



Pooled Platelet-Rich Plasma Lysate Therapy Increases Synoviocyte Proliferation and Hyaluronic Acid Production While Protecting Chondrocytes From Synoviocyte-Derived Inflammatory Mediators

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Platelet-rich plasma (PRP) preparations are being used with moderate success to treat osteoarthritis (OA) in humans and in veterinary species. Such preparations are hindered, however, by being autologous in nature and subject to tremendous patient and processing variability. For this reason, there has been increasing interest in the use of platelet lysate preparations instead of traditional PRP. Platelet lysate preparations are acellular, thereby reducing concerns over immunogenicity, and contain high concentrations of growth factors and cytokines. In addition, platelet lysate preparations can be stored frozen for readily available use. The purpose of this study was to evaluate the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. Our hypothesis was that PRP-L treatment of inflamed synoviocytes would protect chondrocytes challenged with synoviocyte conditioned media by reducing synoviocyte pro-inflammatory cytokine production while increasing synoviocyte anti-inflammatory cytokine production. Synoviocytes were stimulated with either interleukin-1 β (IL-1 β) or lipopolysaccharide (LPS) for 24 h followed by no treatment or treatment with platelet-poor plasma lysate (PPP-L) or PRP-L for 48 h. Synoviocyte growth was evaluated at the end of the treatment period and synoviocyte conditioned media was assessed for concentrations of hyaluronic acid (HA), IL-1 β , tumor necrosis factor alpha (TNF- α), and interleukin-6 (IL-6). Chondrocytes were then challenged for 48 h with synoviocyte conditioned media from each stimulation and treatment group and examined for gene expression of collagen types I (COL1A1), II (COL2A1), and III (COL3A1), aggrecan (ACAN), lubricin (PRG4), and matrix metalloproteinase 3 (MMP-3) and 13 (MMP-13). Treatment of inflamed synoviocytes with PRP-L resulted in increased synoviocyte growth and increased synoviocyte HA and IL-6 production. Challenge of

chondrocytes with conditioned media from PRP-L treated synoviocytes resulted in increased collagen type II and aggrecan gene expression as well as decreased MMP-13 gene expression. The results of this study support continued investigation into the use of pooled PRP-L for the treatment of osteoarthritis and warrant further *in-vitro* studies to discern the mechanisms of action of PRP-L.

Keywords: platelet-rich plasma lysate, osteoarthritis, IL-1 β , LPS, HA, collagen type II, aggrecan, MMP-13

INTRODUCTION

Intra-articular injections of autologous platelet-rich plasma (PRP) are commonly used to treat osteoarthritis (OA) in humans and veterinary species, including horses and dogs (1–16). There is tremendous variability, however, in the composition of PRP generated based on the systemic health and hydration status, sex, and age of the patient, the quality of the venipuncture technique, the system or processing methods used, and whether or not the PRP is activated prior to injection (4, 17–21). The end result of such variability is large differences in platelet concentration, and therefore, growth factor and cytokine concentrations as well as leukocyte concentration within the products being used (4, 17–21). While classification systems have been put in place to define leukocyte-poor and leukocyte-rich PRPs (22–25), there is still controversy over which preparation is most efficacious for the treatment of OA and other musculoskeletal diseases (1, 4, 26–30). In addition, the optimal concentration of platelets within these preparations has yet to be elucidated (4, 26).

There has been increasing interest in the use of platelet lysate (PL) instead of PRP as PL is an acellular preparation containing high concentrations of growth factors and cytokines (31–48). The acellular nature of PL is important because it has the potential to be used in an allogeneic manner with further processing to remove immunoglobulins and also because it can be quality tested and then stored frozen to have available for immediate patient use (31–48). Furthermore, the use of pooled PL, or PL generated from multiple healthy donors, is being explored to capitalize on the natural variability that exists between individuals and the growth factors and cytokines that are released from their platelets upon lysis. This concept of optimal pooled PL has been investigated both for the use of PL as a non-immunogenic serum substitute for cell culture as well as for the use of PL as a clinical treatment (34, 41, 49).

A recent study evaluating the ability of equine PL preparations to modulate the innate immune responses of equine monocytes found interesting results when comparing data obtained from six individual PL preparations to data obtained from a pooled PL preparation created from those same six PL preparations (41). Notably, while none of the six individual PL preparations lead to significantly reduced tumor necrosis alpha (TNF- α) production from monocytes compared to fetal bovine serum (FBS), the pooled PL preparation did. Similarly, the pooled PL preparation dramatically reduced the variability observed in individual PL preparations for monocyte production of interleukin-1 β (IL-1 β) and interleukin-10 (IL-10). Lastly, the pooled PL preparation in this study was found to significantly decrease the production of

both TNF- α and IL-1 β by lipopolysaccharide (LPS) stimulated monocytes compared to controls (41). These results suggest that pooled PL preparations reduce variability and increase efficacy compared to individual PL preparations and that pooled PL preparations should be further examined as a means to suppress inflammation (41).

The aim of this study was to examine the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. Our hypothesis was that PRP-L treatment of inflamed synoviocytes would protect chondrocytes challenged with synoviocyte conditioned media by reducing synoviocyte pro-inflammatory cytokine production and increasing synoviocyte anti-inflammatory cytokine production. In particular, we expected chondrocytes challenged with conditioned media from IL-1 β or LPS stimulated synoviocytes treated with PRP-L to have increased gene expression of collagen type II and decreased gene expression of MMP-3 and MMP-13 compared to conditioned media from non-treated or platelet-poor plasma lysate (PPP-L) treated synoviocytes.

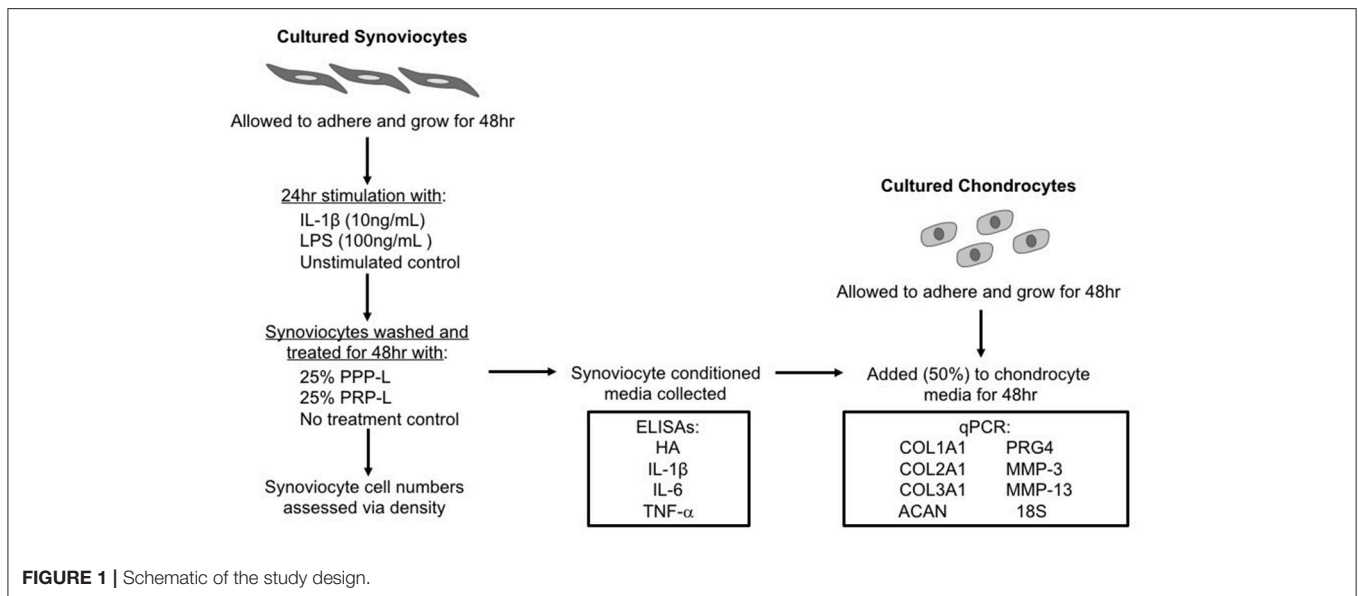
MATERIALS AND METHODS

Study Design

A schematic of the study design is shown in **Figure 1**. The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

Platelet-Poor Plasma Lysate and Platelet-Rich Plasma Lysate Preparation

Whole blood was collected via jugular venipuncture from 6 healthy horses in our closed research herd into four 60 mL syringes containing 6 mL of acid citrate dextrose (ACD) each for a total volume of 240 mL per horse. These horses included 3 geldings and 3 nonparous mares between the ages of 6 and 19 years. Routine automated complete blood counts and platelet counts were performed on each whole blood sample. Erythrocytes were allowed to settle for 30 min in the syringes and the layer above the erythrocytes containing the leukocytes, platelets, and plasma (approximately 120 mL) was then transferred to a 50 mL conical tube and centrifuged at 250 g for 15 min. From each conical, the supernatant above the leukocyte pellet containing the platelets and plasma was then harvested and centrifuged at 1,500 g for 15 min. From this spin, the supernatant containing the platelet-poor plasma (PPP) was removed and saved. The platelet pellet was then



resuspended in 12 mL of PPP to generate platelet-rich plasma (PRP) of approximately 10x the concentration of whole blood. Platelet numbers in PPP and PRP samples were determined by staining platelets with 1 μ M Calcein-AM (Invitroge™ Molecular Probes™, ThermoFisher Scientific, Waltham, MA, USA), incubating for 20 min, and then counting the number of fluorescent cells using a Cellometer® Auto 2000 (Nexcelom Bioscience LLC, Lawrence, MA, USA). White blood cell (WBC) counts in PPP and PRP samples were determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). To generate PPP and PRP lysate, (PPP-L and PRP-L, respectively), the PPP and PRP then underwent five freeze/thaw cycles in liquid nitrogen. The majority of cell debris was removed from all PPP-L and PRP-L samples by centrifugation at 20,000 g for 20 min. PPP-L and PRP-L samples were then clarified by depth filtration using the ZetaPlus™ BC25 Capsule Filter, Medi 90ZB (3M Purification Inc., St Paul, MN, USA). The resultant PPP-L and PRP-L samples were then pooled, respectively, from all 6 horses and frozen at -80°C until use.

Synoviocyte Isolation

Synovium was harvested from the femoropatellar joints of 5 systemically healthy horses (ages 2–14 years) euthanized for reasons other than this study and free of femoropatellar joint disease. The isolated synovium was weighed and digested for 2 h at 37°C under constant rotation with synoviocyte media [high glucose (4.5 g/L) DMEM medium with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, penicillin (100 units/mL), and streptomycin (100 μ g/ml)] added at 10 mL/g tissue and containing 1.5 mg/mL Gibco® collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (50, 51). The resulting digest was passed through a 100 μ m filter and centrifuged at 800 g for 10 min. The cell pellet was then washed twice with fresh synoviocyte media and

live synoviocyte count was determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). Synoviocytes were frozen in aliquots of 10×10^6 cells/mL in liquid nitrogen until use.

Chondrocyte Isolation

Cartilage was harvested from the femoral trochlear ridges of a 2-year-old Thoroughbred gelding free of orthopedic disease and euthanized for reasons other than this study. The isolated cartilage was weighed and digested overnight (16–18 h) at 37°C under constant rotation with chondrocyte media [Ham's F12 medium with 10% FBS, 25 mM HEPES, ascorbic acid (50 μ g/mL), α -ketoglutarate (30 μ g/mL), L-glutamine (300 μ g/mL), penicillin (100 units/mL), and streptomycin (100 μ g/ml)] containing 0.75 mg/mL of Gibco® collagenase type II (ThermoFisher Scientific, Waltham, MA, USA) (52, 53). The resulting digest was passed through a 100 μ m filter and centrifuged at 800 g for 10 min. The cell pellet was then washed twice with fresh chondrocyte media. Cells were resuspended in chondrocyte media and live chondrocyte count was determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution. Chondrocytes were frozen in aliquots of 10×10^6 cells/mL in liquid nitrogen until use.

Synoviocyte Stimulation and Treatment

Cryopreserved synoviocytes were thawed, seeded onto a 12-well plate at 4×10^5 cells/well in synoviocyte media (50), and maintained at 5%CO₂, 90% humidity, and 37°C . Cells were brought to confluency over 48 h before media exchange and stimulated with either recombinant human IL-1 β at 10 ng/mL (R&D Systems, Minneapolis, MN, USA) (54) or *E. Coli* O55:B5 LPS at 100 ng/mL (Sigma-Aldrich, St. Louis, MO, USA) (55, 56). Unstimulated control wells underwent media exchange only. After 24 h of stimulation, the stimulation media was removed and the cellular monolayer was washed twice with phosphate

buffered saline (PBS). Fresh media was then added to the cells either alone (no treatment controls) or with 25% PPP-L or 25% PRP-L. Synoviocytes were treated for 48 h and the resultant synoviocyte conditioned media was collected, centrifuged at 2,000 g for 15 min, and divided into aliquots for cytokine analyses and chondrocyte treatments. Aliquots for cytokine analyses were frozen at -80°C until use while aliquots for chondrocyte treatments were used fresh.

Synoviocyte Quantification

After removal of the synoviocyte conditioned media as described above, 1 mL of PBS was added to each synoviocyte well. Spectrophotometric quantification of synoviocyte cell numbers in each monolayer was then measured by density using a multiple detection plate reader set at an absorbance of 800 nm (SynergyTM 2, BioTek Instruments Inc., Winooski, VT, USA) (57). Fold change in cell density was determined as a change from the unstimulated, non-treated synoviocytes.

Synoviocyte Conditioned Media Analyses

Hyaluronic acid (hyaluronan; HA) concentrations were quantified in duplicate aliquots of all synoviocyte conditioned media samples using the commercially available Hyaluronan Quantikine ELISA kit (R&D Systems Minneapolis, MN, USA). Standards in each kit were used to generate standard curves and samples were analyzed for optical density on a multiple detection plate reader (SynergyTM 2, BioTek Instruments Inc., Winooski VT) at 450 nm with wavelength correction set at 540 nm.

Pro-inflammatory IL-1 β and TNF- α and pro/anti-inflammatory IL-6 concentrations in synoviocyte conditioned media were determined using a truncated version of the commercially available equine multiplex assay (MILLIPLEX MAP Equine Cytokine/Chemokine Magnetic Bead Panel, EMD Millipore, Burlington, MA, USA) on a MAGPIX[®] System (Luminex Corp., Austin, TX, USA). All samples were analyzed in duplicate using a 96-well platform performed per manufacturer's instructions. A minimum bead count of 50 for each cytokine was acquired for data analysis. Data were analyzed using Milliplex Analyst 5.1 software (Luminex Corporation, Austin, TX, USA).

Chondrocyte Challenge With Synoviocyte Conditioned Media

Cryopreserved chondrocytes were thawed, seeded onto a 24-well plate at 2×10^5 cells/well in chondrocyte media (53) and maintained at 5%CO₂, 90% humidity, and 37°C. Cells were brought to confluency over 48 h before media exchange with an equal volume of synoviocyte conditioned media to chondrocyte media for each well. Challenge experiments were carried out for 48 h.

Chondrocyte RNA Extraction and qPCR

Total cellular RNA was extracted from chondrocytes using the RNeasy Mini Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions. The RNA purity and quantity were evaluated using UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA, USA). RNA was stored at -80°C until

cDNA construction by RT-PCR using the QuantiTect Reverse Transcription Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions.

Previously published equine primers were used to amplify collagen types I (COL1A1), II (COL2A1), and III (COL3A1), aggrecan (ACAN), lubricin (PRG4), and matrix metalloproteinase 3 (MMP-3) and 13 (MMP-13) with 18S used as a housekeeping gene (Table 1). Quantitative real time RT-PCR (qPCR) was performed using the QuantiFast[®] SYBR[®] Green PCR Kit (Qiagen Inc., Germantown, MD, USA) according to the manufacturer's instructions with the QuantStudio[®] 6 Flex System (applied biosystems[®], ThermoFisher Scientific, Waltham, MA, USA). Relative gene expression, $2^{-\Delta\Delta\text{Ct}}$, was generated using Real-Time PCR Software v1.2 (applied biosystems[®], ThermoFisher Scientific, Waltham, MA, USA). Chondrocytes cultured with the unstimulated, non-treated synoviocyte conditioned media were used as controls.

Statistical Analyses

All results were assessed for normality by means of Shapiro-Wilk test. Normally distributed data was analyzed by the analysis of covariance (ANCOVA) with horse as covariate, followed by the Tukey's test for multiple comparisons. Non-normally distributed data was analyzed by the non-parametric Wilcoxon rank sum test. Statistical analyses were performed within the non-treated group across stimulations to assess the effects of stimulation and then within each stimulation group to assess for treatment effects. Analyses were performed using JMP[®] Pro11 (SAS Institute Inc., Cary, NC, USA) and significance set at $p < 0.05$. All graphs were generated with GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).

RESULTS

Verification of Platelet-Poor Plasma and Platelet-Rich Plasma Lysate Preparations

White blood cell (WBC) and platelet counts verified the generation of leukocyte-reduced PPP and PRP. The mean \pm standard deviation ($n = 6$) WBC count in whole blood was $5.60 \times 10^3/\mu\text{L} \pm 0.62 \times 10^3/\mu\text{L}$ compared to $0.05 \times 10^3/\mu\text{L} \pm 0.02 \times 10^3/\mu\text{L}$ in PPP and $1.39 \times 10^3/\mu\text{L} \pm 0.26 \times 10^3/\mu\text{L}$ in PRP. The mean \pm standard deviation ($n = 6$) platelet count in whole blood was $134.50 \times 10^3/\mu\text{L} \pm 35.67 \times 10^3/\mu\text{L}$ compared to $9.38 \times 10^3/\mu\text{L} \pm 3.46 \times 10^3/\mu\text{L}$ in PPP and $1226.38 \times 10^3/\mu\text{L} \pm 55.32 \times 10^3/\mu\text{L}$ in PRP. As such, the platelet concentration in each PRP sample was very close to our target of 10x the platelet concentration of whole blood.

Effects of IL-1 β or LPS Stimulation on Synoviocytes and Chondrocytes Challenged With Synoviocyte Conditioned Media

Decreased synoviocyte growth compared to unstimulated controls was observed following 24 h of stimulation with either IL-1 β ($p < 0.002$) or LPS ($p < 0.001$; Figure 2A). Synoviocytes stimulated with either IL-1 β ($p < 0.03$) or LPS ($p < 0.03$) also

TABLE 1 | Equine primer sequences used for gene expression analyses.

Gene	Primer sequences
COL1A1, Collagen type I (58)	Forward, 5'-AAGGACAAGAGGCACGTCTG-3' Reverse, 5'-GCAGGAAAGTCAGCTGGATG-3'
COL2A1, Collagen type II (53)	Forward, 5'-GCTACACTCAAGTCCCTCAAC-3' Reverse, 5'-ATCCAGTAGTCTCCGCTCTT-3'
COL3A1, Collagen type III (58)	Forward, 5'-GGGTATAGCTGGTCCCTCGTG-3' Reverse, 5'-GCGCCTCTTTCTCCTTTAGC-3'
ACAN, Aggrecan (59)	Forward, 5'-CAACAACAATGCCCAAGACTAC-3' Reverse, 5'-AGTTCTCAAATTGCAAGGAGTG-3'
PRG4, Proteoglycan 4 (Lubricin) (60)	Forward, 5'-TGCGGTGCTTCCCCATAC-3' Reverse, 5'-AAACAGGAACCCATCAGAAAGTG-3'
MMP-3, Matrix metalloproteinase 3 (53)	Forward, 5'-ATGGACCTTCTTCAGGACTACC-3' Reverse, 5'-GACCGACATCAGGAACCTCCG-3'
MMP-13, Matrix metalloproteinase 13 (53)	Forward, 5'-ACAAGCAGTTCCAAAGGCTAC-3' Reverse, 5'-CTCGAAGACTGGTGATGGCA-3'
18S, 18 small ribonucleic acid (53)	Forward, 5'-GCCGCTAGAGGTGAAATTCT-3' Reverse, 5'-TCGGAACACGACGGTATCT-3'

had increased production of IL-1 β compared to unstimulated synoviocytes (**Figure 3A**). Increased production of TNF- α , however, was only observed following stimulation with LPS ($p < 0.02$; **Figure 3B**), and increased production of IL-6 was only observed following stimulation with IL-1 β ($p < 0.03$; **Figure 3C**). Chondrocytes challenged with synoviocyte conditioned media stimulated with either IL-1 β ($p < 0.03$) or LPS ($p < 0.03$) had increased gene expression of MMP-13 (**Figure 6B**), while only those challenged with synoviocyte conditioned media stimulated with LPS ($p < 0.03$) had increased gene expression of MMP-3 (**Figure 6A**) compared to control chondrocytes challenged with unstimulated synoviocyte conditioned media.

PRP-L Increases Growth and Hyaluronic Acid Production in Naïve and Inflamed Synoviocytes

Both PRP-L and PPP-L treatment increased the growth of unstimulated synoviocytes ($p < 0.0003$ and $p < 0.05$, respectively), but only PRP-L treatment was able to rescue the growth of synoviocytes stimulated with either IL-1 β ($p < 0.0001$) or LPS ($p < 0.0001$; **Figure 2A**). Furthermore, only PRP-L treatment was able to increase total synoviocyte HA production in unstimulated synoviocytes ($p < 0.005$), synoviocytes stimulated with IL-1 β ($p < 0.0001$) and synoviocytes stimulated with LPS ($p < 0.009$; **Figure 2B**). These results indicate that PRP-L treatment has powerful proliferative effects and is able to increase total synoviocyte HA production both under naïve conditions and in the face of inflammatory stimulation.

PRP-L Increases Anti-inflammatory IL-6 Production in Naïve and Inflamed Synoviocytes

PRP-L treatment increased the production of IL-1 β from unstimulated synoviocytes ($p < 0.05$) but did not cause any further increase in IL-1 β following stimulation either IL-1 β or LPS compared to non-treated synoviocytes (**Figure 3A**). Similarly, both PRP-L and PPP-L treatments increased the production of TNF- α from unstimulated synoviocytes ($p < 0.05$) compared to non-treated synoviocytes (**Figure 3B**), but neither caused any further increase in TNF- α following stimulation with IL-1 β and only PPP-L treatment caused a further increase

in TNF- α following LPS stimulation ($p < 0.02$). Only PRP-L treatment was able to further increase production of IL-6 from synoviocytes under all conditions compared to non-treated synoviocytes ($p < 0.005$) and compared to PPP-L treated synoviocytes ($p < 0.05$; **Figure 3C**). These results indicate that PRP-L treatment does not reduce pro-inflammatory cytokine production from stimulated synoviocytes but does increase anti-inflammatory IL-6 production from both unstimulated and stimulated synoviocytes compared to both non-treated and PPP-L treated synoviocytes.

Conditioned Media From PRP-L Treated Synoviocytes Increases Anabolic Gene Expression in Cultured Chondrocytes

The anabolic effects of PPP-L treated, PRP-L treated, or non-treated synoviocytes either unstimulated or stimulated with IL-1 β or LPS on cultured chondrocytes were assessed by measuring relative chondrocyte gene expression of collagen type I (**Figure 4A**), collagen type II (**Figure 4B**), collagen type III (**Figure 4C**), aggrecan (**Figure 5A**), and lubricin (**Figure 5B**). Chondrocytes challenged with conditioned media from PRP-L treated synoviocytes had increased collagen type II expression compared to chondrocytes challenged with conditioned media from non-treated synoviocytes when the synoviocytes were unstimulated ($p < 0.03$), IL-1 β stimulated ($p < 0.003$), or LPS stimulated ($p < 0.001$) and compared to chondrocytes challenged with conditioned media from PPP-L treated synoviocytes when the synoviocytes where either IL-1 β stimulated ($p < 0.01$) or LPS stimulated ($p < 0.01$; **Figure 4B**). No significant differences in chondrocyte gene expression of either collagen type I and collagen type III were found for any stimulation or treatment group of synoviocyte cultured media (**Figures 4A,C**). Chondrocytes challenged with conditioned media from PRP-L treated synoviocytes had increased aggrecan expression compared to chondrocytes challenged with conditioned media from either non-treated or PPP-L treated synoviocytes when the synoviocytes were unstimulated ($p < 0.02$), IL-1 β stimulated ($p < 0.01$), or LPS stimulated ($p < 0.02$) (**Figure 5A**). Lubricin (proteoglycan 4) gene expression was largely unaffected apart from increased gene expression in chondrocytes challenged with PRP-L treated, IL-1 β stimulated synoviocyte conditioned media ($p < 0.05$) compared to those challenged with non-treated, IL-1 β stimulated synoviocyte conditioned media (**Figure 5B**). These

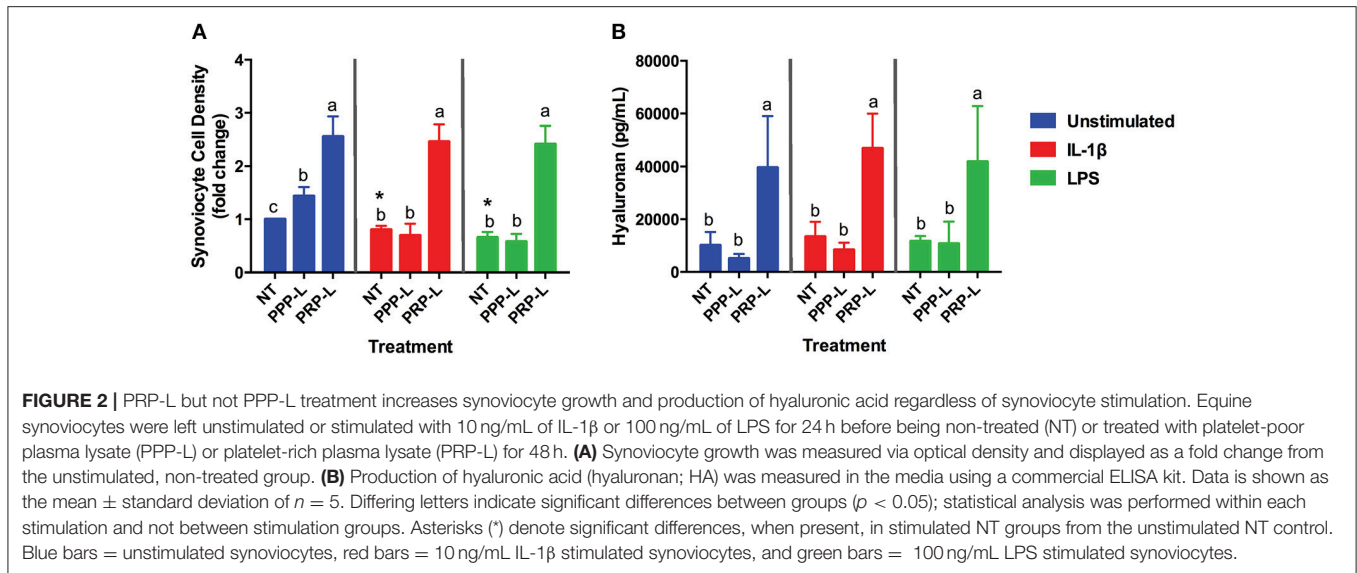


FIGURE 2 | PRP-L but not PPP-L treatment increases synoviocyte growth and production of hyaluronan acid regardless of synoviocyte stimulation. Equine synoviocytes were left unstimulated or stimulated with 10 ng/mL of IL-1 β or 100 ng/mL of LPS for 24 h before being non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L) for 48 h. **(A)** Synoviocyte growth was measured via optical density and displayed as a fold change from the unstimulated, non-treated group. **(B)** Production of hyaluronan acid (hyaluronan; HA) was measured in the media using a commercial ELISA kit. Data is shown as the mean \pm standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1 β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

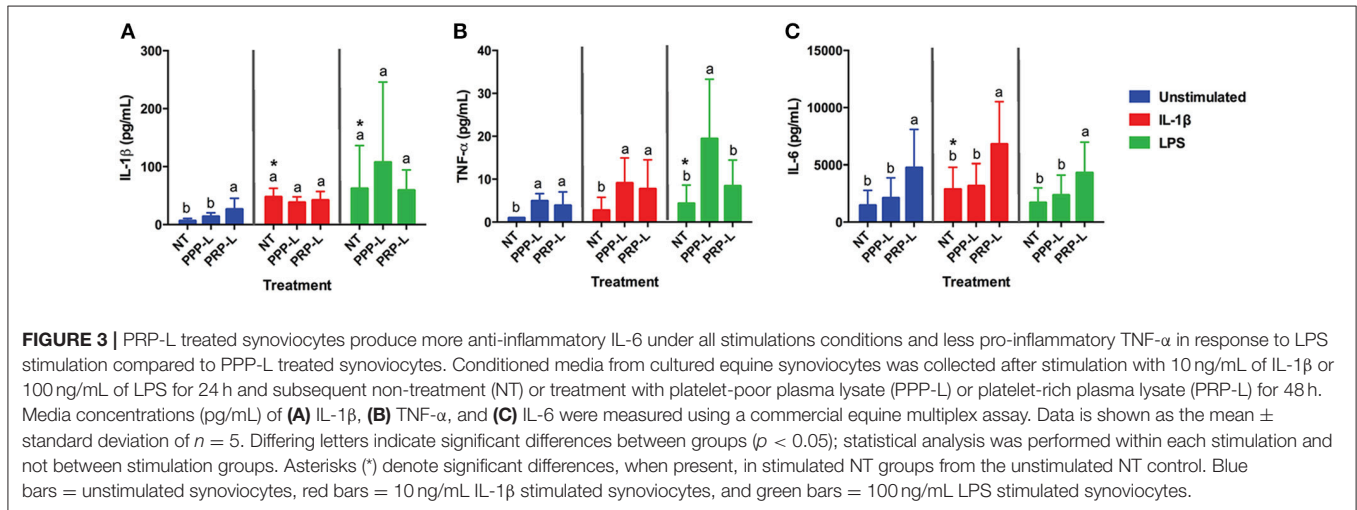


FIGURE 3 | PRP-L treated synoviocytes produce more anti-inflammatory IL-6 under all stimulations conditions and less pro-inflammatory TNF- α in response to LPS stimulation compared to PPP-L treated synoviocytes. Conditioned media from cultured equine synoviocytes was collected after stimulation with 10 ng/mL of IL-1 β or 100 ng/mL of LPS for 24 h and subsequent non-treatment (NT) or treatment with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L) for 48 h. Media concentrations (pg/mL) of **(A)** IL-1 β , **(B)** TNF- α , and **(C)** IL-6 were measured using a commercial equine multiplex assay. Data is shown as the mean \pm standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1 β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

results indicate that PRP-L treatment increases the production of normal collagen type II found in mature articular cartilage rather than inferior collagen type I found in fibrocartilage or collagen type III found in cartilage undergoing repair. In addition, PRP-L treatment increases the production of aggrecan, the major structural proteoglycan of cartilage extracellular matrix.

Conditioned Media From Both PRP-L and PPP-L Treated Inflamed Synoviocytes Decreases Catabolic Gene Expression in Cultured Chondrocytes

The catabolic effects of PPP-L treated, PRP-L treated, or non-treated synoviocytes either unstimulated or stimulated with IL-1 β or LPS on cultured chondrocytes were assessed by measuring relative chondrocyte gene expression of MMP-3 (**Figure 6A**) and MMP-13 (**Figure 6B**). Although no significant differences in chondrocyte gene expression of MMP-3 were

found for conditioned media from PRP-L or PPP-L treated synoviocytes stimulated with either IL-1 β or LPS compared to non-treated synoviocytes, there was a trend toward reduced MMP-3 gene expression under LPS stimulation conditions ($p = 0.09$) and in particular the PRP-L treated group decreased back down to the same level of MMP-3 gene expression observed in control chondrocytes challenged with conditioned media from unstimulated synoviocytes (**Figure 6A**). Chondrocytes challenged with conditioned media from both PRP-L and PPP-L treated synoviocytes had decreased MMP-13 gene expression compared to chondrocytes challenged with conditioned media from non-treated synoviocytes when the synoviocytes were unstimulated ($p < 0.03$) and also when the synoviocytes were stimulated with IL-1 β ($p < 0.05$) or LPS ($p < 0.02$; **Figure 6B**). Similar to the trend observed for chondrocyte gene expression of MMP-3 under LPS stimulation conditions, there was a trend toward chondrocytes challenged with conditioned media from PRP-L treated synoviocytes that were stimulated with LPS to

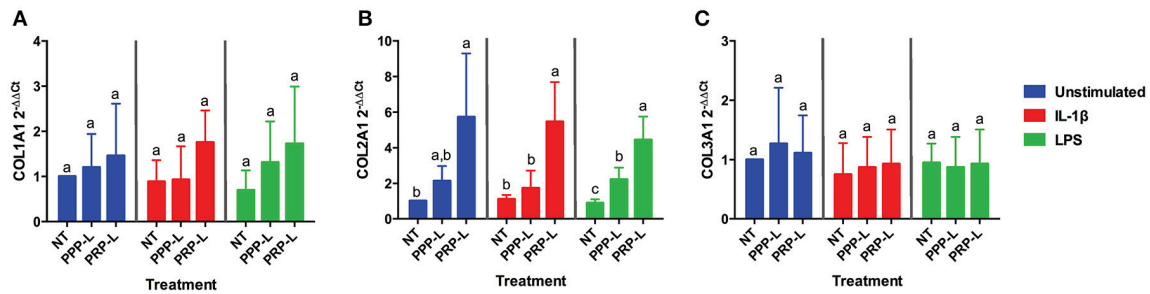


FIGURE 4 | Conditioned media from synoviocytes treated with PRP-L under all stimulation conditions increases collagen type II but not collagen type I or type III in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression is represented as the 2^{-ΔΔCt} of (A) collagen type I (COL1A1), (B) collagen type II (COL2A1), and (C) collagen type III (COL3A1). Data is shown as the mean ± standard deviation of n=5. Differing letters indicate significant differences between groups (p < 0.05); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

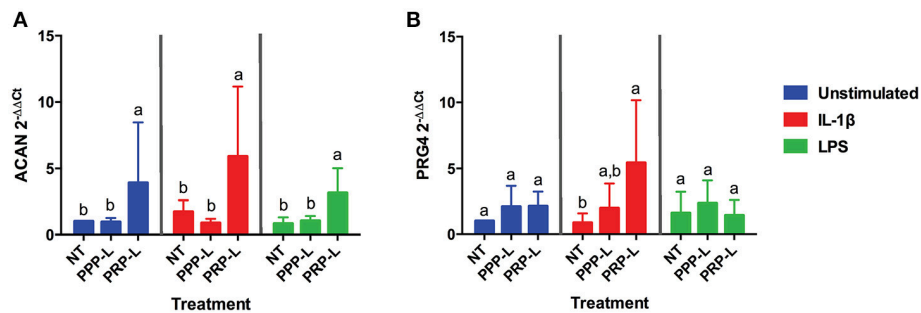


FIGURE 5 | Conditioned media from synoviocytes treated with PRP-L under all stimulation conditions increases aggrecan expression in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression represented as the 2^{-ΔΔCt} of (A) aggrecan (ACAN), and (B) lubricin (PRG4) was measured in cDNA made from extracted chondrocyte RNA. Fold changes were generated from the chondrocytes cultured with unstimulated, non-treated synoviocyte conditioned media. Data is shown as the mean ± standard deviation of n = 5. Differing letters indicate significant differences between groups (p < 0.05); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

have further reduced MMP-13 gene expression compared to chondrocytes challenged with conditioned media from PPP-L treated synoviocytes that were stimulated with LPS (Figure 6B). These results indicate that both PRP-L and PPP-L treatments are able to downregulate MMP-13 production in chondrocytes under LPS stimulation conditions, but with PRP-L treatment trending toward a closer return to unstimulated control levels of MMP-13.

DISCUSSION

The aim of this study was to examine the effects of a pooled allogeneic platelet-rich plasma lysate (PRP-L) preparation on equine synoviocytes and chondrocytes challenged with inflammatory mediators *in-vitro* to mimic the OA joint environment. The findings of this study support our overall hypothesis that PRP-L treatment of inflamed synoviocytes protects chondrocytes challenged with synoviocyte conditioned

media. The protective effect of PRP-L, however, appears to be more through increase of synoviocyte anti-inflammatory cytokine production rather than through reduction of synoviocyte pro-inflammatory mediators. Such findings are consistent with those of previous studies on the anti-inflammatory effects of PL on other cell types (35, 41, 61) and are also consistent with previous studies on the anabolic and anti-catabolic effects of PRP preparations on chondrocytes (62–65). A particularly interesting, and unexpected finding of this study, however, was that treatment with PRP-L stimulated the growth of synoviocytes and the production of HA from synoviocytes even when challenged with IL-1β or LPS.

Numerous studies evaluating the efficacy of PL as a serum replacement for FBS in cell culture media have supported the proliferative effect of PL preparations on different cell types including bone marrow-derived stromal cells, adipose-derived stromal cells, synovial fluid stromal cells, and corneal endothelium cells (31, 38–40, 42, 45, 47). It is therefore

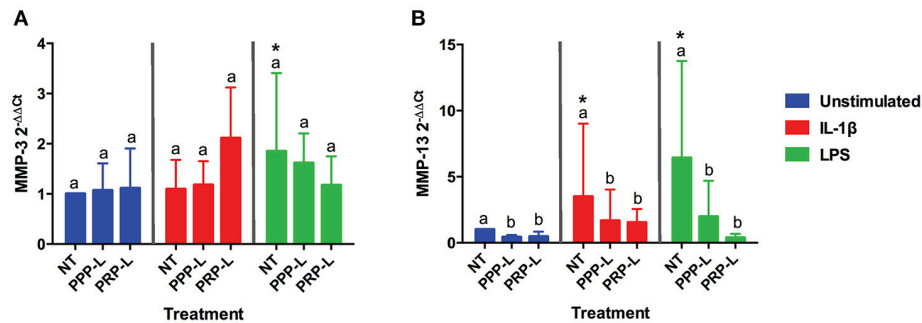


FIGURE 6 | Conditioned media from synoviocytes treated with PRP-L or PPP-L under all stimulation conditions decreases MMP-13 but not MMP-3 gene expression in cultured chondrocytes. Equine chondrocytes were challenged for 48 h with conditioned media from synoviocytes stimulated with IL-1 β or LPS and either non-treated (NT) or treated with platelet-poor plasma lysate (PPP-L) or platelet-rich plasma lysate (PRP-L). Relative gene expression, $2^{-\Delta\Delta C_t}$, was generated using the unstimulated, non-treated conditioned media cultured chondrocytes as the control for (A) MMP-3 and (B) MMP-13. Data is shown as the mean \pm standard deviation of $n = 5$. Differing letters indicate significant differences between groups ($p < 0.05$); statistical analysis was performed within each stimulation and not between stimulation groups. Asterisks (*) denote significant differences, when present, in stimulated NT groups from the unstimulated NT control. Blue bars = unstimulated synoviocytes, red bars = 10 ng/mL IL-1 β stimulated synoviocytes, and green bars = 100 ng/mL LPS stimulated synoviocytes.

not surprising that the PRP-L used in this study would lead to enhanced cell growth of naïve synoviocytes under normal tissue culture conditions. It is surprising, though, that very similar fold changes in synoviocyte growth were observed for synoviocytes stimulated with either IL-1 β or LPS and treated with PRP-L. The dramatic increases in synoviocyte growth observed under naïve and stimulated conditions when treated with PRP-L were consistent with the dramatic increases observed in HA production by synoviocytes under naïve and stimulated conditions when treated with PRP-L. PRP preparations have been previously reported to increase protein production of HA and to increase gene expression of Hyaluronan synthase-2 (HAS-2) in synoviocytes isolated from OA patients (63, 66). There is also a report of two different platelet gel supernatants isolated from a single horse that were able to increase HA production from synoviocytes under LPS stimulation compared to controls, but not to the same extent as observed in this current study (67). It is possible that the pooled nature of the PRP-L used in this study may have been in part responsible for the differences observed in HA production compared to previous studies evaluating a platelet preparation from a single donor. As discussed earlier, there is existing evidence in the literature to support increased efficacy of pooled PL preparations compared to individual donor preparations, as pooled preparations are able to capitalize on the natural cytokine variability that occurs in donors (31, 41). In this current study, we were unable to discern whether or not the dramatic increases in total HA concentration were due solely to the increases in synoviocyte cell numbers caused by PRP-L treatment or due in part to upregulation of HA production by synoviocytes treated with PRP-L. Future studies examining HAS-2 gene expression in synoviocytes following treatment with PRP-L are certainly warranted to determine all mechanisms involved.

The contribution of such a remarkable increase in synoviocyte HA production following PRP-L treatment on chondrocyte gene expression is unknown and also warrants further exploration. In our current study design, it is not possible to discern if the

increases in collagen type II and aggrecan gene expression and the decrease in MMP-13 gene expression that were observed are due primarily to the high concentration of HA in the conditioned media of synoviocytes treated with PRP-L or due to other variables such as the high concentrations of platelet-derived growth factors found in PRP-L, the significant increase in synoviocyte IL-6 production caused by PRP-L treatment, or other factors that were not examined for. HA is known to bind to the cluster of differentiation 44 (CD44) receptors and thereby inhibit IL-1 β expression resulting in decreased MMP production (68, 69). HA has also been shown to increase the proliferation of chondrocytes in tissue culture as well as to stimulate them to produce more collagen type II and aggrecan (70). Although IL-6 can be pro-inflammatory under certain conditions, it is known to induce the production of IL-1 and TNF antagonists by a variety of cell types including macrophages (71) and has been highlighted as a critical cytokine for the repair of other musculoskeletal tissues such as tendon (72, 73).

Another factor to consider that was not examined in this current study is the role of hypoxia-inducible factor (HIF) in PRP-L mediated chondroprotection and cartilage repair (37, 74–76). Several studies have previously demonstrated the critical role of HIF-1 α in cartilage and in the nucleus pulposus for maintaining proper cellular function, including synthesis of extracellular matrix proteins, in a hypoxic environment (76–79). Both articular cartilage and the nucleus pulposus are highly avascular tissues with low intrinsic healing capacity and low oxygen tensions. This unique environment requires mechanisms adapted to support the survival of the tissue's resident cells, and HIF-1 α is considered to be one of the main elements in such mechanisms. A recent study evaluating the effect of a pooled human PL preparation on growth-arrested progenitor cartilage cells found that PL induced the re-entry of such cells into the cell cycle (37). The cell activation and proliferation observed in this study was shown to correspond to induction of HIF-1 by PL (37). Consequently, we would speculate that PRP-L treatment of

inflamed or damaged cartilage may activate the HIF-1 pathway to increase cell proliferation and matrix synthesis, but this remains to be investigated.

In conclusion, treatment of inflamed synoviocytes with PRP-L *in-vitro* resulted in increased synoviocyte growth and increased total synoviocyte HA and IL-6 production. Challenge of chondrocytes with conditioned media from PRP-L treated synoviocytes then resulted in increased collagen type II and aggrecan gene expression as well as decreased MMP-13 gene expression. The results of this study support continued investigation into the use of pooled PRP-L for the treatment of osteoarthritis and warrant further *in-vitro* studies to discern the mechanisms of action of PRP-L.

AUTHOR CONTRIBUTIONS

We certify that all authors meet the qualifications for authorship as listed below: (1) substantial contributions to conception or design of the work or the acquisition, analysis, or interpretation

of data for the work; (2) drafting the work or revising it critically for important intellectual content; (3) final approval of the version to be published; (4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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