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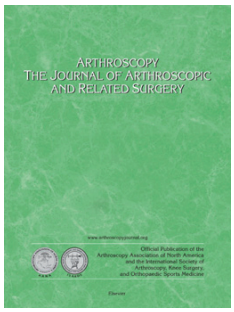

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Growth Factor Delivery to a Cartilage-Cartilage Interface Using Platelet-Rich Concentrates on a Hyaluronic Acid Scaffold

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Abstract:

Purpose: To determine if (1) Human L-PRP or (2) L-PRF delivered on a HA scaffold at a bovine chondral defect, a simulated cartilage tear interface, *in vitro* would improve tissue formation based on biomechanical, histological, and biochemical measures.

Methods: L-PRF and L-PRP was prepared from 3 healthy volunteer donors which was delivered in conjunction with Hyaluronic acid (HA scaffolds) to defects created in full thickness bovine cartilage plugs harvested from bovine femoral condyle and trochlea. Specimens were cultured in vitro for up to 42 days. Treatment groups included an HA scaffold alone and scaffolds containing L-PRF or L-PRP. Cartilage repair was assessed using biomechanical testing, histology, DNA quantification, and measurement of sulfated glycosaminoglycan (sGAG) and collagen content at 28 and 42 days.

Results: L-PRF elicited the greatest degree of defect filling and improvement in other histological measures. L-PRF treated specimens also had the greatest cellularity when compared to L-PRP and control at day 28 (560.4 vs. 191.4 vs. 124.2, p=00.15); at day 48 there remains a difference though not significant between L-PRF vs L-PRP, (761.1 vs 589.3, p=0.219) . L-PRF had greater collagen deposition when compared to L-PRP at day 42 (40.1 vs 16.3, p< 0.0001). L-PRF had significantly higher maximum interfacial strength compared to the control at day 42 (10.92 N vs 0.66 N, p=0.015), but had no significant difference compared to L-PRP (10.92 N vs 6.58, p=0.536). L-PRP facilitated a greater amount of sGAG production at day 42 when compared to L-PRF (15.9 vs. 4.3, p=0.009).

Conclusions: Delivery of leukocyte rich platelet concentrates in conjunction with a HA scaffold may allow for improvements in cartilage healing through different pathways. L-PRF was not

47 superior to L-PRP in its biomechanical strength suggesting that both treatments may be effective
48 in improving biomechanical strength of healing cartilage through different pathways.
49
50 Statement of Clinical Relevance: The delivery of platelet-rich concentrates in conjunction HA
51 scaffolds may augment healing cartilaginous injuries.
52
53 **Keywords: cartilage repair, PRP, leukocyte-rich platelet-rich fibrin, leukocyte rich**
54 **platelet enriched concentrates**
55
56 **Introduction**
57 Focal cartilage defects may be caused by sports injury, trauma, and other activities of daily
58 living[1]. As cartilage has limited capacity for intrinsic repair, such defects often further
59 degenerate, progressing into osteoarthritis with associated symptoms of pain, swelling, and
60 stiffness[1,2]. There are few effective treatments for articular cartilage injuries. Previous efforts
61 have used cell-based therapies (autologous chondrocyte implantation, matrix-induced autologous
62 chondrocyte implantation, bone marrow and adipose-derived stem cells), microfracture, and
63 cytokines to promote cartilage repair; however, these attempts have not achieved ideal
64 osteochondral defect repair with regeneration of morphologically similar hyaline cartilage
65 tissue[3].Autologous chondrocyte implantation as well as matrix-induced autologous chondrocyte
66 implementation has been shown improved clinical outcomes, however its cost is high, and the
67 procedure to obtain the cartilage pieces is invasive[3,4]. These cells can produce hyaline cartilage,
68 however, this procedure is also associated with the formation of fibrocartilage, which typically
69 occurs secondary to de-differentiation of chondrocytes during cell expansion after harvest[3].

Mesenchymal stem cells harvested from bone marrow or adipose tissue have been shown to give rise to a mixture of hypertrophic, cartilaginous, and fibrous tissues and can lead to loss of tissue in the long run[3,5]. Microfracturing has been shown to significantly improve the symptoms of patients and is thought to bring about the recruitment of mesenchymal stem cells, it is only effective for small defects and has been shown to only have a short-term functional improvement due to the formation of fibrocartilage rather than hyaline articular cartilage[3,6].

The use of scaffolds is promising as a viable surgical option in cartilage repair[1,7]. Scaffolds can augment cell migration from the surrounding tissue, stimulate cell proliferation, and aid in maintenance of cell phenotype in order to facilitate regeneration of functional tissue[7,8]. Specifically, hyaluronic acid (HA) scaffolds may facilitate cellular migration by providing both biochemical and biophysical cues improving articular cartilage repair[9,10]. HA scaffolds have been shown to increase early-stage gene expression of SOX-9 and collagen type II as well as cartilage matrix production[11]. Although HA has been found to be useful in reducing pain and symptoms and recovering articular function, Kon et al. found that autologous platelet rich concentrates showed more and longer efficacy than HA, suggesting that using HA scaffold alone may have limited therapeutic effects[12].

Platelet-rich concentrates have been proposed to improve healing of different tissues due to their abundance of growth factors and cytokines. Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) have both been shown to promote the repair of articular cartilage defects[4,13,14]. PRP is a concentrate of platelet-rich plasma derived from whole blood, centrifuged to reduce the number

of red blood cells in a liquid form, whereas PRF is composed of autologous platelets which are present in a complex stable fibrin matrix (gel-like consistency).

There is significant variability in the methods of preparation and composition of platelet-rich concentrates[15-17]. Previous studies that have evaluated platelet-rich concentrates have used a variety of protocols for preparation of platelet-rich concentrates with variation in initial blood volume, velocity and time of centrifugation, number of centrifugation cycles, and inclusion or exclusion of leukocytes, which can alter the effect on tissue repair by changing its composition[18]. Specifically, platelet-rich concentrates with different compositions have different contents of growth factors and numerous other plasma proteins, which may alter its efficacy[19,20]. The optimal preparation method of platelet-rich concentrates has not yet been identified, and will likely differ for various tissues and pathologies. Leukocyte-platelet-rich concentrates have been shown to contain higher levels of growth factors and cytokines, as well as inflammatory cytokines that may have a catabolic effect, all of which is currently thought to be related to the presence of leukocytes[21]. Number of centrifugations of the whole blood has been found to alter the cytokine and growth factors release as well as the number of platelets, which has been found to be higher in double spin protocols to prepare PRP[22-24]. Leuckocyte-platelet-rich plasma (L-PRP) have not been found to be effective in stimulating proliferation of new cartilage tissue[25,26]. It is not currently clear if L-PRF can consistently release growth factors given its microstructure[22]. Current literature is inconclusive as to the ability of L-PRP and L-PRF to improve cartilage integration[23].

Both intraarticular injections of platelet rich concentrates and hyaluronic acid have been found independently to improve knee functional status and symptoms over time, however evidence of the superiority of one treatment over another remains mixed[27-30]. The combination of these two therapies (platelet-rich concentrates and HA scaffolds)may allow for optimized cytokine delivery of the platelet concentrates and promote tissue regeneration by allowing cells to migrate into the scaffold, adhere, and produce new matrix to fill the defect. However, minimal research has been performed to investigate the use of scaffold materials in combination with platelet-rich concentrates to enhance cell migration. Therefore, the purpose of this study is to determine if (1) Human L-PRP or (2) L-PRF delivered on a HA scaffold at a bovine chondral defect, a simulated cartilage tear interface, *in vitro* would improve tissue formation based on biomechanical, histological, and biochemical measures. We hypothesized that the use of L-PRP and L-PRF in conjunction with an HA scaffold will improve biomechanical strength, improve cellular migration, have increased sulfated glycosaminoglycan (sGAG), and have increased collagen content when compared to an HA scaffold alone.

Materials and Methods

Preparation of the Cartilage Plugs:

The explant model used in this study has previously been used to evaluate cartilage repair, scaffold integration, and cartilage interface development *in vitro*[31]. Three operators performed the specimen preparations. 24 full-thickness osteochondral plugs were harvested from a bovine femoral condyle and trochlea in a sterile manner using a 7mm diameter osteochondral graft harvester (single use OATS set 7mm, Arthrex, Naples, Fl, USA). The diameter of these plugs

were 7mm and thickness was 2.5mm. Osteochondral plugs were kept in Advanced DMEM (ADMEM)/F12 with 100 nM dexamethasone, 50 μg/mL ascorbate-2-phosphate, and penicillin/streptomycin (Life Technologies, Carlsbad, CA) within 5% CO_2 and air-humidified incubator at 37 degrees Celsius for 6 hours. They were then washed briefly using phosphate-buffered saline and antibiotics – (penicillin/streptomycin, Life Technologies, Carlsbad, CA). A 5.5mm diameter biopsy punch was used to create a circular defect through the center of the explant (the defect had a diameter 5.5mm and a depth of 2.5mm) to allow for a press fit of a 6mm diameter scaffold with a thickness of 2.5mm placed within the core. **Figure 1, Figure 2A, Figure 2B**).

Preparation of HA Scaffolds:

HA scaffolds (Hyalofast, USA) were used for this study. The scaffolds were punched out using sterile 6 mm biopsy punches (McKesson, San Francisco, USA). The size of the scaffolds was 0.5 mm larger than the diameter of the cartilage defect to allow for a press-fit. The thickness of the scaffold was the same as the plug, 2.5mm.

Groups and Timepoints:

Eight specimens were used at each time point in each group: Six for biomechanical testing of interface strength and biochemical analysis of DNA, collagen, and sGAG content, and two specimens used for histology (total number of specimens: 24). The specimen test and timepoint allocation was determined at the time of preparation. The specimens used for biomechanical testing were processed for biochemical evaluation after the biomechanical testing was complete. Biochemical testing was done on specimens from days 28 and 42 as we expected the largest

differences between groups at these time points. The early (28 day) time-point was chosen based on prior studies indicating that improvements in functional properties can be seen as early as 28 days[26]. The later time point was selected as we expected there to be greater differences between groups over time.

Blood Collection:

L-PRP and L-PRF was produced from blood samples from three fasting healthy male human donors after they provided informed consent (average age: 34.6, range: 27-39). In each subject a total of 37mL of peripheral venous blood was collected in order to prepare the two platelet concentrates. From these 37mL of whole blood, 1 mL was used to measure platelet- and leukocyte-counts with a cell counter Sysmex KX-21N (Sysmex-Suisse AG, Horgen, Switzerland). Of the addition 36 mL, 27 mL of the blood was collected in a 30-mL syringe containing 3 mL of ACD-A to prepare the L-PRP and 9 mL was collected in 10-mL glass-coated to prepare the L-PRF. In the whole blood specimen the average number of platelets was 188.3 x 10^3cells/ µL (± 19 x 10^3cells/ µL) ; and the average number of leukocytes was 4.9 x 10^3cells/ µL (± 0.5 x 10^3cells/ µL). This protocol was approved by the Institutional Review Board.

L-PRP Preparation:

Given that previous literature supports that double-centrifugation results in a greater number of platelets and growth factor release we opted to use a previously described double-centrifugation method[20,22-24]. 27 mL of the blood was collected in a 30-mL syringe containing 3 mL of ACD-A. The blood was separated into its different cell elements by centrifugation at 160 G for 20 minutes at room temperature (Beckman J-6M Induction Drive Centrifuge, Beckman Instruments Inc.,

Palo Alto, CA, USA). This resulted into three basic components: an upper layer referred to as the supernatant that contains most platelets, an intermediate thin layer that is known as the buffy coat which is rich in white blood cells and some platelets, and a bottom layer that contains all the red blood cells. A mark was made 6mm below the line that separated the red blood cell component from the buffy coat and the serum component. All of the content above this mark was removed in order to increase the total amount of platelets collected after the second centrifugation. The sample was then centrifuged again at 400 G for 15 minutes resulting in two components: the supernatant containing the serum, buffy coat containing the platelets and the leukocytes. The L-PRP (approximately 2.7 ml) was separated out. Of this, 500 uL was taken to measure platelet- and leukocyte-counts with a cell counter Sysmex KX-21N (Sysmex-Suisse AG, Horgen, Switzerland).

L-PRF Preparation:

To prepare L-PRF, 9 mL of blood was collected in 10-mL glass-coated, plastic tubes (Vacutainer; BD Biosciences, Allschwil, Switzerland). Using a similar protocol performed by Schär et al., immediately after collection the blood was centrifuged at 400 x g for 12 minutes at room temperature using a table-top centrifuge specifically designed for this application (EBA 20; Andreas Hettich GmbH & Co KG, Tuttlingen, Germany)[23]. The L-PRF was then allowed to congeal on a sterilized custom draining system and was cored using a 6 mm biopsy punch, ensuring a consistent volume for each specimen (**Figure 2C**).

Preparation of Test Groups:

The following groups were tested: (1) control group where only a HA scaffold was applied (**Figure 2D**), (2) L-PRP with HA scaffold (**Figure 2E**), (3) L-PRF with HA scaffold (**Figure 2F**). In the L-PRP groups, the scaffold was soaked for 5 minutes, a time used by previous studies to ensure that PRP had been completely soaked up by the scaffold, in 100uL of undiluted PRP at day 0 prior to being placed into the defect in the culture plates[32,33] (**Figure 2E**). In the L-PRF groups, a 6mm biopsy punch of the L-PRF was carefully layered around the scaffold and then placed within a bovine cartilage defect in Corning plates taking care to ensure that the scaffold now layered with L-PRF had the same thickness of the defect of 2.5mm(Corning Inc, Corning, NY). There were 8 specimens per plate with a total of 3 plates per timepoint; specimens cultured on the same plate were in the same treatment group. Each dish was then filled with 30ml of media. Therefore, the initial PRP concentration was 3% (amount of PRP/total media volume in the dish). In the control group, only a HA scaffold was placed in the defect. Scaffold–cartilage constructs were cultured in individually wells in culture media the consisted of 30 mL Advanced DMEM (ADMEM)/F12 with 100 nM dexamethasone, 50 μg/mL ascorbate-2-phosphate, and penicillin/streptomycin (Life Technologies, Carlsbad, CA) within 5% CO_2 and air-humidified incubator at 37 degrees Celsius. Media was changed every three days by replacing half of the media in the culture dishes for 28 days and 42 days, respectively. The primary outcome measure was interface strength assessed by a pushout test. The secondary outcome measurements included scaffold DNA content (picogreen assay, Invitrogen, Carlsbad, CA), scaffold sulfated-glycosaminoglycan (sGAG) content (Dimethylmethylene Blue Assay described by Farndale et al.)[34], collagen content (hydroxyproline assay described by Stegmann et al.)[35], and histology. All biochemical tests were performed on the tissue formed at the scaffold-explant interface.

228 Biomechanical Testing

229 The cartilage interface strength was determined using a push-out test. A 2.75-mm stainless steel
230 indenter applied load to the scaffold by advancing at 10 μm/s using a custom built compression
231 testing system previously described by Ng et al.[25]. The test was considered completed when there
232 was a consistent drop in force. The maximum load was recorded and normalized to the
233 interfacial surface area for each sample to determine the maximum interfacial strength.

234

235 Biochemical Analysis

236 After biomechanical testing samples were digested in papain for analysis of DNA, sGAG, and
237 collagen content. DNA content per specimen was determined using the dsDNA Picogreen Assay
238 (Molecular Probes, Eugene, OR). sGAG content was determined using the 1,9-
239 dimethylmethylene blue dye binding assay with chondroitin-6 sulfate as a standard[20]. Digested
240 aliquots were also hydrolyzed for 16 h in 12 N HCl at 110°C, and the hydroxyproline (HP)
241 content was quantified via colorimetric reaction with chloramine T (Sigma-Aldrich, St Louis,
242 MO) and 4-Dimethylamino benzaldehyde (Sigma-Aldrich, St Louis, MO), against an HP
243 standard curve[35].

244

245 Histological Analysis

246 Histological analysis was undertaken to evaluate the scaffold–cartilage interface. The explants
247 were fixed in neutral buffered formalin with 0.5% cetylpyridinium chloride for 4 hours at room
248 temperature, washed briefly in phosphate-buffered saline (PBS) to remove residue formalin,
249 cryoprotected in a 30% sucrose solution at 4^{o}C overnight, incubated for 2 hours in 5% gelatin +
250 5% sucrose embedding medium, and then embedded in the gelatin–sucrose medium. Blocks

were cut on a cryotome for histological analysis of interfacial sGAG deposition and cellular infiltration using Safranin-O/Fast Green histological stain. Slides were reviewed at a magnification of 10x. Consensus grading was done by two evaluators in a blinded fashion, with grading of surface regularity, cellularity, stain uptake, and adherence/bonding of the scaffold to adjacent cartilage.

Statistical Analysis

Two-way ANOVA was performed using Graphpad PRISM 6 software (GraphPad Software, Inc, San Diego, CA, USA, 2015) to compare different conditions, with Tukey's post-hoc testing to identify differences between groups. Significance was set at $p < 0.05$.

Results:

Blood Collection:

The number of platelets within the L-PRP was 188.4 x 10^4cells/ µL, representing a 10-fold increase from the original blood specimens collected. The average number of white blood cells was 15.5 x 10^3cells/ µL, which was a 3.2 fold increase from the original blood specimens collected. The hematocrit value was 36, which was a 0.12 fold decrease from the original blood specimen collected. We did not evaluate changes in cellular concentrations in L-PRF given its gelatinous form.

Mechanical Strength of the Interface:

Following 42 days in culture, L-PRF demonstrated a maximum interfacial strength was significantly greater than the control (10.92N±9.25 vs. 0.66N±0.35, p=0.015, **Figure 3, Table 1**).

L-PRF though did had higher mean interfacial strength compared to that of L-PRP it was not significantly different (10.92N ± 9.25 vs. 6.58N ±10.87 , p=0.536). There were no significant differences seen at day 28 between L-PRF, L-PRP, and the control (3.08N±1.23, 1.74N ±1.07, 0.63N ±0.29 p = 0.055) In all groups, the maximum interfacial strength increased at each time point, though not significantly.

Biochemical Analysis**:**

The number of cells and thus DNA content of the constructs varied between groups and with culture time (**Figure 4**). The L-PRF group had a significantly greater number of cells compared to the L-PRP and control groups at both 28 (560.5µg±142.6, 124.2µg±65.8, 191.4µg±44.6 p<0.001, **Table 1, Figure 4**) and 42 days (761.1µg±270.7, 589.3µg±224.2, 296.4µg±101.8, p< 0.01, **Figure 4, Table 1**). Across all groups the DNA content increased between days 28 and 42, however only the L-PRP group had a significant increase (124.2µg±65.8µg vs. 589.3µg±224.2µg, p=0.0004, **Figure 4, Table 1**).

Across groups, the total sGAG production increased from day 28 to day 42 (**Figure 5, Table 1**). L-PRP had larger total sGAG production compared to L-PRF at 28 and 42 days, but only had a significantly greater amount at day 42 (4.3µg± 11.5 vs 11.6µg±7.2, p=0.0095, **Figure 5, Table 1**).

Hydroxyproline assay demonstrated significantly greater collagen content in the L-PRF group compared to the control group on day 28 (31.2µg±5.3 vs. 17.6µg± 7.5, p=0.017, **Figure 6, Table 1**) , but not to the L-PRP treatment group on day 28(31.2µg±5.3 vs. 21.1µg±10.2, p=0.092,

297 **Figure 6, Table 1**). On day 42 there was a significant difference between L-PRF and the control
298 group (40.1μg±9.7 vs. 16.3μg± μg, 20.4μg±6.8 vs. p=0.0006, **Figure 6, Table 1**). There was no
299 significant difference observed between L-PRP and the control group at either timepoint
300 (21.1μg±10.2 vs. 17.6μg±7.5μg, p=0.723, 16.3μg±7.6 vs. 20.4μg±6.8, p=0.659, **Figure 6, Table**
301 **1**).
302
303 Histological evaluation:
304 Most specimens had at least some positive staining for proteoglycans. In all groups the Safranin-
305 O staining increased from day 28 to day 42, suggesting increased cellular infiltration and
306 increased proteoglycan synthesis as demonstrated by the intensity of the staining (**Figure 7**). All
307 the specimens started to form disorganized cartilage-like tissue at the edge with ECM deposition
308 with more apparent cellular integration and extracellular matrix deposition at the edges of the
309 construct in the L-PRP and L-PRF groups. At the interface of the L-PRP group, lacuna
310 structures, typical of cartilage, are observed at the interface. There was more intense Safranin-O
311 staining in the L-PRF and L-PRP groups compared to control on day 42. The L-PRP group had
312 the most intense staining of Safranin-O on day 42.
313
314 **Discussion:**
315 The two principal findings of our study are that L-PRF elicited the greatest degree of cellularity,
316 collagen production, while L-PRP facilitated the greatest amount of sGAG production. There
317 was no superiority observed in biomechanical strength between the platelet concentrates utilized
318 in this study.
319

An important finding was that L-PRF had the greatest number of cells. The significant increase in the L-PRF group may be artificially elevated because of the presence of leukocytes in the preparation compared to the control at both day 28 and 42. However, there were quantitatively more cells in the cartilage interface for L-PRF compared to L- PRP (**Figure 4**), which suggests that it may promote cell migration and/or proliferation to a greater extent than L-PRP. The gelatinous nature of L-PRF may allow for sustained release of growth factors over a greater period of time when compared to L-PRP and the control. PRF has been previously been shown to more gradually release growths factors especially in comparison to PRP[20,36]. Schär et al. specifically found that more TGF-ß1 and VEGF was released from L-PRF compared with L-PRP. The release of TGF-ß1 was bimodal in nature and peaked at 8 hours and 7 days, while L-PRF had a single higher peak at 7 days. VEGF release from L-PRP peaked at 8 hours and 3 days in contrast with L-PRF in which it peaked at 7 days[23]. This sustained release may also offset the known catabolic response cells have in response to leukocyte-rich platelet concentrates[21] as there is clearly an increase in cell number in the L-PRF group. This is supported by the fact that IL-1B, which is known to play a role in inflammation and/or matrix degradation, has been shown to have a more sustained concentration in L-PRP when compared to L-PRF[23]. Schär et al. also found better migration *in vitro* for MSC and endothelial cells in L-PRF compared to L-PRP[23]. Further, even the preparation of PRP can alter the extent and duration of cytokine release[20] and as a result, great care must be taken in ensuring the proper protocol is used for L-PRP given the significant variability in its preparation in the clinical setting.

Interestingly, our findings demonstrated that L-PRP elicited the greatest amount of sGAG production. Kazemi et al. found that L-PRF and L-PRP demonstrated similar macroscopic and

343 microscopic improvements in articular cartilage defects in a dog model[37]. However, it should be
344 noted that both centrifuge spin velocity and time differed between our study and theirs making it
345 difficult to truly make comparisons.
346
347 It is important to highlight that although L-PRF seemed to have increased biomechanical
348 strength and collagen content, such improvements were not seen in sGAG accumulation. Instead,
349 L-PRF had the lowest amount of sGAG accumulation compared to the control and L-PRP. This
350 may be secondary to the fact that L-PRP and L-PRF release cytokines and growth factors at
351 different rates and amounts[20,23,36]. Histological analysis demonstrated that L-PRP-treated
352 specimens had more intense Safranin-O staining at day 42 compared to the other two groups.
353 This finding was expected, as the production of sGAG helps stimulate the production of
354 proteogylcans[38]. L-PRP appeared to successfully incorporate newly-synthesized sGAG into the
355 matrix in the L-PRP group, resulting in increased staining compared to the HA only group.
356 Further study is required to identify how these platelet concentrates affect production and
357 incorporation of a proteoglycan-rich matrix.
358
359 The mechanism for the differences between groups is not known. We hypothesize that the
360 differences relate to the kinetics of cytokine production and release. The fibrin matrix in the L-
361 PRF may entrap platelets and lead to differences in the kinetics of cytokine release. Schär et al.
362 have shown that growth factor release is different between L-PRF and L-PRP[23]. There may also
363 be differences in the platelet number and platelet activation status between the different
364 preparations. There are also differences regarding the presence or absence of leukocytes[39].
365 Additionally, given that PRP has a liquid consistency, with media changes there is more likely to

366 be an incremental decrease in residual PRP concentration in the media. Further studies are
367 required to identify the underlying biological mechanisms for the observed differences.
368
369 Currently, while a number of strategies have been suggested, there has been no consistent
370 method for augmenting biological integration of scaffold materials with host cartilage tissue. In
371 addition to stem cells and/or growth factors used experimentally and clinically, several blood-
372 derived products are currently used in clinical practice for the treatment of articular cartilage
373 defects. The most well-known and commonly used are the platelet-rich concentrates. They are
374 prepared by differential centrifugation of autologous whole blood and contain a higher
375 concentration of platelets compared with whole blood. There is an established minimum number
376 of required platelets (250,000) to ensure efficacy of PRP by the FDA[40], however, the remains a
377 wide variety of protocols currently used to create platelet-rich concentrates suggests that the
378 optimal protocol to promote repair and regeneration of cartilage and other tissues is not yet fully
379 known.
380
381 **Limitations:**
382 This study has a several limitations. We used *in vitro* analysis only for this study. In cell culture
383 cells are grown in artificial non-physiological conditions without mechanical stimulation,
384 limiting the *in vivo* relevance. Additionally, each group was co-cultured within the same dish,
385 which may have allowed for additive effects of circulating growth factors or for individual
386 specimens to influence each other. We did not include gross analysis regarding the extent to
387 which the defect was filled in our study in that all of the scaffolds remained fully present in the
388 defects as it takes at least 10 weeks for the scaffolds to dissolve in culture[41]. In regards to our

mechanical testing we only measured interfacial shear strength, and we did not evaluate additional mechanical properties of the new tissue (i.e. compressive modulus). When evaluating cellularity via the picogreen assay, we did not specifically investigate chondrocyte proliferation and migration, both of which are important in evaluating healing cartilaginous tissue. We did not determine the platelet or leukocyte count for L-PRF. Prior studies have indirectly assessed the PRF clot platelet count by subtracting the platelet count in the residual serum[42]. Additionally, the choice of using a double centrifugation technique for preparing L-PRP can result in an extended force on the platelets which could possibly alter the microstructure of the platelets[24]. Finally, this results of this study may be dampened by use of both bovine and human derived factors and its applicability, however, this methodology has been previously used[43,44] and our study has produced data consistent regarding the ability of L-PRP and L-PRF to facilitate cartilage healing via a scaffold.

Conclusions:

Delivery of leukocyte rich platelet concentrates in conjunction with a HA scaffold may allow for improvements in cartilage healing through different pathways. L-PRF was not superior to L-PRP in its biomechanical strength suggesting that both treatments may be effective in improving biomechanical strength of healing cartilage through different pathways.

The authors declare no conflicts of interest

Table and Figure Legends:

Table 1: Analysis of Biomechanical and Biochemical Data for Day 28 and Day 42; NS = not significant

Figure 1: *In vitro model of c*artilage plug with scaffold +/- L-PRP/L-PRF

Figure 2: Photographs of the experimental specimens including: A) osteochondral with punch biopsy defect, B) hyaluronic acid scaffold punchouts, C) L-PRF after preparation prior to punch biopsy, D) hyaluronic acid scaffold plug within the defect of the osteochondral specimen, E) hyaluronic acid scaffold plug saturated with PRP within the defect of the osteochondral specimen F) placement of a hyaluronic acid scaffold plug with PRF layered on it within the defect of the osteochondral specimen

Figure 3: Treatment with L-PRF and L-PRP led to improved maximum interfacial strength compared to the control; At day 42 L-PRF demonstrated a maximum interfacial strength was significantly greater than the control ($P = 0.015$); (*P <0 .05); Footnote: Two-way ANOVA was performed with a significance set to $P< 0.05$

Figure 4: DNA content increased between days 28 and 42 in all groups, with L-PRF having a significantly larger increase at day 28 ($P<0.001$) and day 42 ($P<0.01$); (*P < 0.05; **P <0 .01; ***P < .0001); Footnote: Two-way ANOVA was performed with a significance set to $P< 0.05$

Figure 5: Total sGAG production increased from day 28 to day 42, with L-PRP demonstrating the greatest production, which a significant increased compared to L-PRF at day 42 ($p=0.0095$); (**P <0 .01); Footnote: Two-way ANOVA was performed with a significance set to $P< 0.05$

Figure 6: Collagen content was significantly higher in the L-PRF group compared to both the control and L-PRP groups on day 28 and day 42 (*P < 0.05; **P <0 .01); Footnote: Two-way ANOVA was performed with a significance set to $P< 0.05$

Figure 7: Day 42 Safranin-O histological specimens at 10x original magnification from Left to Right: L-PRP, L-PRF, Control; All the specimens started to form disorganized cartilage-like tissue at the edge with ECM deposition with more apparent cellular integration and extracellular matrix deposition at the edges of the construct in the L-PRP and L-PRF groups. L-PRP group had the greatest amount of ECM deposition and cellular integration compared to L-PRF and the control group.

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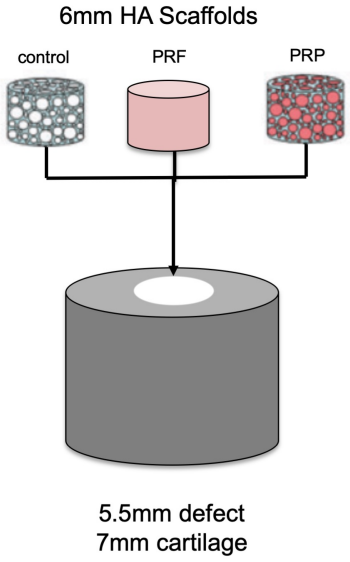
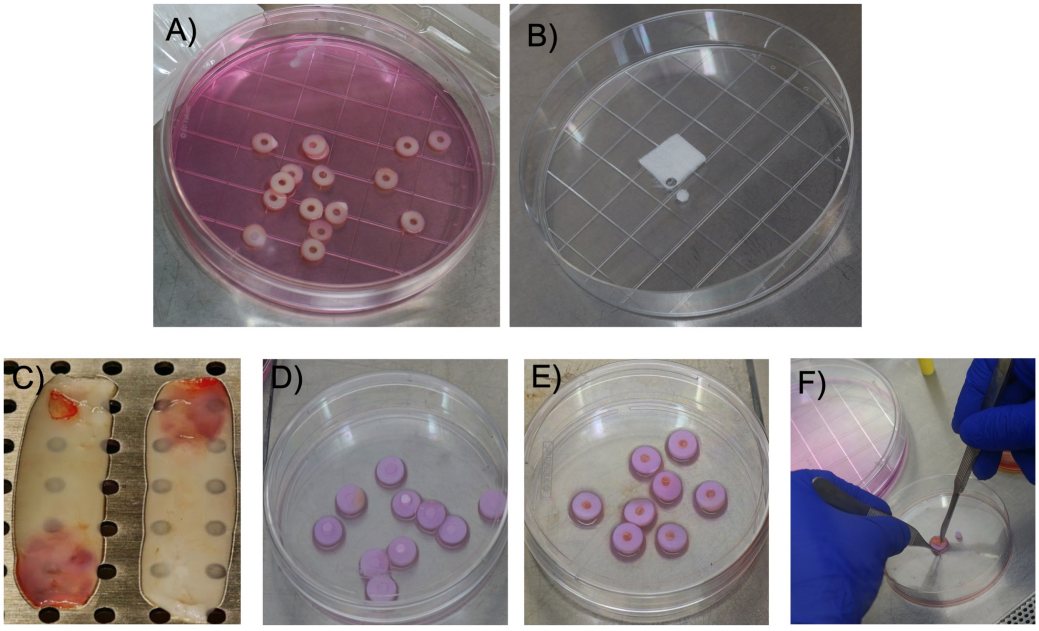
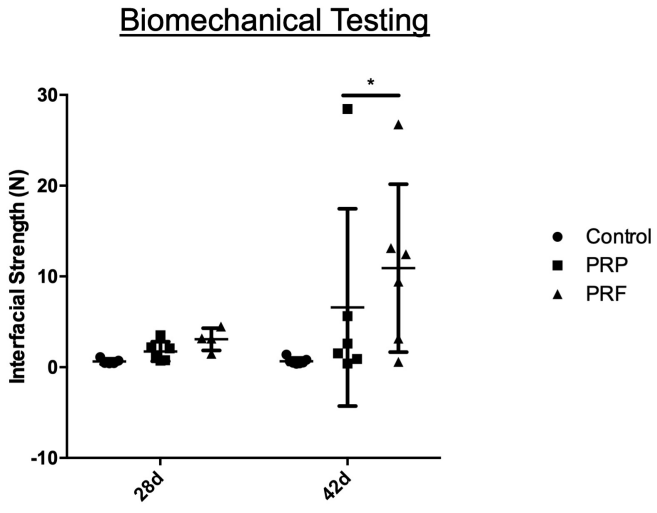
Comparison Group 1 / Comparison Group 2	Day 28 Group 1 Mean ± SD / Group 2 Mean ± SD	P-Value	Day 42 Group 1Mean ± SD / Group 2 Mean ± SD	P-Value
Interfacial Strength (N)				
L-PRP/L-PRF	1.74±1.1/3.08±1.2	NS	6.58±10.9/10.92±9.3	NS
L-PRP/HA Only	1.74±1.1/0.63 ±0.3	NS	6.58±10.9/0.66±0.4	0.015
L-PRF/HA Only	3.08±1.2/0.63 ±0.3	NS	10.92±9.3/0.66±0.4	NS
DNA Content (µg)				
L-PRP/L-PRF	124.2±65.8/560.5±142.6	0.0002	589.3±224.2/761.1±270.7	NS
L-PRP/HA Only	124.2±65.8/191.4±44.6	NS	589.3±224.2/296.4±101.8	0.0125
L-PRF/HA Only	560.5±142.6/191.4±44.6	0.0015	761.1±270.7/296.4±101.8	<0.0001
sGAG (µg)				
L-PRP/L-PRF	0.5±1.1/0.0±0.0	NS	16.0±11.5/4.3±7.2	NS
L-PRP/HA Only	0.5±1.1/2.1±2.5	NS	16.0±11.5/9.7±7.1	NS
L-PRF/HA Only	0.0±0.0/2.1±2.5	NS	4.3±7.2/9.7±7.1	0.0095
Collagen Content (µg)				
L-PRP/L-PRF	21.1±10.2/31.2±5.3	NS	16.3±7/40.1±9.7	<0.0001
L-PRP/HA Only	21.1±10.2/17.6± 7.5	NS	16.3±7/20.4±6.8	NS
L-PRF/HA Only	31.2±5.3/17.6± 7.5	NS	40.1±9.7/20.4±6.8	0.0006



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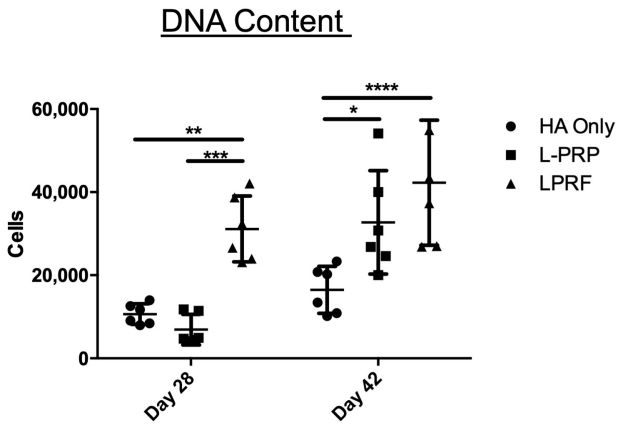
42d
*
Control
PRP
PRF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Cells
**
***
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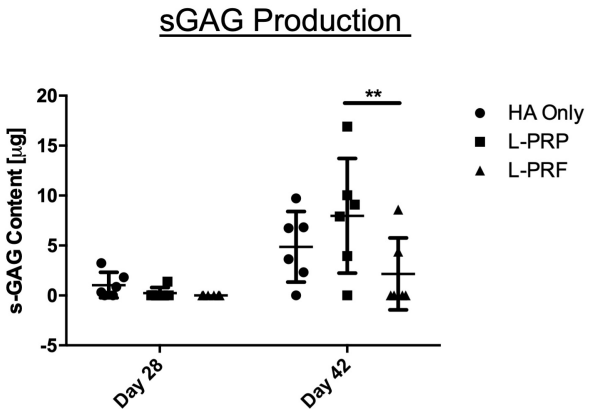
*
Day 28
Day 42
HA Only
L-PRP
LPRF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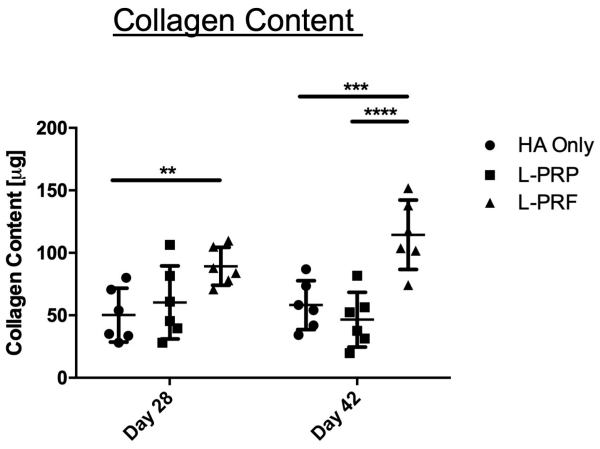
15
10
5
0
-5
**
Day 28
Day 42
HA Only
L-PRP
L-PRF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Collagen Content [µg]
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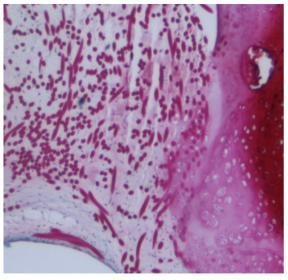
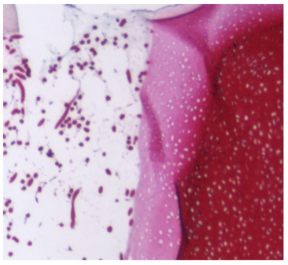
Day 28
Day 42
HA Only
L-PRP
L-PRF

L-PRP



Scaffold Plug

L-PRF



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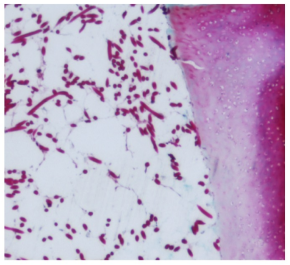

Scaffold Plug