growth factors for treating musculoskeletal injuries. The justification of the clinical use of PRP is derived by an attempt to augment the natural biological healing process through its potential anabolic effects. Despite many studies on the effects of PRP, there remains a paucity of literature on its mechanism of action. The alpha granules of PRP have been reported to contain growth factors that are important for musculoskeletal healing, as stimulators of cell proliferation and via chemoattraction, such as transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), epidermal cell–derived factor (EDF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF).[31] The growth factor concentration in platelets, coupled with the platelet concentration in blood, is sufficient to yield nanograms of specific growth factors per milliliter of blood.[14] Basic science and preclinical research studies support the potential of PRP to have a beneficial effect on connective tissue repair.

PRP injections for injured ligaments are used worldwide by physicians based on many anecdotal studies and a few randomized clinical control studies, which have suggested an acceleration of healing.[27,37,41,42] However, there are many conflicting reports in the literature, and debate continues surrounding the efficacy and role of PRP in accelerating ligament healing. Potential factors that may affect the efficacy of PRP include platelet concentration (thus, growth factor concentration), leukocyte count, timing of the treatment, and activation of platelets within PRP.[8,12,32,44] In vitro studies have confirmed that PRP has beneficial effects on ligament fibroblast migration, differentiation, and collagen production.[35,38] Furthermore, PRP has been reported to enhance the gene expression of collagen type 1, decorin, and cartilage oligomeric matrix protein (COMP) and the collagen type 1:3 ratio in ligament explants.[21] The effects of PRP treatment on in vivo ligament healing have been most commonly studied in the anterior cruciate ligament (ACL). Some studies have reported an improvement in ACL healing when biomechanical, magnetic resonance imaging, or pain assessment outcomes have been measured.[9,29,41] Conversely, other studies have reported no beneficial effects on ACL healing when these same parameters were analyzed.[24,27]

Isolated medial collateral ligament (MCL) injuries are the most common knee ligament injuries and usually heal with nonoperative treatment. However, the mechanical and histological properties have been reported to not return to normal.[28,34] For this reason, the use of PRP to advance MCL healing has been advocated.[2] Surprisingly, despite the frequent use of PRP to treat ligament injuries, there is limited information in basic science and clinical trials to determine if ligament biomechanical properties could be enhanced with the use of this biological approach.[13] In addition, the enhancement of healing via a higher concentration of platelets would supply more growth factors, which could increase cell stimulation. Thus, the purpose of this study was to determine whether a single dose of PRP at different platelet concentrations could accelerate healing and correspondingly improve histological characteristics and biomechanical properties when injected immediately postoperatively into the injured MCL of New Zealand White rabbits. Therefore, we utilized an MCL injury model mimicking that of humans and clinically relevant PRP concentrations. Our central hypothesis was that PRP would accelerate healing in an MCL injury model after acute trauma and correspondingly enhance the histological and biomechanical properties when compared with platelet-poor plasma (PPP) or saline.

METHODS

Experimental Protocol

In Vivo Injury Model. Approval for this study was obtained from the Institutional Animal Care and Use Committee of Colorado State University (protocol No. 15-6192A). Eighty skeletally mature New Zealand White rabbits (160 knees) were used. One rabbit had to be euthanized during the study, and this rabbit was replaced. Each rabbit was anesthetized with 3.5 mL of an intramuscular injection of 80% ketamine (30 mg/kg), 8% xylazine (6 mg/kg), and 12% acepromazine (0.90 mg/kg) for surgery. After the knees were prepared for surgery, a 4 cm–long longitudinal incision was made 1 cm medial and parallel to the patellar tendon to expose the MCL.

A simulated MCL injury was created using a previously described model.[16] Briefly, the MCL was torn midbody,

*Address correspondence to Robert F. LaPrade, MD, PhD, The Steadman Clinic, 181 West Meadow Drive, Suite 400, Vail, CO 81657, USA (email: Drlaprade@sprivail.org).
†Steadman Philippon Research Institute, Vail, Colorado, USA.
‡The Steadman Clinic, Vail, Colorado, USA.
§Orthopaedic Research Center, Colorado State University, Fort Collins, Colorado, USA.
‖Charter Preclinical Services, Hudson, Massachusetts, USA.
R.F.L. and L.R.G. are co–first authors.
Presented at the annual meeting of the AOSSM, Toronto, Ontario, Canada, July 2017.
One or more of the authors has declared the following potential conflict of interest or source of funding: This study was supported by the Kenneth and Anne Griffin Foundation. R.F.L. is a paid consultant for and receives intellectual property royalties and research support from Arthrex, Ossur, and Smith & Nephew and receives research support from Linvatec.

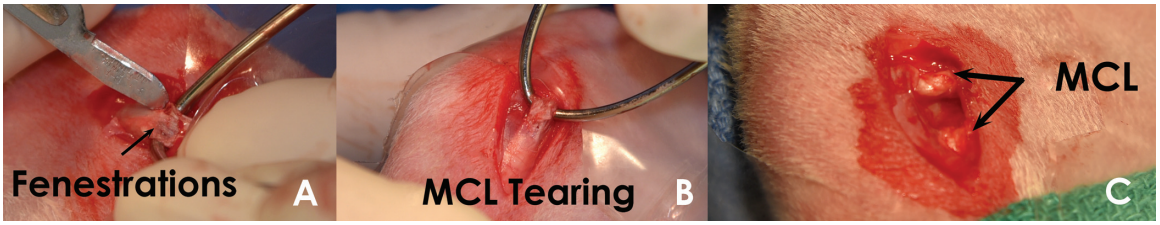

Figure 1. Photographs depicting a medial collateral ligament's (MCL) midbody tear, mimicking a grade 3 tear in a right knee. (A) Incisions were made with a No. 15 blade. (B) Brisk medial traction was applied on the previously placed Kirschner wire. (C) A midsubstance tear was created.

mimicking a grade 3 tear.[1] A Kirschner wire was placed deep to the MCL, several small longitudinal stab incisions were made in the MCL, and brisk medial traction resulted in a midsubstance mop-end tear of the MCL; these ends were left in situ (Figure 1).

The skin was closed with a 5-0 subcuticular absorbable suture. After skin closure, 0.5 mL of PPP or PRP at 2 different platelet concentrations (2× PRP or 4× PRP) was injected into one knee randomly at the mid-MCL tear site, while the contralateral knee was injected with saline at the same location. One group of rabbits underwent sham surgery. That is, the surgical opening was made, and the Kirschner wire placed underneath the MCL and then removed. Closure was similar in all rabbits. The sham-operated rabbits served as the control population that provided a normal biomechanical reference.

Rabbits were administered intramuscular injections of buprenorphine (0.05 mg/kg) for analgesic relief. Enrofloxacin (5 mg/kg intramuscularly) was given immediately after surgery and continued for 3 days postoperatively for prophylaxis against infections. Acetaminophen (1-2 mg/mL) was supplied in the drinking water of each animal for 3 days postoperatively to provide further pain control. All rabbits were allowed unrestricted cage activity over the course of the study and checked daily for infections, the extent of use of the limbs, and any signs of discomfort. Humane euthanasia approved by the Institutional Animal Care and Use Committee was performed at 6 weeks after surgery.

Production and Preparation of PRP. Autologous PRP was processed within 2 hours of blood collection. Blood from each animal was spun at 400*g* for 5 minutes, and the plasma portion of the sample was transferred to a new tube. For animals receiving PPP, the sample was spun a second time at 1000*g* for 5 minutes to pellet all cells. The resulting PPP supernatant was set aside to be used as a treatment. For animals receiving PRP, the plasma portion of the sample was transferred to a new tube and spun a second time at 1000*g* for 5 minutes. However, the PPP supernatant was removed, and 1 mL of PPP was used to suspend the pellet. Platelet and white blood cell (WBC) concentrations were determined for each concentrated PRP solution using a hemocytometer. The concentrated PRP was diluted to either 0.6×10^6 platelets/uL (2 times greater than baseline) or 1.2×10^6 platelets/uL (4× greater than baseline). Each animal received an injection of 0.5 mL of saline on one MCL and then PPP, 2× PRP or 4× PRP on the contralateral MCL. Platelets were not activated with calcium chloride or thrombin because these factors have been reported to possibly influence the biological effects of PRP.[3] Furthermore, preparing PRP in this manner led to an absence of WBCs or minimal WBCs within the preparation; the mean circulating WBC count in the rabbit samples was 6.8×10^3/uL ± 1.2×10^3/uL, and the mean WBC count in the PRP concentrated preparations was 0.3×10^3/uL ± 0.3×10^3/uL.

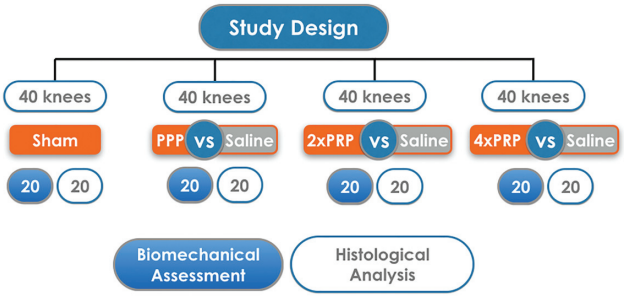

Figure 2. Flowchart demonstrating the study design. One hundred sixty knees were studied. The 4 study groups were (1) sham surgery, (2) platelet-poor plasma, (3) platelet-rich plasma (PRP) with 2 times the baseline number of platelets in circulating blood (2× PRP), and (4) PRP with 4 times the baseline number of platelets in circulating blood (4× PRP). Subsequently, half (n = 20) of the samples were evaluated biomechanically, and half (n = 20) underwent histological analysis.

The study design for the 80 rabbits is shown in Figure 2. Twenty rabbits (40 knees) were utilized in each of the 4 groups (sham surgery, PPP, 2× PRP, and 4× PRP).

Outcome Measures

Gross Postmortem Assessment. All specimens had the skin removed, and gross changes of the MCL were scored and photographed. Proximal, middle, and distal MCL widths were recorded as an indicative measure of topographic scar formation and tissue remodeling for each group. In this regard, a lesser width was indicative of a more efficient remodeling process because it has been reported that straightening of crimped fibers is a functionally relevant phenomenon in healing ligaments.[17]

Histological Analysis. Rabbits designated to undergo histological analysis had their MCLs fixed in 10% neutral-

TABLE 1
Modified Ligament Tissue Maturity Index[25]

	Total = 28 Points
Cellularity subscore (total = 10 points)	
Presence of inflammatory cells	
• Necrosis	0 points
• Polymorphonuclear cells or chronic inflammation	1 point
• No inflammatory cells	2 points
Number of fibroblasts	
• None	0 points
• >2 times the normal ligament	1 point
• <2 times the normal ligament	2 points
Nuclear aspect ratio of fibroblasts	
• No cells	0 points
• Average nuclear aspect ratio <2	1 point
• Average nuclear aspect ratio >2	2 points
Orientation: long axis of the nucleus parallel with normal fascicles	
• No cells	0 points
• <30% of cells oriented	1 point
• >30% of cells oriented	2 points
Arrangement of cells into columns	
• No cells	0 points
• Cells in columns of 2-3	1 point
• Cells in columns of >3	2 points
Collagen subscore (total = 12 points)	
Width of bundles	
• No bundles	0 points
• Width <50 mm	2 points
• Width >50 mm	4 points
Bundle orientation	
• No orientation	0 points
• Presence of bundles perpendicular to the long axis of the ligament	2 points
• Presence of bundles parallel to the long axis of the ligament	4 points
Crimp	
• None present	0 points
• Crimp length <0.5 times the normal length	2 points
• Crimp with normal length present	4 points
Vascularity subscore (total = 6 points)	
Density of blood vessels	
• None present	0 points
• Twice as many as normal present	1 point
• Less than twice normal present	2 points
Orientation of vessels with the long axis of the ligament	
• No vessels oriented	0 points
• <30% oriented	1 point
• >30% oriented	2 points
Vessel maturity	
• No vessels seen	0 points
• Capillaries only present	1 point
• Arterioles present	2 points

buffered formalin. Longitudinal 5 um–thick sections were cut and stained using hematoxylin and eosin and trichrome stain, and polarized light was utilized to grade the sections. The sections were scored by an experienced veterinary pathologist (M.L.H.) unaware of the treatment group. Normal ligament tissue was noted to have fibroblasts arranged in columns, parallel fibers, and no evidence of inflammation. Scoring followed a modification of the grading system of Murray et al[25] (Table 1). Briefly, a composite ligament tissue maturity index was used in which a total score equaled 28. Subscores within this system included those rating cellularity, collagen (including crimp), and vascularity (Figure 3). Each classification within the subscore received 0 to 2 points for worst to best, respectively. Fibroblast maturity was assessed based on the maturity of the nucleus, with more mature nuclei being long with a nuclear aspect ratio of >2. Cell arrangement into columns was assessed, with more mature tissue having long columns of cells and less mature tissue having columns of only 2 to 3 cells. Bundle orientation was also used to assess maturity, with more mature tissue having bundles parallel to the long axis of the ligament and less mature tissue having more bundles

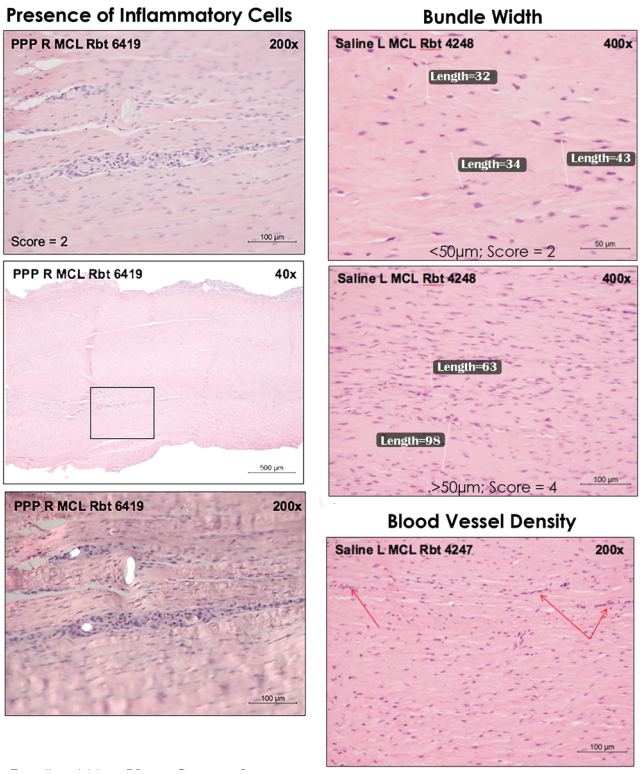

Figure 3. Histological examples of the subscores within the grading system. Left column: the presence of inflammatory cells (cellularity subscore). The top panel shows a platelet-poor plasma–treated sample with minimal lymphoplasmacytic and histiocytic inflammation sometimes around refractile foreign material. The inset box is shown at a lower magnification in the middle panel and also under polarized light in the bottom panel. Right column, top 2 panels: bundle width. Samples with different bundle widths are shown as well as their respective scores. Right column, bottom panel: blood vessel density. High numbers of blood vessels (red arrows) are present in a saline-treated medial collateral ligament sample .

perpendicular to the long axis of the ligament. Crimp was also assessed, with more mature tissue having increased crimp patterns. Blood vessel density was assessed, with normal tissue having fewer blood vessels and less mature tissue having increased arterioles.

Biomechanical Assessment. -The bone-MCL-bone complexes of the rabbit knees were biomechanically evaluated via tensile testing. Each hindlimb specimen was disarticulated at the hip joint and dissected free of all soft tissue on the entire femur and tibia, and the ankle and foot were removed. Meticulous dissection and isolation of the MCL of each knee were performed in a blinded fashion by 2 orthopaedic surgeons (J.C. and R.F.L.). The intact femur and tibia of each knee were successively potted in polymethyl methacrylate (PMMA; Fricke Dental International Inc).

The prepared femur-MCL-tibia complex of each rabbit was rigidly attached via a custom fixture to the actuator

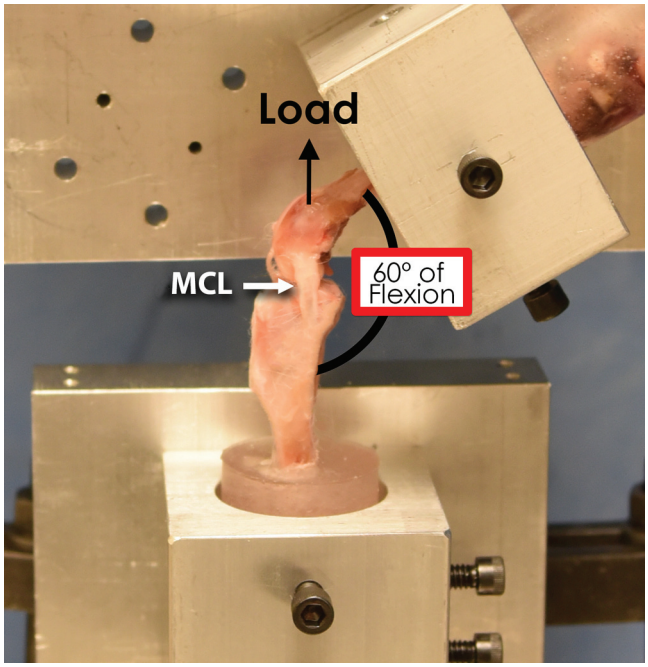

Figure 4. Photograph of a right knee demonstrating the biomechanical testing set-up. Each knee was rigidly fixed in custom clamps at 60° of flexion to align the medial collateral ligament (MCL) fibers with the tensile load.

and base of a dynamic tensile testing machine (ElectroPuls E10000; Instron Systems) (Figure 4). Measurement errors of the testing machine were certified by Instron to be $<\pm 0.01$ mm and $<\pm 2$ N. The custom fixture positioned the knee at 60° of flexion to align the MCL fibers with the tensile load, as previously described.[19,26] The femur-MCL-tibia complex was cyclically preconditioned between 1 and 5 N at 0.1 Hz for 5 cycles and then pulled to failure at 10 mm/min. Load and displacement data were continuously recorded during the pull to failure at a rate of 500 Hz, and the mechanism of failure was noted. Maximum load (N), stiffness (N/mm), displacement at maximum load (mm), and work to maximum load (N·mm) were calculated via a custom script (MATLAB; The MathWorks Inc). Stiffness was computed between 10 N and the elastic limit load, rather than the maximum load, to remove the toe region of pull-to-failure loading and to standardize calculations.

Statistical Analysis

Statistical power of the comparison between paired knees (saline vs treatment) was considered a priori for determining the sample size. Assuming an alpha level of .05, 8 rabbits per group were found to be sufficient to detect a between-limb difference of 30 N in tensile strength with 80% power. To accommodate for unforeseen complications, we included 10 rabbits in each group.

First, comparisons were made between paired contralateral specimens injected with either PRP (PPP, 2× PRP, or 4× PRP) or saline. Wilcoxon signed-rank tests

were used to compare the gross morphology measurement (MCL width) and histological variables, while paired *t* tests were used to compare biomechanical measurements. Second, independent between-group comparisons were made among different PRP formulations and the knees treated with sham surgery. Kruskal-Wallis analyses with Nemenyi post hoc pairwise comparisons were performed for MCL width and histological variables, while parametric analyses of variance with Tukey post hoc comparisons were conducted for biomechanical variables. Statistical significance was declared for P values $<.05$. The statistical computing software R version 3.3.1 (with packages PMCMR and ggplot2; R Core Team) was used to conduct all analyses and plots.

RESULTS

Gross Postmortem Assessment

All animals tolerated surgery, and no sacrifice was needed after the procedures. There was no macroscopic inflammation in any of the knees. MCL healing was grossly evident in all rabbits at 6 weeks. The MCL width was not significantly different among the sham surgery, PPP, 2× PRP, and 4× PRP groups proximally (Kruskal-Wallis, P = .572), in the middle (P = .959), or distally (P = .800). However, 2× PRP resulted in a significantly lower proximal MCL width compared with the control treatment of saline (4.0 ± 1.1 mm vs 4.9 ± 2.0 mm, respectively; P = .044). The width measured in the middle (4.0 ± 0.8 mm vs 4.6 ± 0.5 mm, respectively; P = .014) and distal (3.6 ± 0.9 mm vs 4.4 ± 0.5 mm, respectively; P = .008) aspects of the MCL was significantly lower among knees treated with PPP than their paired counterparts treated with saline.

Histological Assessment

Cellularity Subscore. When compared with the sham surgery group, PPP, 2× PRP, and 4× PRP had a significantly lower cellularity subscore, but no differences were observed between the 3 treatments (Figures 5 and 6). Ligaments treated with PPP had a significantly higher cellularity subscore than their saline-treated contralateral knees (Wilcoxon signed-rank, P = .037). No significant difference was found for 2× PRP or 4× PRP compared with saline.

Collagen Subscore. The PPP (P = .001) and 4× PRP (P = .002) groups had a significantly lower collagen subscore than the sham surgery group (Figures 7 and 8). No other differences were observed among the biological treatments or in comparison to the sham surgery group. Additionally, no significant differences were found for any type of biological treatment compared with their contralateral saline-injected knees.

Vascularity Subscore. All treatment groups (PPP, 2× PRP, and 4× PRP) as well as the saline-treated controls had a significantly lower vascularity subscore than the sham surgery group. No significant differences in tissue vascularity were observed between the 3 biological treatment groups, nor when comparing each biological treatment group to the contralateral saline-injected knees.

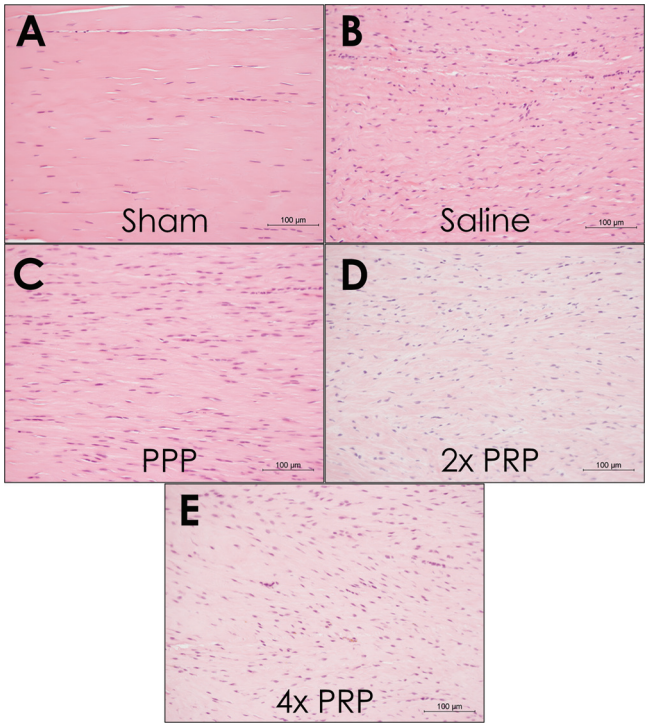

Figure 5. Panels of hematoxylin and eosin staining (200× magnification) represent (A) sham-operated, (B) saline-treated, (C) platelet-poor plasma (PPP)–treated, (D) 2× platelet-rich plasma (PRP)–treated, and (E) 4× PRP–treated medial collateral ligaments (MCLs). (A) The sham-operated ligament reveals a typical number, arrangement, orientation, and nuclear aspect ratio (NAR) of fibroblasts for a normal ligament. (B) The saline-treated ligament shows an increased number of fibroblasts and a decreased NAR of fibroblasts compared with the sham-operated ligament. (C) In the PPP-treated MCL, there is an increased number of fibroblasts and a higher NAR (more elongated) than the saline-treated ligament, and there is a significant ($P > .03$) difference between the saline and PPP groups for the cellularity subscore. (D, E) The 2× PRP– and 4× PRP–treated MCLs reveal cellularity and NARs that are not significantly different than their contralateral saline controls.

Overall Ligament Tissue Maturity Index Score (Compilation of Cellularity, Collagen, and Vascularity Subscores). The PPP, 2× PRP, and 4× PRP groups had a significantly lower ligament tissue maturity index score than the sham surgery group. However, no differences were observed between the 3 treatment groups. Furthermore, no significant differences were found when comparing each biological treatment group with the contralateral saline-injected MCLs.

Biomechanical Assessment

Biomechanical testing results are summarized in Table 2, showing maximum load (N), stiffness (N/mm), displacement at maximum load (mm), and work to maximum load (N·mm). Four knees were lost to premature failure

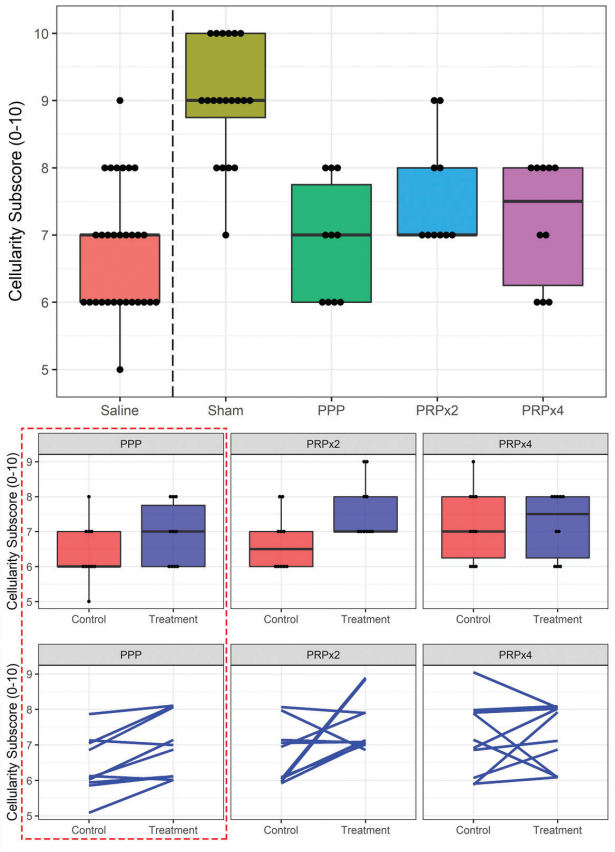

Figure 6. Top: boxplot comparing the cellularity subscore among the biological treatment groups, saline-injected control group, and sham surgery group. All 3 treatment groups (platelet-poor plasma [PPP], 2× platelet-rich plasma [PRP], and 4× PRP) and the saline control group had significantly lower cellularity subscores compared with the sham surgery group. Saline-injected knees are included for reference. Middle: boxplots comparing each biological treatment to the contralateral saline-injected controls. Bottom: line plots connecting the contralateral paired specimens injected with either biological treatment or saline (control). Dots represent individual specimens' measurements. Red-dashed boxes indicate statistical significance.

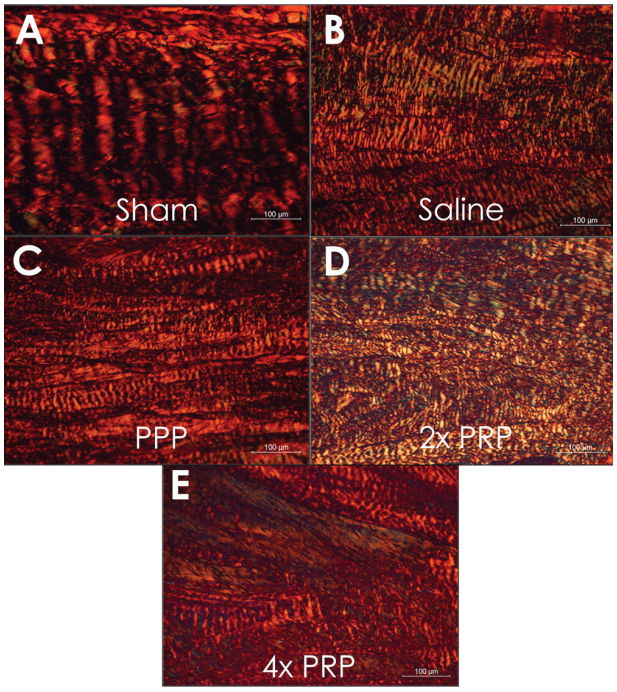

Figure 7. Picrosirius red staining (200× magnification) revealing the median scores of (A) sham-operated, (B) saline-treated, (C) platelet-poor plasma (PPP)–treated, (D) 2× platelet-rich plasma (PRP)–treated, and (E) 4× PRP–treated medial collateral ligaments (MCLs). The sham-operated MCL reveals the typical crimp pattern of ligament fibers under polarized light. In all treatment groups as well as the saline group, the crimp pattern is altered compared with the sham surgery group. The PPP- and 4× PRP–treated MCL scores are significantly decreased compared with the sham-operated MCL.

during specimen preparation or early testing failure (1 sham surgery, 1 PPP, and 2 saline controls for 4× PRP). Thus, only 36 paired comparisons were performed.

Maximum Load. Tukey post hoc comparisons found that the PPP, 2× PRP, and 4× PRP groups had a significantly lower maximum load than the sham surgery group (all $P < .001$), but no differences were observed among the 3 biological treatment groups (all $P > .80$). No significant difference was found in maximum load for the PPP and 2× PRP groups when compared with the saline-injected contralateral knees ($P = .788$ and .325, respectively). Maximum load for knees treated with 4× PRP was significantly less than that for the saline-treated contralateral knees (mean difference, 66 N; 95% CI, 26-105; $P = .006$).

Stiffness. All 3 PRP preparation groups resulted in significantly lower stiffness than the sham surgery group (all $P < .001$). No significant differences were found between the biological treatment groups for stiffness of the MCL (all $P > .40$). In concordance with maximum load testing, significantly lower stiffness was observed in the 4× PRP group compared with the saline-injected contralateral MCLs (mean difference, 11.9 N/m; 95% CI, 6.4-17.3; $P = .001$). No significant difference in stiffness was found for PPP or 2× PRP compared with the saline controls ($P = .085$ and .569, respectively).

Displacement at Maximum Load. All treatment groups were significantly inferior to the sham surgery group for displacement at maximum load (all $P < .001$). There were no significant differences among the 3 biological treatment groups (all $P > .80$), nor were there significant differences between PPP, 2× PRP, and 4× PRP ($P = .108$, .289, and .175, respectively) and the contralateral saline controls with respect to displacement at maximum load.

Work to Maximum Load. All treatment groups were significantly inferior to the sham surgery group in terms of work to maximum load (all $P < .001$). There were no significant differences among the 3 biological treatment groups

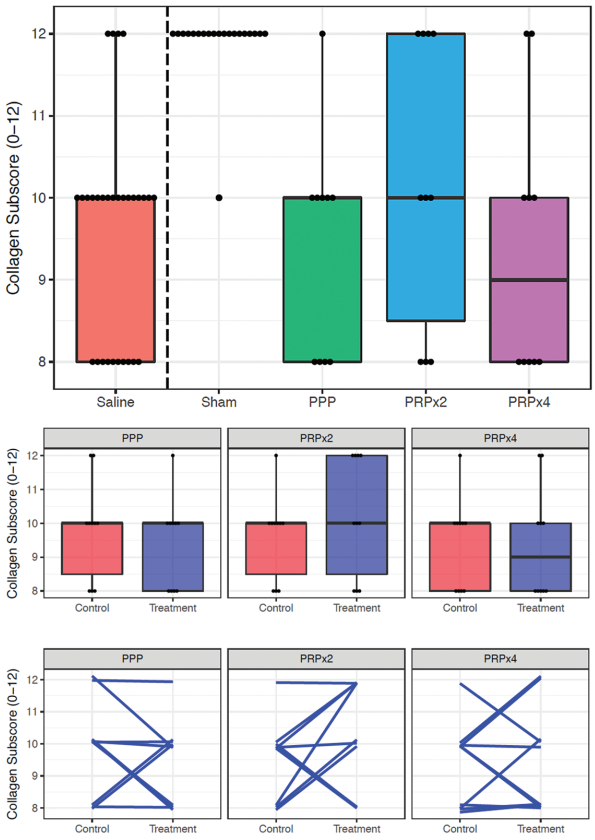

Figure 8. Top: boxplot comparing the collagen subscore among the biological treatment groups, saline-injected control group, and sham surgery group. The platelet-poor plasma (PPP) (P = .001) and 4× platelet-rich plasma (PRP) (P = .002) groups had a significantly lower collagen subscore than the sham surgery group. Saline-injected knees are included for reference. No other differences are observed among the biological treatment groups or in comparison to the sham surgery group. Additionally, no significant differences are found for any type of biological treatment compared with the contralateral saline-injected knees. Middle: boxplots comparing each biological treatment to the contralateral saline-injected controls. Bottom: line plots connecting contralateral paired specimens injected with either biological treatment or saline (control). Dots represent individual specimens' measurements.

(all $P > .90$). Additionally, there were no significant differences between PPP and 2× PRP (P = .501 and .271, respectively) and the matched saline controls with respect to work to maximum load; however, the 4× PRP group exhibited significantly lower work to maximum load compared with the saline control group (mean difference, 175 N·mm; 95% CI, 12-338; P = .038).

DISCUSSION

The most important finding of this study was that one single dose of either PPP or 2× PRP at the time of injury did not accelerate ligament healing. In addition, 4× PRP demonstrated a significant negative effect on ligament strength as well as collagen orientation (relative to sham surgery) at 6 weeks after an injury. Thus, our hypothesis that PRP would accelerate healing in an MCL injury model after acute trauma and correspondingly enhance the histological and biomechanical properties when compared with PPP or saline was not supported. This raises concern that the current practice of treating knee ligament injuries, specifically MCL tears, with PRP immediately after an injury or surgery may not improve healing at low doses of PRP but could be harming ligament healing at higher PRP doses. We strongly recommend that further in vivo studies be performed to determine the dosing and timing of PRP administration after a ligament injury before the widespread use of PRP to treat ligament injuries.

This study helps to provide guidance in a gap in clinical practice. PRP is currently used with little fundamental understanding of its clinical mode of action. Platelets are important in the injury response because they release growth factors, which initiate and modulate wound healing. The justification of the clinical use of PRP is derived by an attempt to augment the natural biological healing process. Despite many studies on the effect of PRP, there has been a paucity of information regarding its in vivo healing potential as well as possible detrimental effects. Conflicting reports on the potential beneficial effects of PRP on healing damaged ligaments, coupled with the lack of basic science research, have left a large gap in evidence that is necessary to justify the use of this commonly sought-after treatment. Unfortunately, anecdotal case reports in peer-reviewed journals and claims of success with this treatment in high-profile sports teams continue to fuel the hype that PRP strongly improves ligament healing.

We found that 4× PRP resulted in a significant decrease in maximum load when compared with saline controls. Furthermore, when compared with sham surgery, 4× PRP had significantly lower collagen subscores. The efficacy of the platelet concentration is of constant debate in the literature.[4,40] In this regard, a recent study suggested that higher platelet concentrations led to more favorable results.[39] Conversely, a recent study by Fleming et al[15] reported that only a baseline concentration of platelets improved healing over traditional ACL reconstruction. Increasing the platelet concentration up to 5 times the baseline concentration did not further improve the graft mechanical properties in their study. In addition, a study by Yoshida et al[43] reported that increasing the platelet concentration above that found in whole blood inhibited wound healing by lowering cell metabolism, increasing cell apoptosis, and decreasing collagen gene expression. Lastly, a study by Boswell et al[5] revealed that increasing the platelet concentration in leukocyte-reduced preparations resulted in decreased synthesis of both COL1A1 and COL3A1 in tendons cultured in vitro. Furthermore, PPP, which contains very few platelets, has also been observed to have a beneficial effect on tendon and muscle repair,[10] and indeed, PPP in our study appeared to have a significant beneficial effect on the cellularity subscore. While this did not ultimately cause the final ligament

TABLE 2
Biomechanical Summary of All Groups[a]

Group	Sample Size	Maximum Load, N	Stiffness, N/mm	Displacement at Maximum Load, mm	Work to Maximum Load, N·mm
Sham	19	423 ± 66 (280-552)	71 ± 11 (53-89)	6.5 ± 1.3 (3.4-8.5)	1399 ± 433 (444-2196)
Saline	28	160 ± 83 (19-376)	48 ± 16 (12-75)	3.6 ± 1.2 (1.4-6.8)	314 ± 255 (16-1107)
PPP	9	156 ± 80 (73-333)	42 ± 10 (27-53)	4.1 ± 1.2 (2.2-6.2)	345 ± 287 (78-1014)
2× PRP	10	167 ± 101 (23-331)	47 ± 17 (25-69)	3.7 ± 1.3 (1.0-5.8)	347 ± 278 (11-949)
4× PRP	10	134 ± 80 (54-307)	39 ± 7 (30-48)	3.6 ± 1.5 (1.9-6.6)	288 ± 300 (47-983)

[a]Values are presented as mean ± SD (range). All knees treated with saline were combined. PPP, platelet-poor plasma; PRP, platelet-rich plasma.

tissue maturity index score (made up of cellularity, collagen, and vascularity subscores) to be significantly improved, it may suggest a small beneficial effect. This further supports the theory that the dose response curve of most growth factors is not linear, and higher concentrations of some growth factors have been reported to be inhibitory to connective tissue cells.[5] In addition, the in vivo results found in this study for MCL healing are similar to those found for intra-articular healing of the ACL in which platelet concentrations greater than 1× were not as effective at promoting ACL healing.[20,43]

Recombinant growth factors supplemented to soft tissue have been reported to improve the repair of musculoskeletal tissue,[33,36] and such studies strongly suggest a "proof of concept" that if various or multiple growth factors are added after a ligament tear, repair mechanisms will be enhanced, leading to accelerated healing and improved function. The primary cell line in the MCL, fibroblasts, has been reported to have receptors for many of the growth factors released by the alpha granules in platelets. The platelet concentrate has important growth factors including those in the TGF-beta superfamily, PDGF, IGF-1, and FGF.[13] The ease of PRP's use and relative affordability make its administration both practical and provocative. Significant hype in the world of regenerative medicine, especially in the orthopaedic arena, has driven many patients to present to their orthopaedic surgeons and demand this treatment. If patients are faced with clinician reluctance to administer treatment, many will seek other clinicians who will administer PRP whether its indications are evidence based or not. However, as is clear from the findings in our study, further studies to determine the timing and dosing frequency of PRP to treat ligament healing are required.

To the best of our knowledge, there exists only one other work studying a preclinical model of surgically created mop-end tears in rabbits.[44] In that article, MCL healing was compared between rabbits that were treated with either nothing or a commercial product in which leukocyte-reduced platelet concentrations were approximately 2 times the circulating blood concentrations. Biomechanical analysis revealed significantly improved structural properties of the MCLs of rabbits treated with PRP, as measured by ultimate load and stiffness. However, the native ligament biomechanical properties were not analyzed, as these authors only compared the plasma rich in growth factor (PRGF) group to the untreated group. Additionally, they reported that histological characteristics were improved with 2× PRP; however, the numbers in each treatment or control group were small ($n \leq 3$ in treatment or control), and therefore, no conclusions could be drawn regarding histological improvements.

We recognize that our study had some limitations. First, we investigated PRP therapy in a rabbit MCL model, and the results cannot be directly applied to the human MCL. However, it has been previously reported that the rabbit model closely resembles healing of the human MCL[16,26]; therefore, the use of this ligament healing model was chosen to mimic current clinical use in humans.[19] We also recognize that the platelet concentration, dosing, timing, and method of application may have influenced these findings. In addition, this study only administered PRP immediately after an injury, and results were assessed only at one time point to keep every arm sufficiently powered. Further studies should be conducted to assess the effect of multiple PRP injections and the optimal length of time between injury and PRP injection. Information from such studies will elucidate whether additional injections are necessary and the optimal time period to wait to administer PRP injections after an injury.

CONCLUSION

One single dose of PPP and 2× PRP at the time of injury did not improve ligament healing. In addition, 4× PRP negatively affected ligament strength and histological characteristics at 6 weeks after an injury.

ACKNOWLEDGMENT

The authors acknowledge Elisa French, BS, LATG, CVT, at the Laboratory Animal Resource Center, for her care of the rabbits and John Schwartz, MS, and Alyssa Ball, MS, for their help with tissue harvest and processing.

REFERENCES

1. American Medical Association. Committee on the Medical Aspects of Sports. Subcommittee on Classification of Sports Injuries. *Standard*

Nomenclature of Athletic Injuries. Chicago: American Medical Association; 1966.
2. Andia I, Maffulli N. Use of platelet-rich plasma for patellar tendon and medial collateral ligament injuries: best current clinical practice. *J Knee Surg*. 2015;28(1):11-18.
3. Anitua E, Sanchez M, Nurden AT, Nurden P, Orive G, Andia I. New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol*. 2006;24(5):227-234.
4. Araki J, Jona M, Eto H, et al. Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen. *Tissue Eng Part C Methods*. 2012;18(3):176-185.
5. Boswell SG, Schnabel LV, Mohammed HO, Sundman EA, Minas T, Fortier LA. Increasing platelet concentrations in leukocyte-reduced platelet-rich plasma decrease collagen gene synthesis in tendons. *Am J Sports Med*. 2014;42(1):42-49.
6. Campbell KA, Saltzman BM, Mascarenhas R, et al. Does intra-articular platelet-rich plasma injection provide clinically superior outcomes compared with other therapies in the treatment of knee osteoarthritis? A systematic review of overlapping meta-analyses. *Arthroscopy*. 2015;31(11):2213-2221.
7. Cassano JM, Kennedy JG, Ross KA, Fraser EJ, Goodale MB, Fortier LA. Bone marrow concentrate and platelet-rich plasma differ in cell distribution and interleukin 1 receptor antagonist protein concentration [published online February 1, 2016]. *Knee Surg Sports Traumatol Arthrosc*. doi:10.1007/s00167-016-3981-9.
8. Cavallo C, Filardo G, Mariani E, et al. Comparison of platelet-rich plasma formulations for cartilage healing: an in vitro study. *J Bone Joint Surg Am*. 2014;96(5):423-429.
9. Cervellin M, de Girolamo L, Bait C, Denti M, Volpi P. Autologous platelet-rich plasma gel to reduce donor-site morbidity after patellar tendon graft harvesting for anterior cruciate ligament reconstruction: a randomized, controlled clinical study. *Knee Surg Sports Traumatol Arthrosc*. 2012;20(1):114-120.
10. de Mos M, van der Windt AE, Jahr H, et al. Can platelet-rich plasma enhance tendon repair? A cell culture study. *Am J Sports Med*. 2008;36(6):1171-1178.
11. Di Matteo B, Kon E, Filardo G. Intra-articular platelet-rich plasma for the treatment of osteoarthritis. *Ann Transl Med*. 2016;4(3):63.
12. Duif C, Vogel T, Topcuoglu F, Spyrou G, von Schulze Pellengahr C, Lahner M. Does intraoperative application of leukocyte-poor platelet-rich plasma during arthroscopy for knee degeneration affect postoperative pain, function and quality of life? A 12-month randomized controlled double-blind trial. *Arch Orthop Trauma Surg*. 2015;135(7):971-977.
13. Engebretsen L, Steffen K, Alsousou J, et al. IOC consensus paper on the use of platelet-rich plasma in sports medicine. *Br J Sports Med*. 2010;44(15):1072-1081.
14. Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg*. 2004;114(6):1502-1508.
15. Fleming BC, Proffen BL, Vavken P, Shalvoy MR, Machan JT, Murray MM. Increased platelet concentration does not improve functional graft healing in bio-enhanced ACL reconstruction. *Knee Surg Sports Traumatol Arthrosc*. 2015;23(4):1161-1170.
16. Frank CB, Loitz BJ, Shrive NG. Injury location affects ligament healing: a morphologic and mechanical study of the healing rabbit medial collateral ligament. *Acta Orthop Scand*. 1995;66(5):455-462.
17. Hurschler C, Provenzano PP, Vanderby R Jr. Scanning electron microscopic characterization of healing and normal rat ligament microstructure under slack and loaded conditions. *Connect Tissue Res*. 2003;44(2):59-68.
18. LaPrade RF, Geeslin AG, Murray IR, et al. Biologic treatments for sports injuries II think tank-current concepts, future research, and barriers to advancement, part 1: biologics overview, ligament injury, tendinopathy. *Am J Sports Med*. 2016;44(12):3270-3283.
19. Liang R, Woo SL, Takakura Y, Moon DK, Jia F, Abramowitch SD. Long-term effects of porcine small intestine submucosa on the healing of medial collateral ligament: a functional tissue engineering study. *J Orthop Res*. 2006;24(4):811-819.
20. Mastrangelo AN, Vavken P, Fleming BC, Harrison SL, Murray MM. Reduced platelet concentration does not harm PRP effectiveness for ACL repair in a porcine in vivo model. *J Orthop Res*. 2011;29(7):1002-1007.
21. McCarrel T, Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. *J Orthop Res*. 2009;27(8):1033-1042.
22. Mishra A, Tummala P, King A, et al. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Eng Part C Methods*. 2009;15(3):431-435.
23. Murray IR, LaPrade RF, Musahl V, et al. Biologic treatments for sports injuries II think tank-current concepts, future research, and barriers to advancement, part 2: rotator cuff. *Orthop J Sports Med*. 2016;4(3):2325967116636586.
24. Murray MM, Palmer M, Abreu E, Spindler KP, Zurakowski D, Fleming BC. Platelet-rich plasma alone is not sufficient to enhance suture repair of the ACL in skeletally immature animals: an in vivo study. *J Orthop Res*. 2009;27(5):639-645.
25. Murray MM, Spindler KP, Ballard P, Welch TP, Zurakowski D, Nanney LB. Enhanced histologic repair in a central wound in the anterior cruciate ligament with a collagen-platelet-rich plasma scaffold. *J Orthop Res*. 2007;25(8):1007-1017.
26. Musahl V, Abramowitch SD, Gilbert TW, et al. The use of porcine small intestinal submucosa to enhance the healing of the medial collateral ligament: a functional tissue engineering study in rabbits. *J Orthop Res*. 2004;22(1):214-220.
27. Nin JR, Gasque GM, Azcarate AV, Beola JD, Gonzalez MH. Has platelet-rich plasma any role in anterior cruciate ligament allograft healing? *Arthroscopy*. 2009;25(11):1206-1213.
28. Niyibizi C, Kavalkovich K, Yamaji T, Woo SL. Type V collagen is increased during rabbit medial collateral ligament healing. *Knee Surg Sports Traumatol Arthrosc*. 2000;8(5):281-285.
29. Radice F, Yanez R, Gutierrez V, Rosales J, Pinedo M, Coda S. Comparison of magnetic resonance imaging findings in anterior cruciate ligament grafts with and without autologous platelet-derived growth factors. *Arthroscopy*. 2010;26(1):50-57.
30. Raeissadat SA, Rayegani SM, Hassanabadi H, et al. Knee osteoarthritis injection choices: platelet-rich plasma (PRP) versus hyaluronic acid (a one-year randomized clinical trial). *Clin Med Insights Arthritis Musculoskelet Disord*. 2015;8:1-8.
31. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets*. 2001;12(5):261-273.
32. Riboh JC, Saltzman BM, Yanke AB, Fortier L, Cole BJ. Effect of leukocyte concentration on the efficacy of platelet-rich plasma in the treatment of knee osteoarthritis. *Am J Sports Med*. 2016;44(3):792-800.
33. Rodeo SA, Suzuki K, Deng XH, Wozney J, Warren RF. Use of recombinant human bone morphogenetic protein-2 to enhance tendon healing in a bone tunnel. *Am J Sports Med*. 1999;27(4):476-488.
34. Scheffler SU, Clineff TD, Papageorgiou CD, Debski RE, Ma CB, Woo SL. Structure and function of the healing medial collateral ligament in a goat model. *Ann Biomed Eng*. 2001;29(2):173-180.
35. Schnabel LV, Sonea HO, Jacobson MS, Fortier LA. Effects of platelet rich plasma and acellular bone marrow on gene expression patterns and DNA content of equine suspensory ligament explant cultures. *Equine Vet J*. 2008;40(3):260-265.
36. Seeherman HJ, Archambault JM, Rodeo SA, et al. rhBMP-12 accelerates healing of rotator cuff repairs in a sheep model. *J Bone Joint Surg Am*. 2008;90(10):2206-2219.
37. Silva A, Sampaio R. Anatomic ACL reconstruction: does the platelet-rich plasma accelerate tendon healing? *Knee Surg Sports Traumatol Arthrosc*. 2009;17(6):676-682.
38. Smith JJ, Ross MW, Smith RK. Anabolic effects of acellular bone marrow, platelet rich plasma, and serum on equine suspensory ligament fibroblasts in vitro. *Vet Comp Orthop Traumatol*. 2006;19(1):43-47.
39. Spang JT, Tischer T, Salzmann GM, et al. Platelet concentrate vs. saline in a rat patellar tendon healing model. *Knee Surg Sports Traumatol Arthrosc*. 2011;19(3):495-502.

40. Thanasas C, Papadimitriou G, Charalambidis C, Paraskevopoulos I, Papanikolaou A. Platelet-rich plasma versus autologous whole blood for the treatment of chronic lateral elbow epicondylitis: a randomized controlled clinical trial. *Am J Sports Med*. 2011;39(10):2130-2134.
41. Vogrin M, Rupreht M, Crnjac A, Dinevski D, Krajnc Z, Recnik G. The effect of platelet-derived growth factors on knee stability after anterior cruciate ligament reconstruction: a prospective randomized clinical study. *Wien Klin Wochenschr*. 2010;122 Suppl 2:91-95.
42. Vogrin M, Rupreht M, Dinevski D, et al. Effects of a platelet gel on early graft revascularization after anterior cruciate ligament reconstruction: a prospective, randomized, double-blind, clinical trial. *Eur Surg Res*. 2010;45(2):77-85.
43. Yoshida R, Cheng M, Murray MM. Increasing platelet concentration in platelet-rich plasma inhibits anterior cruciate ligament cell function in three-dimensional culture. *J Orthop Res*. 2014;32(2):291-295.
44. Yoshioka T, Kanamori A, Washio T, et al. The effects of plasma rich in growth factors (PRGF-Endoret) on healing of medial collateral ligament of the knee. *Knee Surg Sports Traumatol Arthrosc*. 2013;21(8):1763-1769.