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Reduced platelet concentration does not harm PRP effectiveness for ACL repair in a porcine in vivo model

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Abstract

Enhanced primary repair of the ACL using a collagen scaffold loaded with platelets has been shown to improve the functional healing of suture repair in animal models. In this study, our objectives were to determine if lowering the platelet concentration would reduce the structural properties of the repaired ACL and increase postoperative knee laxity. Eight Yucatan minipigs underwent bilateral suture repair. In one knee, the repair was augmented with a collagen scaffold saturated with platelet-rich plasma containing five times the systemic baseline of platelets (5X) while the contralateral knee had a collagen scaffold saturated with platelet-rich plasma containing three times the systemic baseline of platelets (3X). After thirteen weeks of healing, knee joint laxity and the structural properties of the ACL were measured. The 3X platelet concentration resulted in a 24.1% decrease in cellular density of the repair tissue ($p < 0.05$), but did not significantly decrease the structural properties [3X vs 5X: 314 vs 298 N ($p = 0.596$) and 65 vs 64 N/mm ($p = 0.532$) for the yield load and linear stiffness, respectively]. The 3x platelet concentration also did not significantly change the mean anteroposterior knee laxity at 30° and 90° of flexion [5X vs. 3X: 3.5 vs. 5.1 mm ($p = 0.140$), and 6.1 vs. 6.3 mm ($p = 0.764$)] but did result in a lower AP laxity at 60° [5X vs. 3X: 8.6 vs. 7.3 mm ($p = 0.012$)]. The decrease in platelet concentration from 5X to 3X to enhance suture repair of the ACL did not significantly harm the mechanical outcomes in this animal model.

Keywords

ACL; Ligament; Collagen; Scaffold; Fibroblast; Tissue Regeneration

INTRODUCTION

Biological enhancement of primary ACL suture repair using a combination of growth factors and scaffolds to stimulate and sustain a wound healing response has been suggested as an alternative to ACL reconstruction. Platelet-rich plasma (PRP) is an autologous source of growth factors. Collagen, a clinically used and reliably safe biomaterial, lends itself as a scaffold to localize and activate the platelets in the PRP, thus initiating growth factor release.

Pediatric patients with open physes and ACL injury are one group of patients likely to benefit from such a technique. Traditionally, surgical reconstruction of ACL injuries has been avoided due to fear of physeal injury and ensuing growth disturbances¹⁻³. However, a

non-operative approach leads to a high rate of secondary injury to the joint (cartilage and meniscus) in these young patients⁴. Enhanced ACL repair would offer a valuable alternative that does not require full-sized transphyseal tunnels on the one hand and could potentially restore the native ACL structure. Thus, study of such a technique is particularly interesting in a skeletally immature population.

Use of a combination of the two, a collagen-platelet composite (CPC), containing five times the systemic concentration of platelets (5X), has been shown to improve suture repair of the ACL, resulting in significant improvements in yield load, maximum failure load and linear stiffness of the repair at four⁵ and 15 weeks of healing⁶ in the porcine model. Some of the improvement in healing may be due to the stimulatory effects of the PRP; however, the optimal concentration of platelets to use in PRP is not yet known. Furthermore, recently reported evidence has uncovered potentially detrimental effects of PRP, mostly dose-related in nature^{7, 8}. These findings suggest that patients might benefit from a reduction of the platelet concentration in PRP, which would both potentially reduce the potential risk of negative effects, and also reduce the amount of blood needed from the patient.

In this study, we hypothesized that reducing the platelet concentration in PRP from 5X baseline to 3X baseline in a collagen-platelet composite would not result in a significant decrease in mechanical properties of the repaired tissue. We additionally hypothesized that reducing the platelet concentration would not result in significant changes in scar cellularity, cell shape, cell orientation and collagen production, all of which are potential surrogates for long-term functional outcome beyond the duration of the 13 week observational period.

MATERIALS AND METHODS

Experimental Design

The study was approved by the Institutional Animal Care and Use Committee at Children's Hospital, Boston. Based on an a-priori sample size calculation, eight adolescent (44 ± 7 kg, 14 ± 1 month old) Yucatan mini-pigs (five male and three female) underwent bilateral ACL transection and suture repair. A randomized complete block design was employed. All animals were treated with enhanced suture repair in both knees, with one knee receiving PRP containing 5 times the baseline systemic platelet count (5X) and the second knee receiving platelet rich plasma containing 3 times the baseline systemic platelet count (3X). The knees receiving each treatment were randomized preoperatively. All animals were euthanized after thirteen weeks of healing.

Scaffold Preparation

The collagen scaffold was made by solubilizing bovine fascia. Fresh bovine fascia was harvested from the hindlimbs, minced, and solubilized in a pepsin solution to create a bovine atelocollagen solution. The resulting solution was then frozen, lyophilized, and rehydrated with a specified amount of water to create a solution with a collagen content >10 mg/ml. The resulting collagen slurry was neutralized using HEPES buffer (Mediatech Inc, Herndon, VA), sodium hydroxide (Fisher Scientific, Fair Lawn, NJ), PBS (HyClone, Logan UT), and calcium chloride (Sigma-Aldrich, St. Louis, MO) and kept on ice until use. The neutralized atelocollagen solution was placed into cylindrical molds with an inner diameter of 16 mm. The solution was then frozen and lyophilized. The resulting scaffolds were 14 mm in diameter and 40 mm in length and were stored frozen and under vacuum until use.

Surgical Procedure

After the induction of general anesthesia, the pig was placed in the supine position on the operating table. Both lower extremities of the animals were scrubbed and sterilely draped.

The enhanced ACL repair procedure was performed as previously described⁹. A medial arthrotomy was made at the medial border of the patellar tendon. Part of the fat pad was resected and the ligamentum mucosum released. A Lachman test was performed and knee stability verified. The ACL was then cut at the junction of the proximal and middle thirds using a knife. The Lachman test was repeated to verify functional loss of the ACL. The knee was irrigated with 500 cc sterile saline using a bulb syringe. Using a 6mm off-set guide, a drill pin was placed in the femoral attachment site of the ACL and overdrilled with an Endobutton drill. Using an Acufex Gold Guide (Smith and Nephew, Andover, MA) a tibial tunnel was drilled through the tibial attachment site. After drilling, a variable depth suture was placed in the tibial stump with sutures exiting through the proximal cut end of the tibial stump of the ACL. An Endobutton loaded with three #1 Vicryl sutures, was passed up into and through the femoral tunnel and engaged on the femoral cortex so that the suture ends, 6 in total, exited from the tunnel into the joint (Figure 1). Four of these six suture limbs were threaded through the collagen scaffold and passed through the tibial tunnel out of the joint. The collagen scaffold was then soaked with 2 cc of either 3x or 5x PRP and slid into the notch. The sutures were tensioned and tied over a tibial button with the knee in full extension. The two limbs of the remaining suture running through the femur were then tied to the end of the variable depth suture in the tibial ACL stump. On the contralateral knee, the identical procedure was performed and the alternate concentration of PRP used to saturate the scaffold. Both knees were closed in layers and painted with iodine. All animals were kept under anesthesia for 1 hour postoperatively to allow complete clotting of the collagen-platelet composite. The animals were allowed to heal for thirteen weeks and were then euthanized with an injection of pentobarbital solution (Fatal Plus; Vortech Pharmaceuticals, Dearborn, Michigan). The knees, including the proximal femur and distal tibia, were harvested and frozen at -20°C until testing.

Biomechanical Testing

The specifics of biomechanical testing have been previously described⁶. The knees were thawed at room temperature for 18h and soft tissues surrounding the knee were removed, leaving the capsule intact. The specimens were potted in Schedule 40 PVC pipe tubes filled with Smooth Cast (urethane potting compound, Smooth On, Easton, PA) and oriented such that the long axes of the bone and tubes were parallel.

Anteroposterior laxity testing was done at 30°, 60°, and 90° of knee flexion by applying fully-reversed, sinusoidal anterior-posterior directed shear loads of ± 40 N at 0.0833 (1/12) Hz for 12 cycles as previously described⁹⁻¹¹. Anteroposterior laxity tests were done with axial rotation locked in a neutral position, but the varus-valgus angulation and the coronal plane translations were left unconstrained. Data for load and displacement were collected at 20 Hz.

After laxity testing, tensile test to failure was done as previously described⁹⁻¹¹. Briefly, joint capsule, menisci, collateral ligaments and the PCL were removed leaving the femur-ACL scar mass-tibia complex intact. For tensile testing, the knee flexion was set to 30°. The tibia was mounted to the base of the MTS via a sliding X-Y platform with the femur unconstrained to rotation. At the beginning of tensile testing, the femur was lowered to a position of 5N compression across the joint. From this position, the knee was distended at 20 mm/min and the load-displacement data were recorded at 100Hz. The structural properties displacement to 5N and 50N, displacement to yield, displacement to failure, load at yield, load at failure, linear stiffness, and energy to failure were calculated from the load-displacement tracing.

All testing was done using a MTS 810 servohydraulic load frame (MTS Systems Corporation, Eden Prairie, MN). All evaluators were blinded during mechanical testing as to sample allocation.

Histology

In addition to mechanical testing, four ligaments receiving 3X PRP and four ligaments receiving 5X PRP were retrieved and fixed in neutral buffered formalin for 1 week, sectioned longitudinally in the sagittal plane of the ACL, dehydrated and embedded in paraffin. Seven-micron sections were placed onto pretreated glass slides and stored at 4°C. Representative sections from each ligament were stained with hematoxylin and eosin (H&E). The healing ACL was divided into 5 evenly spaced areas for analysis: site 1, femoral stump; site 2, proximal ACL wound; site 3, central ACL wound; site 4, distal ACL wound; and site 5, tibial stump. At each of the five areas, cell density was analyzed with methods previously described¹². Briefly, the number of cells within three 0.1-square-millimeter areas were measured by two independent, blinded, reviewers and the results averaged. At each location, the total number of cells were counted and divided by the area of analysis to yield the cell density (#/mm²). Additionally, cell size was assessed by the length-to-width ratio of the nuclei of the counted cells. Cell orientation was determined in relation to the orientation of the ligamentous fibers.

Maturity Index of Wound Site

A scoring technique, adapted from Murray et al 2007¹², was used to quantitatively assess H&E stained sections of the central wound area (Site 3). The ligament was assessed in three different categories: cellularity, collagen and vascularity, where each section was given a rating of 0, 1, or 2 by two independent scorers. The average of both raters scores were used to generate a total for each major group by adding the scores together for each scoring category.

Statistical Methods

The primary endpoint of this study was biomechanical outcome. Based on standard deviations from earlier published data⁹, a sample size of 8 would allow detection of an effect size of at least 1.5 with 90% power at an alpha of 5% for matched pairs. To address the primary study hypotheses, paired two-tailed t-tests were used to evaluate the differences between means of the two treatment groups (5X vs. 3X) for post-mortem AP laxity testing and the structural properties (yield load, maximum failure load, linear stiffness, displacement to yield, displacement to failure, or displacement to 5N of tensile load). For the secondary endpoint, histological outcome, paired two-tailed t-tests were used for the continuous endpoints cell number and cell shape. All results are given as mean with standard deviations. To test for an association between the primary outcome biomechanical strength and the secondary outcome histology, linear regression was performed for the effect of cellularity of the scar on the biomechanical properties of the repair tissue. Since we test for an effect of cellularity on biomechanical outcomes on different scales (mm and N), the regression coefficients were standardized by dividing the coefficient by its standard deviation. These so-called beta coefficients are on an absolute scale, because units of measure (mm, N) cancel each other out and illustrate the relative importance of the predictor cellularity on all biomechanical outcomes. All statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL). The level of significance was set at a two-tailed α -level of $p < 0.05$.

RESULTS

Animal welfare

There were no significant post-operative complications for any animal. All animals were able to weight-bear within 12 hours of surgery, and were walking normally within one week and continued to do so until the time of euthanasia. There was no evidence of infection in any of the knees either during the study or at the time of euthanasia.

Lab parameters

The systemic platelet count for the animals (mean $\pm$ SD) was $391 \pm 48 \times 10^9/L$. The average platelet count in the 5X PRP was $1951 \pm 304 \times 10^9/L$ (mean $\pm$ SD), with an average enrichment factor of 5.0X. The average platelet count in the 3X PRP was $1161 \pm 179 \times 10^9/L$ (mean $\pm$ SD), with an average enrichment factor of 3.0X. The enrichment factor is the ratio between the platelet concentration in the platelet rich plasma and that in the systemic whole blood of the animal.

Biomechanical Testing

There was no significant difference in AP laxity at 30° ($p=0.140$) and 90° ($p=0.764$) of knee flexion. There was significantly less AP laxity at 60° in the 3x group compared to the 5x group ($p=0.012$). (Table 1a)

There were no significant differences in structural properties, i.e. in the outcomes of tensile testing for displacement to 5N or 50N, displacement to yield, displacement to failure, load at yield, load at failure, linear stiffness, or energy to failure. (Table 1b).

Histology

Cellularity—By 13 weeks, the healing ACL was completely populated with fibroblasts, with the distal wound having the greatest number of fibroblasts throughout the ligament. Animals treated with 5X PRP had a significantly greater cellular density than animals treated with 3X PRP at the femoral insertion site (431 ± 127 cells/mm² vs. 385 ± 107 cells/mm², $p<0.05$) and the central wound (391 ± 87 cells/mm² vs. 279 ± 80 cells/mm², $p<0.01$) (Table 2).

Cellular orientation—Fibroblasts were oriented along the length of the ligament for both groups. There were no acellular or necrotic areas seen. For both groups, areas that had uniform collagen crimp had cells running parallel within those fibers. For areas that did not have uniform crimp, fibroblasts were still oriented along the length of the ligament.

Cell Shape—For both groups, the fusiform shape was most commonly seen (189 ± 85 cells/mm² and 137 ± 77 cells/mm², for 3X and 5X groups); however, the 3X group showed more areas of disorganization and defects characterized by both non-uniform crimp and no crimp. For those areas, the cell nuclei appeared more ovoid and larger than cells nestled within the organized collagen crimp.

Collagen formation—At thirteen weeks, animals treated with 5X PRP showed uniform collagen crimp at the central wound site. There was large waveform present with fibroblasts spaced evenly throughout (Figure 2). The collagen crimp was organized along the length of the axis. Animals treated with 3X PRP showed less organization at the central wound site with non-uniform crimp present in smaller wavelengths when compared to the 5X group (Figure 2). Fibroblasts were oriented within this crimp when it was present.

Maturity Index of Wound Area—The Maturity Index of the wound area in the 5X group was significantly higher in cellularity and vascularity subscores when compared with the 3X (Figure 3 and Table 3, $p < 0.05$ for both comparisons). Significantly greater numbers of vessels were found in the 5X group (Figure 3, $p < 0.05$). The largest difference was seen in the cellularity subscore, with 5X group having a considerably higher cellularity subscore (5.8 ± 1.2 vs. 4.4 ± 1).

Effect of Central Wound Site Cellularity on Biomechanical Outcome—The largest beta-coefficients were seen for displacement at yield and AP laxity at 30° and 60° degrees. However, there was no statistically significant association between scar site cellularity and biomechanical outcomes. (Table 4)

DISCUSSION

Platelet concentrates, as a source for growth factors, are very popular tools in regenerative medicine. Their power has successfully been employed in tissue-engineering enhanced primary ACL repair^{6; 13; 14}, in enhanced ACL reconstruction^{6; 15}, as well as in other fields of surgery¹⁵. However, recent studies have discovered negative effects of PRP and attaining a more differentiated view of what PRP is and does seems prudent and necessary^{7; 8}. In this study we asked whether reducing the platelet concentration in PRP used for primary ACL repair would affect outcomes in two categories, biomechanics and histology. Not only would such a reduction in platelet concentration be expected to cause a substantial reduction in risk of negative effects, it would also decrease the amount of blood that has to be drawn from the patient..

At 13 weeks, we found no evidence that reducing the platelet concentration from 5X to 3X compromises mechanical outcome after tissue-engineered enhanced primary ACL repair. Stiffness, which might very well be the most important biomechanical parameter, showed virtually identical results. Displacement and load, maximum load and yield load, showed only small differences in absolute values. Interestingly, these means for yield and maximum load showed a slight trend in favor of the lower PRP concentration group, although these differences had no statistical significance. It is noteworthy that the absolute values of the mean differences were very small, suggesting widely identical clinical outcomes for both PRP concentrations.

In the histological analysis, we observed significant differences in cellular density at the central wound site and the femoral insertion, with significantly larger cell numbers per area in the 5X group. Findings based on the Ligament Maturity Index highlighted significant histological differences between the 5X and 3X groups. The cellularity subscore showed not only the increased cellularity but the difference in cell shape. 5X fibroblasts were more elongated whereas the 3X wound area was populated by a mix of both fusiform and spheroid cell shapes. The fibroblasts in the 5X specimens were also better organized within the larger collagen bundles compared to the cells in the 3X specimens (Figure 2).

The relationship between tissue cellularity and biomechanical function has yet to be clarified. In a recent series of studies of the effects of skeletal maturity on ligament healing, the young animals had a greater cellularity of the wound site at 1 to 2 weeks after injury in comparison with adult animals^{16; 17}, and the yield load of the ligaments in that age group were significantly higher than the adults at the 15 week time point¹¹. This suggests that a higher cellularity early in the wound healing process may be beneficial in the porcine model. In addition, a recent study in equine tendon healing reported that use of PRP resulted in both an increase in tendon cellularity and tendon strength at 24 weeks after injection¹⁸, and a second study of MCL healing in rabbits found that use of porcine SIS resulted in both

increased cellularity and increased mechanical properties of the ligament after 12 weeks of healing. This suggests that a higher cellularity early in the wound healing process may be beneficial in some situations and animal models. Other in vivo studies of tendon and ligament healing have not found an association between cellularity and biomechanical properties^{19; 20}. Thus, the relationship between cellularity and mechanical properties is not as yet completely defined.

In this study, while we did find significant differences for secondary histological outcomes, we found no statistically significant difference between the groups in biomechanical outcomes. Having a larger sample size or smaller standard deviation might have led to statistically different result; but would have been less likely to show a clinically meaningful decrease in mechanical properties with a reduction in platelet concentration. This is due to the fact that for most outcomes, the differences in the means of the two groups were relatively small, and in all cases, the differences favored the group with a lower platelet concentration. While the differences were not statistically significant, this trend makes it even less likely that increased sample sizes would reveal a detrimental effect of reducing the platelet concentration.

ACL healing has been shown to go through a revascularization phase between six and nine weeks after suture repair, during which time, the biomechanical properties of the healing ligament hit a low point¹³. For this study, we selected a time point that was beyond this nadir in tissue properties. Differences at earlier time points would not necessarily correlate to survival of the repairs through the revascularization process, and thus, results at those time points might be mechanistically interesting, but less translational in their applicability. We have used a similar time point in previously published studies for this reason^{9; 13; 21}. The study of longer time points would have been more justifiable, again, if the decrease in platelet concentration were seen to be significant at 13 week time point.

This study used a randomized complete block experimental design to minimize inter-animal variability, a problem which plagues in vivo studies. We have used this approach with success in other in vivo studies^{13; 22}. The strength of this matched design is that it allows us to limit the number of animal lives required to answer specific questions while maintaining strict control over confounding biologic variables (i.e. weight, sex, activity level). However with this design we are inherently limited to study only one research question per experiment (in this case, 3X vs. 5X concentration of platelets).

In conclusion, our data suggest that there is little functional difference in ligament healing with the use of 3X or 5X PRP. The use of a lower concentration of platelets allows for a proportionally smaller amount of blood required to make the PRP, thus making the procedure easier for the patient.

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Figure 1.

Schematic diagram depicting the primary suture repair with the collagen scaffold in place. Sutures are fixed proximally with an Endobutton device. The scaffold is threaded onto four of the trailing suture ends (RED) which are then passed through the tibial tunnel and tied over a button to provide initial knee stability. The remaining two suture ends (GREEN) are tied to the sutures in the tibial stump of the ACL. All sutures used in this study were resorbable and there was no evidence of suture material remaining at the time of knee retrieval. (Figure from Murray et al, JBJS, 2010¹¹).

Figure 2. Photomicrograph of the ACL at site three, thirteen weeks after rupture using both 3X and 5X PRP, demonstrating the differences in collagen wave crimp. Size bar 100 μ M.

Figure 3.

Ligament Maturity Index scores for the wound area. Values are expressed as mean $\pm$ SD, where n=8. # represents a significant decrease between 5X and 3X in CELLULARITY, $p < 0.05$ † represents a significant decrease between 5X and 3X in VASCULARITY, $p < 0.05$

Table 1a

Biomechanical properties (anteroposterior laxity) of the repairs using 3X and 5X concentrations of platelets in the scaffold. Data represents mean $\pm$ one standard deviation.

Laxity	5X PRP n=8	3X PRP n=8	p-value
30 degrees of flexion	5.1 $\pm$ 2.8	3.5 $\pm$ 3.2	<i>0.140</i>
60 degrees of flexion	7.3 $\pm$ 1.3	8.6 $\pm$ 1.7	<i>0.012</i>
90 degrees of flexion	6.3 $\pm$ 1.2	6.1 $\pm$ 1.7	<i>0.7642</i>

Table 1b

Biomechanical properties (tensile testing) of the repairs using 3X and 5X concentrations of platelets in the scaffold. Data represents mean $\pm$ one standard deviation.

	5X PRP n=8	3X PRP n=8	p-value
Displacement at Yield (mm)	6.1 $\pm$ 1.5	6.3 $\pm$ 0.8	0.824
Displacement at Failure (mm)	6.9 $\pm$ 1.8	7.1 $\pm$ 1.3	0.771
Load at Yield (N)	291 $\pm$ 76	362 $\pm$ 144	0.242
Maximum Load (N)	302 $\pm$ 72	376 $\pm$ 138	0.198
Stiffness (N/mm)	53 $\pm$ 17	70 $\pm$ 29	0.189

Table 2

Cellular density within the ACL at 13 weeks after repair.

	Cellular Density (cells/mm ²)				
	FS	DW	CW	PW	TS
3X	385±107*	412±77	279±80 [†]	409±138	387±149
5X	431±127*	480±124	391±87 [†]	434±125	444±110

Values are expressed as mean ± SD, where n=8. Repairs performed with 5X PRP had significantly higher cellularity than those using 3X PRP ($p < 0.05$).

* represents a significant decrease between 5X and 3X at the femoral insertion site, $p < 0.05$.

[†] represents a significant difference between 3X and 5X at the central wound site, $p < 0.01$.

Table 3

Ligament Maturity Index scores for the wound area.

	<u>Cellularity</u>	<u>Collagen</u>	<u>Vascularity</u>
3X	4.4 ± 1 *	2.4 ± 0.8	1.5 ± 1.1 **
5X	5.8 ± 1.2 *	3.3 ± 0.9	1.9 ± 1.4 **

Values are expressed as mean ± SD, where n=8.

* represents a significant decrease between 5X and 3X in CELLULARITY, p<0.05

** represents a significant decrease between 5X and 3X in VASCULARITY, p<0.05

Table 4

Effect of central wound site cellularity on biomechanical outcomes

	Beta-coefficient	p-value
Displacement at Yield (mm)	0.41	<i>0.115</i>
Displacement at Failure (mm)	-0.01	<i>0.978</i>
Load at Yield (N)	0.01	<i>0.981</i>
Maximum Load (N)	-0.06	<i>0.823</i>
Stiffness (N/mm)	-0.18	<i>0.517</i>
AP laxity (30° flexion)	-0.23	<i>0.386</i>
AP laxity (60° flexion)	0.38	<i>0.150</i>
AP laxity (90° flexion)	0.06	<i>0.830</i>