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The Properties of 3 Different Plasma Formulations and Their Effects on Tendinopathic Cells

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Background: Tendinopathies are attributed to failure of the healing process and inadequate tissue remodeling. Plasma injections can trigger regenerative responses by modifying the molecular microenvironment.

Purpose: To examine the differences in the mitotic, chemotactic, anabolic, and inflammatory effects between leukocyte- and platelet-rich plasma (L-PRP), platelet-rich plasma (PRP), and platelet-poor plasma (PPP).

Study Design: Controlled laboratory study.

Methods: Tendinopathic cells were cultured in 3-dimensional (3D) hydrogels formed using PPP, PRP, and L-PRP. Cell migration was evaluated using a μ-Slide chemotaxis chamber with video microscopy. Proliferation was assessed using XTT assays. Expression of genes associated with matrix turnover, including type 1 collagen (*COL1A1*), *COL3A1*, aggrecan, decorin, fibronectin, matrix metalloproteinase 1 (*MMP-1*), *MMP-3*, A Disintegrin-Like And Metalloprotease With Thrombospondin Type 1 Motif proteins 4 (*ADAMTS-4*), and *ADAMTS-5*, was assessed using real-time reverse-transcription polymerase chain reaction. Secreted inflammatory proteins, including interleukin (IL)–1β, IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), and regulated on activation, normal T cell expressed and secreted (RANTES), as well as vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF), were quantified using enzyme-linked immunosorbent assay.

Results: Tendinopathic cells migrate at a higher velocity along L-PRP and PRP than along PPP gradients. PRP and L-PRP promote hypercellularity. PPP and PRP showed more pronounced anabolic properties, as demonstrated by enhanced *COL1A1* and *COL3A1* and reduced *MMP-1* expression. Decorin, fibronectin, and aggrecan were downregulated in L-PRP compared with PPP and PRP. L-PRP and PRP were shown to be more proinflammatory than PPP in terms of IL-6 secretion, but cells in PPP showed MCP-1^{high} phenotype. CTGF secretion was significantly reduced in L-PRP compared with PPP and PRP.

Conclusion: The main advantages of L-PRP and PRP use, compared with PPP, include their stronger chemotactic and proliferative properties. While PPP and PRP stimulate matrix anabolism, L-PRP is more proinflammatory. Emphasis should be placed on the temporal needs and biological characteristics of injured tendons, and plasma formulations need to be tailored accordingly.

Clinical Relevance: Versatile systems allowing the preparation of different plasma formulations, such as PPP, PRP, or L-PRP, can help refine clinical applications by taking advantage of their different biological properties.

Keywords: tendinopathy; PPP; PRP; leukocyte-rich PRP; chemotaxis; anabolism; inflammation; tissue healing

Surprisingly little is known about the progressive trajectory of tendinopathy, including how to stop and reverse it, considering the prevalence of this chronic condition in sports medicine. Crucial characteristics of human tendon cells are yet to be unveiled, including for example how to recover their ability to maintain extracellular matrix homeostasis and tendon health.

New biological interventions such as platelet-rich plasma (PRP), used for the treatment of tendinopathy, are proving not to be as effective as previously reported.[1-3,8] Nevertheless, some success has been reported in several clinical trials, but effectiveness has not been validated for a broad spectrum of tendinopathies. The limited success is partially a consequence of the differences in PRP formulations (ie, leukocyte- and

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platelet-rich plasma [L-PRP] vs PRP) and different application protocols (single vs multiple injection, volumes used, and injection procedures).

A common problem in tailoring of the second-line interventions with PRP is the absence of stringent criteria on which to base the decision about PRP formulation choice. There exists a possibility that the plasma formulation choice is less important than the number of injections or intervals between them, precise targeting of the degenerated area, or "targeting-by-time" strategy, which includes the use of PRP formulations adapted to the needs of the injured tissue.

The aim of the present study was to examine the biological effects of different PRP formulations in vitro, to evaluate the treatment in cells isolated from tendinopathic tissue (tendinopathic cells) to better mimic in vivo situations. A previous study using 3-dimensional (3D) PRP hydrogels suggested that this represents a suitable model that can be used in studies concerned with tendinopathic cell behavior in the molecular context of PRPs.[31]

A given plasma formulation (eg, L-PRP) can be useful in the initial healing stages (ie, migration, proliferation, and inflammation), but it is perhaps not the most favorable formulation in subsequent stages (ie, extracellular matrix [ECM] synthesis and remodeling). To differentiate between plasma formulations according to well-established benchmarks, we have established 3 different experimental conditions based on 3 plasma formulations: 3D-platelet-poor, 3D-platelet-rich, and 3D-leukocyte and platelet-rich hydrogels, which differed in the presence of leukocytes and the number of platelets. A crucial process in tissue healing, cell migration toward chemotactic stimuli provided by different plasma formulations, was assessed. In addition, we investigated the differences in the expression of representative ECM molecules and in the inflammatory/angiogenic response by measuring proteins secreted by tendinopathic cells. Abbreviations used throughout the article are defined in Table 1.

TABLE 1
Abbreviations Used

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
COL	Collagen
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
IL	Interleukin
L-PRP	Leukocyte- and platelet-rich plasma
MCP	Monocyte chemotactic protein
MMP	Matrix metalloproteinase
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RANTES	Regulated on activation, normal T cell expressed and secreted
VEGF	Vascular endothelial growth factor

METHODS

Tendon Cell Isolation

Supraspinatus tendon biopsy samples were obtained from 3 patients undergoing arthroscopic shoulder surgery. The study and all experimental procedures were approved by the local Ethics Committee of the Basque Country (CEIC-E, PI2014108), and written informed consent was obtained from all donors. Tendon cells were isolated from chronic tendinopathy tissue as previously described[5] and cultured using 10% fetal bovine serum (FBS; Hyclone, GE Healthcare). Cells were starved in Dulbecco's modified Eagle's medium (DMEM)–F12 (Gibco, Life Technologies)—that is, serum deprived for 24 hours in T75 flasks (Corning)—to synchronize their cell cycles.[13] After, they were harvested by trypsinization (TryPLE select; Gibco, Life Technologies) and seeded at a concentration of 4×10^4 cells/well in 3D-plasma hydrogels into 6-well plates, as explained further below. Experiments were performed at passage number 2, so an adequate cell number was available to perform all in vitro experiments.

Preparation of Plasma Formulations

Three different plasma formulations were prepared from 190 mL of peripheral blood obtained from 4 healthy donors (2 men and 2 women; mean age, 34.8 ± 14.2 years).

To obtain plasma with negligible blood cell levels, designated as platelet-poor plasma (PPP), peripheral blood was centrifuged at 1500*g* for 15 minutes. PRP was prepared by a single spin at 570*g* for 7 minutes using a certified clinical centrifuge (Sorvall ST16R TX200; Thermo Fisher Scientific), and we used this plasma formulation in the tendon trials.[26] L-PRP was prepared by a double-spin process. First, whole blood was centrifuged at 100*g* for 8 minutes; the upper plasma layer containing platelets and leukocytes was collected and the volume (V) measured. To concentrate the platelets and the leukocytes, a second spin was performed at 1500*g* for 15 minutes, and the pellet was resuspended in V/2 mL of PPP. All plasma formulations followed 3 freeze-and-thaw cycles to mimic the clinical situation and filtration through 0.22-μm filters (Merck Millipore) prior to preparation of plasma hydrogels.

Chemotaxis (Directed Cell Migration)

Chemotaxis experiments were performed in biological triplicates (ie, using cells from 3 donors in the experiments performed individually). Tendinopathic cells were counted using an automatic counter (TC10 Automated Cell Counter; Bio-Rad) and suspended at a density of 3×10^6 cells/mL in DMEM supplemented with 2% FBS to allow cell adhesion. Viability was assessed using Trypan Blue dye (Sigma). Afterward, 12,000 viable cells suspended in 6 μL were added to the μ-Slide chemotaxis 3D chamber (Ibidi GmbH).[38] The chambers were incubated for 2 hours at 37°C, in a 5% CO_2 atmosphere. After cell attachment, reservoirs were filled with DMEM (–), except for 1 reservoir loaded with the selected plasma formulation (+). To compare the chemotactic potential of the 3 plasma formulations, 3 independent experiments were set: (1) 10% PRP/DMEM, (2) 10% PPP/DMEM, and (3) 10% L-PRP/DMEM. In addition, positive (+/+) and negative controls, DMEM/DMEM (–/–), were used. Video microscopy

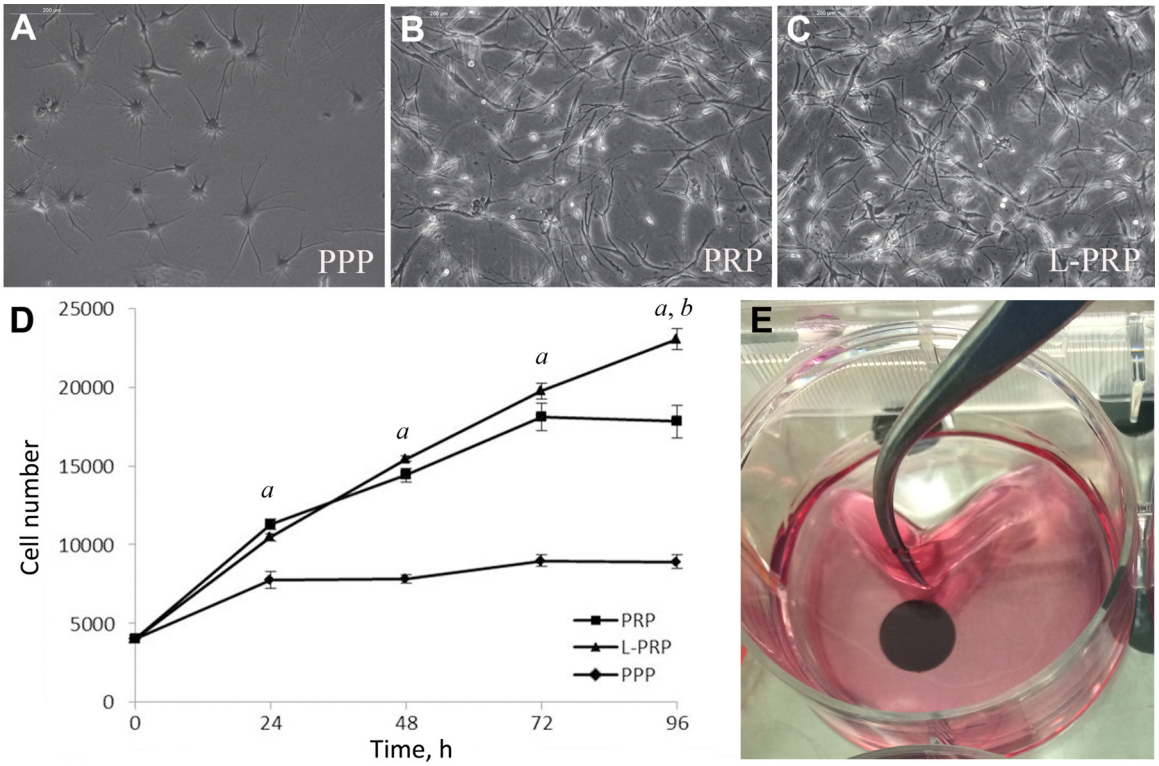

Figure 1. Photomicrographs representing tendinopathic cell proliferation in (A) PPP, (B) PRP, and (C) L-PRP 3-dimensional (3D) hydrogel cultures. (D) Graphs showing differences in tendinopathic cell proliferation between 3 different plasma hydrogels. (E) Macroscopic view of 3D-plasma hydrogel. $^{a}P < .005$ L-PRP and PRP compared with PPP. $^{b}P < .005$ L-PRP compared with PRP.

was performed over a period of 24 hours for each experimental condition and controls. Time-lapse microscopy was performed using a Nikon Eclipse TE2000-E microscope (Nikon), at 10× magnification, and ORCA ER camera, with a 10-minute interval between images. Data were imported into ImageJ software (National Institutes of Health), and cells were tracked using the "Manual Tracking" plugin (Fabrice P. Cordelières, Institut Curie). A minimum of 30 cells were tracked in each experiment. Tracking was further analyzed with the ImageJ "Chemotaxis and Migration Tool" plugin (Ibidi GmbH). The center of mass (COM), forward migration indices (FMIs) in directions parallel and perpendicular to the gradient, velocity (μm/h), directness (μm), and significance using the Rayleigh test were calculated. In addition to trajectory and velocity, these parameters describe the tendency of cells to travel toward the chemotactic gradient.

Cell Cultures in 3D-Plasma Hydrogel

For cell cultures, 3 types of hydrogels (PPP, pure PRP, and L-PRP) were prepared as described previously.[31] Briefly, the cells were resuspended in culture medium with 10% of PPP, pure PRP, or L-PRP, at a density of 4×10^4 cells per well into 6-well plates, and the mixture was incubated at 37°C in 5% CO_2 for 30 minutes. The presence of Ca^{2+} in the culture media induced plasma coagulation, and the cells were captured within the 3D hydrogels (Figure 1). To examine the biology of tendinopathic cells, we set up technical triplicates of 12 independent cultures for each hydrogel type (3 cell donors and 4 blood donors). Cells were cultured in these conditions for 96 hours, after which the hydrogels were centrifuged to obtain the aqueous phase. In parallel, cells were harvested for gene expression analyses. We performed gene expression assays and quantification of secreted proteins (measurements were done in the conditioned media). To normalize protein secretion to the cell number, we quantified the total proteins in each experimental condition. Briefly, cells were harvested by centrifugation, and 100 μL radio-immunoprecipitation assay (RIPA) lysis buffer, together with 1% protease inhibitor cocktail, was added. Samples were centrifuged at 15,000 rpm for 15 minutes at 4°C, and the supernatants were transferred to a fresh tube. Protein content was quantified using the Pierce BCA protein assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Cell Proliferation Assays

The cells were seeded at a density of 4×10^3 cells/well in 3D-PRP hydrogels prepared from 4 donors as explained above.[31] Cell proliferation was measured at 0, 24, 48, 72, and 96 hours using a colorimetric assay, XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide), which detects metabolically active cells (Cell Proliferation Kit II; Roche). Population doubling times were calculated in the exponential phase (up to 72 hours)

(http://www.doubling-time.com/compute.php). In addition, total cell numbers in different 3D hydrogels were compared after 96 hours of culture.

RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from tenocytes using TRIZOL (Invitrogen, Life Technologies). RNA concentrations were measured with the NanoDrop 2000 (Thermo Scientific). A sample of 1 μg RNA was reverse transcribed into complementary DNA (cDNA) using random hexamers in a total volume of 20 μL (SuperScript III First-Strand Synthesis System; Invitrogen Life Technologies).

For the real-time reverse-transcription polymerase chain reaction (RT-PCR), cDNA from each sample was diluted 5 times and 2 μL cDNA (20 ng) was mixed with Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and 5 pmol of primers in a 20-μL final volume. Real-time RT-PCR reactions were performed using ABI-7900 (Applied Biosystems, Life Technologies) in triplicate for each sample.

The expression levels of *COL1A1*, *COL3A1*, aggrecan, decorin, and fibronectin were assessed in comparison with constitutive genes (ie, *GAPDH* and *TBP*). In addition, we measured the expression levels of proteins involved in the turnover of the ECM, including metalloproteinases (MMP1 and MMP3) and ADAMTS proteins (ADAMTS-4 and ADAMTS-5). Gene expression data were analyzed using the $2^{-\Delta Ct}$ method.

Quantification of Secreted Cytokines by Enzyme-Linked Immunosorbent Assay

Reagents used for the enzyme-linked immunosorbent assay (ELISA), including VEGF, CTGF, MCP-1, IL-6, IL-8, IL-1β, and RANTES, were purchased from PeproTech House. SureBlue TMB Microwell peroxidase was purchased from KPL.

Statistical Analysis

All data are expressed as mean ± standard deviation, 95% CIs of technical triplicates of 12 independent cultures, or median and 25th to 75th percentiles. For analysis of cell proliferation 1-way analysis of variance (ANOVA) was used, followed by Bonferroni post hoc comparisons. Comparisons between velocity of migration, accumulated and Euclidean distances, gene levels, and protein secretions were performed using the Kruskal-Wallis test, followed by the Wilcoxon test. Correlations were calculated using the Spearman correlation coefficient. $P < .05$ was considered significant. Data were analyzed using SPSS for Windows version 18.0 (SPSS Inc).

RESULTS

Plasma Formulations

PRP and L-PRP contained more platelets than PPP ($P = .029$ and $P = .018$, respectively). In addition, the platelet number was different between them. L-PRP showed a 4.76 ± 0.86–fold increase in the number of platelets compared with peripheral blood, while for PRP, there was a 2.52 ± 0.28–fold increase ($P = .037$). Significant differences were found between PRP and L-PRP in leukocyte numbers ($P = .029$), and L-PRP contained more leukocytes than peripheral blood as well ($P = .020$). All data are presented in Table 2.

TABLE 2
Characteristics of the 3 Plasma Formulations[a]

	Platelet Concentration, $\times 10^3$ cells/μL	Leukocyte Concentration, $\times 10^3$ cells/μL	Erythrocyte Concentration, $\times 10^3$ cells/μL
Whole blood	185 ± 47	5.17 ± 0.87	4.17 ± 0.48
PPP	6 ± 2	ND	ND
Pure PRP	460 ± 103	0.06 ± 0.07	ND
L-PRP	882 ± 251	8.20 ± 1.31	0.05 ± 0.04

[a]ND, nondetected.

Tendinopathic Cells Migrate Along PRP and L-PRP Gradients

Figure 2 shows representative trajectory plots of tendinopathic cells migrating along PPP, PRP, and L-PRP gradients. As demonstrated, cells migrate in the direction of the chemotactic source.

The main parameters describing cell movement in each experimental condition are presented in Table 3.

All 3 plasma formulations had chemotactic properties, determined by the Rayleigh test, which can show the difference between arbitrary movement and directed migration, and P values obtained were $P = .011$ for PPP, $P = 8.56 \times 10^{-12}$ for PRP, and $P = 1.658 \times 10^{-12}$ for L-PRP. Although tendon cells are slow migrating cells, the mean ± SD velocity was 16.6 ± 6.9 μm/h and 20.1 ± 6.8 μm/h along PRP and L-PRP gradients, respectively ($P = .001$). In the PPP gradient, cells moved with an average velocity of 8.62 ± 4.1 μm/h, similar to the negative control and significantly slower compared with their velocities in L-PRP and PRP gradients ($P < .001$) (Table 3).

Platelets Enhance Cell Proliferation

The population doubling time in the exponential phase in PPP hydrogels was 119 hours (95% CI, 58-181 hours). In contrast, cells in PRP hydrogels doubled in number after 33 hours (95% CI, 31-36 hours) and in L-PRP hydrogels after 31 hours (95% CI, 28-33 hours). One-way ANOVA showed that cell proliferation was significantly different between hydrogel types ($P < .001$), and post hoc analyses demonstrated that proliferation rates were reduced in PPP compared with PRP ($P = .002$), and with L-PRP ($P = .001$). There were no significant differences in doubling rates at 72 hours between PRP and L-PRP ($P > .99$), but cells in PRP hydrogels reached the plateau earlier. At 96

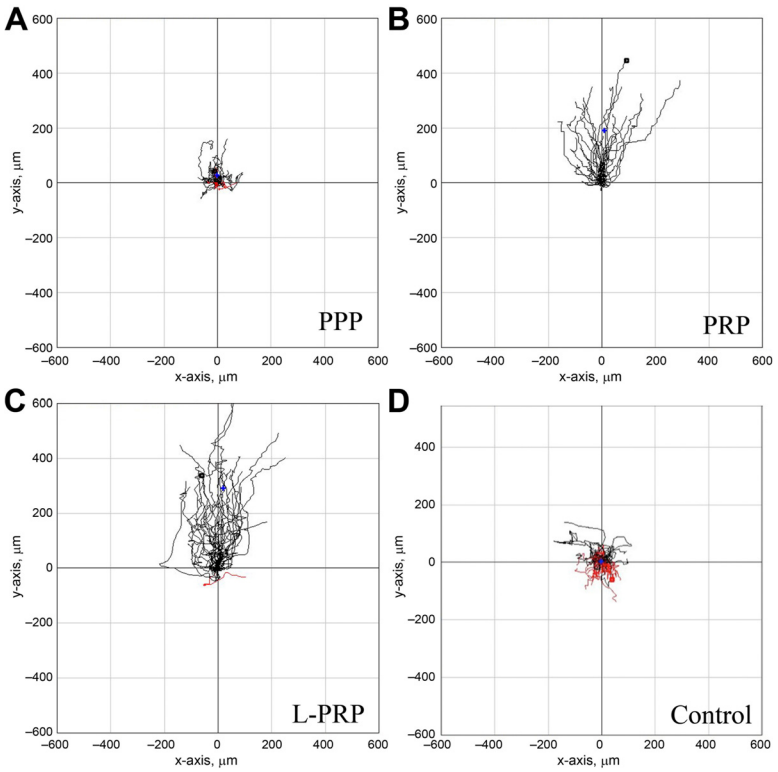

Figure 2. Representative plots of tendinopathic cell migration along (A) PPP gradient (PPP/DMEM), (B) PRP gradient (PRP/DMEM), and (C) L-PRP gradient (L-PRP/DMEM). (D) Negative control (DMEM/DMEM). Video microscopy was performed over a period of 24 hours, and a minimum of 30 cells was tracked in each experiment. All cell trajectories are transformed by setting each starting point to (x, y) (0,0) at time zero.

TABLE 3
Parameters Describing Cell Movement in the Time Period From 0 to 24 Hours: Euclidean Distance, Distance Between 2 Points, Cell at Time Zero, and Cell After 24 Hours[a]

	Euclidean Distance, μm	Accumulated Distance, μm	Velocity, μm/h	Directness
PPP/DMEM (+/–)	73.7 ± 55.6[b] (59.6-87.8)	195.4 ± 97.7[b] (170.6-220.2)	8.16 ± 4.05[b] (7.14-9.20)	0.38 ± 0.21[b,c] (0.33-0.43)
PRP/DMEM (+/–)	247.0 ± 138.6[d] (220.8-289.6)	372.5 ± 158.7[e] (340.3.1-404.7)	15.51 ± 6.61[d] (14.17-16.85)	0.63 ± 0.18 (0.59-0.66)
L-PRP/DMEM (+/–)	307.8 ± 133.5 (280.9-334.7)	482.0 ± 163.6 (449.1-515.0)	20.09 ± 6.82 (18.71-21.47)	0.63 ± 0.15 (0.60-0.66)
DMEM/DMEM (–/–)	49.4 ± 40.1 (39.1-59.8)	186.0 ± 98.4 (160.6-211.4)	9.3 ± 4. 9 (8.02-10.57)	0.29 ± 0.20 (0.24-0.34)

[a]Data are reported as mean ± SD (95% CI). Accumulated distance represents the sum of all incremental movements. Directness $D_i = 1/n \Sigma (d_{i\ Euclid}/d_{i\ accum})$.
[b]$P < .001$ PPP compared with PRP and L-PRP.
[c]$P = .018$ compared with negative control (–/–).
[d]$P = .002$.
[e]$P < .001$ PRP compared with L-PRP.

hours, the number of cells was higher in L-PRP than in PRP hydrogels ($P = .003$) (Figure 1).

The origin of plasma did not affect the cell proliferation rate ($P = .901$), and neither did the origin of cell recipients ($P = .073$).

Downregulation of ECM Constituents in L-PRP Hydrogels

The presence of leukocytes (in L-PRP) decreased tendon metabolism, as evidenced by reduced matrix synthesis,

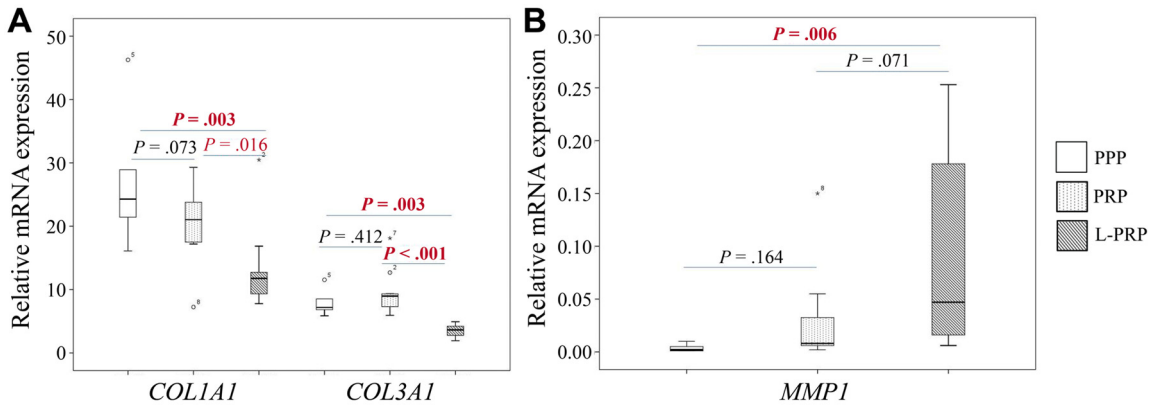

Figure 3. Anabolic/catabolic properties of the different plasma formulations: box plots show median and 25th to 75th percentiles. (A) Relative expression (log $2^{-\triangle Ct}$) of *COL1A1* and *COL3A1*. (B) *MMP-1* expression levels. *MMP-3* expression was not detectable in any experimental condition.

that is, downregulation of *COL1A1* ($P = .003$ and $P = .016$) and *COL3A1* ($P = .003$ and $P < .001$) compared with PPP and PRP. Expression levels of both type 1 and type 3 collagens were downregulated when leukocytes were present in the hydrogel but were similar in PPP and PRP hydrogels (Figure 3A).

Consistently, the expression of the *MMP-1* gene was upregulated in L-PRP hydrogels ($P = .006$ compared with PPP and $P = .071$ compared with PRP) (Figure 3B), while *MMP-3* expression was not detected in any experimental condition.

Collagen is interlaced with nonfibrillar small proteins, such as decorin and fibronectin, and we found that PPP and PRP hydrogels induce a similar expression pattern of ECM molecules (Figure 4). However, decorin was downregulated in L-PRP in comparison with PPP ($P = .003$) and PRP ($P < .001$). Concomitantly, fibronectin was downregulated in L-PRP compared with PPP ($P = .003$), while aggrecan was downregulated in PRP ($P = .006$) and highly downregulated in L-PRP ($P = .003$) in comparison with PPP (Figure 4B).

Significant Spearman correlations were found between all ECM components; all were positive except a negative significant correlation found for *MMP-1*.

ADAMTS-4 and *-5* expression levels were low in all plasma formulation experiments and did not show any significant association with any of the other ECM molecules we analyzed (Figure 4C).

Plasma Formulation Influences the Pattern of Inflammatory Protein Secretion

Tendinopathic cells cultured in 3D hydrogels synthesize proinflammatory proteins, including MCP-1, RANTES, IL-6, IL-8, and IL-1β (Figure 5, A and B). The secretion of MCP-1 by tendinopathic cells was significantly lower in PRP and L-PRP hydrogels compared with PPP ($P < .001$ for both comparisons), whereas the secretion of T cell–specific protein RANTES was higher in L-PRP compared with PPP and PRP ($P = .045$ and .001, respectively) (Figure 5A).

No differences were found in IL-8 secretion among plasma formulations, although IL-6 secretion was higher in PRP and L-PRP compared with PPP hydrogels ($P = .010$ for both comparisons; Figure 5B). IL-1β was not detected in the conditioned media; we were able to detect it in L-PRP hydrogels, but it could not be quantified precisely (detection limit was 30 pg/mL).

VEGF secretion by tendinopathic cells was lower in PRP and L-PRP hydrogels than in PPP ($P = .001$ and .005, respectively) (Figure 5C).

CTGF, which is involved in precursor tendon cell proliferation and differentiation, was less secreted in L-PRP compared with PRP and PPP hydrogels ($P = .045$ and .013, respectively) (Figure 5D).

Overall, all investigated plasma formulations showed proinflammatory potential, but PPP was more angiogenic compared with other hydrogels, in terms of VEGF secretion, but less proinflammatory as demonstrated by IL-6 secretion, and it shows greater ability to attract monocytes (Table 4).

DISCUSSION

In the present study, we revealed functional differences between 3 plasma formulations. We demonstrate that L-PRP and PRP promote hypercellularity by enhancing cell migration and proliferation. We also revealed that PPP and PRP have a stronger effect than L-PRP stimulating matrix anabolism. L-PRP and PRP were shown to be more proinflammatory than PPP, but cells in PPP-hydrogels showed the MCP-1^{high} phenotype.

Controversial clinical data have been previously reported regarding the efficacy of PRP injections in the conservative management of tendinopathies.[1-3,5] Previous studies have demonstrated that the presence of leukocytes, platelet count, and the number of injections can influence the variability and clinical outcomes of tendinopathy treatment.[6,7,11,15,36] By comparing tendinopathic cells cultured in 3 different 3D hydrogels (PPP, PRP, and L-PRP), we obtained information regarding their differential effects on chemotaxis, proliferation, ECM anabolism, and inflammation.

A sensible approach to the enhancement of tendon repair is to direct mesenchymal cell migration within the

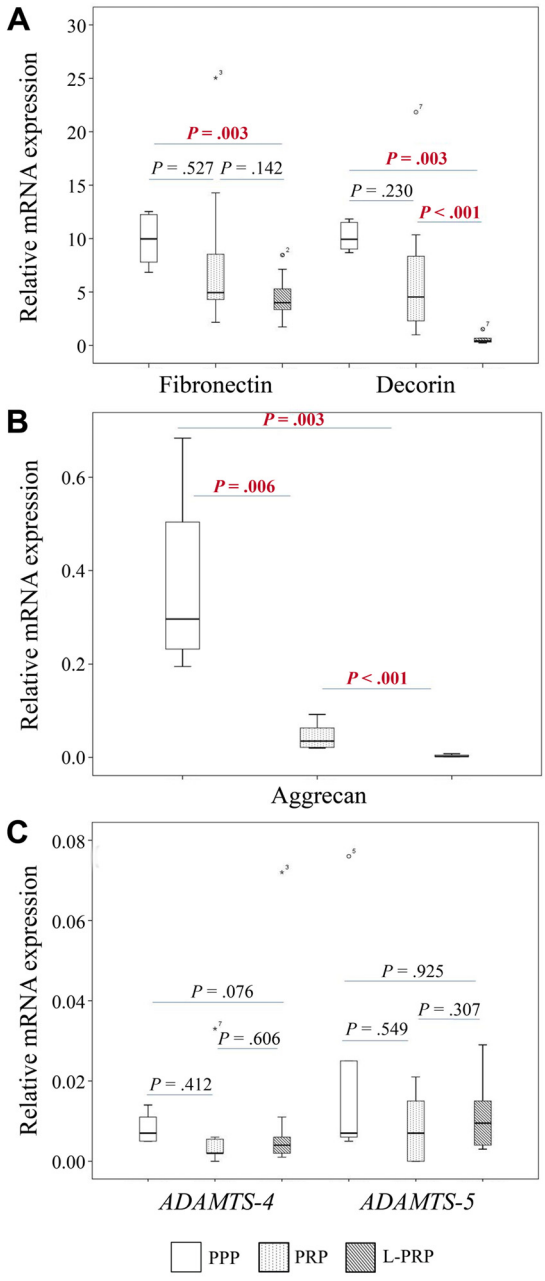

Figure 4. Relative expression (log $2^{-\triangle Ct}$) of proteoglycans: box plots show median and 25th to 75th percentiles. (A) Differences in fibronectin and decorin expression levels between the different plasma formulations. (B) Differences in aggrecan expression levels. (C) *ADAMTS-4* and *-5* expression levels.

tendinopathic areas. The inability of PPP to stimulate cell migration and the increased efficacy of L-PRP ((882 ± 251) × 10^3 platelet/μL) in comparison with PRP ((460 ± 103) × 10^3 platelet/μL) are in agreement with studies showing that platelet-secreted molecules enhance cell migration.[24] Both L-PRP and PRP can form platelet-derived cytokine gradients (eg, stromal cell-derived factor 1α [SDF-1α] and platelet-derived growth factor [PDGF]), favoring not only tendon fibroblast migration but also tissue infiltration by precursor cells.[24] Cell migration is crucial in the degenerated tendons with reduced cellularity. Proliferation of tendon progenitor cells is enhanced in L-PRP compared with PRP[39] and in tenocytes from degenerated human rotator cuffs.[21] These differences could be attributed both to platelet count and to the presence of leukocytes.[21]

In a degenerated tendon, a plasma formulation triggering an increased anabolic response in the tendon anabolism may be the best choice. Our results revealed that L-PRP hydrogels could have catabolic properties, based on the results showing that *COL1A1* and *COL3A1* expression was downregulated, and *MMP-1* expression was upregulated in tendinopathic cells. These results are in accordance with previous studies that were performed with healthy equine tendon cells.[9] Boswell et al[9] showed that a reduction in leukocyte numbers appears to be more important in the minimization of catabolic signaling than the increase in platelet numbers. *MMP-1* mainly degrades type 1 collagen, and its upregulation was shown to accompany full-thickness tears in patients.[34] In addition, its upregulation was highly correlated with failed healing of the rotator cuff in a case-control study.[29]

The collagen structure is interlaced with various nonfibrillar proteins that enable tendons to support high loads and provide stability.[16,19,37] Along with the catabolic features of L-PRP, we found that its use is coupled with a significant reduction in decorin expression. Decorin is a small leucine-rich proteoglycan, and experiments involving *Dcn*(–/–) mice revealed that decorin deficiency results in an aberrant tendon collagen fibril structure with inferior mechanical properties.[18] Decorin content mostly affects the viscoelasticity of tendons.[16,17,30] Furthermore, the expression of fibronectin, labeled as a tissue "master organizer"[32] because of its involvement in the initiation, progression, and maturation of fibrillogenesis, is reduced in L-PRP hydrogels.

PPP is a multimolecular mixture, although less complex than PRP and L-PRP, and its clinical applications have not been explored yet. It contains considerable amounts of insulin-like growth factor-1 (IGF-1), insulin-like growth factor-binding protein (IGF-BP), and hepatocyte growth factor (HGF) proteins, synthesized mainly in the liver, which can be useful in certain healing stages because of their anabolic and anti-inflammatory properties. In our study, PPP hydrogels showed increased anabolism and influenced the increase in expression of decorin and fibronectin, but PPP is not mitogenic. Previous studies report that tendinopathic cells treated with PPP may serve as a source of paracrine CTGF (also known as CCN2, a member of matricellular proteins), which can induce proliferation and differentiation of endogenous tendon precursor cells, CD146+ perivascular cells.[23,25] In addition, CTGF downregulates the proinflammatory activity of IL-1β in a nuclear factor κB–dependent manner.

The positive aspects of L-PRP and PRP include strong induction of proliferation and migration, which are crucial mechanisms in tendon healing. Cell locomotion and migration toward chemotactic stimuli are important features in early phases of tissue healing. The low cell velocity during

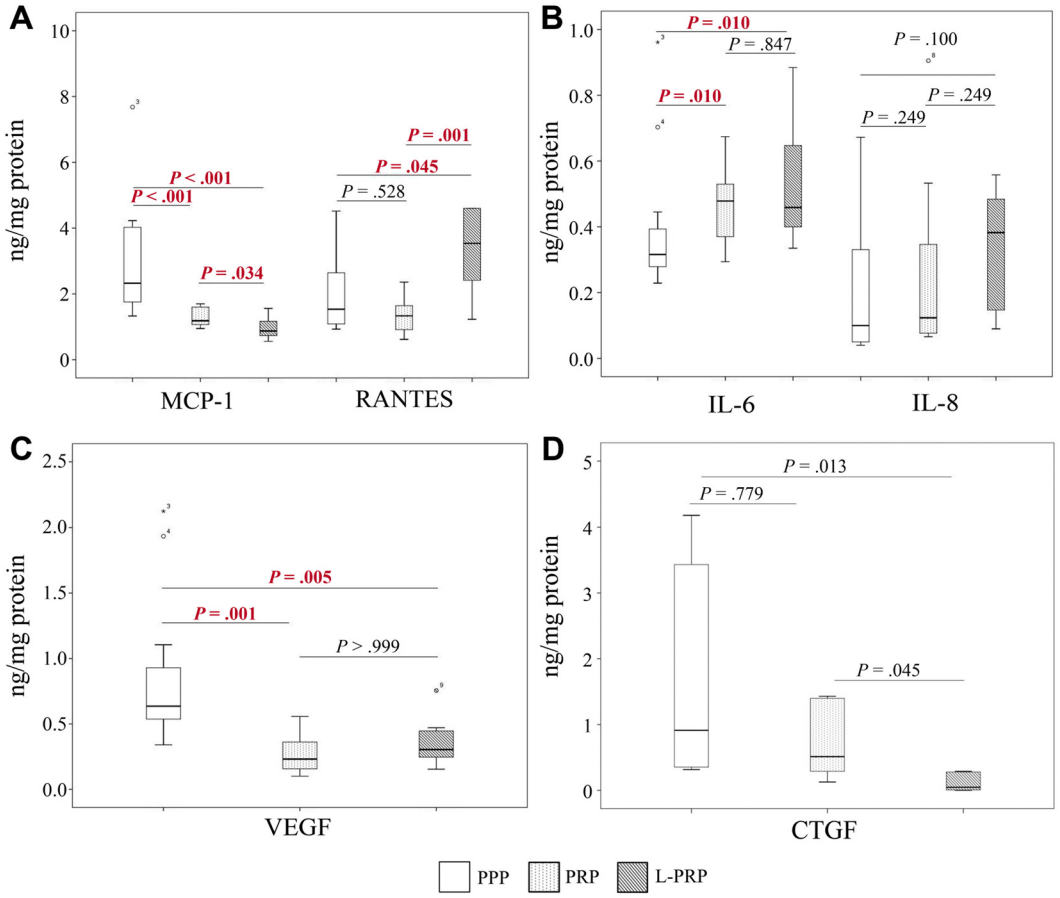

Figure 5. Inflammatory protein secretion patterns. (A) MCP-1 and RANTES secretion. (B) IL-6 and IL-8 secretion. (C) VEGF secretion. (D) CTGF secretion. Boxplots represent median and 25th to 75th percentiles of nanograms of secreted cytokine, normalized by total protein to account for the differences in cell numbers.

TABLE 4
Biological Properties of 3 Different Plasma Formulations: PPP, PRP, and L-PRP[a]

		Chemotaxis			Extracellular Matrix							Inflammation					
	PROLI	Distance	Velocity	Directness	*COL1A1*	*COL3A1*	MMP-1	DCN	FN	ADAMTS-4	ADAMTS-5	MCP-1	RANTES	IL-6	IL-8	VEGF	CTGF
PPP	↔	↓	↓	↑	↑	↑	↔	↑	↔	↔	↔	↑	↔	↔	↔	↑	↑
PRP	↑	↑	↑	↑	↑	↑	↔	↑	↔	↔	↔	↑	↔	↑	↔	↔	↑
L-PRP	↑	↑	↑	↑	↓	↓	↑	↓	↓	↔	↔	↓	↑	↑	↔	↔	↓

[a]Matrix metalloproteinase 3 gene expression and IL-1β release were undetectable. Enhanced/favors ↑; reduced ↓; similar, unmodified ↔. DCN, decorin; FN, fibronectin; PROLI, PROLIFERATION.

migration along PPP gradients shows that chemotaxis is mostly induced by platelet secretome, which may outweigh advantages offered by its anabolic potential.

Tenocytes isolated from tendinopathic tissue play an important role in inflammation, due to their involvement in paracrine signaling to immune cells, through the production and secretion of chemotactic interleukins, including IL-8, IL-6, MCP-1, and RANTES.[4] IL-6 is a proinflammatory protein with complex biological activities, produced by stromal and immune cells as a consequence of Toll-like receptor activation, stress responses, or cell activation by other cytokines, such as IL-1β.[20] In accordance with previous studies, PPP may have several advantages over PRP and L-PRP, as we found that the secretion of IL-6 is lower in the PPP compared with PRP or L-PRP hydrogels.[39] In addition, cells in PPP hydrogels can create a gradient of MCP-1, which precedes nerve regeneration in early tendon healing[35] and attracts monocytes from the bloodstream. This interesting issue warrants additional investigations. Although it remains unclear whether angiogenesis is a poor prognostic

factor for tendinopathy, imaging assays using power Doppler ultrasound showed high blood vessel density in patients with tendon rupture.[33] In contrast to this, a study by Carr et al[10] interpreted reduction in the number of blood vessels and cellularity at 12 weeks after L-PRP–augmented arthroscopic acromioplasty as detrimental for healing tissue. Unfortunately, this study failed to reveal any association between cellularity, angiogenesis, and clinical outcomes.

Here, we investigated the main advantages and disadvantages of PPP, PRP, and L-PRP. Establishing which tissue characteristics (ie, cellularity, vascularity, and hypoechogenicity) can be used as good prognostic parameters for biological interventions is a major challenge in tendinopathy research.

Once the prognostic markers describing the biology of the tendon tissue are established, planning of the best therapeutic approach and taking advantage of the biological properties of different plasma formulations would be easier. A careful description of the target tissue is paramount, since the effects of plasma formulations result from their interactions with it. Location of tendinopathy is crucial, because of the differences in the nature of cells in the main body of the tendon and in the enthesis (fibroblastic vs chondroid cells), as well as the composition of the ECM in these locations. It is currently not possible to describe major deficits of tendinopathic patient tissues, which presents an important problem in therapy development. The research advancements in this direction would enable us to take advantage of different plasma formulations.

The treatment scheme for the application of PRP is not clear. Some authors report that additional plasma injections (ie, 2-3 injection cycles) do not improve clinical outcomes.[22,27] Treatment plans based on sequential administration of different plasma formulations need to be investigated. For example, L-PRP injection, taking advantage of its chemotactic and mitotic properties, can be followed by PPP injection within 1 to 2 weeks to boost ECM anabolism. Information about the biological characteristics of tendinopathic tissue may influence the choice of plasma formulations tailored to fulfill temporal tissue needs. Further elucidation of the basic processes is needed to understand the differences in the molecular patterns of secreted inflammatory proteins between different formulations. Emphasis should be on the temporal needs and biological characteristics of injured tendon, and plasma formulations should be tailored accordingly.

Several limitations should be noted in this study. First, even if our model is a 3D system, it did not account for the contribution of external mechanical forces. Some bioreactors aim to induce certain loading patterns and loading history and to reproduce tensile strain, but compressive and shear stresses have not been replicated in a meaningful in vitro model. Other tissue culture approaches[12] have not been validated, and cell behavior in the diseased tissue explants may reflect artifacts attributable to death and matrix degradation. The second limitation to our study was the fact that we have not used available commercial systems more representative of the clinical practice. However, as PRP can be prepared in blood banks or sterile facilities following good manufacturing practices, the PRP used in this study was prepared by a single-spin procedure in a clean room, which led to the production of PRP formulations similar to ACP (Arthrex, Inc) or Cascade (MTF), which are commonly used in research as representative pure PRPs.[11,28] Furthermore, L-PRP was prepared using a double-spin protocol, which produces L-PRP similar to Magellan (Arteriocyte Medical Systems, Inc), which is a representative formulation of double-spin L-PRPs.[11,14] However, to avoid artifacts induced by erythrocytes in vitro, we did not use the buffy-coat method for L-PRP preparation. The third limitation to this study is that we had higher platelet concentration in L-PRP than in pure PRP; accordingly, differences in biological effects between L-PRP and PRP can be attributed to both higher platelet concentration and the presence of leukocytes in the formulations. However, this L-PRP formulation reflects clinical reality where double-spinning procedures lead to higher platelet and leukocyte concentrations than single spinning.

In conclusion, our findings indicate that the main advantages of L-PRP and PRP use, compared with PPP, include their stronger chemotactic and proliferative properties. While PPP and PRP stimulate matrix anabolism, L-PRP is more proinflammatory. Emphasis should be placed on the temporal needs and biological characteristics of injured tendons, and plasma formulations need to be tailored accordingly.

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