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Platelet-Rich Plasma Enhances Proliferation and Migration and Inhibits Inflammatory Processes in Canine Chondrocytes

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Abstract : This study was performed to assess the anti-inflammatory and cartilage regenerative effects of platelet-rich plasma (PRP) on canine chondrocytes. Proliferation and migration assays under both normal and lipopolysaccharide (LPS)-induced inflammatory conditions were performed with various concentrations of PRP (1% to 10%). The expression levels of genes related to osteoarthritis were evaluated in the following groups: PRP group, LPS group and LPS + PRP group. mRNA expression levels were detected using real-time polymerase chain reaction (RT-PCR). Proliferation assays showed significantly enhanced proliferation in all PRP-treated groups compared with the no serum group. Compared with 10% fetal bovine serum (FBS), PRP concentrations above 3% in the normal condition and 1% to 7% PRP in the LPS-induced inflammatory condition were found to significantly promote chondrocyte proliferation. In the normal condition, all PRP-treated groups showed significantly increased cell migration compared with the no serum group. Chondrocyte migration was decreased with LPS-induced inflammation, but PRP treatment resulted in significantly enhanced migration compared with the other groups in this condition. According to RT-PCR, the LPS + PRP group showed significantly higher levels of COL1A1, IL-6, aggrecan and lower levels of TNF-α, MMP-1, MMP-3 mRNA expression compared to the LPS group. The results of this study suggest that PRP application can enhance the proliferation and migration of canine chondrocytes and improve canine articular cartilage regeneration.

***Key words*:** anti-inflammation, canine osteoarthritis, migration, platelet-rich plasma, proliferation.

Introduction

Osteoarthritis (OA) is a disease of progressive joint degradation resulting in alterations to the joint capsule, cartilage and synovium, and this condition is associated with compromised joint function and mobility impairment (1,27).

OA exhibits catabolic changes to the extracellular matrix (ECM), which consists of proteoglycans, aggrecans and type 2 collagen. ECM catabolic changes include decreased cartilage compressive stiffness, damage to cartilage collagen and proteoglycans, increased cartilage hydration, loss of collagen integrity, loss of tensile strength, fibrillation, and eburnation. These changes lead to damage to the cartilage collagen fibrillar network and upregulation of aggrecanases, matrix metalloproteinases (MMP) and disintegrin (20,27).

The pathology of OA is accompanied by increased mRNA and protein levels of several inflammatory cytokines, including the important cytokines IL-1β and TNF-α. These pro-inflammatory cytokines initiate the inflammatory cascade and facilitate disease progression. Releasing MMPs such as stromelysin 1 (MMP-3), collagenase (MMP-1), aggrecanase (31).

The most common treatment approach for OA is to prevent articular cartilage degeneration and alleviate pain using various drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs) are primarily used for OA treatment; however, long-term NSAID therapy has adverse effects such as gastrointestinal complications as well as renal, hepatic and coagulation disorders (5,14,21). Most current OA treatments focus on palliative care and alleviating pain rather than a curative approach.

PRP is essentially autologous material and thus does not generate problematic immune-mediated responses; PRP also contains various growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β. These factors can enhance angiogenesis and reconstruct damaged tissue at the site of injury and can be helpful for healing damaged cartilage (3,12,19). PRP was also reported as a potent down-regulator of inflammatory cytokines, such as IL-1β in chondrocytes (12,16,30).

Many studies have investigated the effect of PRP treatment in various inflammatory diseases in the context of human medicine. However, few studies have evaluated PRP in veterinary medicine, especially in chondrocytes (19). Therefore, the purpose of this study was to determine the ability of PRP to promote proliferation and migration in canine chondrocytes, and to evaluate the transcriptional expression of OA-related cytokines in normal and inflammatory conditions upon PRP treatment.

Matreials and Methods

Isolation of chondrocytes

Fresh cartilage fragments were aseptically obtained from

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the stifle joint of an approximately one-year-old beagle dog. The cartilage was rinsed two times with phosphate-buffed saline (PBS) and dissected into fragments less than 1 mm^3 in size. For enzymatic digestion of the extracellular matrix, the cartilage was treated with collagenase type I (Collagenase TYPE 1A, Sigma-Aldrich, USA) and dispase II (Dispase II, Sigma-Aldrich, USA) in Dulbecco's modified eagles medium (DMEM) containing 100 μg/ml penicillin and 100 μg/ml streptomycin, and digested for approximately 6 hours in a 37°C water chamber. An equal volume of fresh media was added, and the samples were centrifuged at 2500 rpm for 10 minutes. After removal of the supernatant, tissue debris and cell clumps were removed by passing the suspension through a 100-μm pore size nylon mesh. Another centrifugation was performed, and the remaining cell pellet was seeded in a 100 × 20 mm cell culture dish. Within 24 hours, the non-adherent cells were removed by washing with PBS, and the culture medium was replaced twice per week. Chondrocytes were used between passage 1 and 2 for all experiments.

Preparation of activated PRP

For the *in vitro* experiments, PRP was activated with autologous thrombin and 10% $CaCl_2$ (Sigma-Aldrich, USA), and only the supernatant was used. Autologous thrombin was made with 1 ml of PRP by adding 10% $CaCl_2$. Sixty minutes after clotting, the fluid obtained by squeezing the fibrin gel was considered autologous thrombin. By adding autologous thrombin and 10% calcium chloride, the PRP clotted and formed a fibrin gel. To ensure sufficient secretion of soluble factors, the clotted PRP was incubated for 1 hour in a 37°C water bath. After 3600 rpm centrifugation for 30 minutes, the supernatant was collected and stored at −4°C before use.

Proliferation test

Chondrocytes (1.0×10^4 cell/well) were seeded and cultured in a 96-well plate with DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) for 12 hours to ensure adherence. Then, the medium was replaced with 10% FBS, no serum, 1% PRP, 3% PRP, 5% PRP, 7% PRP or 10% PRP diluted in low-glucose DMEM, according to the specified group. After treatment for 48 hours, proliferation was evaluated with Cell Count Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). Optical density was measured at 450 nm using a microplate spectrophotometer (Epoch, Biotek Instruments, USA). The inflammatory condition was established with the same procedure.

Migration test

Chondrocytes (70 μl of 500,000 cells/ml) were seeded on Culture-Insert plates (Ibidi, GmbH, Martinsried, Germany) and incubated for 24 hours. The migration device was removed 24 hours after cell seeding to create a 500 μm-thick cell-free gap in the middle of the cell monolayer. The cells were rinsed with sterile phosphate-buffered saline (PBS) (Gibco, USA) and incubated for 24 hours with 10% FBS, no serum, 1% PRP, 3% PRP, 5% PRP, 7% PRP or 10% PRP diluted in low-glucose DMEM, according to the specific group. To measure the area of cell migration, phase-contrast and fluorescent photographs of the center of the gap were captured at pre- and post-treatment time points with a digital camera coupled to an inverted microscope (CKX41, Olympus, Japan). The percentage of the gap area occupied by cells was calculated using ImageJ software (National Institites of Health, Maryland, USA). The inflammatory condition was induced with LPS (10 μg/ml, LPS from *E. coli* 0111:B4, Sigma-Aldrich, USA), and three groups were established: no serum, no serum + LPS and LPS + PRP. In the LPS + PRP group, PRP was treated at a 5% concentration.

Real-time PCR analysis

Cell were seeded (1.0×10^4 cell/dish) and grown until confluence in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in 100 × 20 mm cell culture dishes. Cells were then maintained in serum-free medium for 24 hours. PRP at 5% was added to the first group as a control group, and 5% PRP was added to the third group treated with LPS (10 μg/ml). These groups were incubated for 24 hours. Real-time PCR was performed using a Bio-Rad CFX96 (Bio-Rad Laboratories, Inc., USA). RNA was isolated using TRIzol® Reagent (Life technologies, USA). cDNA was synthesized by M-MuLV reverse transcriptase (Cosmogenetech, Seoul, Korea) with RNase Inhibitor (Cosmogenetech, Seoul, Korea) according to the manufacturer`s protocol. The reactions were performed in a 20 μl volume including 10 μl of SYBR Green Q Master, 0.4 μl of each primer (Table 1), 5 μl of RT products (1:20 dilution) and 4.2 μl of distilled water. The standard reaction condition was as follows: 3 minutes of pre-denaturation at 94°C and amplification for 40 cycles of 94°C for 10 seconds, 58°C for 10 seconds and 72°C for 20 seconds. Some reactions were performed under slightly different conditions. IL-1β was 40 cycles at 94°C for 10 seconds and 60°C for 30 seconds;

Table 1. PCR primer sequences

Gene	Primer	Sequence
GAPDH	Forward	GTT TGT GAT GGG CGT GAA CC
	Reverse	TTT GGC TAG AGG AGC CAA GC
COL1A1	Forward	AGA CCT GCG TAT ACC CCA CT
	Reverse	AAT CCG TCG GTC ATG GCT CC
COL2A1	Forward	TCC CAG TCA CTC TAG GGC TC
	Reverse	GTC TGC CCA GTT CAG GTC TC
IL-1β	Forward	GTG ATG CAG CCA TGC AAT CG
	Reverse	TGG AGA GCC CGA AGC TCA TA
TNF-α	Forward	TTC TGC CTG CTG CAC TTT
	Reverse	GGC AAC AGG GCT GAT TAG TT
IL-6	Forward	CTC CTG ACC CAA CCA CAG AC
	Reverse	GTG TGC TTC ACG CAC TCA TC
MMP-1	Forward	TGT AGG CTT GCA GTA GGT GC
	Reverse	TGC TGG CTA TGA AGT GCT CC
MMP-3	Forward	GAG ATC ACG GAG ACT TTA ACC C
	Reverse	TCA AAG TGG GCA TCT CCA TAA A
Aggrecan	Forward	GCC TGA GGA GCC TTT CAC AT
	Reverse	GGG AAA GCA GTG ACC CCT AC

MMP-1 was 40 cycles at 94°C for 10 seconds, 50°C for 10 seconds and 72°C for 20 seconds. MMP-3 was 40 cycles at 94°C for 10 seconds, 56°C for 10 seconds and 72°C for 20 seconds. Aggrecan was 40 cycles at 94°C for 10 seconds, 54°C for 10 seconds and 72°C for 20 seconds. The melting curve analysis was performed by heating the PCR product from 65°C to 95°C in order to confirm a single peak of the fluorescence. The Real-time PCRs were performed in quintuplicate. Relative expressions of canine chondrocyte genes encoding COL1A1, COL2A1, IL-1β, IL-6, TNF-α, MMP1, MMP3, SOX9, and aggrecan were calculated using the comparative threshold cycle method with CFX manager version 3.1 and normalized to expression of the GAPDH housekeeping gene.

Statistical analysis

All data were recorded as the mean ± standard deviation (SD).The non-parametric Mann-Whitney U test was performed for between-group comparisons (SPSS Statistics version 23, IBM SPSS Inc., USA). P values less than 0.05 were considered statistically significant.

Results

Proliferation test

Compared with the no serum group, all PRP-treated groups and the FBS 10% group showed significantly increased chondrocyte proliferation. Compared with 10% FBS, PRP concentrations of 3% to 10% significantly increased chondrocyte proliferation (Fig 1A and 1B). In the LPS-induced inflammation condition, a significant increase in canine chondrocyte proliferation was observed with PRP concentrations of 1% to 7% compared with 10% FBS. No significant difference was found between the 10% FBS and 10% PRP group. Compared with the no serum group, all PRP-treated groups and the FBS-treated group showed increased proliferation in the LPS-induced inflammation condition (Fig 1C and 1D).

Migration test

The FBS 10% group and all PRP-treated groups showed an increased percentage of the gap area occupied by cells compared with the no serum group. However, compared with

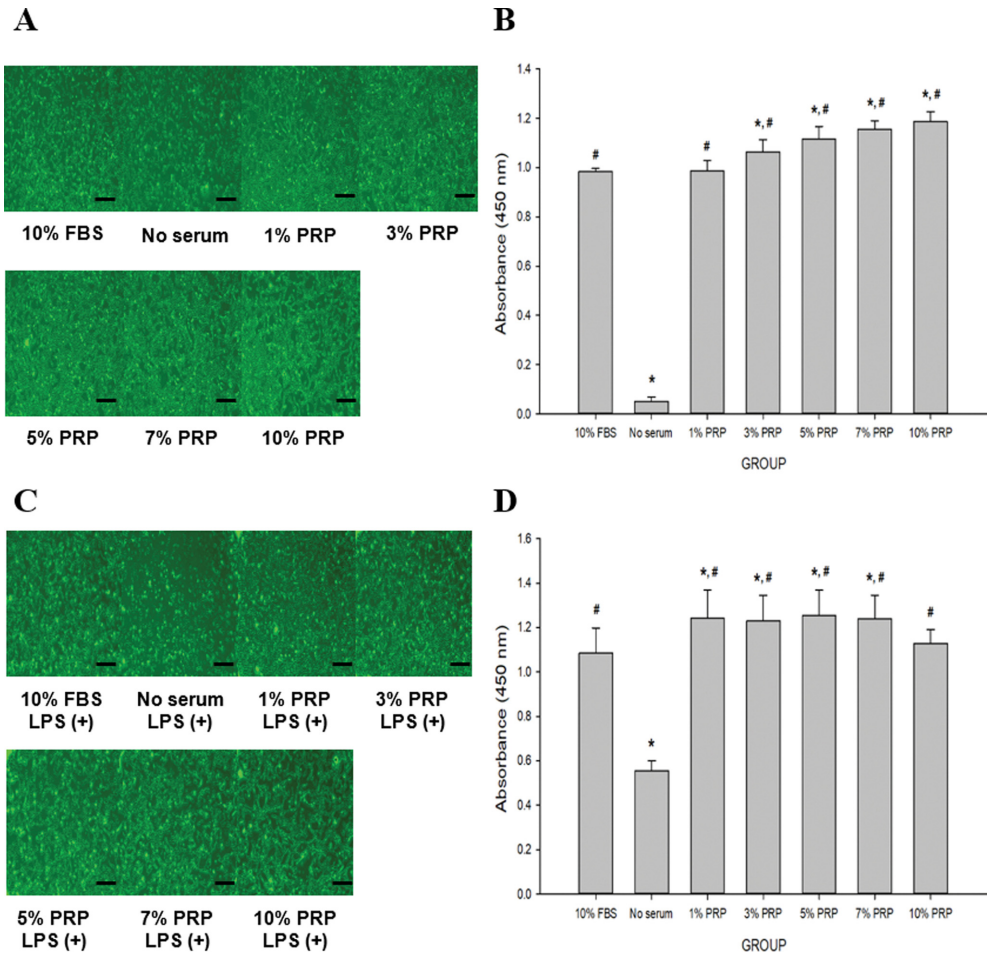

Fig 1. Proliferation of canine chondrocytes. (A) Microscopic feature of chondrocyte proliferation in normal conditions. Canine chondrocytes were cultured for 48 hours in DMEM. (B) Results of the proliferation test on canine chondrocytes in normal conditions. (C) Microscopic features of chondrocyte proliferation in the LPS-induced inflammatory condition. LPS-treated canine chondrocytes were cultured for 24 hours in DMEM. (D) Results of the prolifera tion test in LPS-treated canine chondrocytes were cultured for 24 hours in DMEM. Data were expressed as the mean ± SD. *: Significant differences compared with the FBS 10% group ($P < 0.05$). #: Significant differences compared with the no serum group ($P < 0.05$). Bar = 500 μm.

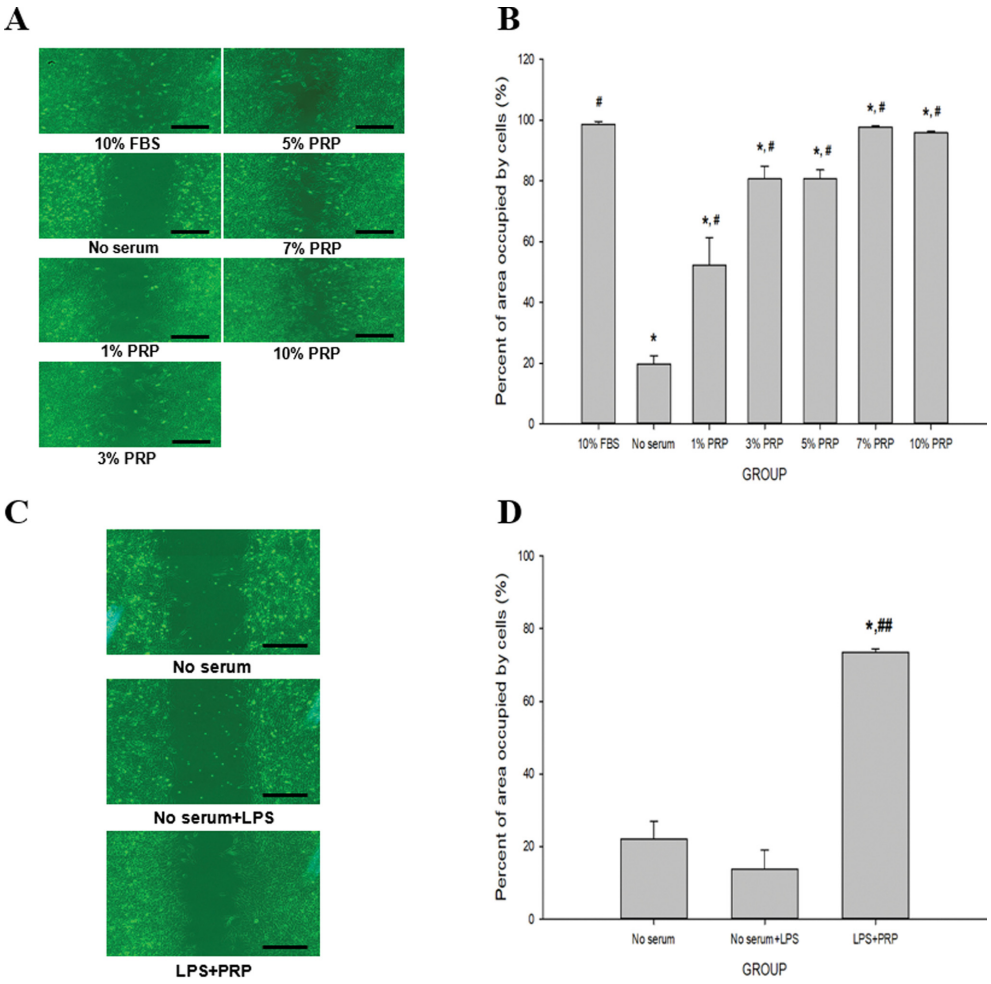

Fig 2. Migration of canine chondrocytes. (A) Microscopic feature of chondrocyte migration in the normal condition. (B) Migration test results of canine chondrocytes cultured for 12 hours in DMEM (C) Microscopic features of chondrocyte migration in the LPS-induced inflammatory condition. (D) Migration test results for canine chondrocytes cultured for 12 hours in the LPS treatment condition. Data were expressed as the mean ± SD. *: Significant differences compared with the DMEM group ($P < 0.05$). #: Significant differences compared with the LPS group ($P < 0.05$). Bar = 250 μm.

FBS 10%, the no serum group and all PRP-treated groups demonstrated a significantly decreased percentage of the area occupied by cells (Fig 2A and 2B). In the LPS-induced inflammation condition, chondrocyte migration was decreased. However, the LPS + PRP group showed a significant increase in migration compared to the no serum and no serum + LPS groups (Fig 2C and 2D).

Real-time PCR analysis

The mRNA expression levels of genes related to canine articular cartilage remodeling, including COL1A1, COL2A1, MMP-1, MMP-3 and aggrecan, were analyzed. In addition, mRNA expression levels of the pro-inflammatory cytokines IL-1β and TNF-α and the inflammation-modulating cytokine IL-6 were evaluated. COL1A1, IL-6 and aggrecan were significantly increased in the LPS + PRP group compared to the LPS group. COL2A1 and IL-1β showed lower expressions levels in the LPS + PRP group than the LPS group, but these differences did not reach statistical significance. TNF-α, MMP-1 and MMP-3 were significantly decreased in the LPS + PRP group compared with the LPS group (Fig 3).

Discussion

In this study, proliferation, migration and gene expression were evaluated in canine chondrocytes treated with various PRP concentrations in normal and LPS-induced inflammatory conditions. These results may provide foundational knowledge for the use of PRP as a treatment for OA in canines.

OA is characterized by the loss of articular cartilage and synovial proliferation (1). These processes are associated with increased expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α as well as proteolytic enzymes such as MMP1 and MMP3 [11].

PRP contains various anabolic growth factors such as VEGF, PDGF, FGF, EGF, IGF-1 and TGF-β (3,12,19). These factors are reported to be beneficial for cartilage repair and chondrocyte differentiation (9,11,19). In humans, these growth factors have been shown to enhance chondrocyte proliferation *in vitro*. In addition, a proliferation-promoting effect was observed for 12 days of monolayer and three-dimensional *in vitro* culture (2,6,15,26). In this study, proliferation promotion was observed within 48 hr.

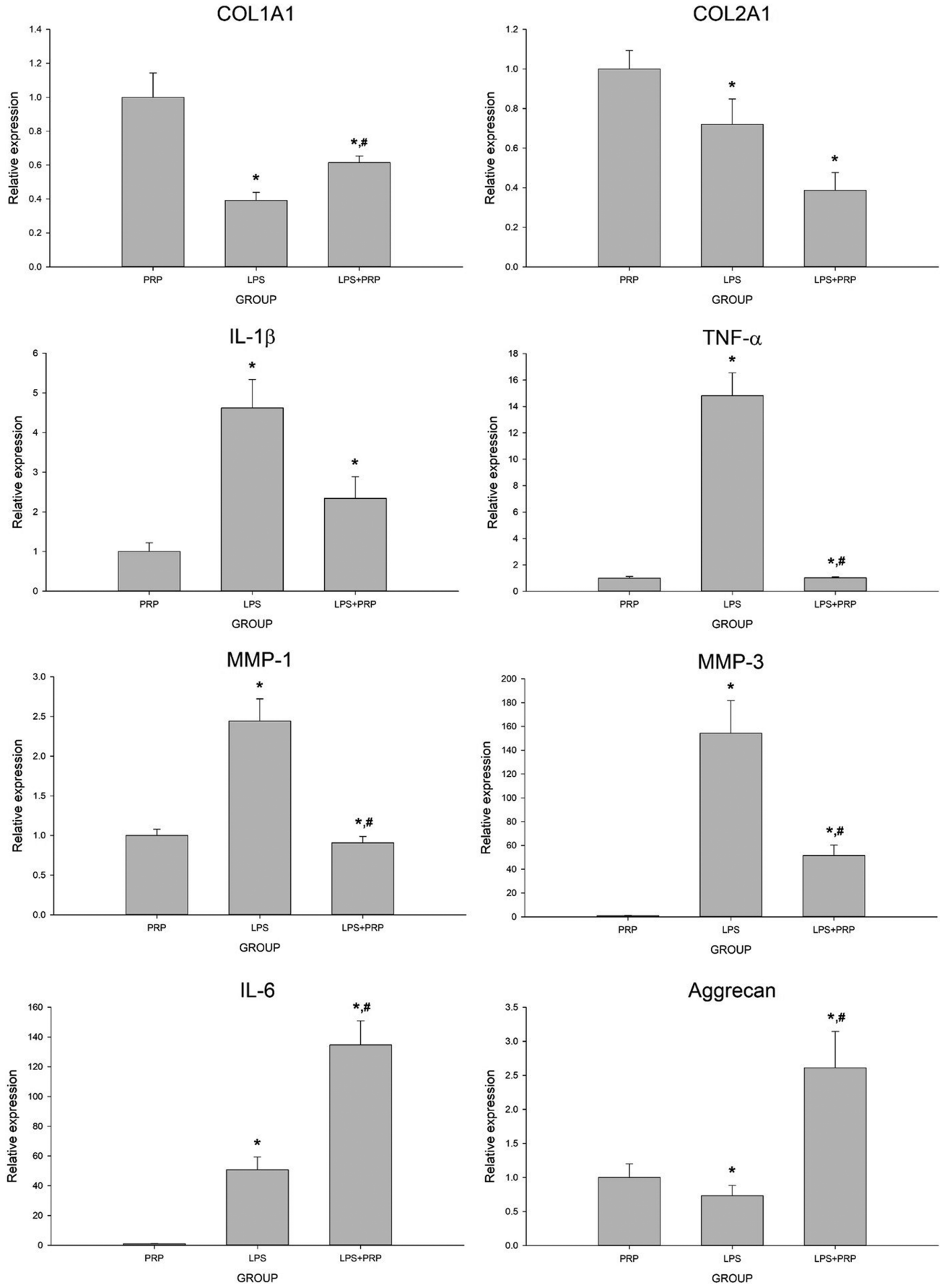

Fig 3. Relative mRNA expression levels of COL1A1, COL2A1, IL-1β, TNF-α, IL-6, MMP-1, MMP-3 and Aggrecan in canine chondrocytes by real-time PCR after 24-hour culture with 5% PRP, LPS, and 5% PRP plus LPS. Date were expressed as the mean ± SD. *: Significant differences compared to the PRP group ($P < 0.05$). #: Significant differences compared to the LPS group ($P < 0.05$).

PRP has anti-inflammatory effects on OA chondrocytes. Inflammatory cytokines such as IL-1β and TNF-α stimulate the production of MMPs, enzymes that can degrade all components of the extracellular matrix (17,29). In human chondrocytes, PRP mitigates gene expression of pro-inflammatory cytokines such as IL-1β and TNF-α (4). In this study, PRP downregulated IL-1β and TNF-α expression in canine chondrocytes and also upregulated IL-6, an inflammation-modulating cytokine. These data demonstrate that PRP has anti-inflammatory effects in canine OA.

Healthy articular cartilage is mainly composed of type 2 collagen. However, when this structure is damaged in OA, this is replaced primarily with type 1 collagen (13,33). In this study, COL1A1 expression was upregulated while COL2A1 was downregulated. This result could be related to leukocyte concentrations in the PRP formulation (34). Leukocytes can enhance catabolic reactions and the expression of pro-inflammatory molecules (22), and activated platelets can also produce IL-1β (32). These pro-inflammatory factors can inhibit COL2A1 expression (24). Additonally, PRP includes de-differentiation factors, such as MMP-9 and IL-1β. These factors facilitate degradation of the collagen extracellular matrix and cause delayed healing effects (2,8). Thus, PRP can affect the expression of COL2A1. Because normal hyaline cartilage mainly consists of type 2 collagen, caution is recommended regarding the use of PRP as the sole treatment for OA.

Regarding the ECM, OA has been shown to negatively impact the articular matrix associated with proteolytic enzymes such as MMP-1, MMP-3 (1,20,27). These proteolytic enzymes are induced by inflammatory cytokines such as IL-1β and TGF-α (17). In addition, MMP-3 contributes to the activation of pro-MMP-1, and thus MMP-3 has a dual role in matrix destruction (29). MMP-1 and MMP-3 expression levels were significantly decreased in our data. This indicates that PRP has a protective function on the articular matrix.

Aggrecan is a major proteoglycan in articular matrix (25). Proteoglycans are composed of repeating glucuronic acid and N-acetylglucosamine disaccharides to create massive proteoglycan aggregates. These aggregates attract water, leading to significant hydration and swelling of the tissue. This structure provides a low friction coefficient on the joint surface and provides the cartilage with protective resiliency (23). In OA, aggrecan catabolism is induced by pro-inflammatory cytokines such as IL-1β and TNF-α along with the activities of proteolytic enzymes, such as aggrecanases. (10). In this study, IL-1β and TGF-α expression was decreased, and aggregan mRNA was upregulated, demonstrating that PRP can have regenerative effects on cartilage ECM.

In conclusion, PRP increases the proliferation and migration of canine chondrocytes, downregulates gene expression of inflammatoy cytokines and proteolytic enzymes, and upregulates the expression of genes related to ECM synthesis. Therefore, these results suggest that PRP may be a beneficial substance for canine OA treatment.

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