

Platelet-Rich Plasma Releasate Inhibits Inflammatory Processes in Osteoarthritic Chondrocytes

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Background: Platelet-rich plasma (PRP) has recently been postulated as a treatment for osteoarthritis (OA). Although anabolic effects of PRP on chondrocytes are well documented, no reports are known addressing effects on cartilage degeneration. Since OA is characterized by a catabolic and inflammatory joint environment, the authors investigated whether PRP was able to counteract the effects of such an environment on human osteoarthritic chondrocytes.

Hypothesis: Platelet-rich plasma inhibits inflammatory effects of interleukin-1 (IL-1) beta on human osteoarthritic chondrocytes.

Study Design: Controlled laboratory study.

Methods: Human osteoarthritic chondrocytes were cultured in the presence of IL-1 beta to mimic an osteoarthritic environment. Medium was supplemented with 0%, 1%, or 10% PRP releasate (PRPr, the active releasate of PRP). After 48 hours, gene expression of collagen type II alpha 1 (COL2A1), aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, ADAMTS5, matrix metalloproteinase (MMP)13, and prostaglandin-endoperoxide synthase (PTGS)2 was analyzed. Additionally, glycosaminoglycan (GAG) content, nitric oxide (NO) production, and nuclear factor kappa B (NFκB) activation were studied.

Results: Platelet-rich plasma releasate diminished IL-1 beta–induced inhibition of COL2A1 and ACAN gene expression. The PRPr also reduced IL-1 beta–induced increase of ADAMTS4 and PTGS2 gene expression. ADAMTS5 gene expression and GAG content were not influenced by IL-1 beta or additional PRPr. Matrix metalloproteinase 13 gene expression and NO production were upregulated by IL-1 beta but not affected by added PRPr. Finally, PRPr reduced IL-1 beta–induced NFκB activation to control levels containing no IL-1 beta.

Conclusion: Platelet-rich plasma releasate diminished multiple inflammatory IL-1 beta–mediated effects on human osteoarthritic chondrocytes, including inhibition of NFκB activation.

Clinical Relevance: Platelet-rich plasma releasate counteracts effects of an inflammatory environment on genes regulating matrix degradation and formation in human chondrocytes. Platelet-rich plasma releasate decreases NFκB activation, a major pathway involved in the pathogenesis of OA. These results encourage further study of PRP as a treatment for OA.

Keywords: platelet-rich plasma; PRP; cartilage; chondrocyte; osteoarthritis; catabolic

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Articular cartilage provides a low-friction and efficient load-bearing joint surface. Cartilage is an avascular and aneural tissue without lymphatic drainage, and has a very limited intrinsic regenerative capacity.⁴⁵ Osteoarthritis (OA) is a degenerative joint disease, characterized by an imbalance of anabolic and catabolic processes in synovial joints.³⁵ This imbalance results in progressive cartilage damage and ultimately patient disability. To stimulate repair processes by endogenous cells, platelet-rich plasma (PRP) has recently been under extensive investigation.^{12,42,53} Platelet-rich plasma is a plasma preparation that can be obtained from whole blood, having an increased platelet concentration above baseline.³⁷ Platelets contain various organelles, including α granules. These granules hold numerous growth factors and other bioactive proteins, including vascular endothelial growth factor (VEGF),

platelet-derived growth factor (PDGF), and transforming growth factor- β (TGF- β), many of which play a pivotal role in hemostasis or tissue healing.³¹ Upon activation, platelets are triggered to release these growth factors to the injured site to stimulate various healing processes. Platelet-rich plasma can be produced by centrifugation of autologous blood, making PRP a highly concentrated, natural, and autologous treatment option for a variety of regenerative medicine fields.¹⁰ Several preclinical and clinical studies using PRP as a treatment for OA have been performed, reporting promising results.^{19,30,41,43} Unfortunately, no randomized controlled clinical trials regarding this subject have been reported, thereby limiting the level of current evidence of benefit for PRP-based treatment of OA. Elucidating the working mechanisms of PRP in a standardized *in vitro* environment could provide tools to further optimize these therapies. Several studies have previously described the effects of PRP on chondrocytes in an *in vitro* model.^{2,14,21,29,44} These studies did not evaluate PRP primarily as a treatment option, but mainly as a culture additive in standard *in vitro* conditions. They describe without exception a stimulating effect of PRP on cell proliferation, although the effects on the maintenance of a chondrogenic phenotype were less concordant. However, to evaluate PRP as a therapy for OA, the *in vitro* environment has to simulate the *in vivo* situation. The joint environment in OA has shifted toward the catabolic side at the moment patients require medical care. Therefore, the emphasis may well be better directed toward understanding not only the anabolic effects, but also the effects of PRP on prevention of degeneration in an osteoarthritic environment. Interleukin (IL)-1 β is one of the most potent inflammatory factors in osteoarthritic joints. This cytokine has been described to induce the production of destructive proteases together with the inhibition of extracellular matrix formation.^{1,50} Treating cells with IL-1 β has been reported to be a useful model to reproduce the mechanisms involved in degenerative arthropathies.⁹ Interleukin-1 β exerts its effects through a diverse spectrum of signaling cascades, including nuclear factor κ B (NF κ B) activation.^{9,32} Nuclear factor κ B is normally located in an inactivated state in the cytosol bound to I κ B, an inhibitory protein. Upon activation, NF κ B translocates to the nucleus and acts on various regulatory genes involved in apoptosis, inflammation, and other immune responses.²⁷ To determine the effects of PRP in a standardized inflammatory environment, we studied the capacity of PRP releasate (PRPr, the active releasate of PRP) to counteract IL-1 β -induced effects on genes involved in matrix formation and degradation in osteoarthritic chondrocytes, including NF κ B as a possible pathway through which these effects occurred.

MATERIALS AND METHODS

PRPr Preparation

Anticoagulated whole blood from 3 healthy male donors was acquired (Sanquin Blood Supply Foundation,

Amsterdam, the Netherlands) after participants gave informed consent. Whole blood was further processed within 2 hours after collection. Platelet-rich plasma was prepared by means of a GPS III System (kindly provided by Biomet Nederland BV, Dordrecht, the Netherlands) according to the manufacturer's protocol. Clotting upon addition of 22.8 mM CaCl₂ 1:10 (v/v) activated platelets to release their growth factors. Supernatant was collected and designated PRPr. Baseline platelet, white blood cell, and red blood cell concentrations of whole blood, PRP, and PRPr were measured on a clinical Sysmex XE-2100 automated hematology system (Sysmex Europe, Norderstedt, Germany). Platelet-rich plasma releasate was subsequently stored in aliquots of 1.5 mL at -80°C for further experiments and growth factor analyses. Cryopreservation has been reported before to maintain the ability of PRPr to influence chondrocyte behavior.² Baseline VEGF, PDGF-AA, and PDGF-AB/BB in PRPr were measured by means of a MILLIPLEX (Millipore BV, Amsterdam, the Netherlands) and TGF- β 1 using the human TGF- β 1 Immunoassay Quantikine (R&D Systems, Abingdon, United Kingdom). Two dilutions of the samples were measured for growth factor analyses. The measurements that were best in the range of the standard are reported.

Chondrocyte Culture With PRPr

Human osteoarthritic cartilage from 6 donors was obtained from patients undergoing total knee replacement surgery (after implicit consent, as approved by the local ethical committee; MEC-2004-322). Full-thickness cartilage was harvested, treated with 0.2% protease (Sigma-Aldrich, Zwijndrecht, the Netherlands) in physiologic saline solution for 90 minutes and subsequently digested overnight in standard culture medium (Dulbecco's modified Eagle medium [DMEM] containing 10% fetal calf serum [FCS], 50 μ g/mL gentamicin, and 1.5 μ g/mL fungizone) supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). After digestion, cells were washed twice with physiologic saline and the harvested cell number was determined using a hemocytometer. Alginate beads were prepared as described previously.²³ In short, chondrocytes were suspended in 1.2% low-viscosity alginate (Keltone LV, Kelco, Surrey, United Kingdom) at a concentration of 4 million cells/mL. Beads were created by dripping the alginate/cell suspension through a 23-gauge needle into a CaCl₂ solution. Chondrocytes in alginate beads were cultured in standard culture medium as described above, supplemented with 1×10^{-4} M ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, Missouri). After chondrocytes were precultured in alginate beads for 3 weeks to allow extracellular matrix formation, beads were washed 3 times with serum-free DMEM. Subsequently, beads were placed in serum-free culture medium (DMEM containing ITS+1 1:100 [v/v], 50 μ g/mL gentamicin, 1.5 μ g/mL fungizone, and 1×10^{-4} M ascorbic acid-2-phosphate) with or without 10 ng/mL IL-1 β and the addition of PRPr at concentrations of 0%, 1%, and 10% (v/v). After 48 hours of culture, beads were harvested for

gene expression analyses. Forty-eight hours of culture has been described previously as a suitable time point to study the effects of IL-1 beta and various treatments on chondrocyte behavior.^{9,33} Additionally, beads were harvested for glycosaminoglycan (GAG) and DNA analyses and medium was harvested for nitric oxide (NO) and GAG analysis.

Gene Expression Analysis

Alginate beads were dissolved in 55 mM sodium citric acid and spun down at 350g for 8 minutes at a temperature of 4°C. Cell pellets were resuspended in RNA-Bee™ (TELTEST, Friendswood, Texas). RNA was extracted with chloroform and purified from the supernatant using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines with on-column DNA digestion. Nucleic acid content was determined spectrophotometrically (NanoDrop ND1000, Isogen Life Science, IJsselstein, the Netherlands). Complementary DNA (cDNA) synthesis was performed using a RevertAid First Strand cDNASynthesis Kit (MBI Fermentas, St. Leon-Rot, Germany), and polymerase chain reactions (PCRs) were performed using TaqMan Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, the Netherlands) as described earlier.²⁴ Real-time PCR primers for collagen type II alpha 1 (COL2A1), aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, ADAMTS5, and matrix metalloproteinase (MMP)13 are reported by Uitterlinden et al.⁴⁸ Prostaglandin-endoperoxide synthase (PTGS)2 gene expression was determined using a commercial assay-on-demand set (Hs01573474.g1, Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), HPRT (hypoxanthine-guanine phosphoribosyltransferase), and beta 2-microglobulin were compared as housekeeping genes. Relative expression levels, normalized to GAPDH as the most stably expressed of the 3 reference genes (data not shown), were calculated using the $2^{-\Delta\Delta Ct}$ method.³⁴

Glycosaminoglycan and DNA Assay

Glycosaminoglycan analysis was performed by a dimethylmethylene blue (DMB) assay. Alginate beads were digested overnight at 60°C in papain buffer (250 µg/mL papain in 50 mM EDTA [ethylenediaminetetraacetic acid] and 5 mM L-cysteine). The GAG amount in the digest was quantified using a modified DMB assay,¹⁶ based on the original description by Farndale et al.¹⁷ The metachromatic reaction of GAG with DMB was monitored using a spectrophotometer, and the ratio A530:A590 was used to determine the amount of GAG present, using chondroitin sulphate C (Sigma-Aldrich) as a standard. The amount of DNA in each papain-digested sample was determined using ethidium bromide with calf thymus DNA (Sigma-Aldrich) as a standard.

Nitric Oxide Assay

Nitric oxide production was determined by quantifying its derived product, nitrite, in medium using a

spectrophotometric method based upon the Griess reaction.²² Briefly, 100 µL of culture medium was mixed with 100 µL of Griess reagent (0.5% sulphanilamide, 0.05% naphthyl ethylenediamine dihydrochloride, 2.5% H₃PO₄). A serial dilution of sodium nitrite (NaNO₂, Fluka, Buchs, Switzerland) was used as a standard. The absorption was measured at 540 nm.

Nuclear Factor Kappa B

To evaluate the involvement of NFκB activation in the working mechanism of PRPr, freshly isolated chondrocytes were seeded in monolayer at a density of 20 000 cells/cm². Cells were precultured in basal medium in 48-well plates until subconfluency. A preliminary study revealed 1 hour of IL-1 beta incubation to be the optimal moment to evaluate NFκB activation (data not shown), confirming a previous study by Largo et al.³² Cells were cultured for 1 hour in the presence or absence of IL-1 beta and PRPr at concentrations of 0%, 1%, and 10% (v/v) and subsequently fixed in formalin 4%. All conditions were simultaneously fixed per donor. Fixation was followed by incubation with NFκB-p65 Antibody (Ab-276) 1:100 (Signalway Antibody, Leusden, the Netherlands) and goat anti-rabbit AP 1:20 (Sigma-Aldrich). Freshly prepared new fuchsin was used as substrate to achieve staining. Negative controls were performed using an immunoglobulin G isotype control. Staining was performed synchronously for all conditions per donor. The percentage of positive cells was determined by 2 independent observers by scoring 3 times 100 cells/well. All separate values from both observers were used for statistical analysis.

Statistical Analysis

Experiments were performed in triplicate samples for all 3 PRPr donors on 2 separate chondrocyte donors each (6 chondrocyte donors in total). The NFκB immunohistochemistry was performed for all 3 PRPr donors on single chondrocyte donors. Statistical analysis was performed using a mixed-model analysis of variance, in which treatment was considered a fixed factor and donor a random factor. The effects of IL-1 beta and PRPr were calculated in separate models. A *P* value < .05 was considered statistically significant.

RESULTS

Baseline Platelet Concentration and Growth Factor Analyses

Whole blood samples contained physiologic values of platelets, white blood cells, and red blood cells for all 3 donors. Platelet-rich plasma contained on average 6.04 to 7.75 times more platelets than whole blood (Table 1). White blood cells increased in a comparable manner (5.87- to 7.24-fold increase), while red blood cell counts decreased.

TABLE 1
Analyses of Blood Components, PRP, and PRP Releasates From 3 Donors^a

Blood Component Analyses					PRPr Analyses					
Blood Components	Donor	Concentration, $\times 10^6/\text{mL}$			Donor	N-Fold Change in PLT Concentration	Concentration, pg/mL			
		PLT	WBC	RBC			VEGF	PDGF-AA	PDGF-AB/BB	TGF- β 1
Whole blood	1	89	5.86	4260	1	7.75	290	73 000	86 000	226 000
	2	190	5.45	4160						
	3	96	5.35	4970						
PRP	1	690	38.86	910	2	6.66	1380	103 000	86 000	391 000
	2	1266	39.48	440						
	3	580	31.38	480						
PRPr	1	13	5.41	290	3	6.04	<5	117 000	141 000	386 000
	2	76	6.93	140						
	3	80	2.56	130						

^aPRP, platelet-rich plasma; PRPr, PRP releasate; PLT, platelets; WBC, white blood cells; RBC, red blood cells; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; TGF- β 1: transforming growth factor- β 1. Two dilutions of each sample were measured; measurements that were best in the range of the standard are shown.

After activating the PRP by clotting, platelet numbers were decreased to 0.15 to 0.83 times the baseline value of whole blood levels. Next to this, the clotting procedure decreased white blood cells to 0.48 to 1.27 times baseline and red blood cell count to 0.03 to 0.07 times baseline whole blood level. The growth factors PDGF-AA, PDGF-AB/BB, and TGF- β 1 were abundantly present in PRPr from all 3 donors (values listed in Table 1). Vascular endothelial growth factor was found in a lower amount on average and was below detection level in 1 donor.

PRPr Diminishes IL-1 Beta Effects on Chondrocyte Gene Expression

The addition of the inflammatory cytokine IL-1 beta diminished expression of COL2A1 and ACAN in chondrocytes, while it increased expression of ADAMTS4, MMP13, and PTGS2 (Figure 1; $P < .03$ for all genes). Upon addition of 10% PRPr, COL2A1 gene expression increased compared with IL-1 beta-only treated samples ($P = .003$) as well as ACAN ($P = .001$), while ADAMTS4 expression was reduced ($P = .001$) as was PTGS2 ($P = .004$). The addition of 1% PRPr to our cultures revealed a trend toward normalizing COL2A1 and ADAMTS4 expression, although these results did not reach statistical significance. Matrix metalloproteinase 13 expression was not changed by PRPr, whereas ADAMTS5 was not influenced by the presence of IL-1 beta or additional PRPr.

Without the addition of IL-1 beta to our cultures, PRPr did not significantly affect COL2A1 gene expression compared with the control containing no PRPr, while ACAN showed a significant downregulation after the addition of 1% and 10% PRPr ($P \leq .003$ for both). ADAMTS4 and MMP13 were significantly upregulated by 10% PRPr ($P \leq .002$ for both), whereas ADAMTS5 was downregulated by 10% PRPr ($P = .002$). Prostaglandin-endoperoxide synthase 2 was not influenced by PRPr in the absence of IL-1 beta.

PRPr Effects on GAG and NO Production

Neither IL-1 beta nor simultaneous addition of PRPr showed an effect on the amount of GAG per cell in our experiments (Figure 2). Next to this, no effects were seen on GAG release in the medium, DNA content per bead, or total GAG per bead (data not shown). Moreover, PRPr did not alter any of these parameters in the absence of IL-1 beta (data not shown). We also determined the effect of PRPr on NO production in IL-1 beta-treated chondrocyte cultures. Platelet-rich plasma releasate was unable to counteract the IL-1 beta-induced production of this inflammatory mediator (Figure 3). In the absence of IL-1 beta, PRPr alone did not induce production of NO by osteoarthritic chondrocytes (data not shown).

PRPr Reduces IL-1 Beta-Induced NF κ B Activation

To determine a possible mechanism through which PRPr exerts its effects, we studied the effect of PRPr on NF κ B activation using immunohistochemistry. This revealed NF κ B activation in our IL-1 beta-treated chondrocytes, which was reduced by PRPr (Figure 3A-C). Manually scoring these cells showed IL-1 beta to cause a marked increase in NF κ B-activated chondrocytes (Figure 3D; $P = .001$). This activation was downregulated by PRPr ($P < .001$ for both doses) in a dose-dependent manner. The highest concentration of PRPr (10%) reduced the amount of NF κ B-activated chondrocytes back to control levels.

DISCUSSION

In this study, we tested our hypothesis that PRPr has anti-inflammatory properties and influences gene expression of matrix-forming and matrix-degrading proteins in an OA-mimicking environment. The osteoarthritic and

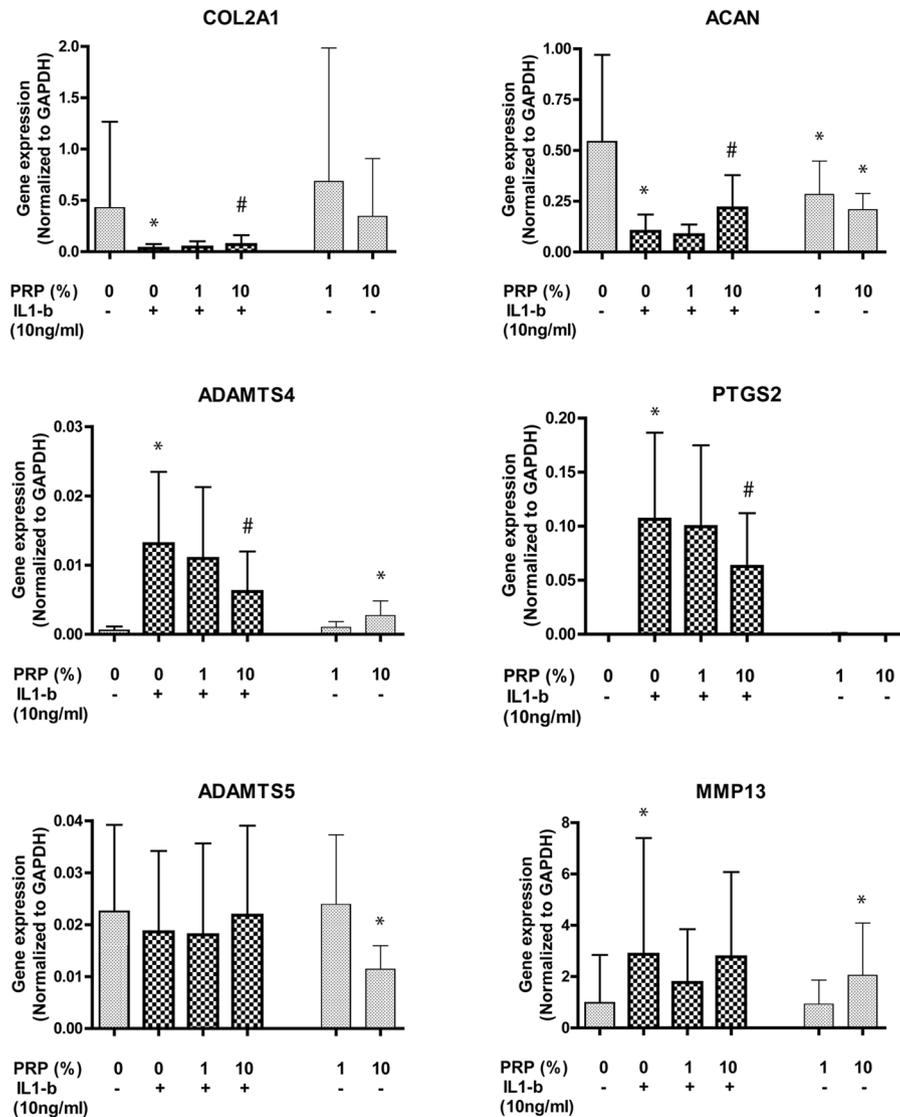


Figure 1. Effects of platelet-rich plasma releasate (PRPr) on osteoarthritic chondrocyte gene expression. PRPr reduced interleukin (IL)-1 beta effects on collagen type II alpha 1 (COL2A1), aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, and prostaglandin-endoperoxide synthase (PTGS)2 gene expression. No PRPr effects were seen on ADAMTS5 or matrix metalloproteinase (MMP)13 in the presence of IL-1 beta. Without the addition of IL-1 beta, PRPr downregulated ACAN gene expression and ADAMTS5, while ADAMTS4 and MMP13 were upregulated. PRPr did not alter COL2A1 or PTGS2 gene expression in the absence of IL-1 beta. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as means \pm standard deviations for 6 experiments. *Indicates $P < .05$ compared with control without IL-1 beta; #indicates $P < .05$ versus control with IL-1 beta.

inflammatory environment was created through addition of IL-1 beta, one of the key players in OA pathogenesis,^{1,7,9,50} to chondrocytes in culture. Exposure of chondrocytes to the inflammatory cytokine IL-1 beta resulted in marked changes in the expression of genes involved in matrix formation and degradation as well as inflammation, many of which were reduced by the addition of PRPr. Platelet-rich plasma releasate ameliorated the IL-1 beta-induced changes on chondrocyte gene expression of COL2A1, ACAN, ADAMTS4, and PTGS2. The inhibition of NF κ B activation was found to be

a possible mechanism through which PRPr exerted these effects.

Various research groups have studied the use of PRP mainly as a culture supplement for chondrocytes. A consistent finding in their reports is that PRP increases cell proliferation.^{2,14,21,29,44} However, some describe an increased gene expression or synthesis of COL2A1 and ACAN upon PRP addition,^{2,44} while others mention PRP to decrease these parameters.^{14,21,29} We consider these contradicting reports and our own results using PRP in the absence of IL-1 beta to hinder a clear statement regarding the use

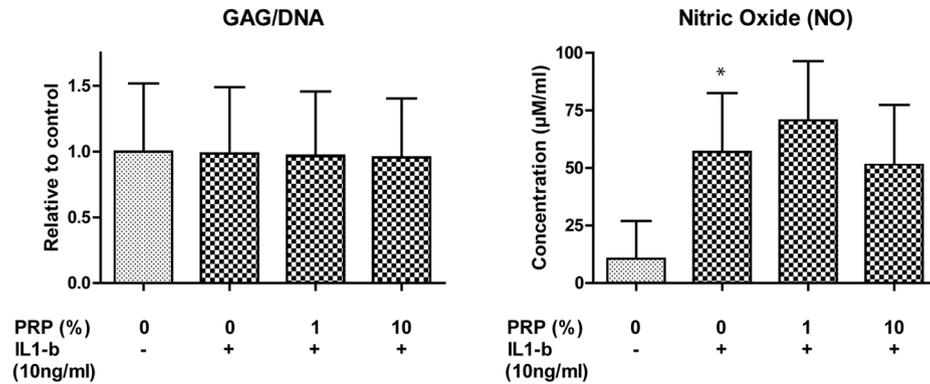


Figure 2. Influence of platelet-rich plasma releasate (PRPr) on glycosaminoglycan (GAG)/DNA and nitric oxide (NO) in the presence of interleukin (IL)-1 beta. No effects on GAG per cell were found by IL-1 beta or additional PRPr. Interleukin-1 beta increased NO release by chondrocytes; additional PRPr did not alter this. Data are shown relative to control as means \pm standard deviations for 6 experiments. *Indicates $P < .05$ compared with control without IL-1 beta.

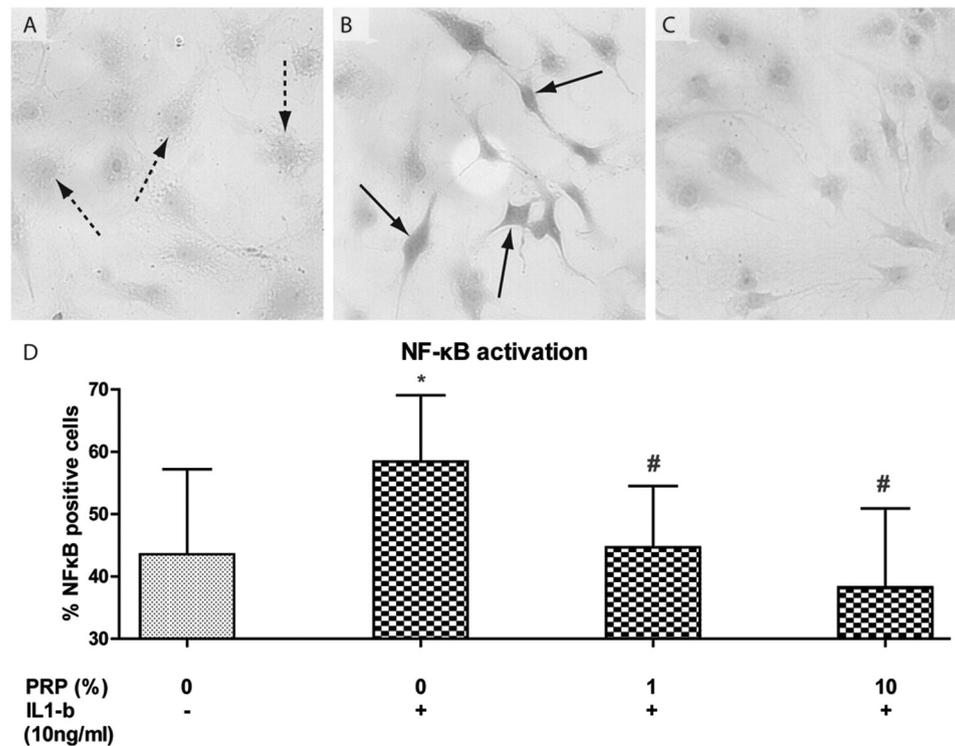


Figure 3. Effect of platelet-rich plasma releasate (PRPr) on interleukin (IL)-1 beta-induced nuclear factor kappa B (NFκB) activation. Nuclear factor kappa B immunohistochemistry showing IL-1 beta-treated chondrocytes in the absence of PRPr (A) and the presence of 10% PRPr (B). Panel C represents immunoglobulin G (IgG) control. Dotted arrows in panel A resemble negative cells, whereas solid arrows in panel B indicate NFκB activated cells. D, interleukin-1 beta induced NFκB activation, which was dose-dependently reduced by PRPr. Data are presented as means \pm standard deviations for 3 experiments. Magnification (A-C) $\times 200$. *Indicates $P < .05$ compared with control without IL-1 beta; #indicates $P < .05$ versus control with IL-1 beta.

of PRP as a culture supplement for chondrocytes. A major difference between these previous reports and the focus of our current study is the fact that we did not evaluate PRPr as a culture additive, but aimed at establishing the potential

of PRP as a possible treatment for OA. For this application, PRP should preferably restore the anabolic-catabolic imbalance found in OA joints. By applying PRPr to osteoarthritic chondrocytes in a standardized IL-1 beta-mediated

inflammatory and catabolic environment, our results indicated consistent anti-inflammatory effects of PRP.

Platelet-rich plasma inhibited the translocation of NF κ B to the nucleus. Nuclear factor kappa B is present as an inactive, inhibitor-bound complex in almost all mammalian cells and gets translocated into the nucleus upon activation.⁴⁰ It regulates more than 150 genes, including those involved in inflammation and other immune responses.³⁹ In chondrocytes, NF κ B has been reported to inhibit COL2A1 gene expression and to regulate MMP1, MMP3, MMP13, IL-8, and MCP-1 (monocyte chemotactic protein 1) expression.⁴⁰ Several pharmacologic agents act as NF κ B inhibitors, including nonsteroidal anti-inflammatory drugs, glucocorticoids, and proteasome inhibitors.^{11,15,46} Nuclear factor kappa B inhibition by PRP has been recently reported in a human immortalized chondrocyte cell line.⁴ We were able to confirm the latter result using primary human osteoarthritic chondrocytes, thereby providing a link toward clinical application.

Nitric oxide production is increased in OA joints, and has been described to inhibit collagen and GAG synthesis while inducing chondrocyte apoptosis and the production of metalloproteinases.^{6,8,18,28,38} Vuolteenaho et al⁵¹ previously reported that both TGF-beta and an NF κ B inhibitor decreased IL-1 beta-induced NO production by immortalized murine chondrocytes. Interleukin-1 beta induced an evident increase in NF κ B activation and NO production in our experiments, but PRPr only counteracted the first effect. Transforming growth factor-beta was abundantly present in our PRPr, and the amount of TGF-beta in the condition where we used 10% PRPr exceeded that used by Vuolteenaho et al. The different origin of our cells or the heterogeneity and multitude of growth factors present in PRP, affecting many regulatory pathways, could be possible explanations for the fact that we did not observe an inhibitory effect of PRPr on NO production. Although PRP could be a promising autologous treatment option with many applications, this heterogeneity and multitude of growth factors in PRP substantially limits the ability to understand and investigate the effects of PRP. This could also explain the different PRP-mediated effects we observed in an environment with versus without IL-1 beta.

A limitation of our study is the use of a short-term in vitro model to study PRP as a possible OA treatment. In our model, we did not detect catabolic effects of IL-1 beta on GAG content at a protein level, although we observed clear downregulation of ACAN gene expression and upregulation of the aggrecanase gene ADAMTS4, indicating IL-1 beta did evoke inflammatory effects. Previous studies from our group did reveal an IL-1 beta effect on GAG content of constructs of immature bovine chondrocytes in alginate.⁴⁷ These bovine chondrocytes had a substantially higher GAG turnover than the human cells used in this present study. This difference could be attributable to the fact that we used human osteoarthritic chondrocytes from relatively aged donors. The lower overall GAG turnover of human chondrocytes in our culture model could be masking IL-1 beta and PRP effects after 48 hours. Furthermore, since PRP-derived growth factors may not all have their optimal effect in an in vitro model, further

in vivo studies are needed to show long-term beneficial PRP effects on cartilage or joints as a whole.

The use of PRP as a highly concentrated autologous source of growth factors has gained wide acceptance in various applications, such as orthopaedic surgery, otolaryngology, and plastic surgery.²⁰ Because of the biologic nature of these PRP products, interdonor variability in the amount of platelets and growth factors is known to occur.^{12,31} This was also seen in our PRP preparations, especially in the variation of VEGF concentration. Anitua et al³ reported this finding previously and mentioned a correlation between VEGF and platelet concentration, which is in line with our findings. Despite these variations, we found consistent anti-inflammatory effects of PRPr from 3 donors on osteoarthritic chondrocytes from 6 donors. Next to this donor variation, multiple platelet- or growth factor-concentration methods are available.^{13,52} These different methods isolate diverse blood fractions with different platelet yields and with or without red and white blood cells.^{13,25} All these dissimilarities complicate the comparison of results between different research groups. Therefore, caution has to be exercised regarding safety and efficacy when considering the translation of PRP as an OA treatment into clinical studies.

We obtained PRP with an approximately 6.8-fold platelet increase above baseline. The amount of growth factors found in PRPr was comparable with those reported by others.^{26,49} Platelet-rich plasma releasate inhibited multiple IL-1 beta-induced effects in osteoarthritic chondrocytes. It beneficially affected genes involved in the formation and degradation of extracellular matrix, as well as genes related to inflammation. Additionally, PRPr diminished IL-1 beta-induced NF κ B activation, a mechanism known to be involved in many pathologic OA processes.^{5,36,40} Revealing the relevant mechanisms and processes could improve the application of PRP in a clinical setting. Although additional studies are needed before proceeding to clinical translation, we consider these encouraging results for the further study of PRP as a conservative, autologous, and low-cost treatment for OA.

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REFERENCES

1. Aigner T, Soeder S, Haag J. IL-1beta and BMPs—interactive players of cartilage matrix degradation and regeneration. *Eur Cell Mater.* 2006;12:49-56.
2. Akeda K, An HS, Okuma M, et al. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis Cartilage.* 2006;14(12):1272-1280.
3. Anitua E, Andia I, Sanchez M, et al. Autologous preparations rich in growth factors promote proliferation and induce VEGF and HGF production by human tendon cells in culture. *J Orthop Res.* 2005;23(2):281-286.
4. Bendinelli P, Matteucci E, Dogliotti G, et al. Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes:

- mechanisms of NF-kappaB inhibition via HGF. *J Cell Physiol.* 2010;225(3):757-766.
5. Berenbaum F. Signaling transduction: target in osteoarthritis. *Curr Opin Rheumatol.* 2004;16(5):616-622.
 6. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol.* 1995;146(1):75-85.
 7. Bondeson J, Blom AB, Wainwright S, Hughes C, Catterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. *Arthritis Rheum.* 2010;62(3):647-657.
 8. Cao M, Westerhausen-Larson A, Niyibizi C, et al. Nitric oxide inhibits the synthesis of type-II collagen without altering Col2A1 mRNA abundance: prolyl hydroxylase as a possible target. *Biochem J.* 1997;324(Pt 1):305-310.
 9. Cardile V, Frasca G, Libra M, et al. Dehydroxymethylepoxyquinomycin inhibits expression and production of inflammatory mediators in interleukin-1beta-induced human chondrocytes. *Cell Physiol Biochem.* 2010;25(4-5):543-550.
 10. Chen FM, Zhang M, Wu ZF. Toward delivery of multiple growth factors in tissue engineering. *Biomaterials.* 2010;31(24):6279-6308.
 11. De Bosscher K, Vanden Berghe W, Haegeman G. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J Neuroimmunol.* 2000;109(1):16-22.
 12. de Mos M, van der Windt AE, Jahr H, et al. Can platelet-rich plasma enhance tendon repair? A cell culture study. *Am J Sports Med.* 2008;36(6):1171-1178.
 13. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leukocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol.* 2009;27(3):158-167.
 14. Drengk A, Zapf A, Sturmer EK, Sturmer KM, Frosch KH. Influence of platelet-rich plasma on chondrogenic differentiation and proliferation of chondrocytes and mesenchymal stem cells. *Cells Tissues Organs.* 2009;189(5):317-326.
 15. Elliott PJ, Zollner TM, Boehncke WH. Proteasome inhibition: a new anti-inflammatory strategy. *J Mol Med.* 2003;81(4):235-245.
 16. Enobakhare BO, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem.* 1996;243(1):189-191.
 17. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986;883(2):173-177.
 18. Farrell AJ, Blake DR, Palmer RM, Moncada S. Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann Rheum Dis.* 1992;51(11):1219-1222.
 19. Filardo G, Kon E, Buda R, et al. Platelet-rich plasma intra-articular knee injections for the treatment of degenerative cartilage lesions and osteoarthritis. *Knee Surg Sports Traumatol Arthrosc.* 2011;19(4):528-535.
 20. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. *Am J Sports Med.* 2009;37(11):2259-2272.
 21. Gaissmaier C, Fritz J, Krackhardt T, Fleisch I, Aicher WK, Ashammakhi N. Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate cultures. *Biomaterials.* 2005;26(14):1953-1960.
 22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannerbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem.* 1982;126(1):131-138.
 23. Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. *Matrix.* 1992;12(2):116-129.
 24. Hellingman CA, Koevoet W, Kops N, et al. Fibroblast growth factor receptors in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. *Tissue Eng Part A.* 2010;16(2):545-556.
 25. Hraha TH, Doremus KM, Mcllwraith CW, Frisbie DD. Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. *Equine Vet J.* March 11, 2011. doi: 10.1111/j.2042-3306.2010.00321 [Epub ahead of print].
 26. Ishida K, Kuroda R, Miwa M, et al. The regenerative effects of platelet-rich plasma on meniscal cells in vitro and its in vivo application with biodegradable gelatin hydrogel. *Tissue Eng.* 2007;13(5):1103-1112.
 27. Ishinaga H, Jono H, Lim JH, et al. Synergistic induction of nuclear factor-kappaB by transforming growth factor-beta and tumour necrosis factor-alpha is mediated by protein kinase A-dependent RelA acetylation. *Biochem J.* 2009;417(2):583-591.
 28. Jarvinen TA, Moilanen T, Jarvinen TL, Moilanen E. Nitric oxide mediates interleukin-1 induced inhibition of glycosaminoglycan synthesis in rat articular cartilage. *Mediators Inflamm.* 1995;4(2):107-111.
 29. Kaps C, Loch A, Haisch A, et al. Human platelet supernatant promotes proliferation but not differentiation of articular chondrocytes. *Med Biol Eng Comput.* 2002;40(4):485-490.
 30. Kon E, Buda R, Filardo G, et al. Platelet-rich plasma: intra-articular knee injections produced favorable results on degenerative cartilage lesions. *Knee Surg Sports Traumatol Arthrosc.* 2010;18(4):472-479.
 31. Lacci KM, Dardik A. Platelet-rich plasma: support for its use in wound healing. *Yale J Biol Med.* 2010;83(1):1-9.
 32. Largo R, Alvarez-Soria MA, Diez-Ortego I, et al. Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. *Osteoarthritis Cartilage.* 2003;11(4):290-298.
 33. Legendre F, Bauge C, Roche R, Saurel AS, Pujol JP. Chondroitin sulfate modulation of matrix and inflammatory gene expression in IL-1beta-stimulated chondrocytes—study in hypoxic alginate bead cultures. *Osteoarthritis Cartilage.* 2008;16(1):105-114.
 34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C[T]) method. *Methods.* 2001;25(4):402-408.
 35. Loeser RF. Molecular mechanisms of cartilage destruction in osteoarthritis. *J Musculoskelet Neuronal Interact.* 2008;8(4):303-306.
 36. Marcu KB, Otero M, Olivetto E, Borzi RM, Goldring MB. NF-kappaB signaling: multiple angles to target OA. *Curr Drug Targets.* 2010;11(5):599-613.
 37. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant Dent.* 2001;10(4):225-228.
 38. Murrell GA, Jang D, Williams RJ. Nitric oxide activates metalloproteinase enzymes in articular cartilage. *Biochem Biophys Res Commun.* 1995;206(1):15-21.
 39. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene.* 1999;18(49):6853-6866.
 40. Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage.* 2006;14(9):839-848.
 41. Saito M, Takahashi KA, Arai Y, et al. Intraarticular administration of platelet-rich plasma with biodegradable gelatin hydrogel microspheres prevents osteoarthritis progression in the rabbit knee. *Clin Exp Rheumatol.* 2009;27(2):201-207.
 42. Salemi S, Rinaldi C, Manna F, Guarneri GF, Parodi PC. Reconstruction of lower leg skin ulcer with autologous adipose tissue and platelet-rich plasma. *J Plast Reconstr Aesthet Surg.* 2008;61(12):1565-1567.
 43. Sanchez M, Anitua E, Azofra J, Aguirre JJ, Andia I. Intra-articular injection of an autologous preparation rich in growth factors for the treatment of knee OA: a retrospective cohort study. *Clin Exp Rheumatol.* 2008;26(5):910-913.
 44. Spreafico A, Chellini F, Frediani B, et al. Biochemical investigation of the effects of human platelet releasates on human articular chondrocytes. *J Cell Biochem.* 2009;108(5):1153-1165.
 45. Steinert AF, Ghivizzani SC, Rethwilm A, Tuan RS, Evans CH, Noth U. Major biological obstacles for persistent cell-based regeneration of articular cartilage. *Arthritis Res Ther.* 2007;9(3):213.
 46. Tegeger I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* 2001;15(12):2057-2072.

47. Uitterlinden EJ, Jahr H, Koevoet JL, et al. Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. *Osteoarthritis Cartilage*. 2007;15(11):1267-1274.
48. Uitterlinden EJ, Jahr H, Koevoet JL, et al. Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. *Osteoarthritis Cartilage*. 2006;14(3):250-257.
49. van den Dolder J, Mooren R, Vloon AP, Stoelinga PJ, Jansen JA. Platelet-rich plasma: quantification of growth factor levels and the effect on growth and differentiation of rat bone marrow cells. *Tissue Eng*. 2006;12(11):3067-3073.
50. van der Kraan PM, van den Berg WB. Anabolic and destructive mediators in osteoarthritis. *Curr Opin Clin Nutr Metab Care*. 2000;3(3):205-211.
51. Vuolteenaho K, Moilanen T, Jalonen U, et al. TGFbeta inhibits IL-1-induced iNOS expression and NO production in immortalized chondrocytes. *Inflamm Res*. 2005;54(10):420-427.
52. Wehling P, Moser C, Frisbie D, et al. Autologous conditioned serum in the treatment of orthopedic diseases: the orthokine therapy. *Bio Drugs*. 2007;21(5):323-332.
53. Wrotniak M, Bielecki T, Gazdzik TS. Current opinion about using the platelet-rich gel in orthopaedics and trauma surgery. *Ortop Traumatol Rehabil*. 2007;9(3):227-238.