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The Anti-inflammatory and Matrix Restorative Mechanisms of Platelet-Rich Plasma in Osteoarthritis

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Investigation performed at the Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, and at the Department of Orthopedics, Midwest Orthopedics at Rush University Medical Center, Chicago, Illinois

Background: Intra-articular (IA) treatment with platelet-rich plasma (PRP) for osteoarthritis (OA) results in improved patient-reported pain and function scores.

Purpose: To measure the effects of PRP and high molecular weight hyaluronan (HA) on the expression of anabolic and catabolic genes and on the secretion of nociceptive and inflammatory mediators from OA cartilage and synoviocytes.

Study Design: Controlled laboratory study.

Methods: Synovium and cartilage harvested from patients undergoing total knee arthroplasty were co-cultured with media of PRP or HA. Tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and IL-1 β were measured in the media by enzyme-linked immunosorbent assay. Hyaluronan synthase-2 (*HAS-2*), matrix metalloproteinase-1 (*MMP-1*), *MMP-13*, and *TNF- α* genes were measured in synoviocytes by reverse transcription polymerase chain reaction (RT-PCR). Collagen type I α 1 (*COL1A1*), *COL2A1*, aggrecan (*ACAN*), and *MMP-13* gene expression were measured in cartilage by quantitative RT-PCR.

Results: Media TNF- α concentration was decreased in PRP and HA compared with control cultures (PRP = 6.94 pg/mL, HA = 6.39 pg/mL, control = 9.70 pg/mL; $P \leq .05$). Media IL-6 concentration was decreased in HA compared with PRP and control (HA = 5027 pg/mL, PRP = 5899 pg/mL, control = 5613 pg/mL; $P \leq .05$). Media IL-1 β was below detectable concentrations (<0.1 pg/mL) in all samples. Synoviocyte MMP-13 expression was decreased in PRP compared with HA and control (PRP = 10.1, HA = 12.8, control = 13.5; $P \leq .05$). Synoviocyte HAS-2 expression was increased in PRP compared with HA and control (PRP = 12.1, HA = 9.8, control = 8.7; $P \leq .05$). Cartilage ACAN expression was increased in PRP compared with HA, but neither was different from control (PRP = 8.8, HA = 7.7, control = 7.6; $P \leq .05$); *COL1A1* expression was increased in HA compared with PRP, but neither was different from control (PRP = 14.9, HA = 13.5, control = 12.9; $P \leq .05$). Neither platelet nor leukocyte concentration had a significant effect on outcome measurements (gene or protein expression data) in cartilage or synoviocytes ($P > .05$).

Conclusion: Both PRP and HA treatments of OA joint tissues result in decreased catabolism, but PRP treatment also resulted in a significant reduction of MMP-13, an increase in *HAS-2* expression in synoviocytes, and an increase in cartilage synthetic activity compared with HA. These results indicate that PRP acts to stimulate endogenous HA production and decrease cartilage catabolism. Platelet-rich plasma showed similar effects as HA in the suppression of inflammatory mediator concentration and expression of their genes in synoviocytes and cartilage.

Clinical Relevance: The antinociceptive and anti-inflammatory activities of PRP support its use in OA joints to reduce pain and modulate the disease process. This study supports further clinical investigations of IA PRP for the treatment of OA.

Keywords: knee; articular cartilage; biology of cartilage; growth factors/healing enhancement; platelet-rich plasma; regenerative medicine; synovium

Osteoarthritis (OA) is a chronic degenerative disease with physical and economic consequences. Because of an active and aging population, OA will continue to be an important and costly disease process. In OA, articular cartilage damage is primarily caused by an alteration of normal

metabolism, which favors a decrease in anabolism and increase in catabolism. This is realized as the breakdown of the primary components of cartilage extracellular matrix, including collagen type II and aggrecan. These changes in articular cartilage homeostasis occur concurrently with inflammation of the synovium and synovial fluid. Surgical treatments for severe knee arthritis, including total knee arthroplasty (TKA), have increased for the past decade; however, such surgical procedures are invasive and have associated morbidity and failure rates.³³ Nonsurgical interventions

aim to slow the disease process, relieve pain, increase function, and prolong life of the joint.²³

Hyaluronan (HA) is a commonly used intra-articular (IA) therapy that results in improved patient pain and function scores.¹⁵ Endogenous, high molecular weight HA is produced in the joint by the synovium and cartilage by hyaluronan synthase (HAS) enzymes.³⁹ Various pathologic joint lesions result in a decreased molecular mass of HA in the synovial fluid.¹² Intra-articular HA therapy has a good safety profile with few reported adverse events or side effects.⁸ Numerous commercial IA HA products are available that differ by molecular weight, ranging from 0.5×10^6 Da to 6.0×10^6 Da.⁸ Repeated IA HA supplementation has been shown to decrease the rate of OA progression and diminish joint inflammation.^{17,37,44}

Platelet-rich plasma (PRP) is used in sports medicine practices to treat bone, tendon, and ligament injuries.^{28,40} Intra-articular administration of PRP has recently emerged as a treatment for OA.^{14,25,40} A few small-scale randomized clinical trials have compared PRP to both HA and sham treatments and have demonstrated that IA PRP improves clinical signs.^{10,34,38}

Platelet-rich plasma is an autologous blood product that contains an increased concentration of platelets in comparison with the baseline platelet concentration in whole venous blood. Different commercial preparations vary in the concentration of platelets and white blood cells and subsequently in the contained concentration of growth factors and inflammatory mediators.⁴¹ Platelet α -granules contain and release numerous growth factors, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor- β (TGF- β).⁵ Platelets have also been found to have anti-inflammatory activity via inhibition of the nuclear factor- κ B (NF- κ B) pathway.³ Results of recent *in vivo* studies of IA PRP in humans and animal model studies suggest that positive clinical results might be caused by the anti-inflammatory effects of PRP rather than the anabolic effects of the growth factors on the articular cartilage or the joint as an organ.²⁷ Some literature specifically evaluated leukocyte-poor PRP and supports its anti-inflammatory and anabolic properties in comparison with leukocyte-rich PRP.³²

MATERIALS AND METHODS

Samples

Human knee cartilage, subchondral bones, and joint capsules ($n = 21$) were procured by a participating institution

from patients undergoing TKA for OA. Institutional review board approval was obtained from the participating institution. Osteoarthritis was diagnosed based on clinical signs and radiographic findings. Samples were de-identified and shipped overnight on ice to another institution for tissue culture and analysis (Figure 1). Samples were grossly inspected to ensure an adequate supply of synovial membrane and full-thickness cartilage.

Co-culture

Cartilage was dissected into 13 to 14 explants ($3 \times 5 \times 5$ mm) and incubated overnight in medium (complete Dulbecco's Modified Eagle Medium [DMEM] supplemented with glutamine, ascorbic acid, α -ketoglutaric acid, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, penicillin, streptomycin, and 10% fetal bovine serum). Synovium was dissected from the joint capsule and digested for 2 hours in 1.5% collagenase type 2 and 0.15% DNase I in Hank's Balanced Salt Solution (HBSS). The resultant slurry was filtered and plated in 6-well plates at a density of 2.0×10^5 cells/cm² in medium.

Synoviocyte and cartilage co-culture systems were created by adding hanging inserts (Millipore, Billerica, Massachusetts) containing 4 cartilage explants to each well containing synoviocytes. Three co-culture systems were established per sample to accommodate 3 treatment groups. The 3 treatment groups were HA (2.5 mL SYN-VISC, Hylan G-F 20 [Genzyme, Cambridge, Massachusetts] and 1.5 mL media), PRP (2.5 mL Autologous Conditioned Plasma [Arthrex Inc, Naples, Florida] and 1.5 mL media), and untreated control (4 mL media). Co-cultures were incubated at 5% CO₂, 37°C, and 90% humidity for 96 hours. At study termination, treatment media were snap frozen and stored at -80°C for later analyses. Cartilage explants were removed from culture, rinsed in HBSS, snap frozen, and stored at -80°C. Synoviocytes were rinsed with HBSS, lifted from the plate using Trizol Reagent (Invitrogen, Carlsbad, California), snap frozen, and stored at -80°C.

PRP Preparation

Venous blood was obtained from 21 healthy volunteers and used to produce PRP using the Double Syringe Autologous Conditioned Plasma System (Arthrex Inc). Automated hemograms were performed on whole blood and PRP to document platelet and leukocyte concentrations. Platelet-rich plasma was produced separately for each sample and not pooled as a treatment.

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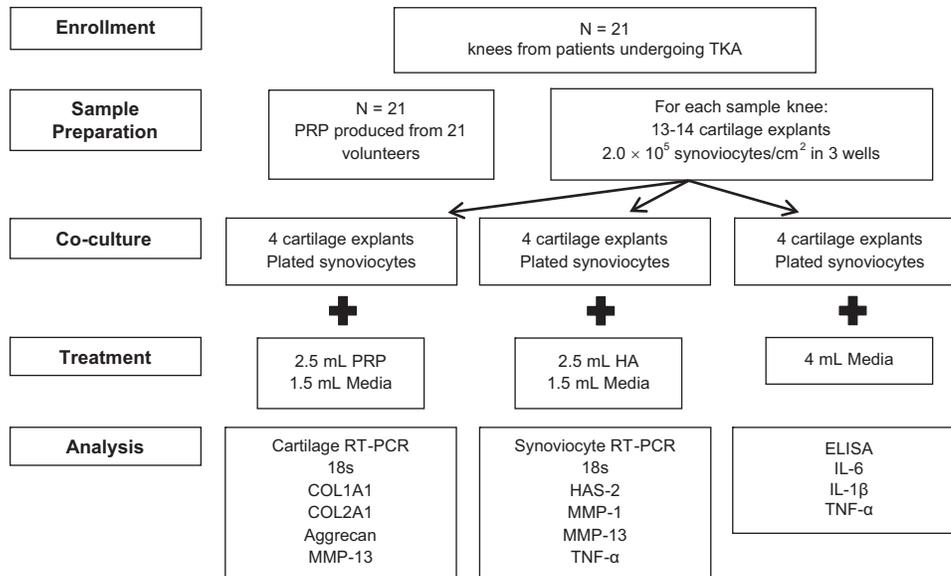


Figure 1. Schematic of study outline.

Tissue Extraction

Frozen cartilage was pulverized in a freezer mill (Spex SamplePrep, Metuchen, New Jersey), and total cellular RNA was extracted using the PerfectPure Fibrous Tissue Kit (5Prime, Gaithersburg, Maryland) and then concentrated using the SpeedVac Plus vacuum concentrator (ThermoFisherScientific, Waltham, Massachusetts). Total cellular RNA was extracted from synoviocytes using the Purelink RNA Mini Kit (Invitrogen). RNA concentration was measured using a spectrophotometer (Nanodrop, Wilmington, Delaware). Samples were diluted to a concentration of 5 ng/uL of RNA.

1-Step Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the ABI PRISM 7900HT Sequence Detection System with One-Step RT-PCR Master Mix and Taqman Gene Expression Assays-Inventoried (Applied Biosystems, Carlsbad, California). Cartilage RNA was assayed for collagen type I $\alpha 1$ (*COL1A1*), *COL2A1*, aggrecan (*ACAN*), matrix metalloproteinase-13 (*MMP-13*), and the 18S ribosomal subunit. Synoviocyte RNA was assayed for *HAS-2*, *MMP-1*, *MMP-13*, tumor necrosis factor- α (*TNF- α*), and the 18S ribosomal subunit. To normalize each treatment group to 18S results, Δ CT analyses were performed.

ELISA Analyses

Enzyme-linked immunosorbent assay (ELISA) analyses of treatment media were performed for interleukin-6 (IL-6) (R&D Systems, Minneapolis, Minnesota), IL-1 β (Affymetrix, Santa Clara, California), and TNF- α (Invitrogen). All kits have been validated to work in the presence of

blood and plasma and therefore the contents of PRP. Because the samples were heterogeneous (PRP, HA, control media), samples were spiked with known concentrations of recombinant IL-6, IL-1 β , or TNF- α according to manufacturers' recommendations to ensure that there were no false-positive or epitope-masking effects by the samples. The ELISA plates were read on a Tecan Safire Absorbance Reader using the Magellan 3.11 analysis program (Tecan Group Ltd, Männedorf, Switzerland).

Histology

Before culture, 1 cartilage explant from each sample was fixed in 4% paraformaldehyde for 24 hours and then embedded in paraffin. Paraffin-embedded sections (6 μ m) were stained with hematoxylin and eosin (H&E) and scored by 2 examiners, who were blinded to treatment group, using a modified Mankin scoring system. The modification was the use of H&E rather than safranin O staining, as described in the original Mankin grading system.³¹

Radiography

Radiographs of the sample knee were taken of all patients before surgery. Anterior-posterior radiographs were graded by the examining surgeon using the Kellgren-Lawrence grading system.²⁴

Statistical Analyses

Descriptive statistics and measures of central tendency and dispersion were performed using SPSS v.19 (IBM Statistical Software, Armonk, New York). A univariate general linear model in SPSS was used to evaluate the significance of association between the putative factors (patient, patient age, Kellgren-Lawrence score, cartilage

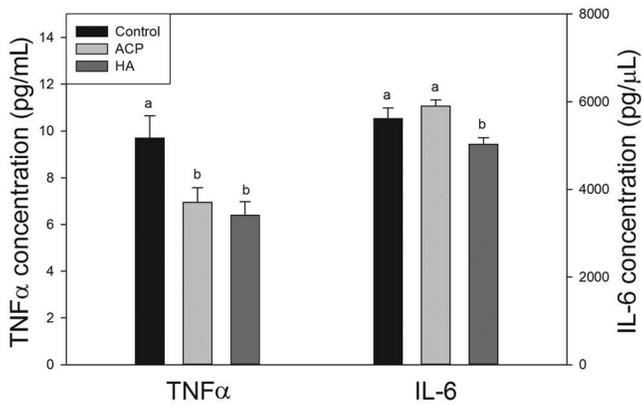


Figure 2. Concentration of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in control media (n = 9), platelet-rich plasma (PRP) (TNF- α , n = 17; IL-6, n = 20), and hyaluronic acid (n = 19) after 96 hours of culture. Bars represent the mean with standard error (SE). Superscript letters indicate a significant difference between treatment groups ($P \leq .05$, ANOVA).

Mankin grade, PRP platelet or leukocyte concentration) and the outcome measures (protein data: TNF- α , IL-6; gene expression: *ACAN*, *COL1A1*, and *COL1A1* in cartilage and *MMP-13* and *HAS-2* in synoviocytes). Appropriate transformations were performed for the outcomes that were not normally distributed. Post hoc multiple comparisons for categorical variables were performed using the Tukey test with $P \leq .05$ considered to be significant.

RESULTS

PRP Preparation

Automated hemograms of venous blood and resultant PRP verified the generation of leukocyte-reduced PRP with a mean $2.36\text{-fold} \pm 1.50\text{-fold}$ concentration of platelets and decreased leukocytes by a mean of $0.70\text{-fold} \pm 0.51\text{-fold}$ compared with venous blood. The mean platelet count was $331 \times 10^3/\mu\text{L} \pm 231 \times 10^3$, and the mean leukocyte concentration was $3.41 \times 10^3/\mu\text{L} \pm 2.47 \times 10^3$. The mean hematocrit value of PRP was $3.8\% \pm 2.4\%$. Neither platelet nor leukocyte concentration had a significant effect on outcome measurements (gene or protein expression data) in cartilage or synoviocytes.

Histological Scores

Cartilage samples exhibited classic signs of OA including surface fibrillation and vertical cracks, areas of chondrocyte clustering or hypocellularity, and marked loss of matrix metachromasia indicative of proteoglycan depletion. The mean modified Mankin score of the articular cartilage histological samples was 6.9 ± 0.8 of 14 (with 0 being normal). The Mankin score was not significantly associated with outcomes of either protein or gene expression in cartilage or synoviocytes.

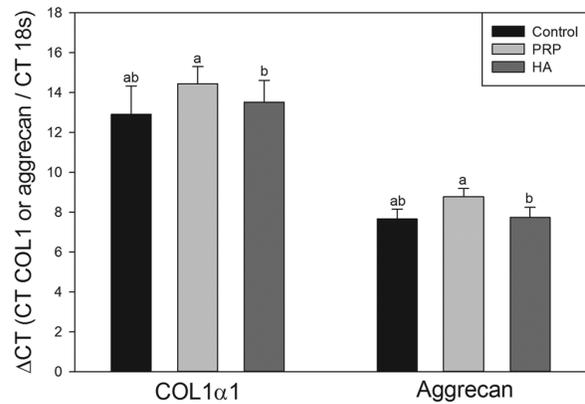


Figure 3. Relative gene expression of cartilage aggrecan in co-cultures treated with control media (control; n = 13), platelet-rich plasma with media (PRP; n = 12), and hyaluronic acid with media (HA; n = 10) and co-cultures treated with cartilage collagen type I α 1 (*COL1A1*) gene expression in control, PRP (n = 9), and HA (n = 9). Bars represent the mean with standard error (SE). Superscript letters indicate a significant difference between treatment groups ($P \leq .05$, ANOVA).

Radiographic Scores

Using the standard 1-to-4 scale of the Kellgren-Lawrence grading system on patient radiographs, 4 of 20 knees were graded as 3 and demonstrated moderate multiple osteophytes, definite narrowing of joint spaces, some sclerosis, and possible deformities of the bone contour. The remaining 16 knees were graded as 4 with the presence of large osteophytes, marked narrowing of joint spaces, severe sclerosis, and definite deformities of the bone contour noted on radiographs. The Kellgren-Lawrence score was not significantly associated with outcomes of either protein or gene expression in cartilage or synoviocytes.

Cytokine Concentration in Media

In the culture media, the concentration of TNF- α in both PRP and HA treatment groups was significantly reduced compared with the control group (Figure 2), but they were not significantly different from each other. Interleukin-6 concentration was decreased in cultures treated with HA compared with both control and PRP treatment groups (Figure 2). In all samples, the concentration of IL-1 β was below the lower limit of detection (0.01 pg/mL), even though a high-sensitivity ELISA was used for the assay.

Cartilage Matrix Gene Expression

Aggrecan gene expression was significantly increased by PRP treatment compared with HA, but neither was significantly different from the control (Figure 3). Similarly, *COL1A1* mRNA expression was significantly increased in PRP treatment groups compared with HA, but again, neither was different from control cultures (Figure 3). There was no difference between the control, PRP, and HA

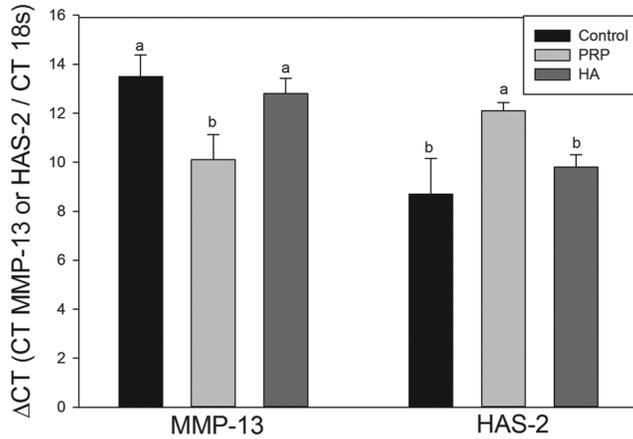


Figure 4. Relative gene expression of matrix metalloproteinase-13 (*MMP-13*) (control, $n = 10$; PRP, $n = 8$; HA, $n = 9$) and hyaluronan synthase-2 (*HAS-2*) (control, $n = 10$; PRP and HA, $n = 9$) from synoviocytes cultured in control media (control), platelet-rich plasma with media (PRP), and hyaluronic acid with media (HA). Results are reported as the mean, standard error (SE), and P value. Bars represent the mean with standard error (SE). Superscript letters indicate a significant difference between treatment groups ($P \leq .05$, ANOVA).

treatment groups in cartilage *MMP-13* expression ($P = .34$) or *COL2A1* expression ($P = .13$).

Synoviocyte Gene Expression

The *MMP-13* and *HAS-2* data were log transformed to achieve normal distribution. *MMP-13* gene expression in synoviocytes was significantly decreased in the PRP-treated cell groups compared with both HA and control groups (Figure 4). There was no difference in *MMP-13* gene expression between the HA and control groups. Similarly, *HAS-2* gene expression was significantly increased in PRP-treated synoviocytes compared with both the HA treatment group and control group (Figure 4). There were no significant differences in the expression of *TNF- α* ($P = .68$) or *MMP-1* ($P = .46$) between the 3 treatment groups.

DISCUSSION

The findings of this study suggest that both PRP and HA act to modulate the OA environment in similar ways but by different mechanisms. Both PRP and HA enhance metabolism and diminish markers of inflammation and nociception in a co-culture system. The use of an ex vivo co-culture system of OA cartilage and synoviocytes allows for interactions of the 2 tissues to better represent the articular environment than an isolated culture of cartilage, chondrocytes, or synoviocytes.

Both PRP and HA significantly decreased the concentration of *TNF- α* in the culture media, which reflects

synovial fluid in this co-culture system by providing a common interface between the cartilage and synoviocytes. In fact, *TNF- α* is a recognized mediator of acute inflammation and an activator of MMPs in many tissues including those of the articular environment.^{23,42,45} Additionally, *TNF- α* is implicated in initiating neuropathic pain pathways in animal models and human disease states.^{26,36} Elevation of *TNF- α* in synovial fluid is common in OA joints and has been shown to correlate with patient pain scores.³⁶ Decreasing *TNF- α* could be one mechanism responsible for the reported benefits of PRP and HA on joint tissues and in clinical application.^{8,16,25,27} Although both PRP and HA decreased *TNF- α* , the results were not directly related to a decrease in MMP expression, as noted by the significant decrease in *MMP-13* by PRP but not HA in synoviocytes. This might suggest that *TNF- α* has a role in pain, independent of MMPs, within the context of an OA joint and that the administration of PRP or HA decreases pain via decreased *TNF- α* synthesis.

The synovial membrane is responsible for nutrition and the removal of waste products, shock absorption, and lubrication and in inflammatory conditions is a source of catabolic cytokines.¹⁸ In synoviocytes, PRP but not HA treatment resulted in significantly decreased (100% compared with control cultures) *MMP-13* expression. Matrix metalloproteinase-13 is recognized as integral in cartilage matrix degradation during the development and continuation of OA.^{4,13,22,35,42,46} Despite reports that HA inhibits *MMP-13* in isolated OA chondrocytes,²¹ there was no effect of exogenous HA on *MMP-13* expression in this ex vivo co-culture system.

The role of IL-6 in inflammation and OA is less clear compared with *TNF- α* and IL-1 β .²³ Interleukin-6 is elevated in acute inflammation and has been found in the synovial membrane and synovial fluid of OA joints.^{6,23,43,45} The concentration of IL-6 appears to decrease with increasing severity of OA.⁶ In contrast, IL-6 has also been shown to modulate the severity of inflammation via the regulation of other proinflammatory cytokines, in particular *TNF- α* .⁴⁷ Therefore, IL-6 generates both proinflammatory and anti-inflammatory signals. Co-cultures treated with HA, but not those treated with PRP, had significantly less IL-6 in the media. It cannot be determined in this culture system if IL-6 was eliciting proinflammatory and/or anti-inflammatory signals, but it is unlikely that IL-6 was functioning to decrease *TNF- α* because IL-6 was higher in PRP-treated groups than in HA-treated groups, but *TNF- α* was decreased in both PRP and HA groups.

The expression of *ACAN* or *COL1A1* in cartilage explants treated with PRP was greater than in HA treatment groups but not different from the control. The expression of cartilage matrix genes can be up or down regulated depending on the stage and severity of OA.^{1,7,11,22,30} Most of the cartilage samples in this study were classified as severe OA, and many had fibrocartilaginous-appearing repair tissue, which is biochemically different from articular cartilage particularly with respect to *COL1A1* and *ACAN*. Type I collagen is expressed in immature articular cartilage, in de-differentiated articular chondrocytes, and in fibrocartilage repair tissue to a greater extent than it

is in normal articular cartilage.^{11,29} The expression of aggrecan in OA cartilage is similarly variable; however, late-stage OA samples demonstrate a loss of aggrecan from the extracellular matrix. Because both aggrecan and collagen type I expression were increased by PRP treatment, it suggests that PRP results in a generalized stimulation of cartilage metabolism rather than targeting specific matrix molecules.

Platelet-rich plasma significantly increased *HAS-2* expression by over 100%, whereas exogenous HA did not. Hyaluronan is produced by HAS in numerous tissues. Three isoforms of the HAS protein are found in synovial fluid and cartilage.³⁹ *HAS-2* produces the largest HA molecules of the 3 isoforms, with an HA product of $\geq 2 \times 10^6$ Da.^{19,20} High molecular weight HA has been found to be anti-inflammatory and antinociceptive in rat models.¹⁶ A hallmark of joint disease is a decrease in the size of HA in synovial fluid, and these smaller HA molecules have been found to be proinflammatory and unable to relieve pain.^{9,16} The finding of increased *HAS-2* gene expression in PRP-treated groups is in agreement with the results of others who have reported that cultured synoviocytes treated with PRP have an increased production of HA in comparison to a platelet-poor product.²

The findings of this study support our hypothesis that both PRP and HA would enhance the metabolism and diminish the marks of inflammation and nociception in a co-culture system. The findings also support our hypothesis that PRP would have the greatest effect in synoviocytes; however, PRP was not superior to HA regarding cartilage metabolism or nociceptive mediators. A limitation of this co-culture model and study design is the number of treatment groups and assays that could be performed because of the limited volume of cartilage remaining in the OA joints. The potentially stimulatory or inhibitory effects of co-culture on the metabolism of isolated cartilage and synoviocytes could not be identified using this study design. The joint-preserving effects of PRP on MMP-13 and *HAS-2* expression in the synovial membrane suggest that PRP can stimulate the endogenous production of HA while minimizing catabolic pathways. The antinociceptive properties of PRP and HA observed in clinical practice are supported in this study through decreased TNF- α concentration. The combination of antinociceptive and anti-inflammatory activity in specific formulations of PRP supports its use in joints with early OA to modulate disease progression. However, the potentially positive effects of HA on cartilage matrix gene expression suggest that a combination of PRP and HA might be better than either PRP or HA alone for the treatment of OA. A level I study comparing PRP, HA, and a combination of PRP + HA would be needed to answer this question for improved nonoperative treatment of severe, chronic OA.

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