The matrix synthesis and anti-inflammatory effect of autologous leukocyte-poor platelet rich plasma in human cartilage explants

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The matrix synthesis and anti-inflammatory effect of autologous leukocyte-poor platelet rich plasma in human cartilage explants.

Running title: Platelet-rich plasma and cartilage explants

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ABSTRACT

Objective. To determine the effects of autologous leukocyte-poor platelet-rich plasma (LP-PRP) on the expression of markers involved in cartilage-extracellular matrix production and inflammation in cartilage explants bearing osteoarthritis. Materials and Methods. Cartilage explants and LP-PRP were obtained from 10 patients who underwent total knee arthroplasty. The explants were cultured in spinner flasks for 28 days in the presence of interleukin (IL)-1β and/or LP-PRP. The gene expression of catabolic (MMP13, ADAMTS5, and IL1β) and anabolic factors (COL2A1, ACAN, and SOX9) was quantified. A histological assessment was performed according to a modified Mankin score, and quantification of type II and I collagen deposition. Results. The gene expression of catabolic factors and the Mankin score were lower in LP-PRP- and LP-PRP/IL-1β- than in IL-1β-treated explants, suggesting less matrix degradation in explants cultured in the presence of LP-PRP. Higher expression of genes involved in cartilage matrix restoration was observed in LP-PRP and LP-PRP/IL-1β- when compared to IL-1β-treated explants. The explants treated with LP-PRP and LP-PRP/IL-1β exhibited a higher deposition of type II collagen as well as a lower deposition of type I collagen and also better surface integrity and a significant increase in the number of chondrocytes. Conclusion. LP-PRP treatment favored restoration in early osteoarthritic cartilage and reduced the pro-inflammatory effect of IL-1β. LP-PRP is a promising therapy for early osteoarthritis, as it promotes extracellular matrix repair, reduces inflammation, and slows cartilage degeneration.

Key Words: leukocyte-poor platelet-rich plasma, cartilage explants, extracellular matrix, interleukin-1β.
INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by an imbalance of anabolic and catabolic processes in synovial joints (Loeser, 2008). Current treatments for OA are limited to symptomatic relief or surgical replacement of the affected joints; therefore, there is considerable interest in developing effective treatments that can halt or reverse its progression. A promising alternative is the application of biological therapies. In this particular field, platelet-rich plasma (PRP) is the main object of attention.

The importance of PRP lies in the fact that α granules from platelets store numerous growth factors and other bioactive proteins, many of which play a pivotal role in homeostasis and tissue healing (Marx, 2004). Due to its biological properties, PRP has been widely used in orthopedics for the treatment of different soft-tissue lesions (Sheth et al., 2012). The major evidence of the clinical usefulness of PRP addresses the treatment of degenerative OA. A recent meta-analysis suggested that a series of intra-articular injections of PRP may provide short-term clinical benefits in symptomatic knee arthritis (Dold et al., 2014).

Some studies have indicated that leukocyte-poor PRP (LP-PRP) offers more beneficial effects than leukocyte-rich PRP (Kisiday et al., 2012; Boswell et al., 2014). In this vein, Sundman et al. (2011) pointed out that leukocytes increase the catabolic profile of PRP, and that the catabolic cytokine concentration positively correlates with leukocyte concentration. Indeed, it is increasingly accepted that inflammation plays a critical role in the development of OA (Kapoor et al., 2011). The best understood pro-inflammatory cytokine is interleukin-1 beta (IL-1β), which appears to be critical in the susceptibility to and progression of OA (Pelletier et al., 2001). It is well known that IL-1β stimulates the expression of cartilage-associated enzyme matrix
metallopeptidases (MMPs), and that along with tumor necrosis factor-alpha (TNF-α), it can also increase chondrocyte expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) (Sawaji et al., 2008). The changes mainly induced by pro-inflammatory cytokines such as IL-1β, result from active processes involving matrix destruction and inefficient repair. Using an explant-based in vitro culture model for OA, we evaluated the effect of LP-PRP in cartilage explants bearing early OA stimulated with IL-1β, on gene expression and deposition of type I and type II collagen.

**MATERIALS AND METHODS**

**Patients**

Twelve knee cartilage- and 12 autologous LP-PRP-samples were obtained from 10 patients (6 men and 4 women) who underwent total knee arthroplasty due to degenerative joint disease (OA). Six independent experiments were performed for gene expression (n=6) and histological analysis (n=6). The mean age of the participants was 65.8 ± 7.1 years (Fig. 1). The study was authorized by the Ethics Committee and the Internal Review Board of our University Hospital and Faculty of Medicine (BI13-006) and all donors signed written informed consent.

**LP-PRP preparation**

A 54 mL-venous blood sample was taken from each patient. The blood sample was collected in 12 sterile vacuum tubes with sodium citrate 0.109 M (369714, BD Vacutainer; Franklin Lakes, NJ, USA). An extra tube with EDTA-anticoagulated blood was obtained for the initial platelet count (368171; BD Vacutainer). LP-PRP was prepared using a previously described manual
double-centrifugation method (Simental-Mendía et al., 2016). Briefly, blood samples were centrifuged for 10 minutes at 1800 rpm. The upper plasma layer was collected in a new tube while attempting not to remove the erythrocyte and leukocyte layer. The plasma collected was centrifuged again for 12 minutes at 3400 rpm. The superficial layer consisting of platelet-poor plasma was discarded to obtain LP-PRP. A sample of LP-PRP (1 mL) was sent to the laboratory for analysis with an automated platelet and leukocyte counting method (Cytoflex, Beckman Coulter, CA, USA); the remaining LP-PRP (6 mL) was activated by adding 0.15 mL of 10% calcium gluconate (Laboratorios PISA, Guadalajara, Mexico)/per mL of LP-PRP. The activated LP-PRP was divided into 1.5 mL aliquots and stored at –80°C until used.

**Procurement of cartilage explants**

Human cartilage tissue was obtained from the same 10 participants from who LP-PRP was obtained. A minimum of 27 explants were obtained from each sample. The cartilage explants (6–8 mm in diameter) were obtained from zones with grade I and II damage according to the Outerbridge classification system (Cameron et al., 2003) with an Osteochondral Autograft Transfer System (OATS®; Arthrex Inc., Santa Barbara, CA, USA). Before setting them in culture, the explants were previously washed several times as previously reported (Moo et al., 2011).

**Culture of cartilage explants**

After 2 days of stabilization, the baseline characteristics of 3 randomly chosen explants were analyzed; the remaining 24 explants were randomly divided into four 25-mL spinner flasks (Wheaton Celstir, Wheaton Industries Inc., Milville, NJ, USA) containing 15 mL of complete medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 [DMEM/F12, Thermo
Fisher Scientific) and 50 μg/mL of gentamicin). Two flasks were supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific). One of the spinner flasks was left as the non-treated control (Control group). Human recombinant IL-1β, 20 pg/mL (R&D Systems Inc., Minneapolis, MN, USA), was added to a second flask (IL-1β group). Autologous LP-PRP 10% (v/v) was added to the remaining two flasks (LP-PRP group); 20 pg/mL of IL-1β (LP-PRP/IL-1β group) was added to one of the flasks. All flasks were incubated at 37°C in a 5% CO₂ atmosphere, with 100% humidity, and constant shaking. Culture medium was changed every 7 days. The cartilage explants were collected at days 0 (baseline), 14, and 28 for further study.

**Analysis of gene expression**

Total RNA was extracted from the cartilage explants following the method of Ali and Alman (2012) with minor modifications (Ali and Alman, 2012). Briefly, the cartilage explants (3 for each treatment and time point of analysis) were entirely cut into pieces with a scalpel. Then, the cartilage–explant RNA was extracted with TRIzol reagent (Thermo Fisher Scientific). Retrotranscription was performed using M-MLV reverse transcriptase (Thermo Fisher Scientific). Total RNA (100 ng) was treated with 0.5 U of deoxyribonuclease I (DNase I, Thermo Fisher Scientific) to digest genomic DNA. A set of TaqMan probes was used to evaluate gene expression by quantitative polymerase chain reaction (qPCR) of type II collagen (COL2A1, Hs00264051_m1), aggrecan (ACAN, Hs00153936_m1), SOX9 (SOX9, Hs01001343_g1), type I collagen (COL1A2, Hs00264051_m1), type X collagen (COL10A1, Hs00166657_m1), MMP13 (MMP13, Hs00233992_m1), TIMP1 (TIMP1, Hs00171558_m1), ADAMTS5 (ADAMTS5, Hs00199841_m1), and IL-1β (IL1β, Hs01555410_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1) and β-2-microglobulin (B2M, Hs99999907_m1) were used to normalize data, calculating the geometric mean for accurate averaging of the control
genes as a basis for obtaining a normalization factor (Vandesompele et al., 2002). The qPCR reactions were performed with a 7500 Fast Real-Time PCR System using MicroAmp 96-well reaction plates and TaqMan Universal PCR Master Mix. Each sample was run in triplicate. Gene expression was analyzed using the comparative $C_T$ method ($\Delta\Delta C_T$). All instruments and chemicals used during the gene expression experiments were purchased from Applied Biosystems (Foster City, CA, USA).

**Histological assessment**

The explants (three for each treatment and time-point analysis) were fixed in 10% formaldehyde for 48 h and successively dehydrated in xylene and absolute ethanol, 96% ethanol, and 70% ethanol for 5 min. The samples were then embedded in paraffin, cut with a microtome in histological sections of 5 µm, and stained with safranin-O, green fast and ferric hematoxylin for histochemical analysis. In addition, cartilage explants were subjected to immunohistochemical analyses and were labeled with anti-type II (ab34712) and anti-type I (ab23446) collagen monoclonal antibodies (Abcam plc, Cambridge, MA, USA). Positive staining was detected using a mouse- and rabbit-specific horseradish peroxidase (HRP)/3,3’-diaminobenzidine (DAB) detection immunohistochemistry kit (Abcam plc) and hematoxylin counterstaining was performed for nuclei visualization, according to the manufacturer’s instructions. The explants stained with safranin-O were examined and scored according to a modified Mankin score (Mankin et al. 1971) for OA (Table 1). In accordance with this score, a value close to zero represents a histology closer to normal cartilage.

For the immunohistochemical staining analysis, eight fields in each preparation were randomly chosen and imaged. The color photomicrographs were stored in the NIS-elements BR 2.30
software (Nikon Instruments, Tokyo, Japan). The background was uniformly eliminated from the photomicrographs with a digital filter, and the tissue-staining intensities were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The threshold for the intensities of stained areas marked with the primary antibody was manually setup from a sample of images for each antibody in order to avoid artifacts that could interfere with the analysis. The intensity of immunolabeling is reported as the percentage of total pixels normalized to the measurement area.

To obtain the number of chondrocytes in the sections analyzed, we performed an automated counting and analysis with the Object Count function of the NIS-Elements BR 2.30 software (Nikon Instruments, Tokyo, Japan) to provide the number of objects (chondrocyte-stained nuclei) in the same set of safranin-O images previously used for the Mankin score. The method uses the thresholding and restriction components to detect stained nuclei by determining characteristics such as intensity, area, and circularity of the selected objects. The software displays the number of objects with the selected parameters in the selected area.

**Statistical analysis**

Data are presented as the mean ± standard error of the mean (SEM) of six independent experiments performed in triplicate for gene expression and histological assessment. For gene expression analysis, a one-way ANOVA and a post hoc Tukey test for multiple comparisons was used. For the histological analysis, the Mankin score was analyzed with the Mann-Whitney test or Kruskall-Wallis with Dunn’s posttest either to compare all groups or selected pairs. The number of chondrocytes was analyzed with one-way ANOVA and a post hoc Tukey test for multiple comparisons. Finally, data from immunohistochemistry intensity was analyzed with the
Mann-Whitney test or Kruskall-Wallis with Dunn’s posttest either to compare all groups or selected pairs. A value of \( P \leq 0.05 \) was considered statistically significant. Data were analyzed with GraphPad Prism Software v5.00 (GraphPad Software, La Jolla, CA, USA).

RESULTS

LP-PRP Analysis

The mean \( \pm \) standard deviation (SD) of the platelet number in the peripheral blood and LP-PRP was \( 273.5 \pm 61.4 \times 10^3/\mu L \) and \( 910.91 \pm 199.5 \times 10^3/\mu L \), respectively (it was 3.6 times higher in LP-PRP than in whole blood). The mean \( \pm \) SD of leukocytes in LP-PRP was \( 0.539 \pm 0.76 \times 10^3/\mu L \), and in peripheral blood, \( 8.24 \pm 1.87 \times 10^3/\mu L \) (it was 15.2 times lower in LP-PRP than in the whole blood).

Gene expression

At days 14 and 28, the expression of \( COL2A1 \) and \( ACAN \) (Fig. 2A) was significantly higher in the LP-PRP group than in the Control group (2.4 and 2.7 times for \( COL2A1 \) and 4.0 and 2.7 times for \( ACAN \), respectively). A significant overexpression of \( COL2A1 \) in the LP-PRP group (4.3 times) and the LP-PRP/IL-1\( \beta \) group (3.1 times) was observed at day 28 compared with expression in the IL-1\( \beta \) group. A similar behavior was observed for the gene expression of \( ACAN \) (18.3 and 11.3 times, respectively) at day 28. \( SOX9 \) was significantly overexpressed in the LP-PRP group (4.9 times) and the LP-PRP/IL-1\( \beta \) group (3.0 times) compared with the Control group at day 28 (Fig. 2A). This effect was also observed in both the LP-PRP and the LP-PRP/IL-1\( \beta \) group, when the relative expression of \( SOX9 \) was compared with that of the IL-1\( \beta \) group at day
28. COL1A2 or COL10A1 did not show significant changes in expression between any of the treatment groups at any time (Fig. 2B).

The expression of TIMP1 did not change significantly among any group or incubation time (Fig. 2C). At day 14, the expression of MMP13 in IL-1β group was significantly higher than that of the Control group and the LP-PRP group (14.4 and 5.1 times, respectively). At day 28, the expression of MMP13 in the IL-1β group was significantly higher than that of the Control, LP-PRP, and LP-PRP/IL-1β groups (6.4, 33.8, and 11.1 times, respectively). No significant differences were observed in the expression of ADAMTS5 among any group. Nevertheless, at day 28, the expression of ADAMTS5 in the IL-1β group was significantly higher than that of the LP-PRP group and the LP-PRP/IL-1β group (9.4 and 8.4 times, respectively). At day 14 and 28, the expression of IL1β in the IL-1β group was significantly higher than that of the Control group (14.2 and 5.9 times, respectively) and the LP-PRP group (18.2 and 10.5 times, respectively). An important reduction in the expression of IL1β was observed in the LP-PRP/IL-1β group (11.0 times) compared with the IL-1β group at day 28.

**Histological assessment**

The distal zones in the Baseline, Control, and IL-1β-preparations showed a blue–reddish color with safranin-O staining and a more intense red staining in the proximal zone (Fig. 3A, 3B1-3B2 and 3B5-3B6). At day 14, the LP-PRP and LP-PRP/IL-1β groups showed that their distal zone was intensely stained compared to the control and IL-1β groups (Fig. 3B3 and 3B4). At day 28, the LP-PRP group had a strong reddish staining in its distal zones compared to the rest of the treatment groups (Fig. 3B7); this is revealed by a lower Mankin score (LP-PRP, 1.7 ± 0.8 vs Control, 3.1 ± 1.2; IL-1β, 2.8 ± 1.3; and LP-PRP/IL-1β, 2.0 ± 0.6). The Mankin score of the LP-PRP and LP-PRP/IL-1β groups was lower than that of the other two groups; however, no
significant difference was observed (Fig. 3C). Fig. 3A shows the appearance of surface fibrils, fissures, and erosion in cartilage surfaces at Baseline. These surface irregularities were present at days 14 and 28 in the Control and IL-1β groups.

Figure 4 shows that the number of nuclei in the explants stained with safranin-O were significantly higher in the Control, LP-PRP, and LP-PRP/IL-1β groups than at Baseline (1.7, 2.3, and 2.1 times, respectively). A significantly higher number of chondrocytes were noted in the LP-PRP groups with respect to IL-1β group (1.6 times). At day 14 and 28, lagoons were more visible and numerous in the LP-PRP group and in the LP-PRP/IL-1β group (Fig. 3B1–3B4).

LP-PRP explants showed the highest intensity of type II collagen at both 14 and 28 days (Fig. 5B3 and 5B7). Figure 5C shows that at day 14, type II collagen deposition on the Baseline cartilage explants was significantly lower than that of the LP-PRP group (23.1%) and the LP-PRP/IL-1β group (16.1%). At day 14, the deposition of type II collagen in the Control group was significantly lower than in the IL-1β group (12.5%), the LP-PRP group (29.8%), and the LP-PRP/IL1β group (22.8%) (Fig. 5C). In addition, the type II collagen immunolabel in the LP-PRP and LP-PRP/IL-1β groups was 17% and 13% higher than the IL-1β group.

Type I collagen was preferentially stained for Baseline, Control, and IL-1β groups (Fig. 6A, 6B1, 6B2 and 6B6). It was preferentially deposited in the distal zones of all explants. The label intensity of type I collagen was greater at day 14 in Controls and at days 14 and 28 in IL-1β group. At day 14, the most intense immunolabeling was observed in the IL-1β group, as this labeling was significantly higher than Baseline (13.5%), the LP-PRP group (12.1%), and the LP-PRP/IL-1β group (14.4%) (Fig. 6C). At day 28, a tendency of higher immunolabeling intensity was observed in IL-1β and Control groups with respect to LP-PRP group.
DISCUSSION

OA is a chronic degenerative joint disease. It is the most common cause of pain and one of the leading causes of disability and dependence among adult populations and generates enormous expense in health systems (Woolf, 2003). We have shown that LP-PRP enhanced the expression of genes devoted to restoring the matrix of hyaline cartilage and did not favor the expression of those genes involved in cartilage degradation. We also noted that the effects induced by the proinflammatory cytokine IL-1β were weakened in the presence of LP-PRP. We selected to test the concentration of 20 pg/mL of IL-1β in order to better represent a chronic pro-inflammatory effect, rather than an acute pro-inflammatory effect (using a concentration of 10 ng/mL or more), based on the concentrations that have been reported for this protein in synovial fluid from patients with OA (Westacott et al., 1990; Kokebie et al., 2011).

The expression of COL2A1 and ACAN in LP-PRP/IL-1β, two genes involved in the synthesis of cartilage extracellular matrix, were comparable to that of LP-PRP at day 28. Similarly, the expression of SOX9, a transcription factor importantly involved in cartilage matrix formation, increased significantly in LP-PRP and LP-PRP/IL-1β. This finding is in accordance with the COL2A1 overexpression observed in the same LP-PRP or LP-PRP/IL-1β, since it is widely accepted that SOX9 positively regulates the expression of COL2A1 (Akiyama and Lefebvre, 2011). The enhanced expression of COL2A1 and ACAN induced by LP-PRP strongly suggests that, under appropriate conditions, LP-PRP can promote the neosynthesis of cartilage extracellular matrix in early OA. The absence of changes in the expression of COL1A2 in the presence or absence of LP-PRP and IL-1β have been reported previously by other investigators (Sundman et al., 2014; Osterman et al., 2015). In addition, the expression of COL10A1 did not
vary greatly among the explants treated under all our experimental conditions. This fact indicates that neither LP-PRP nor IL-1β influence COL10A1 expression in initial stages of OA.

Regarding the genes associated with inflammation and cartilage degeneration in OA, the lack of significant differences among all groups indicates that the expression of TIMP1 is not inducible by IL-1β or LP-PRP. TIMP1 has been described as being capable of inhibiting many MMPs (including MMP13) (Brew and Nagase, 2010), and it is also thought to regulate extracellular matrix turnover by modulating the degradation activity of MMPs (Troebge and Nagase, 2012). Nonetheless, a previous study from Assirelli et al. (2014) has shown that the expression of TIMP1 in human osteoarthritic cartilage is not modulated by IL-1β, but promotes expression of MMP13 and ADAMTS4. We showed here that IL-1β induced the expression of MMP13 in IL-1β-treated cartilage explants. Conversely, LP-PRP inhibited MMP13 expression due to IL-1β in either LP-PRP or LP-PRP/IL-1β. This finding is consistent with the fact that IL-1 stimulates the expression of some MMPs, such as MMP1 and MMP13 in osteoarthritic cartilage (Bau et al., 2002; Kobayashi et al., 2005). Furthermore, studies in chondrocytes from mice have shown that the expression of SOX9, along with the presence of FGF-2 inhibit proteinase expression, such as MMP13 and ADAMTS5 (Troebge and Nagase, 2012).

In cartilage, two different aggrecanases (ADAMTS4 and ADAMTS5) have been identified; however, which aggrecanase is responsible for aggrecan degradation during human articular cartilage destruction remains debatable (Verma and Dalal, 2011). The expression of ADAMTS5 was not regulated by IL-1β. On the other hand, ADAMTS5 expression was inhibited by LP-PRP. These results are in agreement with those of other investigators, who pointed out that ADAMTS5 expression is not regulated in human chondrocytes by catabolic cytokines such as IL-1β or TNF-
α, and that it is expressed constitutively (Koshy et al., 2002; Pratta et al., 2003). It has yet to be determined how LP-PRP inhibits \textit{ADAMTS5} expression.

LP-PRP appears to partially neutralize the undesirable effects of IL-1β, since all explants stained with safranin-O contained proteoglycans. However, the highest concentration and the most uniform distribution of proteoglycans were observed in the explants treated with only LP-PRP or LP-PRP/IL-1β (had the lowest Mankin score). This improvement was also observed in the normalization of the cartilage surface, in the number and distribution of lagoons, and in the increase in chondrocyte number. We observed a lower concentration of proteoglycans in the distal zones of the Control and IL-1β groups and a slender number of lagoons and chondrocytes (represented by a higher Mankin score). Our results show that LP-PRP can inhibit the expression of \textit{IL1β}, and that explants have a considerable expression of \textit{TIMP1}, which explains the improvements in the structure of the extracellular cartilage matrix in LP-PRP and IL-1β. The persistence of surface irregularities in the IL-1β group correlated with the high induction of endogenous \textit{IL1β}, \textit{MMP13}, and \textit{ADAMTS5}.

The chondrocyte proliferation in the LP-PRP and LP-PRP/IL-1β groups agrees with results from other studies where LP-PRP showed strong positive effects on chondrocyte proliferation (Kaps et al., 2002; Gaissmaier et al., 2005; Sprefico et al., 2009). The presence of growth factors involved in the proliferation of chondrocytes, like platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β), were demonstrated in PRP preparations similar to ours (Amable et al., 2013).

The highest deposition of type II collagen was observed in LP-PRP at both 14 and 28 days, followed by LP-PRP/IL-1β. On the other hand, at day 14, the deposition of type II collagen in IL-1β was...
1β was higher than in non-treated explants. This relationship was inverted at day 28, possibly due to the deleterious effect of IL-1β. Small proportions of type I collagen are present in healthy and osteoarthritic cartilage. At days 14 and 28, the greater deposition of type I collagen was observed in the Control and IL-1β group, and minor depositions were observed in explants treated with LP-PRP, in the presence or not of exogenous IL-1β.

Although the etiology of OA is not completely understood, the extent of extracellular matrix degradation is highly associated with an imbalance between MMPs and TIMPs (Pathak et al., 2015), and they are proposed to play a vital role in extracellular matrix turnover and breakdown under normal and disease conditions (Burrage et al., 2006). Our results suggest that this imbalance could be restored in favor of cartilage matrix preservation with the treatment of LP-PRP in early stages of the disease.

Some investigations have reported the effect of different PRP formulations from healthy donors in cartilage or chondrocytes from patients undergoing total knee arthroplasty (Cavallo et al., 2014; Sundman et al., 2014; Osterman et al., 2015). In this study, each of the LP-PRP and cartilage explants that were tested was derived from the same donor. As far as we know, this would be the first time that human cartilage explants have been treated with autologous LP-PRP in vitro.

CONCLUSIONS

LP-PRP induces the satisfactory repair of early-osteoarthritic cartilage via four main effects: 1) by inducing the expression of genes involved in the synthesis of the hyaline–cartilage matrix; 2) by inducing the deposition of type II collagen, but not type I collagen; 3) by partially disabling
the undesirable effects of IL-1β; and 4) by enhancing chondrocyte proliferation. Accordingly, LP-PRP therapy can favorably induce a satisfactory restoration of osteoarthritic cartilage in the initial stages of the disease.

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REFERENCES


Table 1. Modified Mankin scoring for cartilage degradation

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<td>Superficial irregularities</td>
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</tr>
<tr>
<td>Pannus and surface irregularities</td>
<td>2</td>
</tr>
<tr>
<td>Clefts to transitional zone</td>
<td>3</td>
</tr>
<tr>
<td>Clefts to radial zone</td>
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<tr>
<td><strong>Cellularity</strong></td>
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<tr>
<td>Slight loss of chondrocyte clusters</td>
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<tr>
<td>&lt;25% of the clusters</td>
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<tr>
<td>Hypocellularity</td>
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*Modified from Mankin et al. (Mankin et al. 1971)*
Figure Legends

Figure 1. Schematic representation of the distribution and handle of cartilage and LP-PRP samples employed. Twelve knee cartilage- and 12 autologous LP-PRP-samples were obtained from 10 patients. A minimum of 27 explants were obtained from each sample. Control, non-treated control; IL-1β, explants treated with IL-1β; LP-PRP, explants treated with LP-PRP; and LP-PRP/IL-1β, explants treated with LP-PRP and IL-1β. Genes analyzed by RT-qPCR: COL2A1, ACAN, SOX9, COL1A2, COL10A1, MMP13, TIMP1, ADAMTS5, IL1β. Histological analysis: Safranin-O (Mankin Score) and Immunohistochemistry (type I and type I collagens).

Figure 2. Relative gene expression in cartilage explants. (A) Relative expression of genes involved in cartilage matrix formation. (B) Genes expressed in osteoarthritis. (C) Genes associated with inflammation and cartilage degeneration. Control/white bars, non-treated control; IL-1β/blue bars, explants treated with IL-1β (20 pg/mL); LP-PRP/green bars, explants treated with LP-PRP; LP-PRP/IL-1β/orange bars, explants treated with LP-PRP and IL-1β (20 pg/mL). These preparations were incubated for 0, 14, and 28 days. All bars represent Log10 of change times in gene expression with respect to their correspondent expression at time 0 (basal). #P<0.05 and ## P<0.01 versus control. The differences between groups are indicated with lines (*P<0.05, **P<0.01, and ***P<0.001). Data are presented as the mean ± SEM of six independent experiments performed in triplicate.

Figure 3. Analysis of the safranin-O staining. Cartilage explants were incubated for 0 days (baseline and square A; black bar), 14 days (B1–B4), or 28 days (B5–B8) in complete medium (controls; B1 and B5; white bars), IL-1β (20 pg/mL) (B2 and B6; blue bars), LP-PRP (B3 and B7; green bars), and LP-PRP/IL-1β (20 pg/mL) (B4 and B8; orange bars). Each image is
representative of six independent experiments. (C) graphic of the Mankin score. Each bar corresponds to the average ± SEM of six independent experiments. No significant differences were observed. All images had the same amplification (200X); the scale bar represents 200 µm.

Figure 4. Number of chondrocytes per field in cartilage explants. The number of stained nuclei in safranin-O preparations was counted in eight randomly chosen fields for each group (baseline, black bar; control, white bar; IL-1β (20 pg/mL), blue bar; LP-PRP, green bar; and LP-PRP/IL-1β, orange bar (20 pg/mL) (n=48/bar data). ##P<0.01 versus baseline (black bar). Significant differences between groups are indicated with lines (*P<0.05).

Figure 5. Analysis of type II collagen immunohistochemical staining. Cartilage explants were incubated for 0 days (baseline and square A; black bar), 14 days (B1–B4), or 28 days (B5–B8) in complete medium (Control; B1 and B5; white bars), IL-1β (20 pg/mL) (B2 and B6; blue bars), LP-PRP (B3 and B7; green bars), and LP-PRP/IL-1β (20 pg/mL) (B4 and B8; orange bars). Each image is representative of six independent experiments. All images had the same magnification (400X); the scale bar represents 50 µm. (C) Graphic of a densitometric analysis. Each bar represents the average ± SEM of 48 determinations. ##P<0.01 versus baseline (black bar). The differences between groups are indicated with lines (*P<0.05 and **P<0.01).

Figure 6. Analysis of the type I collagen immunohistochemical staining. Cartilage explants were incubated for 0 days (baseline and square A; black bar), 14 days (B1–B4), or 28 days (B5–B8) in complete medium (Control; B1 and B5; white bars), IL-1β (20 pg/mL) (B2 and B6; blue bars), LP-PRP (B3 and B7; green bars), and LP-PRP/IL-1β (20 pg/mL) (B4 and B8; orange bars). Each image is representative of six independent experiments. All images had the same magnification (400X); the scale bar represents 50 µm. (C) Graphic of a densitometric analysis. Each bar
represents the average ± SEM of 48 determinations. ##\textit{P}<0.01 versus baseline (black bar). The differences between groups are indicated with lines (*\textit{P}<0.05 and **\textit{P}<0.01).
n=10 patients undergoing total knee arthroplasty

For each cartilage sample (n=12 in total):
27 cartilage explants, 4 treatment groups (after 2 days of stabilization)

- Control
  - Complete medium
  - 14 days
  - 28 days

- IL-1β
  - IL-1β (20 pg/mL)
  - 14 days
  - 28 days

- LP-PRP
  - Autologous LP-PRP (10%)
  - 14 days
  - 28 days

- LP-PRP/IL-1β
  - Autologous LP-PRP (10%)
  - IL-1β (20 pg/mL)
  - 14 days
  - 28 days

Baseline
3 explants

RT-qPCR (n=6)
(6 independent experiments)

Histological Analysis (n=6)
(6 independent experiments)