


# Effects of Platelet-Rich Plasma Composition on Anabolic and Catabolic Activities in Equine Cartilage and Meniscal Explants

Cartilage  
3(3) 245-254  
© The Author(s) 2012  
Reprints and permission:  
sagepub.com/journalsPermissions.nav  
DOI: 10.1177/1947603511433181  
http://cart.sagepub.com  


John D. Kisiday<sup>1</sup>, C. Wayne McIlwraith<sup>1</sup>,  
William G. Rodkey<sup>2</sup>, David D. Frisbie<sup>1</sup>, and J. Richard Steadman<sup>2</sup>

## Abstract

**Objective:** To evaluate the effects of single- and double-spin preparations of platelet-rich plasma (PRP) on anabolic and catabolic activities of cartilage and meniscal explants *in vitro*. **Methods:** Single- and double-spin PRP was prepared using laboratory processing or commercial kits. The cellular contents were quantified, and each PRP was mixed in equal quantities with cell culture medium and added to cartilage or meniscus explant cultures, with or without interleukin 1 $\beta$  (IL-1 $\beta$ ). Extracellular matrix synthesis was quantified over 24 hours via <sup>35</sup>S-sulfate and <sup>3</sup>H-proline incorporation, while gene expression of catabolic enzymes was evaluated using real-time PCR. **Results:** The platelet concentration in single-spin laboratory PRP was 59% higher than blood. Platelet and white blood cell concentrations in single-spin laboratory and kit PRP were not significantly different, while the double-spin kit resulted in approximately 2.5-fold higher platelet and approximately 400-fold higher white blood cell concentrations. In cartilage cultures without IL-1 $\beta$ , radiolabel incorporation in single-spin PRP cultures was significantly higher than in double-spin cultures. Similar results were obtained for <sup>35</sup>S-sulfate incorporation in meniscus cultures without IL-1 $\beta$ . In IL-1 $\beta$ , radiolabel incorporation was largely similar among all PRPs. After 24 hours of culture, ADAMTS-4 gene expression in cartilage was lowest for single-spin PRP, while expression in the double-spin kit was not significantly different from double-spin laboratory PRP in which platelets were concentrated 6-fold. **Conclusions:** This study suggests that single-spin PRP preparations may be the most advantageous for intra-articular applications and that double-spin systems should be considered with caution.

## Keywords

articular cartilage, growth factors, meniscus, osteoarthritis disease modification, other

## Introduction

Platelets represent an accessible and inexpensive source of growth factors that have recently received considerable attention for the treatment of injured or damaged musculoskeletal tissues.<sup>1</sup> Enthusiasm for the therapeutic potential of platelets is based on the  $\alpha$ -granules that contain growth factors such as transforming growth factor  $\beta$  (TGF $\beta$ ) and platelet-derived growth factor (PDGF).<sup>2</sup> The growth factor concentration in platelets, coupled with the platelet concentration in blood, is sufficient to yield nanograms of growth factor per milliliter of blood without increasing the native platelet concentration.<sup>3</sup>

Platelet growth factors are typically administered in plasma, the combination of which is known as platelet-rich plasma (PRP). PRP is prepared from whole blood using centrifugation steps that eliminate the majority of the red blood cells and concentrate platelets based on the specific gravities of each cell type.<sup>4</sup> In some cases, PRP protocols

result in a significant white blood cell content.<sup>5</sup> While extensive efforts are underway to understand the therapeutic benefit of PRP for tendon, ligament, and bone,<sup>1</sup> relatively few studies have evaluated the effect of PRP on articular cartilage and the meniscus. In a laboratory study, PRP was shown to stimulate chondrocyte extracellular matrix (ECM) synthesis.<sup>6</sup> When administered via intra-articular injection in ovine subjects, PRP increased cartilage healing in a microfracture treatment model for chondral defects.<sup>7</sup> In addition, PRP delivered in a gelatin carrier stimulated superior healing relative to platelet-poor plasma in a

<sup>1</sup>Department of Clinical Science, Orthopaedic Research Center, Colorado State University, Fort Collins, CO, USA

<sup>2</sup>Steadman Philippon Research Institute, Vail, CO, USA

### Corresponding Author:

John D. Kisiday, Orthopaedic Research Center, Colorado State University, 300 West Drake Road, Fort Collins, CO 80523  
Email: wli@csu.edu

meniscal defect model.<sup>8</sup> In human cases of chronic osteoarthritis, intra-articular injections of PRP resulted in improvements in clinical scores.<sup>9</sup> Based on these data, PRP may hold promise for stimulating tissue growth in degenerative or damaged cartilage and meniscus.

An important factor in characterizing the potential of PRP to heal joint tissues is to determine the dose of platelets that best stimulates cartilage or meniscal growth. PRP containing less than a 2-fold increase in platelet concentration relative to blood may be obtained by a single-spin protocol in which the red blood cells are pelleted and the entire plasma supernatant is collected.<sup>10</sup> To further concentrate the platelets, a second higher force spin may centrifuge the platelets into a pellet, after which the platelets are resuspended in a reduced plasma volume.<sup>4</sup> The platelet concentration in PRP can be at least 10-fold greater than that in whole blood, which results in TGF $\beta$ 1 concentrations in excess of 50 ng/mL.<sup>11,12</sup> Such TGF $\beta$ 1 concentrations may be favorable for stimulating ECM synthesis in articular joint tissues, as laboratory studies have demonstrated increasing proteoglycan synthesis with increasing TGF $\beta$ 1 concentration up to 10 or 100 ng/mL in cartilage<sup>13</sup> or meniscal<sup>14</sup> explants, respectively. Based on these data, we hypothesized that double-spin PRP preparations will stimulate higher ECM synthesis without a concomitant increase in catabolic activities in cartilage and meniscal explants than single-spin protocols in which the platelets are minimally concentrated. We tested this hypothesis using an explant laboratory model that used tissues harvested from adult horses, an ideal species for modeling human cartilage resurfacing.<sup>15</sup>

## Materials and Methods

### Equine Tissues

Tissues were obtained from 5 healthy 2- to 5-year-old horses. Prior to sedation for euthanasia, 50 mL of blood was drawn into 60-mL syringes preloaded with 5 mL of anticoagulant citrate dextrose using a 14-gauge medical-grade catheter. Following euthanasia, articular cartilage and menisci were retrieved from the femorotibial joint, equivalent to the human knee. Each femorotibial joint was grossly normal, and tissue samples were harvested within 3 hours of euthanasia. Full-thickness (approximately 1-2 mm) sections of articular cartilage were removed from the main load-bearing region of one medial femoral condyle and stored in sterile phosphate-buffered saline (PBS). Medial menisci were removed from each subject joint and stored in sterile PBS. Cartilage sections were divided into approximately 5-mm by 5-mm explants and placed in wells of a 48-well plate in low glucose DMEM (Invitrogen, Carlsbad, CA). Approximately 3-mm-thick meniscal explants were harvested from the femoral articulating surface of the avascular

portion (inner two thirds) of the medial menisci. As performed for cartilage, approximately 50-mg samples were cut from the explants and stored in 48-well plates in low glucose DMEM. Once all explants were distributed into wells (approximately 1 hour), the medium was replaced with PRP medium made as described below.

### Preparation of PRP and Culture Medium

PRP was processed within 2 hours of blood collection.

#### Commercial Kit Preparations of PRP

**Single spin.** PRP was prepared using Arthrex ACP (Naples, FL) by centrifuging the kit at 160g for 5 minutes and collecting the entire plasma supernatant above the red blood cell pellet. Hereafter, this PRP is referred to as “single-spin kit”.

**Double spin.** PRP from Harvest Technologies SmartPrep 2 (Plymouth, MA) was prepared according to the manufacturer’s protocol. Hereafter, this PRP is referred to as “double-spin kit”.

**Laboratory Preparations of PRP.** Blood was transferred to centrifuge tubes and spun at 200g for 18 minutes. The plasma was harvested down to the interface between the pelleted red blood cells. A portion of this PRP, referred to hereafter as “single-spin laboratory”, was set aside for experimentation. The remaining PRP was centrifuged at 1,000g for 15 minutes, which pelleted approximately 98% of the platelets (data not shown) and resulted in a platelet-poor plasma (PPP) supernatant. PPP was removed from each tube and was added back to the centrifuged platelets in reduced volumes that resulted in 3x, 6x, and 9x concentrations of platelets relative to single-spin laboratory PRP. Hereafter, these PRP preparations are referred to as “double-spin 3x”, “double-spin 6x”, and “double-spin 9x”, respectively. The platelets were resuspended by gently pipetting with a 5-mL pipette. All PRP preparations appeared yellow in color and absent of red discoloration that would indicate hemolysis.

**Cell Counts.** The platelet concentration in whole blood was determined via automated counts performed in the Diagnostic Laboratory at the Orthopaedic Research Center, Colorado State University. The concentration of platelets in PRP was determined by diluting 1:20 in PBS and manually counting on a hemocytometer. We confirmed that manual counts were not significantly different than automated counts (data not shown). White blood cells were counted using a hemocytometer. White blood cell differential counts were not performed.

**Culture Medium.** PRP was mixed with equal volumes of low glucose DMEM (containing 1.8 mM calcium chloride) supplemented with 10 mM HEPES, 0.1 mM nonessential amino acids (Sigma Chemical, St. Louis, MO), 20  $\mu$ g/mL ascorbate-2-phosphate (Wako Chemicals, Richmond, VA),

100 U/mL penicillin G, 100 ug/mL streptomycin, and 0.25 mg/mL amphotericin B. For each tissue and medium formulation, half of the cultures received 10 ng/mL of recombinant human IL-1 $\beta$  (R&D Systems, Minneapolis, MN) resuspended in PBS plus 0.1% bovine serum albumin (Sigma Chemical) to produce a proinflammatory environment, as previously performed for equine cartilage.<sup>16</sup>

### Evaluation of ECM Synthesis

For each condition and horse, one explant was evaluated for <sup>35</sup>S-sulfate and <sup>3</sup>H-proline incorporation as measures of proteoglycan and protein synthesis, respectively. Radiolabel medium was prepared by adding 5 uCi/mL of each isotope in volumes that were less than 1% of the total. After 24 hours of incubation in a cell culture environment, the explants were washed 5 times over 90 minutes in PBS plus 1 mM unlabeled proline and sulfate. Each explant was transferred to 1 mL of proteinase K–TRIS HCl solution and digested overnight at 60 °C. From the digests, radiolabel incorporation was measured via scintillation counting. These data were normalized to total DNA, which was evaluated by mixing aliquots of digest with Hoescht dye and quantifying against calf thymus DNA standards on a spectrofluorometer.<sup>17</sup>

### Gene Expression of Catabolic Enzymes

After 24 hours of culture, 4 additional explants per condition and horse were pooled, snap frozen and pulverized in liquid nitrogen, stabilized in TRIzol reagent (Invitrogen), and then homogenized for 50 seconds on ice. The samples were centrifuged for 10 minutes at 10,000g, and the supernatant was saved. Nucleic acids were extracted from the supernatant by adding 20% chloroform (volume/volume) and then centrifuging for 15 minutes at 12,000g. This step was repeated 3 to 5 times to ensure separation of the nucleic acids from the residual ECM.<sup>18</sup> The nucleic acids were precipitated by adding a 5x volume of isopropanol and then centrifuging for 10 minutes at 10,000g. The pellet was resuspended in nuclease-free water, and absorbance was measured at 260/280 nm to determine concentration and purity. For samples that resulted in 260/280 ratios less than 1.7, the RNA was further purified using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using 50  $\mu$ M random hexamers (Superscript III cDNA synthesis kit, Invitrogen). cDNA solution and TAQMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) were mixed with primers/probes for the genes of interest, and expression levels were determined using semi-quantitative real-time PCR conducted on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

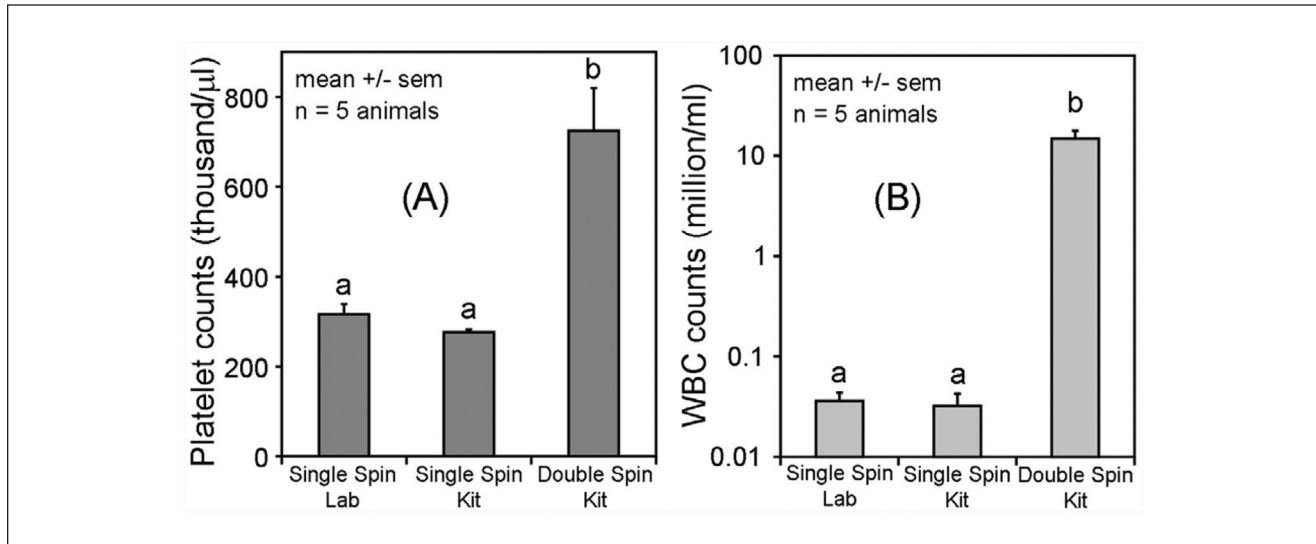
Primers/probes for ADAMTS-4, ADAMTS-5, MMP1, and GAPDH were designed and validated at our institution (ADAMTS-4: forward-TGTGATCGTGCATTGGCTCC, reverse-TGTTTGCTGCAGCTAGAACCATC, probe-AGTTTGACAAGTGCATGGTGTGCGGT; ADAMTS-5: forward-AAGGTGACTGATGGGACCGAATGT, reverse-TTTGAGCCAATGATGCCGTACAG, probe-AGGCCATACAGTAATTCCGTCTGCGT; MMP1: forward-ACTGCCAAATGGACTTCAAGCTGC, reverse-TCTTACAGTGCTAGGAAAGCCG, probe-CAGGATGTGCTCTACGGATACCCCAAGGAC; GAPDH: forward-AAGTGGATATTGTCGCCATCAAT, reverse-AACTTGCCATGGGTGGAATC, probe-TGACCTCAACTACATGGTCTACATGTTTCA). Primers and probes for MMP13 were purchased from the Lucy Whitter Molecular Core Facility (Davis, CA). Gene expression was normalized to the housekeeping gene GAPDH. For each gene, relative expression was calculated by calibrating to the average expression in single-spin laboratory cultures.<sup>19</sup>

### Evaluation of Growth Factors Released into the Medium

TGF $\beta$ 1 released to the culture medium in IL-1 $\beta$ -free cultures was evaluated using a commercial kit (R&D Systems, Minneapolis, MN) that has been validated for equine proteins.<sup>20</sup> Prior to the start of culture, baseline TGF $\beta$ 1 concentrations were evaluated for medium supplemented with single-spin laboratory, single-spin kit, and double-spin kit PRP. Following 24 hours of culture, media from IL-1 $\beta$ -free cartilage and meniscus cultures were evaluated for TGF $\beta$ 1 content.

### Data Analysis

The cellular content of single-spin laboratory PRP relative to blood was compared for 4 donor horses using a paired *t* test. The analysis of cartilage and meniscal explants was repeated for blood and tissue samples from 5 donor horses, with blood and tissues paired in an autologous fashion. TGF $\beta$ 1 concentration and gene expression data were log transformed to meet the assumption of normality as determined by residual plots. All data were analyzed using a mixed-model analysis of variance (Glimmix procedure, SAS 9.2, SAS Institute, Cary, NC), with the donor animal used as a random effect. Individual comparisons were made using a least-squares means procedure. For cell and TGF $\beta$ 1 concentration outcomes, PRP preparation was considered as the main effect. For radiolabel and gene expression outcomes, PRP preparation and IL-1 $\beta$  were considered main effects as well as their interactions. Individual comparisons of main effects or interactions were indicated based on a protected *F* test. *P* values less than 0.05 were



**Figure 1.** Cell content of laboratory, ACP, and SmartPrep preparations of PRP. **(A)** Platelet concentration. For each assay, different letters indicate significant differences ( $P < 0.05$ ). **(B)** White blood cell concentration. For each assay, different letters indicate significant differences ( $P < 0.05$ )

considered significant. Data are reported as mean  $\pm$  standard error of the mean.

## Results

### Cellular Content of PRP Preparations

Preliminary tests were performed to determine the extent to which platelets in single-spin laboratory PRP were concentrated relative to whole blood. The platelet concentration in blood was  $172 \pm 12$  thousand/ $\mu$ L, which is consistent with a previous report for equine blood (mean = 165 thousand/ $\mu$ L<sup>11</sup>). Following centrifugation, the platelet concentration was  $275 \pm 27$  thousand/ $\mu$ L, representing a 59% increase over blood ( $P < 0.01$ ). The packed cell volume, calculated as a percentage of the volume of the red blood cell pellet following centrifugation at 1,000g for 10 minutes relative to the total volume, was  $43\% \pm 1\%$ .

For PRP preparations used for cartilage and meniscus cultures, the concentration of platelets and white blood cells was determined for single-spin laboratory, single-spin kit, and double-spin kit PRP. The mean concentration of platelets in single-spin laboratory PRP ( $317 \pm 22$  thousand/ $\mu$ L) (**Fig. 1A**) was not significantly different from single-spin kit PRP ( $276 \pm 8$  thousand/ $\mu$ L) ( $P = 0.20$ ). Double-spin kit PRP contained 2.3- and 2.6-fold higher platelet concentrations ( $725 \pm 95$  thousand/ $\mu$ L) relative to single-spin laboratory and single-spin kit PRP, respectively. White blood cell counts in single-spin laboratory PRP ( $0.04 \pm 0.001$  thousand/ $\mu$ L) were not significantly different from single-spin kit PRP ( $0.03 \pm 0.01$  thousand/ $\mu$ L) ( $P = 0.98$ ) (**Fig. 1B**). Double-spin kit PRP contained at least a 400-fold higher concentration of white blood cells

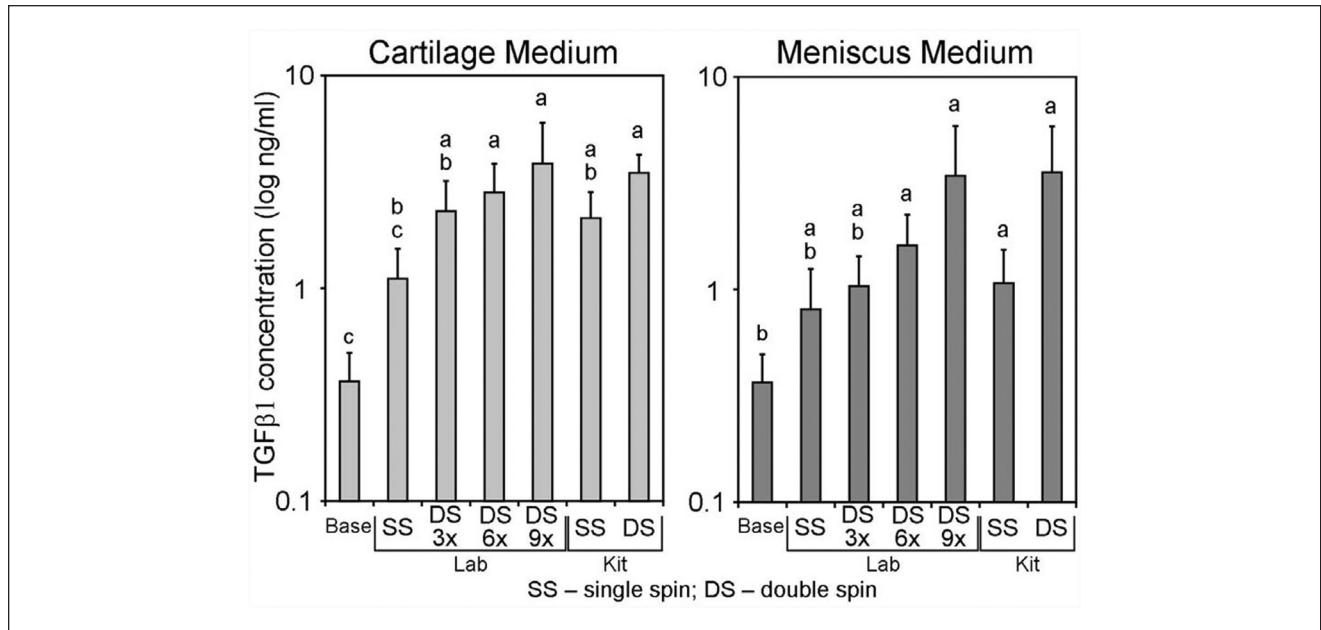
( $14.8 \pm 3.0$  thousand/ $\mu$ L) relative to single-spin laboratory and single-spin kit PRP.

### Concentration of TGF $\beta$ 1 in Culture Medium

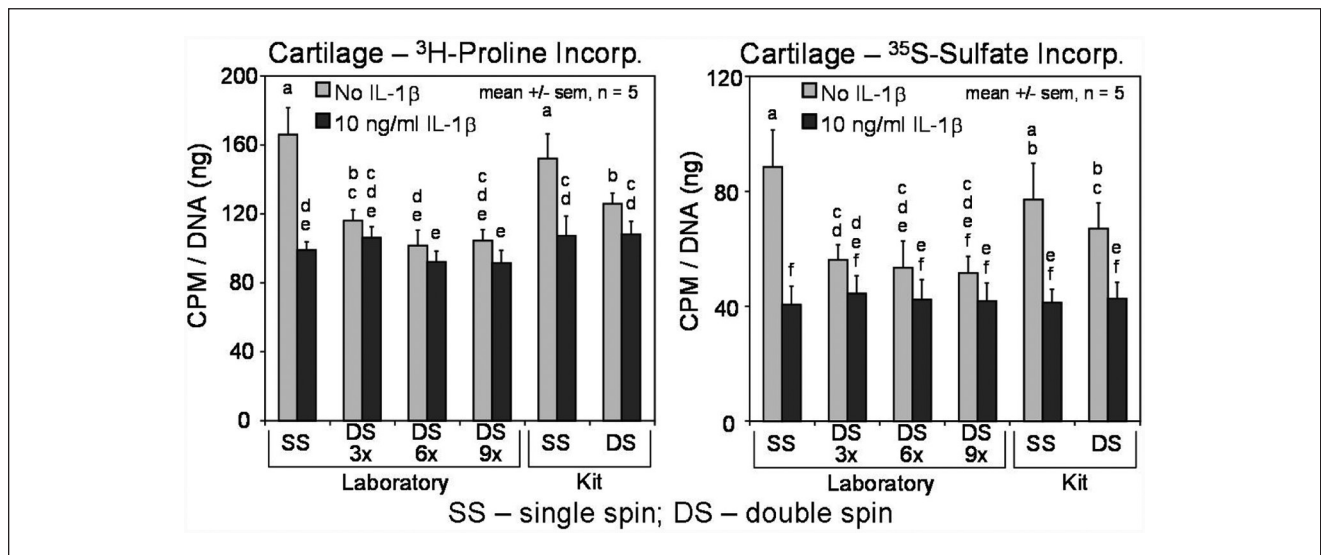
**Baseline TGF $\beta$ 1 in Uncultured Medium.** Prior to adding to explant cultures, samples of single-spin laboratory, single-spin kit, and double-spin kit medium were centrifuged to remove the cells, and the baseline TGF $\beta$ 1 content was measured. The concentrations of TGF $\beta$ 1 in uncultured single-spin laboratory ( $365 \pm 130$  pg/mL, abbreviated as "Base" in **Fig. 2**), single-spin kit ( $463 \pm 241$  pg/mL, data not shown), and double-spin kit ( $577 \pm 93$  pg/mL, data not shown) media were not significantly different ( $P = 0.42$ ).

**Cartilage Cultures.** Following 24 hours of culture in the presence of cartilage explants without IL-1 $\beta$ , all media contained significantly higher TGF $\beta$ 1 concentrations than baseline except for single-spin laboratory ( $P = 0.09$ ). TGF $\beta$ 1 concentrations in single-spin laboratory ( $1,108 \pm 426$  pg/mL) and kit ( $2,134 \pm 695$  pg/mL) media were not significantly different ( $P = 0.06$ ). The TGF $\beta$ 1 concentration in single-spin laboratory medium was significantly less than all double-spin PRP media except for double-spin 3x ( $2,302 \pm 891$  pg/mL) ( $P = 0.06$ ). The TGF $\beta$ 1 concentration in single-spin kit medium was not significantly different from all double-spin PRP media ( $P = 0.16-0.98$ ). The TGF $\beta$ 1 concentrations in double-spin 3x, 6x ( $2,818 \pm 1,028$  pg/mL), 9x ( $3,852 \pm 2,152$  pg/mL), and kit ( $3,552 \pm 2,283$  pg/mL) media were not significantly different ( $P = 0.17-0.75$ ).

**Meniscus Cultures.** In IL-1 $\beta$ -free meniscus cultures, TGF $\beta$ 1 concentrations increased between single-spin laboratory ( $806 \pm 442$  pg/mL) and double-spin 9x ( $3,427 \pm 2,432$  pg/mL) media, although only double-spin 6x and 9x media were



**Figure 2.** TGFβ1 concentration in media following 24 hours of culture in the absence of IL-1β. “Base” represents the concentration of TGFβ1 in laboratory PRP prior to adding to explant cultures. For each tissue, different letters indicate significant differences ( $P < 0.05$ ).



**Figure 3.** Radiolabel incorporation for cartilage explant cultures. The combined effects of PRP formulations and IL-1β conditions were evaluated for  $^3\text{H}$ -proline or  $^{35}\text{S}$ -sulfate. For each assay, different letters indicate significant differences ( $P < 0.05$ ).

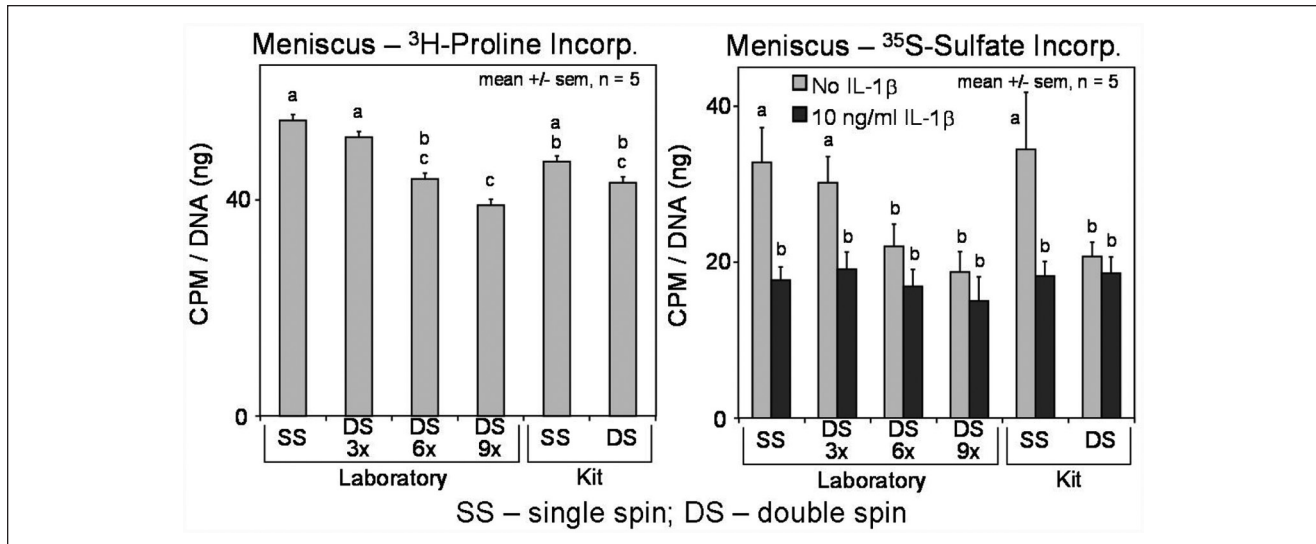
higher than baseline (**Fig. 2**). TGFβ1 concentrations in single-spin ( $1,071 \pm 465$  pg/mL) and double-spin ( $3,552 \pm 2,283$  pg/mL) kit media were not significantly different from each other ( $P = 0.39$ ) and the laboratory preparations ( $P = 0.07$ - $0.98$ ).

## ECM Synthesis

### Cartilage

**$^3\text{H}$ -proline.** In the absence of IL-1β,  $^3\text{H}$ -proline incorporation in single-spin laboratory cultures was not significantly

different from single-spin kit cultures ( $P = 0.22$ ) (**Fig. 3**).  $^3\text{H}$ -proline incorporation in single-spin laboratory PRP was 31% to 64% higher than double-spin 3x, 6x, and 9x and double-spin kit cultures. In single-spin kit cultures,  $^3\text{H}$ -proline incorporation was 31% and 20% higher than double-spin 3x and double-spin kit cultures, respectively. In double-spin kit cultures,  $^3\text{H}$ -proline incorporation was not significantly different from double-spin 3x PRP ( $P = 0.25$ ). No significant differences were found among double-spin 3x, 6x, and 9x cultures ( $P = 0.07$ - $0.58$ ).  $^3\text{H}$ -proline incorporation decreased with the addition of IL-1β for



**Figure 4.** Radiolabel incorporation for meniscus explant cultures. For <sup>3</sup>H-proline, the main effect of PRP formulation was evaluated. The combined effects of PRP formulations and IL-1 $\beta$  conditions were evaluated for <sup>35</sup>S-sulfate. For each assay, different letters indicate significant differences ( $P < 0.05$ ).

single-spin laboratory cultures only. In IL-1 $\beta$  medium, <sup>3</sup>H-proline incorporation was not significantly different among single-spin laboratory, double-spin 3x, and single- and double-spin kit cultures ( $P = 0.22-0.98$ ). <sup>3</sup>H-proline incorporation in double-spin 6x and 9x cultures was approximately 15% lower than single- and double-spin kit cultures.

**<sup>35</sup>S-sulfate.** In the absence of IL-1 $\beta$ , <sup>35</sup>S-sulfate incorporation in single-spin laboratory cultures was not significantly different from single-spin kit cultures ( $P = 0.28$ ) (**Fig. 3**). <sup>35</sup>S-sulfate incorporation in single-spin laboratory cultures was 32% to 72% higher than double-spin 3x, 6x, and 9x and double-spin kit cultures. <sup>35</sup>S-sulfate incorporation in single-spin kit cultures was 38% to 50% higher than that in double-spin 3x, 6x, and 9x cultures. No significant differences were detected among double-spin 3x, 6x, and 9x PRP ( $P = 0.47-0.97$ ). <sup>35</sup>S-sulfate incorporation in double-spin kit cultures was not significantly different from single-spin kit and double-spin 3x, 6x, and 9x PRP cultures ( $P = 0.07-0.27$ ). <sup>35</sup>S-sulfate incorporation decreased with the addition of IL-1 $\beta$  for single-spin laboratory, single-spin kit, and double-spin kit cultures. In IL-1 $\beta$  cultures, <sup>35</sup>S-sulfate incorporation among all media was not significantly different ( $P = 0.47-0.99$ ).

### Meniscus

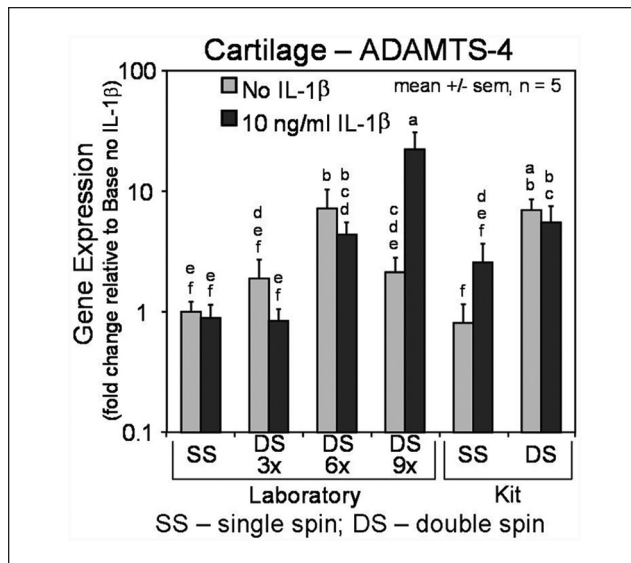
**<sup>3</sup>H-proline.** Interactions between PRP preparations and IL-1 $\beta$  were not significant ( $P = 0.78$ ). When considering the effect of PRP independently of IL-1 $\beta$ , <sup>3</sup>H-proline incorporation in single-spin laboratory and double-spin 3x cultures was not significantly different ( $P = 0.46$ ) (**Fig. 4**). <sup>3</sup>H-proline incorporation in double-spin 6x and 9x cultures was not

significantly different ( $P = 0.15$ ), and both were significantly lower than single-spin laboratory and double-spin 3x cultures. Single-spin kit cultures were not significantly different from all other cultures ( $P = 0.06-0.35$ ) except for double-spin 9x PRP. <sup>3</sup>H-proline incorporation in double-spin kit cultures was less than single-spin laboratory and double-spin 3x cultures only.

**<sup>35</sup>S-sulfate.** In the absence of IL-1 $\beta$ , <sup>35</sup>S-sulfate incorporation was not significantly different among single-spin laboratory, double-spin 3x, and single-spin kit cultures ( $P = 0.28-0.67$ ) (**Fig. 4B**). <sup>35</sup>S-sulfate incorporation in double-spin 6x and 9x and double-spin kit cultures was not significantly different ( $P = 0.40-0.74$ ). <sup>35</sup>S-sulfate incorporation in double-spin 6x and 9x and double-spin kit cultures was approximately 40% less than single-spin laboratory, double-spin 3x, and single-spin kit cultures. <sup>35</sup>S-sulfate incorporation decreased with the addition of IL-1 $\beta$  for single-spin laboratory, double-spin 3x, and single-spin kit PRP. In IL-1 $\beta$  cultures, <sup>35</sup>S-sulfate incorporation among all media was not significantly different ( $P = 0.30-0.89$ ).

### Gene Expression of Catabolic Enzymes

**Cartilage.** Interactions between PRP formulation and IL-1 $\beta$  were not significant for MMP1 ( $P = 0.47$ ), MMP13 ( $P = 0.12$ ), and ADAMTS-5 ( $P = 0.19$ ). When considering the effect of IL-1 $\beta$ , MMP1 expression increased (1.6-fold,  $P < 0.001$ ) and ADAMTS-5 expression decreased (3.0-fold,  $P < 0.001$ ) with IL-1 $\beta$  treatment (data not shown). In the absence of IL-1 $\beta$ , ADAMTS-4 expression in double-spin 6x cultures was 3.4- to 8.9-fold higher than single-spin laboratory, single-spin kit, and double-spin 3x cultures (**Fig. 5**). ADAMTS-4



**Figure 5.** Normal tissue: Gene expression of ADAMTS-4 in cartilage explant cultures. Expression levels were normalized to single-spin laboratory cultures, with values less than 1 representing a relative decrease in expression. Different letters indicate significant differences ( $P < 0.05$ ).

expression in double-spin kit cultures was not significantly different from double-spin 6x cultures ( $P = 0.78$ ) and significantly higher than all other IL-1 $\beta$ -free cultures. The addition of IL-1 $\beta$  to the culture medium increased ADAMTS-4 expression for double-spin 9x cultures (11-fold) only. In IL-1 $\beta$  medium, ADAMTS expression was not significantly different between single-spin laboratory and double-spin 3x cultures ( $P = 0.97$ ). ADAMTS-4 expression increased approximately 5-fold for double-spin 6x and 22-fold for double-spin 9x cultures. ADAMTS-4 expression in single-spin kit cultures was not significantly different from all laboratory preparations ( $P = 0.16$ - $0.18$ ) except for double-spin 9x. ADAMTS-4 expression in double-spin kit cultures was 2.1-fold higher than single-spin kit, approximately 5-fold higher than single-spin laboratory and 3x, and not significantly different from single-spin 6x ( $P = 0.68$ ) cultures.

**Meniscus.** Expression levels of MMP1 and MMP13 were not analyzed, as many samples resulted in expression levels near the PCR detection limit. Interactions between PRP formulations and IL-1 $\beta$  as well as both main effects were not significant for ADAMTS-4 ( $P = 0.19$ - $0.48$ ) and ADAMTS-5 ( $P = 0.07$ - $0.33$ ) (data not shown).

## Discussion

We evaluated the influence of different PRP formulations on anabolic and catabolic activities in cartilage and meniscus

explants in different PRP formulations created using laboratory processing and commercial kits. In single-spin laboratory PRP, the increase in platelet concentration over whole blood (59%) was consistent with previous reports for single-spin processing of human blood (51%).<sup>10</sup> Given that the vast majority of the packed cell volume (43% here) consists of red blood cells, the increase in platelet concentration in single-spin laboratory PRP over blood may be largely attributed to the replacement of the red blood cell volume with plasma and platelets during low speed centrifugation. For example, the ideal exchange of these volumes would increase the percentage of plasma and platelets in the supernatant region from 57% to 100%, for a corresponding 75% increase in platelet concentration. The platelet concentration in double-spin kit PRP ( $725 \pm 95$  thousand/ $\mu$ L) exceeded that of both single-spin PRPs and was comparable to a previous study ( $513 \pm 85$  thousand/ $\mu$ L) that produced equine PRP using the SmartPrep system (Harvest Technologies).<sup>20</sup> The double-spin 6x and 9x PRP preparations represent higher platelet concentrations than were achieved by either commercial kit but may be obtained using other methods.

The centrifugation protocols used for laboratory processing ( $\leq 1,000g$ ) and the single-spin kit (260g) generate forces that are at least 3 times less than a well-established protocol for human blood that does not harm platelets.<sup>21</sup> Furthermore, the pipetting used to process each preparation was less vigorous than injections through small bore needles that were found not to harm equine platelets.<sup>22</sup> Therefore, it is likely that little platelet activation was induced during processing of laboratory and single-spin kit PRP. Information on the centrifugation protocols used for the double-spin kit was not available from the manufacturer, although previous evaluating of PRP from this kit<sup>23</sup> and the lack of significant differences in TGF $\beta$ 1 concentration in uncultured PRP medium here suggest that the double-spin kit stimulates little platelet activation. The PRP medium was added to the explant cultures without performing additional steps to ensure that all growth factors were released from the platelets. We elected to test PRP in this manner, as collagen induces platelet degranulation,<sup>20</sup> and the large collagenous articular surfaces *in vivo* may render an *ex vivo* activation step unnecessary. Also, the small amount of calcium chloride in the culture medium may have contributed to platelet activation, although typically 500-fold higher concentrations of calcium chloride (10% w/v) are used to rapidly activate platelets. The approximately 10-fold increase in TGF $\beta$ 1 concentration between single-spin laboratory and double-spin 9x PRP cultures suggested that platelet degranulation occurred in explant cultures. However, TGF $\beta$ 1 concentration proved to be a poor indicator of the influence of platelet growth factors on cartilage and meniscus biosynthesis given the limited number of significant differences in TGF $\beta$ 1 concentration

among PRP preparations. High variability in TGF $\beta$ 1 concentrations in PRP has been previously reported. TGF $\beta$ 1 concentration showed a poor correlation with platelet concentration in human PRP.<sup>3</sup> In addition, our data were consistent with TGF $\beta$ 1 concentrations in unactivated equine PRP following exposure to tendon explants.<sup>20</sup>

In cartilage cultures in the absence of IL-1 $\beta$ , radiolabel incorporation was highest for single-spin laboratory PRP and decreased in double-spin laboratory PRP formulations. A similar trend was found for <sup>35</sup>S-sulfate incorporation in meniscus explants cultured in the absence of IL-1 $\beta$ , with biosynthesis decreasing between double-spin 3x and 6x PRP. In the presence of IL-1 $\beta$ , no significant differences in radiolabel incorporation in cartilage and meniscal explants were detected among PRP preparations. Taken together, these data do not support the hypothesis that concentration of platelets in PRP stimulates cartilage and meniscal biosynthesis over PRP in which the platelets are minimally concentrated.

When considering the effect of IL-1 $\beta$ , only single-spin laboratory PRP consistently resulted in a significant decrease in biosynthesis upon the addition of IL-1 $\beta$ . These results were somewhat unexpected given that <sup>35</sup>S-sulfate incorporation in cartilage explants has been reported to decrease in the presence of IL-1 $\beta$ ,<sup>24-26</sup> although biosynthesis in meniscal explants has been less sensitive to IL-1 $\beta$ .<sup>27</sup> While additional studies are necessary to define more detailed interactions between PRP formulation and IL-1 $\beta$ , it is possible that high densities of platelets produced an inflammatory response in chondrocytes, meniscus cells, or white blood cells within the PRP preparations to which 10 ng/mL of IL-1 $\beta$  was minimally additive. For example, <sup>35</sup>S-sulfate incorporation in cartilage has been shown to decrease relative to IL-1 $\beta$ -free controls at concentrations as low as 0.01 ng/mL<sup>25</sup> and 0.1 ng/mL.<sup>24,26</sup> In each of these dose titration studies, modest to no reduction in <sup>35</sup>S-sulfate incorporation was reported between 1 and 10 ng/mL of IL-1 $\beta$ . Given the 10-ng/mL concentration of IL-1 $\beta$  used here, it appears possible that a modest proinflammatory effect of high platelet concentrations may have been sufficient to decrease biosynthesis to nearly that of the recombinant IL-1 $\beta$  cultures.

With the exception of <sup>3</sup>H-proline incorporation in meniscal explants, radiolabel incorporation in the absence of IL-1 $\beta$  for commercial PRP preparations coincided with biosynthesis in laboratory PRP that contained the most similar platelet concentration. Based on these data, we speculate that the single-spin procedure may be more the appropriate closed system kit of the 2 evaluated in this study for intra-articular applications. In addition, the measures of biosynthesis for double-spin 6x and 9x PRP cultures suggest that commercial kits that concentrate platelets to a greater degree than that found in this study would not provide benefit over minimally concentrated platelet preparations for

intra-articular applications. These data are relevant to the choice of commercial PRP kits, as the majority of closed system kits that have been characterized for cellular content have been reported to contain at least a 2-fold concentration of platelets over whole blood.<sup>28,29</sup>

The different PRP formulations had little effect on gene expression of catabolic enzymes associated with a proinflammatory environment,<sup>30</sup> with significant interactions between PRP formulations and IL-1 $\beta$  detected for ADAMTS-4 in cartilage only. The role of ADAMTS-4 and -5 in human cartilage degradation has not been determined,<sup>31</sup> although ADAMTS-4 and -5 have been associated with diseased equine cartilage<sup>32</sup>; therefore, the observed differences in ADAMTS-4 expression here may be suggestive of an important catabolic process in equine cartilage. Based on the laboratory preparations resulting in an increase in ADAMTS-4 gene expression between double-spin 3x and 6x PRP, these data further suggest that high platelet concentrations in PRP may actually produce a proinflammatory environment for cartilage.

In single-spin kit PRP cultures, ADAMTS-4 expression was not significantly different from single-spin laboratory PRP. Conversely, double-spin kit PRP cultures resulted in ADAMTS-4 expression that was higher than double-spin 3x PRP in which the platelet concentration was most similar. A major difference in cellular composition of PRP preparations that may account for this result is the high concentration of white blood cells in double-spin kit PRP ( $14.8 \pm 3.0$  thousand/ $\mu$ L) that exceeds the normal white blood count that we have historically recorded for young adult horse blood in our laboratory ( $<5$  thousand/ $\mu$ L). It has been suggested that the leukocyte population in PRP enriched with white blood cells is proinflammatory,<sup>33</sup> a theory that is supported by a report of increased MMP expression in tendon explants cultured in white blood cell-rich PRP.<sup>20</sup> While the influence of white blood cells in PRP for clinical applications remains controversial,<sup>34</sup> and additional studies are necessary to better understand the influence of leukocytes in PRP on catabolism, our data suggest that white blood cells in PRP may be proinflammatory for cartilage.

This study evaluated PRP formulations across a range of platelet concentrations as an indicator of whether differences exist among the various PRP kits that are commercially available. A limitation of this study was the lack of a synovial fluid control group, which would have helped support or refute the hypothesis that PRP enhances biosynthesis over the natural joint environment. In addition, when we project the results of *in vitro* studies to clinical applications, certain limitations of laboratory models must be considered. While the dilution of PRP by 50% was intended to mimic the dilution of an intra-articular injection in synovial fluid, tissue culture does not allow for clearance of the growth factors<sup>35</sup> as may occur in joints. Therefore, the



laboratory model may overestimate the concentration of growth factors that is present *in vivo*. Based on our growth factor analysis, equine platelets contain less TGF $\beta$ 1 than has been reported for human preparations, although the extent to which other platelet-derived factors may differ among species is unknown. Most importantly, the acute response of cartilage and meniscal explants does not necessarily recapitulate the response that occurs over weeks of clinical healing. While *in vivo* testing will be necessary to address these limitations, the present study rejects the hypothesis that the concentration of platelets in PRP stimulates ECM synthesis in cartilage and the meniscus; furthermore, these findings suggest that high platelet concentrations for intra-articular injection should be considered with caution.

### Acknowledgments and Funding

This work was funded by the Steadman Philippon Research Institute and by discretionary funds from the Orthopaedic Research Center at Colorado State University. The authors thank Dr. David Karli of the Steadman Clinic for his technical assistance.

### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Drs. McIlwraith and Frisbie are consultants for Arthrex, the company that manufactures the ACP kit used in this study.

### References

- Rodeo SA, Delos D, Weber A, Ju X, Cunningham ME, Fortier L, et al. What's new in orthopaedic research. *J Bone Joint Surg Am.* 2010;92(14):2491-501.
- Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets.* 2001;12(5):261-73.
- Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg.* 2004;114(6):1502-8.
- Gonshor A. Technique for producing platelet-rich plasma and platelet concentrate: background and process. *Int J Periodontics Restorative Dent.* 2002;22(6):547-57.
- Everts PA, van Zundert A, Schonberger JP, Devilee RJ, Knape JT. What do we use: platelet-rich plasma or platelet-leukocyte gel? *J Biomed Mater Res A.* 2008;85(4):1135-6.
- Akeda K, An HS, Okuma M, Attawia M, Miyamoto K, Thonar EJ, et al. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis Cartilage.* 2006;14(12):1272-80.
- Milano G, Sanna Passino E, Deriu L, Careddu G, Manunta L, Manunta A, et al. The effect of platelet rich plasma combined with microfractures on the treatment of chondral defects: an experimental study in a sheep model. *Osteoarthritis Cartilage.* 2010;18(7):971-80.
- Ishida K, Kuroda R, Miwa M, Tabata Y, Hokugo A, Kawamoto T, 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):1103-12.
- Kon E, Buda R, Filardo G, Di Martino A, Timoncini A, Cenacchi A, et al. Platelet-rich plasma: intra-articular knee injections produced favorable results on degenerative cartilage lesions. *Knee Surg Sports Traumatol Arthrosc.* 2010;18(4):472-9.
- Tamimi FM, Montalvo S, Tresguerres I, Blanco Jerez L. A comparative study of 2 methods for obtaining platelet-rich plasma. *J Oral Maxillofac Surg.* 2007;65(6):1084-93.
- Sutter WW, Kaneps AJ, Bertone AL. Comparison of hematologic values and transforming growth factor-beta and insulin-like growth factor concentrations in platelet concentrates obtained by use of buffy coat and apheresis methods from equine blood. *Am J Vet Res.* 2004;65(7):924-30.
- Zimmermann R, Jakubietz R, Jakubietz M, Strasser E, Schlegel A, Wiltfang J, et al. Different preparation methods to obtain platelet components as a source of growth factors for local application. *Transfusion.* 2001;41(10):1217-24.
- Morales TI, Roberts AB. Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. *J Biol Chem.* 1988;263(26):12828-31.
- Imler SM, Doshi AN, Levenston ME. Combined effects of growth factors and static mechanical compression on meniscus explant biosynthesis. *Osteoarthritis Cartilage.* 2004;12(9):736-44.
- Frisbie DD, Bowman SM, Colhoun HA, DiCarlo EF, Kawcak CE, McIlwraith CW. Evaluation of autologous chondrocyte transplantation via a collagen membrane in equine articular defects: results at 12 and 18 months. *Osteoarthritis Cartilage.* 2008;16(6):667-79.
- Little CB, Flannery CR, Hughes CE, Goodship A, Caterson B. Cytokine induced metalloproteinase expression and activity does not correlate with focal susceptibility of articular cartilage to degeneration. *Osteoarthritis Cartilage.* 2005;13(2):162-70.
- Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem.* 1988;174(1):168-76.
- Upton ML, Chen J, Guilak F, Setton LA. Differential effects of static and dynamic compression on meniscal cell gene expression. *J Orthop Res.* 2003;21(6):963-9.
- 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):402-8.
- McCarrel T, Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. *J Orthop Res.* 2009;27(8):1033-42.
- Slichter SJ, Harker LA. Preparation and storage of platelet concentrates, I: factors influencing the harvest of viable platelets from whole blood. *Br J Haematol.* 1976;34(3):395-402.

22. Textor JA, Norris JW, Tablin F. Effects of preparation method, shear force, and exposure to collagen on release of growth factors from equine platelet-rich plasma. *Am J Vet Res.* 2011;72(2):271-8.
23. Schnabel LV, Mohammed HO, Miller BJ, McDermott WG, Jacobson MS, Santangelo KS, *et al.* Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J Orthop Res.* 2007;25(2):230-40.
24. Pratta MA, Di Meo TM, Ruhl DM, Amer EC. Effect of interleukin-1-beta and tumor necrosis factor-alpha on cartilage proteoglycan metabolism in vitro. *Agents Actions.* 1989;27(3-4):250-3.
25. Takafuji VA, McIlwraith CW, Howard RD. Effects of equine recombinant interleukin-1alpha and interleukin-1beta on proteoglycan metabolism and prostaglandin E2 synthesis in equine articular cartilage explants. *Am J Vet Res.* 2002;63(4):551-8.
26. Tyler JA. Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. *Biochem J.* 1989;260(2):543-8.
27. Shin SJ, Fermor B, Weinberg JB, Pisetsky DS, Guilak F. Regulation of matrix turnover in meniscal explants: role of mechanical stress, interleukin-1, and nitric oxide. *J Appl Physiol.* 2003;95(1):308-13.
28. Castillo TN, Pouliot MA, Kim HJ, Dragoo JL. Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. *Am J Sports Med.* 2010;39(2):266-71.
29. Leitner GC, Gruber R, Neumuller J, Wagner A, Kloimstein P, Hocker P, *et al.* Platelet content and growth factor release in platelet-rich plasma: a comparison of four different systems. *Vox Sang.* 2006;91(2):135-9.
30. Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms involved in cartilage proteoglycan catabolism. *Matrix Biol.* 2000;19(4):333-44.
31. Fosang AJ, Rogerson FM. Identifying the human aggrecanase. *Osteoarthritis Cartilage.* 2010;18(9):1109-16.
32. Kamm JL, Nixon AJ, Witte TH. Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine Vet J.* 2010;42(8):693-9.
33. Anitua E, Sanchez M, Orive G, Andia I. Shedding light in the controversial terminology for platelet rich products. *J Biomed Mater Res A.* 2009;90(4):1262-3.
34. Ehrenfest DM, Bielecki T, Del Corso M, Inchingolo F, Sammartino G. Shedding light in the controversial terminology for platelet-rich products: platelet-rich plasma (PRP), platelet-rich fibrin (PRF), platelet-leukocyte gel (PLG), preparation rich in growth factors (PRGF), classification and commercialism. *J Biomed Mater Res A.* 2010;95(4):1280-2.
35. Sprugel KH, McPherson JM, Clowes AW, Ross R. Effects of growth factors in vivo, I: cell ingrowth into porous subcutaneous chambers. *Am J Pathol.* 1987;129(3):601-13.