

Comparison of Platelet-Rich Plasma Formulations for Cartilage Healing

An in Vitro Study

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Background: Platelet-rich plasma (PRP) has been advocated as one treatment for cartilage tissue regeneration. To date, several different platelet-rich formulations have been available, but a deep knowledge of their composition and mechanism of action in a specific clinical use is needed. The aim of this study was to investigate the effect of various PRP formulations on human chondrocytes in vitro.

Methods: Blood from ten human volunteers was used to prepare three formulations: (1) PRP with a relatively low concentration of platelets and very few leukocytes (P-PRP), (2) PRP with high concentrations of both platelets and leukocytes (L-PRP), and (3) platelet-poor plasma (PPP). Selected growth factors in the formulations were measured, and the in vitro effects of various concentrations were tested by exposing chondrocytes isolated from osteoarthritic cartilage of four different men and measuring cell proliferation, matrix production, and gene expression.

Results: L-PRP contained the highest levels of growth factors and cytokines. All three formulations stimulated chondrocyte proliferation throughout the culture period evaluated; the only significant difference among the formulations was on day 7, when P-PRP induced greater cell growth compared with the other two formulations. P-PRP stimulated chondrocyte anabolism, as shown by the expression of type-II collagen and aggrecan, whereas L-PRP promoted catabolic pathways involving various cytokines. However, L-PRP induced greater expression of the hyaluronic acid synthase-2 gene and greater production of hyaluronan compared with P-PRP.

Conclusions: L-PRP and P-PRP induced distinct effects on human articular chondrocytes in vitro, possibly because of differences in the concentrations of platelets, leukocytes, growth factors, and other bioactive molecules. The identification of the optimal amounts and ratios of these blood components could ideally lead to a formulation more suitable for the treatment of cartilage lesions.

Clinical Relevance: PRP formulations that are suitable for the treatment of degenerative joint diseases will likely contain balanced concentrations of platelets and leukocytes.

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Articular cartilage lesions are difficult to treat and remain one of the most challenging problems for orthopaedic surgeons^{1,2}. Most available nonsurgical treatments have controversial and unproven efficacy. They primarily offer temporary clinical relief, and surgical treatment is often unavoidable

when they fail³. Various surgical options are available, but there is no agreement regarding the effective superiority of any technique over the others^{4,5}. Even though some surgical options offer a satisfactory clinical outcome at intermediate-term follow-up^{6,7}, rehabilitation is long and improvement is often unpredictable,

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incomplete, and of limited duration⁸⁻¹¹. The search for a less invasive solution to improve the status of the chondral surface and allow a swift return to full activity is highly desirable.

One area of interest in cartilage repair involves the complex network of growth factors involved in tissue homeostasis, such as transforming growth factor (TGF)- β , platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF)- β , and hepatocyte growth factor (HGF)¹²⁻¹⁶. These factors and other bioactive molecules are contained in platelet alpha granules, and the use of intra-articular injections of platelet concentrate has been proposed as a minimally invasive solution to promote cartilage healing¹⁷.

Platelet-rich plasma (PRP) is a blood derivative containing autologous growth factors and cytokines¹⁸. Its use in regenerative medicine represents a simple, low-cost, and minimally invasive approach that might promote healing processes^{19,20}. Initial studies primarily documented the safety and feasibility of PRP use²¹⁻²⁹, but subsequent publications have reported on the potential benefit of multiple intra-articular PRP injections for the treatment of cartilage lesions of traumatic origin and those of degenerative origin³⁰⁻³³.

However, the optimal method of preparing PRP for use in cartilage repair is unknown, and different methods may result in formulations that differ with regard to platelet and leukocyte concentrations, potentially influencing cell responses³⁴. The aim of the present study was to investigate the mechanism of action of two different PRP formulations on human chondrocytes through the evaluation of cell proliferation, matrix production, and expression of specific genes. Both of the tested formulations have been reported to offer clinical benefits.

Materials and Methods

The study was approved by the local ethics committee and by the institutional review board, and each donor signed a written informed-consent form.

Plasma Preparation

PRP and platelet-poor plasma (PPP) were obtained from ten healthy male volunteers (mean age [and standard deviation], 29.9 ± 3.4 years) who underwent collection of a 200-mL blood sample. Exclusion criteria were a systemic disorder, smoking, infection, nonsteroidal anti-inflammatory drug use in the five days before blood donation, a hemoglobin level of <11 g/dL, or a platelet concentration of $<150 \times 10^3/\mu\text{L}$.

The two strategies for PRP preparation were a one-step method, which provided a pure platelet concentrate without leukocytes but with a limited platelet concentration through one centrifugation, and a two-step method, which provided high platelet and high leukocyte concentrations through two centrifugations^{35,36}. In the one-step method, 45 mL of venous blood was divided into five tubes containing 1 mL of 3.8% trisodium citrate solution and was centrifuged at $460 \times g$ for eight minutes. Pure PRP (P-PRP) was then obtained by collecting 1 mL of the platelet-rich supernatant above the red blood-cell pellet in each tube, carefully avoiding harvesting of leukocytes. In the two-step method, 150 mL of venous blood was collected in a bag containing 21 mL of sodium citrate and was centrifuged at $730 \times g$ for fifteen minutes. Most of the red blood cells were eliminated, and the resulting plasma and buffy coat were transferred to a separate bag through a closed circuit and were centrifuged again for ten minutes at $3800 \times g$. PPP was obtained from the superficial layer of the supernatant, and leukocyte PRP (L-PRP) was obtained from the supernatant above the red blood cells. Samples of P-PRP, L-PRP, and PPP were analyzed with an automated blood-cell counter (COULTER LH 750 Hematology Analyzer; Beckman Coulter, Milan, Italy) to determine the concentrations of platelets and nucleated cells. (The linearity range of the instrument was 5 to $1000 \times 10^3/\mu\text{L}$ for the platelet count and 0.1 to

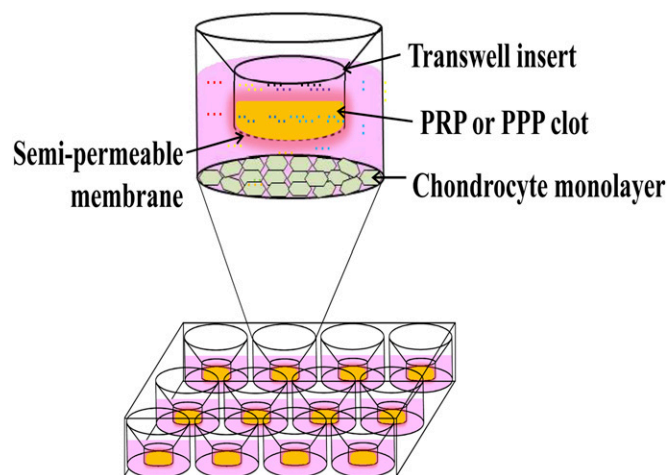


Fig. 1
Schematic representation of the Transwell system used in the experiments.

$100 \times 10^3/\mu\text{L}$ for the white blood-cell count.) Each sample was divided into two aliquots, one for cell culture experiments and the other for growth factor evaluation.

Measurement of Growth Factors and Cytokines

The PRP and PPP formulations were evaluated for the release of interleukin (IL)-1 β , vascular endothelial growth factor (VEGF), TGF- β 1, PDGF-AB/BB, and FGF- β . First, each formulation was activated with 10% CaCl_2 (final concentration, 22.8 mM) and was incubated for seven days at 37°C . After centrifugation at $2800 \times g$ for fifteen minutes at 20°C , the supernatant was collected and immediately frozen at -30°C until use. Samples were assayed in duplicate with use of commercially available immunoassay kits (Millipore, Billerica, Massachusetts, for PDGF-AA/BB, and Bio-Rad Laboratories, Hercules, California, for the other molecules) and were quantified with use of the Bio-Plex Protein Array System (Bio-Rad)³⁷. Standard levels between 70% and 130% of the expected values were considered accurate and were used.

Chondrocyte Isolation

Cartilage samples were obtained from four male patients (age range, sixty-two to seventy-three years) with Kellgren-Lawrence grade II or II osteoarthritis who were undergoing knee replacement surgery. The diagnosis of osteoarthritis was based on clinical, laboratory, and radiographic evaluations and fulfilled the American College of Rheumatology criteria for symptomatic primary knee osteoarthritis³⁸. Chondrocytes were isolated from four different subjects and were randomly distributed among the trials.

The chondrocytes were isolated by an enzymatic procedure as described previously³⁹ and were used at passage 3. The cells were seeded in a 12-well plate at a density of 0.25×10^5 cells/cm² and were cultured for twenty-four hours in Dulbecco Modified Eagle Medium (DMEM; Sigma, St. Louis, Missouri) without fetal bovine serum to permit them to adhere to the wells. The chondrocytes were then cultured for seven days in medium supplemented with 5, 10, or 20% (vol/vol) of P-PRP, L-PRP, or PPP. Each formulation had previously been placed in a Transwell device (Corning, Lowell, Massachusetts) and activated with 10% CaCl_2 (22.8 mM). The medium was not changed during the seven days in order to better simulate the clinical effect of the various formulations. The seven-day time point was chosen to match the time at which the second PRP injection in the clinic would be scheduled, as the clinical protocol comprises three injections at weekly intervals³³. A schematic representation of the culture system is shown in Figure 1.

Proliferation Assay

Chondrocyte growth in the presence of each formulation was evaluated after zero, three, and seven days of culture. Proliferation of the cells grown in the presence of a PRP or PPP formulation was assessed on days zero, three, and seven by the Alamar blue test⁴⁰. Briefly, the cells were incubated with 10% Alamar

blue for three hours, and the fluorescence (excitation wavelength, 490 nm; measurement wavelength, 540 nm) was measured with use of a microplate reader (CytoFluor 2350; Millipore). The results were expressed as the percentage of Alamar blue reduction as indicated by the manufacturer's data sheet (AbD Serotec, Kidlington, United Kingdom).

Gene Expression

Chondrocytes grown in the presence of 5%, 10%, or 20% P-PRP, L-PRP, or PPP were analyzed by real-time PCR (polymerase chain reaction) on day seven to assess the expression of type-II collagen, aggrecan, IL-1 β , IL-6, IL-8, IL-10, tumor necrosis factor (TNF)- α , VEGF, FGF- β , HGF, TGF- β , hyaluronic acid synthase-2 (HAS-2), tissue inhibitor of metalloproteinase (TIMP)-1, and matrix metalloproteinase (MMP)-13. RNA extraction and real-time PCR were performed as described previously³⁹. PCR primers for the selected genes and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control, were obtained from the literature⁴¹⁻⁴⁵ or designed with use of PRIMER3 software (Andreas Untergasser, Michelstadt, Germany) (see Appendix). The calculated mRNA (messenger RNA) level of each target gene was normalized with respect to that of GAPDH (with use of the usual $2^{-\Delta Ct}$ formula) and was expressed as a percentage of that reference gene.

Hyaluronan and Lubricin Production

Hyaluronan and lubricin protein levels (in ng/mL) were measured in the supernatants above chondrocytes that had been treated for seven days with P-PRP, L-PRP, or PPP. The measurements were performed by quantitative sandwich enzyme immunoassay according to the instructions of the manufacturer (R&D Systems, Minneapolis, Minnesota, for the hyaluronan assay; Uscn Life Science, Wuhan, China, for the PRG4 [lubricin] assay).

Statistical Analysis

Results are presented as the mean and the standard error of the mean or as the median and the 25th and 75th percentiles, as appropriate. Because of the relatively small number of subjects, platelet numbers and concentrations of soluble factors were analyzed with use of nonparametric tests. Differences among the formulations were evaluated with use of the Friedman ANOVA (analysis of variance) test and the Wilcoxon matched-pairs test for multiple comparisons. After Bonferroni correction, a *p* value <0.0167 was considered significant for each of these comparisons.

The general linear model for repeated measures was used to test differences over time and the influence of concentration and treatment on these changes; the Sidak correction for multiple comparisons was used to take into account the various concentrations and treatment groups tested. A *p* value of <0.05 was considered significant for these tests. Statistical analysis was performed with SPSS software (version 15.1; SPSS, Chicago, Illinois).

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Results

Characterization of the Formulations

The median number of platelets per cubic millimeter increased progressively from 6000 (interquartile range [IQR], 4000 to 7000) in PPP to 194,000 (IQR, 120,000 to 364,000) in P-PRP to 929,000 (IQR, 720,000 to 965,000) in L-PRP. The platelet concentration differed significantly among the formulations (*p* < 0.0005) and between each pair of formulations (*p* < 0.001). The median concentration of white blood cells per cubic millimeter was 5500 (IQR, 5000 to 6500) in L-PRP, negligible (<200) in P-PRP, and zero in PPP.

The soluble factors analyzed showed a wide variability in concentration among individuals. Overall, the IL-1 β , PDGF-

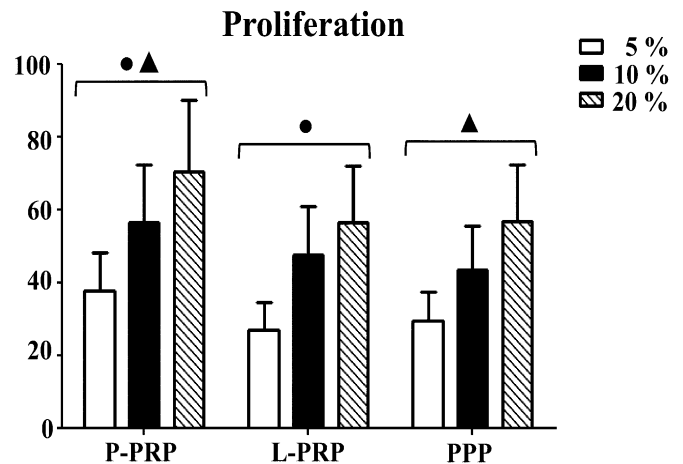


Fig. 2

Proliferation of human chondrocytes grown in presence of three different concentrations of P-PRP, L-PRP, and PPP on day seven. Proliferation was assessed on the basis of the percentage of Alamar blue reduction, and values are shown as the mean and the standard error (*n* = 10). Matching symbols above the plots indicate a significant difference between the indicated results (*p* < 0.05).

AB/BB, TGF- β 1, and VEGF concentrations differed significantly (*p* < 0.0005) among the formulations (Table I). In particular, the IL-1 β and VEGF concentrations were similar in PPP and P-PRP, and both were lower than the concentration in L-PRP. The PDGF-AB/BB concentration increased progressively and significantly from PPP to P-PRP to L-PRP, whereas the TGF- β 1 concentration was lower in PPP than in P-PRP and L-PRP, which did not differ significantly from each other. FGF- β was produced at a similar level in all three formulations (Table I). All of the factors except PDGF-AB/BB showed increased concentrations after seven days of incubation compared with one hour; the concentration of PDGF-AB/BB was similar at the two time points.

Proliferation Assay

The 5%, 10%, and 20% concentrations of each formulation induced a dose-dependent enhancement of chondrocyte proliferation during the culture period evaluated. However, the enhancement reached significance only for the highest concentration of each formulation compared with the lower concentrations on day seven (*p* < 0.05). At each concentration, P-PRP induced greater cell proliferation compared with L-PRP and PPP on day seven (*p* < 0.05) and the difference between L-PRP and PPP was not significant (Fig. 2).

Gene Expression

In general, no significant differences were observed among the various concentrations of each formulation, and the results for the three concentrations are therefore grouped together in Figure 3. Expression of the cartilaginous markers, type-II collagen and aggrecan, was higher in the cells treated with P-PRP and PPP compared with L-PRP (*p* < 0.05). A different trend was observed for all of the inflammatory genes evaluated; in particular, IL-1 β and IL-6 were expressed significantly more in the cells treated with L-PRP compared with P-PRP and PPP (*p* < 0.05), whereas IL-8 and TNF- α

TABLE I Soluble Factor Concentrations in the Plasma Formulations After Seven Days of Incubation

	Formulation* (pg/mL)			P Value†		
	PPP	P-PRP	L-PRP	P-PRP vs. PPP	L-PRP vs. PPP	P-PRP vs. L-PRP
IL-1 β	0.60 (0.02-3.1)	0.77 (0.19-3.4)	65.4 (34.7-233.3)	NS	<0.005	<0.005
VEGF	3.7 (0-15.4)	85.6 (64.6-161.9)	226.8 (145.8-743.3)	NS	<0.005	<0.005
TGF- β 1	7428.4 (3028.6-46,168)	32,787.2 (15,632.9-639,316)	103,553.4 (64,935.7-1,341,400)	<0.01	<0.005	NS
PDGFAB/BB	304.4 (0-974.9)	2042.5 (1081.4-9157.1)	31,670.6 (18,617.6-80,462.3)	<0.005	<0.005	<0.005
FGF- β	5.0 (0-11.0)	3.3 (0-6.1)	9.8 (0-17.1)	NS	NS	NS

*The values are expressed as the median for the ten donors, with the IQR in parentheses. †The p value for the comparison among all three plasma formulations with the Friedman ANOVA test was <0.0005 except for FGF- β .

were expressed similarly by PRP and PPP-stimulated cells. VEGF and FGF- β were expressed significantly more in cells treated with L-PRP ($p < 0.05$), whereas HGF expression was highly stimulated by P-PRP ($p < 0.05$). TGF- β expression did not differ among the

formulations. Expression of the HAS-2 gene was significantly stimulated by L-PRP ($p < 0.05$). TIMP-1 and IL-10 were significantly stimulated by L-PRP ($p < 0.05$), whereas MMP-13 was expressed similarly by cells treated with the three formulations.

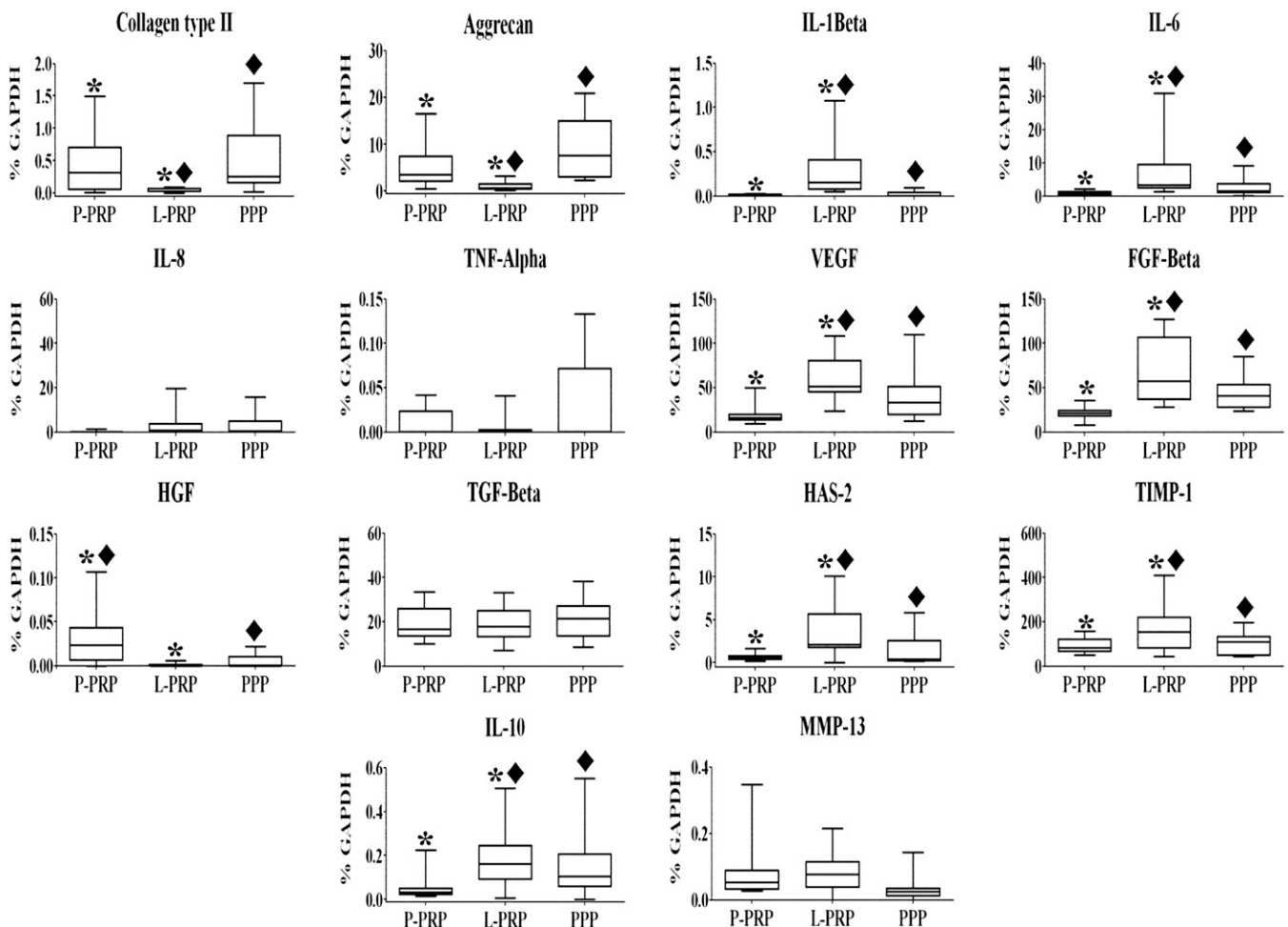


Fig. 3

Messenger RNA expression in chondrocytes grown in the presence of P-PRP, L-PRP, or PPP on day seven. Data were normalized to GAPDH and expressed as a percentage of that reference gene. The top and bottom of each box indicate the 25th and 75th percentiles, the line within the box indicates the median, and the whiskers indicate the minimum and maximum values. Matching symbols above the plots indicate a significant difference between the indicated results ($p < 0.05$).

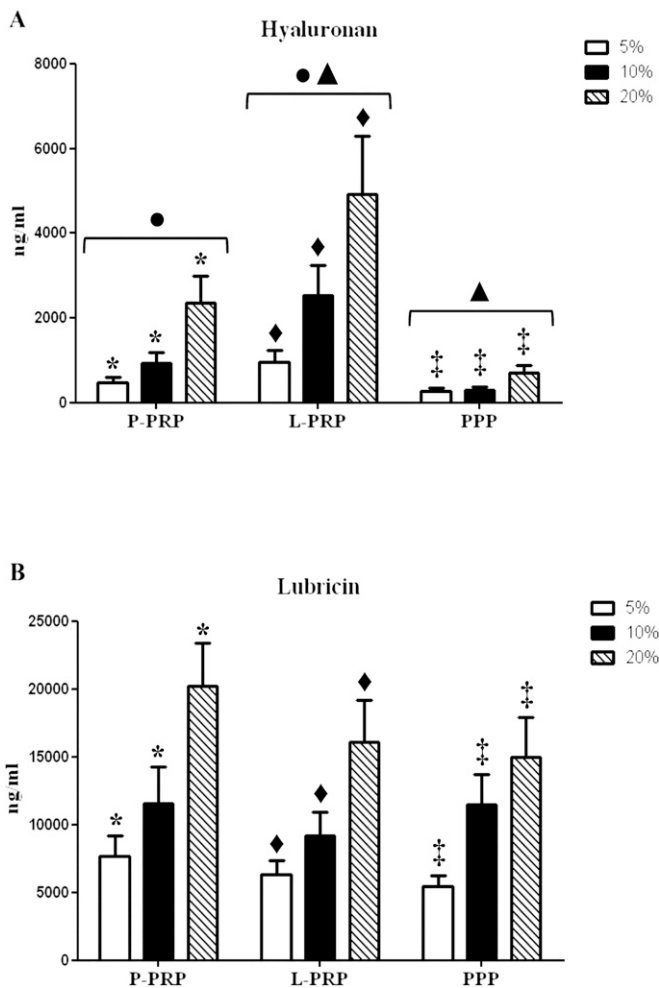


Fig. 4
Hyaluronan (Fig. 4-A) and lubricin (Fig. 4-B) levels in the culture media of human chondrocytes grown in the presence of three different concentrations of P-PRP, L-PRP, or PPP on day seven. The values are shown as the mean and the standard error. Matching symbols above the plots indicate a significant difference between the indicated results ($p < 0.05$).

Hyaluronan and Lubricin Production

Hyaluronan and lubricin proteins were measured in the supernatant above cells grown in the presence of 5%, 10%, or 20% P-PRP, L-PRP, or PPP on day seven. Hyaluronan secretion was significantly enhanced by the addition of 10% and 20% of each formulation compared with 5% ($p < 0.05$) (Fig. 4-A). L-PRP induced a significantly greater increase in hyaluronan production compared with P-PRP and PPP ($p < 0.05$). A significant dose-dependent increase in lubricin production was likewise noticed for each formulation ($p < 0.05$); however, no differences were observed among the formulations (Fig. 4-B).

Discussion

Several blood-derived products are currently used in clinical practice for the treatment of degenerative articular cartilage lesions in osteoarthritis; the majority of these products represent formulations of PRP. PRP is prepared by differential centrifugation

of autologous whole blood and contains a higher concentration of platelets compared with untreated blood, but more specific methods of preparation and attributes have not been uniformly defined. Some authors propose that the blood platelet concentration in PRP should be approximately four times that in the peripheral blood and that a clinically valuable PRP should typically contain 1 million platelets or more per microliter^{44,45}, with anything less than this concentration being considered PRP diluted with PPP. Others consider PRP as having a wide range of possible concentrations, from four to eight times that found in whole blood⁴⁶. Moreover, PRP has been reported by still other authors^{21,23,30} to have clinical efficacy even with a less concentrated formulation. Among the many other differences among the heterogeneous PRP preparation procedures that have been described (e.g., activation modalities, storage methods, and injection protocols), the most debated one is cellularity. In particular, the presence of leukocytes, monocytes, macrophages, and mast cells in many platelet concentrates is controversial; some authors attribute better results to formulations with leukocyte depletion⁴⁷ because of the deleterious effects of proteases and reactive oxygen species released by white blood cells, whereas others consider leukocytes to be a beneficial source of cytokines and enzymes that may be important for the prevention of infection⁴⁸⁻⁵⁰. Leukocytes secrete several molecules into the wound-healing milieu that play a pivotal role in driving appropriate cell populations into the tissue space; these molecules include interleukins, TGF- β , TNF- α , monocyte chemoattractant protein (MCP)-1, and interferon (IFN)- γ .

The characteristics of PRP are important because the growth factors and cytokines released are potent molecules and small variations in their concentrations can result in very different, and even detrimental, results⁵¹⁻⁵⁴. The two PRP formulations used in the present study differed in their preparation methodology and consequently in their platelet and leukocyte concentrations. The comparisons between the two PRP formulations were performed at three different concentrations to assess not only their advantages and disadvantages but also possible dose-response effects and to obtain further indications regarding the choice of the most appropriate formulation for the treatment of cartilage lesions of either traumatic or degenerative origin.

The PRPs used in the present study contained several pro-chondrogenic molecules such as TGF- β 1 and FGF- β but also high levels of anti-chondrogenic molecules such as VEGF, PDGF-AB/BB, and IL-1 β that have effects on tissue metabolism that may oppose the anabolic effects of the former molecules.

The addition of various concentrations of PRP and PPP to human chondrocyte cultures appeared to have various effects in terms of the viability of the cells and the production and expression of several bioactive molecules. All three of the formulations in the present study were able to induce a dose-dependent enhancement of chondrocyte growth at all of the time points evaluated. However, on day seven, P-PRP stimulated greater cell proliferation compared with L-PRP and PPP, thus showing how the composition of PRP can influence this biological process. This finding is in accordance with other studies, including that of Anitua et al.⁵⁵, which showed that fibroblasts responded

differentially to various PRP formulations. The expression of the tested genes generally did not show any dose-response effects for any of the formulations in the present study. However, administration of P-PRP appeared to favor some mechanisms that stimulate chondrocyte anabolism, as demonstrated by the expression of type-II collagen and aggrecan, whereas L-PRP appeared to promote other biological pathways (including catabolic ones) involving various cytokines. This might be due to the presence of leukocytes in L-PRP; the leukocytes may have been responsible for the increased expression of certain molecules such as IL-1 β , IL-6, VEGF, and FGF- β , which in turn could have stimulated TIMP-1 and IL-10. TIMP-1 is a natural inhibitor of MMPs, and IL-10 is an immunosuppressive cytokine that protects articular cartilage from damage.


Unexpectedly, for some of the genes, PPP was able to induce an expression level comparable with that induced by P-PRP. This result may be explained by the presence of plasma proteins and growth factors in PPP that can also induce the expression of chondrogenic markers. In vitro studies have shown that plasma contains approximately 200 proteins (including albumin, immunoglobulins, and clotting factors) that are involved in hemostasis and tissue healing⁵⁶. Moreover, hormones (such as thyroxin, androgens, estrogens, and progesterone) and human growth factors circulate in this biological fluid; one of the most relevant is IGF-1, which improved healing in another cartilage repair model⁵⁷.

The expression of HAS-2, the predominant HAS isoform in articular chondrocytes, was regulated differently by the various formulations. HAS-2 expression was higher in the cells stimulated with L-PRP compared with P-PRP and PPP, which is in agreement with the finding that higher levels of hyaluronan were secreted by chondrocytes grown in the presence of L-PRP compared with the other formulations. However, the effects of L-PRP and P-PRP on the secretion of lubricin were similar; although the levels of lubricin, which is involved in joint lubrication and wear protection, increased in a dose-dependent manner over the seven-day period, no significant differences were observed among the three formulations.

In conclusion, clinical use of the various available PRP formulations requires accurate evaluation of the biological response of human cells to such treatment. In particular, the specific environment of each target tissue must be taken into account

when PRP is used for therapeutic applications. The effects produced by L-PRP in the present study included increased secretion of hyaluronan by chondrocytes from osteoarthritic human cartilage and increased expression of TIMP-1 and IL-10; both of these effects would support use of L-PRP in the treatment of degenerative joint pathology. However, despite these positive features of L-PRP, the increased production of inflammatory cytokines and the decreased production of other matrix molecules compared with the less concentrated P-PRP suggest that L-PRP is far from being an optimal formulation for the treatment of cartilage lesions, and further studies are required to determine the most suitable PRP formulation for this use.

Appendix

 A table showing the PCR primers used in the study is available with the online version of this article as a data supplement at jbjs.org. ■

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