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Research Article

Proteases and Collagenase Enzymes Activity After Autologous Platelet-Rich Plasma, Bio-Physically Activated PRP and Stem Cells for the Treatment of Osteoarthritis in Dogs

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Abstract: The aim of this study was to evaluate the treatment of osteoarthritis (OA) in dogs with intra-articular injection of an (autologous platelet-rich plasma (PRP), Mesenchymal stem cells (MSCs), bio-physically activated platelet-rich plasma (B-PRP/plasma-poor platelets-PPP) and their combinations and to determine the changes in the levels of synovial fluid samples (inflammatory mediator, proteases (MMP-2 and MMP-9) and collagenase enzymes (MMP-1, MMP-8 and MMP-13) obtained from the joints. Total of 36 different breeds, sex, age and weight of dogs with osteoarthritis were used as the material. The groups were divided as Group I (PRP), Group II (MSCs), Group III (PRP + MSCs), Group IV (B-PRP), Group V (B-PRP + MSCs) and group VI (saline control). Only single injection was given. Metalloproteinases were measured by using the ELISA in preoperative and postoperative treatment of 0, 15th, 30th, 60th, and 90th days in synovial fluid samples. Clinical and radiographic examinations were performed on the 0, 15th, 30th, 60th, and 90th days. The results obtained from the PRP + MSCs combination group were more successful. It was also noted that successful results could be obtained with PRP alone or in combination with stem cells, especially if repeated intraarticular injections are required for osteoarthritis. New studies are needed to understand the effectiveness of B-PRP. Only B-PRP or stem cell combination was effective on many enzymes, but varying results were obtained with each case.

Keywords: *Anti-inflammatory effect, Collagenase, Gelatinase, Lameness, Pain scores, Dog*

Osteoartritli Köpeklerde Otolog Trombositten Zengin Plazma, Biyo-Fiziksel Olarak Aktive Edilmiş TZP ve Kök Hücre Tedavilerinin Proteaz ve Kollajenaz Enzim Aktivitesine Etkisi

Öz: Bu çalışmanın amacı, köpeklerdeki OA'in tedavisinde eklem içi enjeksiyonu yapılan, otolog trombositten zengin plazma (PRP), Mezenkimal kök hücreler (MSC'ler), biyo-fiziksel olarak aktive edilmiş trombositten zengin plazma (B-PRP/plazmadan fakir trombositler-PPP) ve kombinasyonlarının etkinliğinin, sinovyal sıvı örneklerinde (yangısel mediator, proteazlar (MMP-2 ve MMP-9) ve kollajenaz enzimlerinin (MMP-1, MMP-8 ve MMP-13) düzeylerindeki değişiklikleri değerlendirmektir. Farklı cins, cinsiyet, yaş ve kiloda OA teşhisi konulan 36 köpek kullanıldı. Gruplar; Grup I (PRP), Grup II (MSC'ler), Grup III (PRP + MSC'ler), Grup IV (B-PRP), Grup V (B-PRP + MSC'ler) ve Grup VI (kontrol) oluşturdu. Sadece tek intra artiküler enjeksiyon yapıldı. Operasyon öncesi ve tedavi sonrası 0, 15, 30, 60 ve 90. günlerde metalloproteinaz seviyeleri ELISA ile ölçüldü. Sinovyal sıvı örneklerinde klinik ve radyografik muayeneler 0, 15, 30, 60 ve 90. günlerde yapıldı. PRP + MSC kombinasyon grubundan elde edilen sonuçların başarılı olduğu gözlendi. Özellikle osteoartritin tedavisinde tekrarlayan intraartiküler enjeksiyonlar gerekiyorsa, PRP tek başına veya kök hücrelerle kombinasyon kullanımı halinde başarılı sonuçlar alınabileceği kaydedildi. B-PRP'nin etkinliğini anlamak için yeni çalışmalara ihtiyaç olduğu gözlendi. Genelde B-PRP veya kök hücre kombinasyonu birçok enzim üzerine etkili olduğu kayıt edidi. Ancak vakalar arasında farklı sonuçlar elde edildi.

Anahtar sözcükler: *Anti-inflamatuar etki, Kollajenaz, Jelatinaz, Topallık, Ağrı skorları, Köpek*

Introduction

Osteoarthritis (OA) is one of the most common joint diseases in dogs. It is observed in about a quarter of the dog population. Joint disease occurs in 20% of the dog population over the age of one [1]. In the pathogenesis of OA,

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cartilage destruction and remodeling in the bone, active chondrocytes in the articular cartilage and inflammatory cells around are effective.

Metalloproteinases (MMPs) can degrade all components of the extracellular matrix which are central role in the disease process of arthritis. The mechanism involved in cartilage degradation is not fully understood. Macrophages, neutrophils and chondrocytes effective in the destruction of the cartilage matrix. Especially, MMPs expressed in connective tissue degrade collagen [2]. Proteases and collagenases (collagenase-1 (interstitial collagenase (MMP-1); collagenase-2 (neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13) have an active role in the destruction of the cartilage structure (collagen and proteoglycan) in osteoarthritis. In addition, MMP-13 serves as a major mediator of type II collagen cleavage and matrix degradation [3]. Increase of MMPs has been demonstrated in synovial fibroblasts, chondrocytes and inflammatory cells [4,5]. MMP-2 and MMP-9 have an active role in cartilage degradation [5]. It was shown that MMPs triggers the release of growth factors that independently affect the extracellular matrix and contribute to all stages of wound healing [2].

PRP has been used frequently in human patient with OA [6,7]. It contains different types of growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGFs) and other cytokines that stimulate healing of soft tissue. Growth factors are effective for healing tissues in cell proliferation and regeneration of matrix metabolism. PRP has begun to be used as a source of autologous growth factor accelerating the healing process in articular cartilage, intervertebral disc and bone [6]. In recent years, intra-articular PRP has also been used in the treatment of OA in dogs [8-12].

Bio-physically activated PRP (Platelet poor plasma) is a biologic treatment that is derived from blood. It is similar to PRP except that it has a lower concentration of platelet cells but number of growth factors are high. B-PRP contains a concentration of growth factors that can help stimulate healing and also provide extended anti-inflammatory relief. Before PRP is applied, the process of revealing the healing and growth factors within the platelets is called the activation of thrombocytes. The activation of platelets is possible by chemical and physical methods. In the chemical method, bovine thrombin or calcium chloride ($CaCl_2$) is added to PRP to activate platelets before application. It has been shown that 70% of the growth factors from α-granules are released within 10 min and almost all within 1 h after the addition of activator [9,13].

The aim of this study was to evaluate the treatment of OA in dogs with intra-articular injection of an (autologous platelet-rich plasma (PRP), Mesenchymal stem cells (MSCs), bio-physically activated platelet-rich plasma (B-PRP/ plasma-poor platelets-PPP) and their combinations and to determine the changes in the levels of synovial fluid samples (inflammatory mediator, proteases (MMP-2 and MMP-9) and collagenase enzymes (MMP-1, MMP-8 and MMP-13) obtained from the joints.

Material and Methods

Ethical Statement

This study was approved by the Selcuk University Animal Experiments Local Ethics Committee (Approval no: 2016/14).

Design of the Study

Thirty-six dogs (different age, breed, gender, weighing 25 to 50 kg (mean 32.1), mean age 5.1 years) diagnosed with unilateral stifle OA were used as the study material. Randomized, controlled trial was designed for study in Small Animal Hospital, Faculty of Veterinary Medicine, University of Selcuk, Türkiye. The groups were divided as Group I plasma rich plasma platelets (PRP), Group II (Mesenchymal stem cells [MSCs]), Group III (PRP + MSCs), Group IV (bio-physically activated platelet-rich plasma (B-PRP/PPP), Group V (B-PRP + MSCs) and group VI control (0.9% isotonic saline). In particular, the dogs with no systemic disease were used as a material. In the last three months, no surgical application or steroid applications were made in the dogs participating in the study. Osteoarthritis was evaluated as primary and secondary according to their cause. A diagnosis of secondary OA was made when abnormalities causing joint instability (e.g., cranial cruciate ligament rupture) were evident. Cases with suspected meniscus damage were excluded from the study. Only dogs who had not received any treatment or nutritional supplement before were included in the study. Clinical examination, radiographic examinations and joint fluid analysis were performed on days 0, 15th, 30th, 60th, and 90th in each group.

Clinical Examination

The diagnosis was made by clinical examination while sitting, walking, running, climbing stairs, and jumping activities especially by tests performed on the affected joint. Pain scoring tests (Canine Brief Pain Inventory, CBPI) and walking scoring tests (Hudson Visual Analog Scale, HVAS) were performed [14,15]. The repeated walking and pain rating tests were examined by the same veterinarian.

Radiographic Examination

Radiographic examination was done before treatment and after at 4, 8, and 12 weeks (Regius 110; Konica Minolta, Tokyo, Japan). The radiograms were checked for enthesophte formation, periarticular osteophytes, subchondral bone

sclerosis, fat pat displacement, intra-articular mineralization, articular surface narrowing and soft tissues were evaluated. Classification of OA was mild (1), moderate (2), moderate to severe (2 to 3), or severe (3) as previously described by according to the Kellgren-Lawrence scoring [16]. The repeated radiological examination was also performed and evaluated by the same veterinarian.

Platelet-Rich Plasma Preparation

Genesis Autologous Cell System 2 (Neo Genesis, Seoul, Republic of Korea) branded PRP (30 mL) preparation kits were used in the study to obtain the standardized platelet counts at the desired level. The acid citrate (3 mL) from the kit was added to a 50 mL injector in order to prevent blood clotting and 27 mL of the blood was taken from the jugular vein. A 30 mL mixture of the blood and acid citrate was injected into the Genesis tube. The tubes were centrifuged in a Genesis centrifuge instrument at 1700 x g for 5 min. The anticoagulated blood revealed three layers after centrifugation: bottom layer (red blood cells, density = 1.09); middle layer (platelets and white blood cells (buffy coat), density = 1.06); top layer (plasma, density = 1.03). After centrifugation, the bottom lid of the tube was replaced with a buffy coat controller cap and the pusher was assembled. The buffy coat controller was turned counter-clockwise until the layer of the red blood cells reached the "0" line. The platelet poor plasma portion was removed, the pusher was turned until the layer of the red blood cells reached the top, and the PRP portion (3-5 mL) was pushed into a Luer lock injector. The platelet count obtained from the Genesis Autologous Cell System has been reported to be 4-8 times greater than the normal. The number of platelets injected for each dog ranged between 1.200.000 and 1.500.000. The injection was given immediately when PRP was prepared [8,9].

Preparation of Bio-Physically Activated Platelet-Rich Plasma

After the PRP was prepared, it was placed on one end of the Biophysics activator instrument. An empty Luer lock injector was mounted on the other end of the instrument. The activation process was achieved by injecting platelets from one syringe to the other via the activator a total of 30 times [9]. The platelet concentrate was injected immediately. The concentrate (PRP and B-PRP) was injected intraarticularly until sufficient resistance was obtained to push back the syringe plunger.

Allogeneic Adipose Mesenchymal Stem Cell (MSCs) Isolation and Expansion

Allogeneic adipose stem cells preparation was produced by Biovalda Ltd. (Ankara, Türkiye). MSCs were isolated from adipose tissue allogeneic origin and reproduced. Adipose tissue donors were recruited from dogs (n = 3) included in the study, which were determined to have no infectious or infectious diseases. Approximately 1000.000-1.300.000 cells were injected intraarticularly.

Joint Fluid Sampling

After centrifugation for 5 min (10.000 G), the joint fluid samples were frozen and stored at -80°C until analysis. The joint fluid was dissolved shortly before the analysis. Various MMPs that work as metalloproteinases (MMP-1, MMP-2, MMP-8, MMP-9, MMP-13) were measured by ELISA.

Lameness and Pain Evaluations

Pain and lameness evaluations were performed on the 1st, 3rd, 5th, 7th, 15th day and at weeks 4, 8 and 12 after the PRP and saline injections. Canine Brief Pain Inventory (CBPI) and Hudson and Visual Analog Scale (HVAS) [14] scoring system were used for lameness and pain assessment [15,17]. The study was conducted in a blind manner. The same veterinarian was assessed for dogs. The same scoring system was used throughout the 12 weeks for all time points.

Pressure Analyzer (Pet Safe Stance Analyzer)

A pressure analyzer (Pet Safe Stance Analyzer, Kruuse, Germany) was used to measure the unequal weight distribution for extremities in dogs. It is based on the principle of proportionally calculating the body weight falling at four different measurement points and transferring them to the computer. In practice, the analyzer was made ready by weight calibration before the cases were examined. Subsequently, the subjects were placed on the analyzer one by one and fixedly held with one extremity in each square compartment. Meanwhile, by taking weight measurements (at least 15 times) from an auxiliary analyzer control, the average pressures were automatically recorded in the computer.

Protease and Gelatinase Zymography

Gelatin zymography technique was used for gelatinase activity levels using a modification of a previously described [4,8,9]. Synovial fluid samples collected on day 0, 30, 60, and 90 after PRP or saline injection were used for analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) for Protease and Gelatin-Degradation

Synovial fluid samples were taken from dogs before the application of groups and on the post-op. 15th, 30th, 60th, and 90th days. ELISA was used to measure degree of disintegration of gelatin. Double-antibody sandwich ELISA was used for measuring MMP-1 (cat.no: 201-15-0945); MMP-2 (cat.no: 201-15-0159); MMP-8 (cat.no: 201-15-0887); MMP-9 (cat.no: 201-15-0038) and MMP-13 (cat.no: 201-15-1978) Sunred Biological Technology Co., Ltd. (Shanghai, China). The intensity of the reaction product was measured at 450 nm in an ELISA plate

reader (MWGt Lambda Scan 200, Bio-Tek Instruments, Winooski, VT, USA). All samples were assayed in duplicate. The calculation was made following the manufacturer's recommendations.

Statistical Analysis

SPSS 20.0 (IBM, USA) was used for The HVAS and the CBPI scoring tests. The data is analyzed by Non-parametric the Mann Whitney U test. The results of the joint fluid ELISA tests run on the six groups were evaluated by Tukey's test.

Results

Results of Clinical Examination

Medium and large breed dogs were evaluated. Their age ranged was from 4 to 8 years. No complications related to platelet concentration, activated platelet concentrate, stem cells or saline were observed.

Finding of Radiographic Examination

Thirty dogs had moderate OA (radiographic score 2), four had moderates to severe OA (radiographic score 2-3), and two had severe OA (radiographic score 3). At the end of the 12th week, the same procedures were performed as before the treatment. Considering the radiological results, no difference was found between the groups.

Preparation of PRP, Bio-Physically Activated PRP, Allogeneic Adipose Mesenchymal Stem Cell (MSC)

The platelet concentration (mean 1.420.000 platelets/μL) was significantly (P<0.001) higher than the platelet counts in the blood samples, representing a 4.0- or 5.0-fold increase in the platelet count. The White Blood Cell count (1.09 s/μL) was significantly decreased. Hematocrit was also significantly lower than Hct for the blood samples.

Lameness and Pain Evaluations

The HVAS and CBPI scores were not affected by the clinicians who evaluated the cases because of blind manner. For the dogs in the PRP group, activated PRP, MSCs scores for all components of the HVAS (mood, attitude, activity, comfort, exercise, playfulness, walking comfort) and all components of the CBPI (general activity, pain, rise, walk, run, climb and the ability to enjoy life) were significantly different between pre-treatment and week 4 (P<0.05), week 8 (P<0.05), and week 12 (P<0.05). Improvements in treatment outcomes were noted in almost all groups.

Pressure Analysis Findings

In the study, unequal weight distributions in the extremities were evaluated in the compression analysis and recorded all. Pre-op and post-op 15th, 30th, 60th, and 90th day results were compared individually. The problematic extremity of the 36 dogs were easy to distinguish from the other limbs as their compression force was insufficient due to the inadequate weight usage. As a result of the treatments, no difference was observed between in the PRP group, activated PRP, MSCs groups. Pressure analysis findings did not support clinical findings in some cases.

Protease and Gelatinase Zymography

MMP-2 activity of 70 kDa enzyme in pre-treatment synovial fluid samples was increased. Enzyme activity was seen at 204 kDa (MMP-9 pro-dimer) and 257 kDa (MMP-9 dimer). MMP-1 was partially inhibited in MSCs, MSCs + PRP and MSCs + B-PRP day 90th. The MMP-2, MMP-8 and MMP-9 concentration was inhibited in the B-PRP group and these enzymes were inhibited partially all groups. MMP-13 concentration was inhibited in the MSCs, MSCs+PRP and MSCs + B-PRP. The effects of 0, 50, and 100 mM EDTA (MMP inhibitor) were also investigated. Enzyme bands were partially inhibited by 50 mM EDTA and totally inhibited at 100 mM.

Metalloproteinases ELISA Analysis Results in Joint Fluid Samples

Gelatin degradation of synovial fluid demonstrated as measured by ELISA. The effects of single and combined administration of PRP, B-PRP and MSCs on joint fluid for MMP-1 *(Table 1, Fig. 1)*, MMP-2 *(Table 2, Fig. 2)*, MMP-8 *(Table 3, Fig. 3)*, MMP-9 *(Table 4, Fig. 4)*, and MMP-13 *(Table 5, Fig. 5)*, levels in dogs with osteoarthritis were presented. MMP-1 levels were started to decrease statistically in B-PRP+MSCs group on the 15th and enzyme

Table 1. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-1 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	46.00±18.98	60.91±21.90^{ab}	69.36±32.51^{a}	47.46±25.93	81.16±27.62^{a}
PRP	41.11±15.76	52.96±18.65^{ab}	10.07±9.72^{b*}	43.87±23.05	43.82±19.05^{ab}
BPRP	63.66±14.97	31.13±22.12^{ab}	67.47±44.77^{a}	50.53±27.47	49.05±17.97^{ab}
MSC	58.35±25.15	57.93±21.85^{ab}	68.83±36.26^{a}	52.12±32.71	32.61±15.83^{b}
PRP+MSC	42.15±28.03	27.63±15.34^{b}	44.07±19.47^{ab}	41.66±24.04	25.33±13.83^{b}
BPRP+MSC	38.63±15.57	71.73±35.35^{a}	90.70±19.36^{a*}	69.80±25.40*	62.31±29.78^{ab}

^{ab} *Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

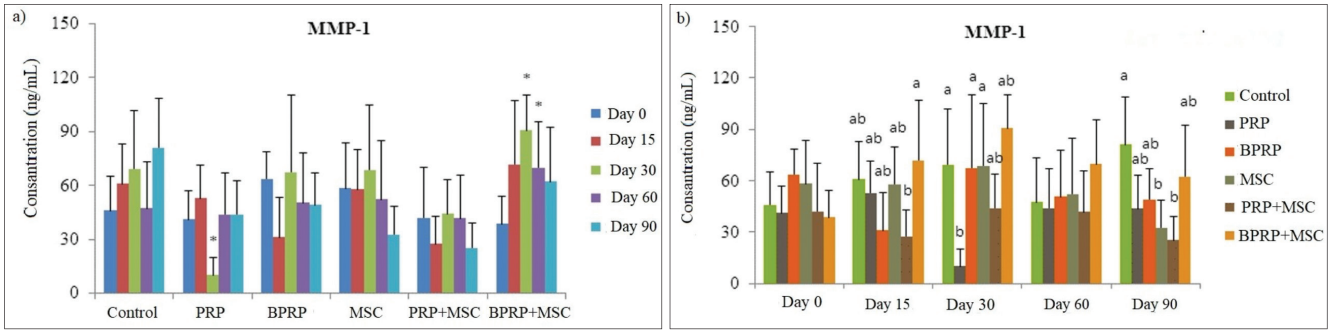

Fig 1. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-1 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 2. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-2 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	165.75±36.13	157.08±45.02^{ab}	159.06±48.19	142.99±44.78^{ab}	254.83±36.09^{a*}
PRP	178.90±58.16	167.34±62.93^{a}	165.82±47.77	196.13±43.97^{a}	143.16±33.94^{b}
BPRP	198.53±34.46	227.70±56.87^{a}	152.07±57.30	105.61±37.08^{b*}	81.99±31.62^{bc*}
MSC	144.46±50.99	145.95±59.13^{ab}	136.18±60.97	81.99±49.86^{b}	87.34±46.57^{bc}
PRP+MSC	141.93±65.51	64.47±52.87^{b}	115.91±48.01	71.86±41.95^{b}	47.42±40.41^{c*}
BPRP+MSC	149.18±29.76	139.03±48.24^{ab}	132.72±28.36	71.13±44.76^{b*}	93.61±42.01^{bc*}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

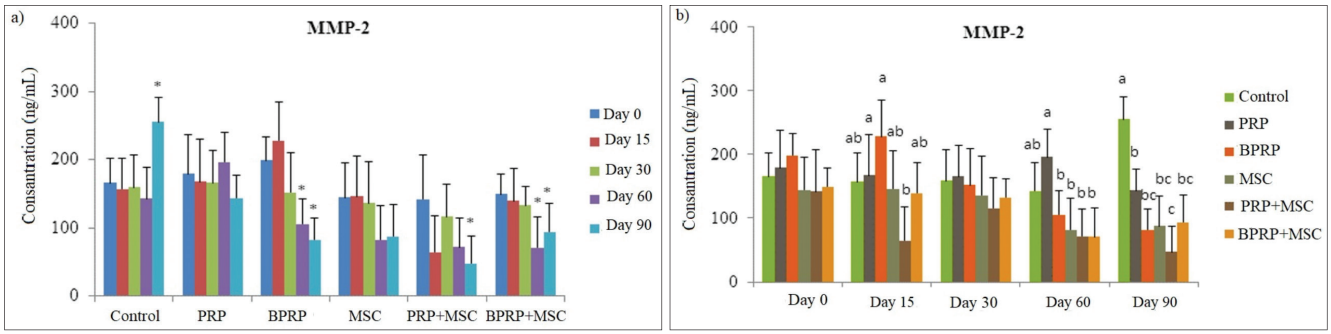

Fig 2. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-2 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 3. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-8 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	36.00±17.11	44.58±19.44^{a}	36.58±15.38	42.99±14.98^{ab}	63.06±13.88^{a*}
PRP	42.07±22.77	40.87±22.87^{ab}	40.05±9.98	51.87±19.46^{a}	31.30±20.04^{b}
BPRP	39.88±9.85	34.15±8.52^{abc}	23.68±15.28*	27.14±5.22^{bc}	19.33±8.07^{b*}
MSC	25.12±10.85	26.00±7.78^{abc}	31.04±13.37	17.73±9.22^{c}	22.47±7.36^{b}
PRP+MSC	29.34±13.19	15.10±8.08^{c*}	20.97±7.73	17.77±11.13^{c}	11.14±8.18^{b*}
BPRP+MSC	25.11±9.44	19.94±8.63^{bc}	35.56±4.70	23.17±9.12^{bc}	24.40±9.09^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

levels decreased in PRP + MSCs on the 30th (P<0.05). MMP-2 level decreased significantly in B-PRP and PRP + MSCs group on the 60th and B-PRP + MSCs 90th day. However, MMP-2 level decreased after treatment in all groups. MMP-8 level decreased significantly in the B-PRP and PRP + MSCs group on the 60th and in the B-PRP +

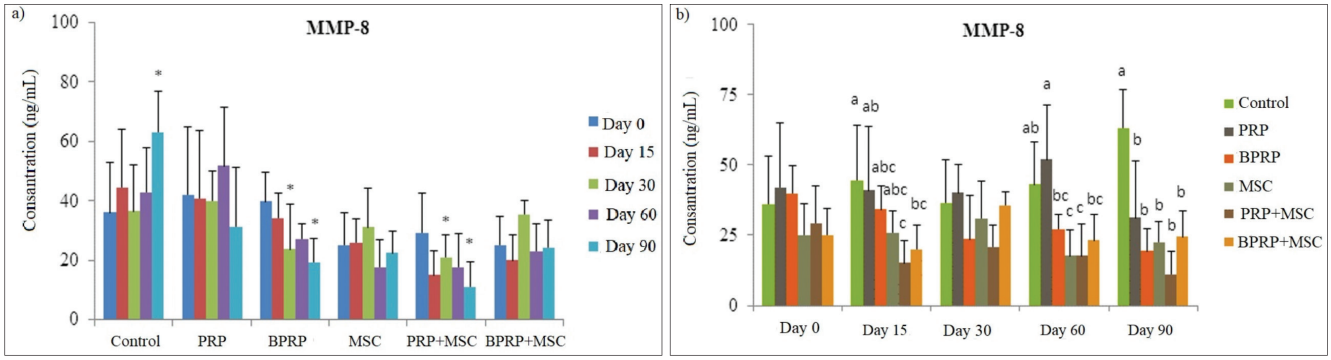

Fig 3. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-8 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 4. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-9 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	172.42±70.37	297.52±94.04^{a*}	257.58±124.22^{a}	284.07±105.03^{a*}	417.01±116.97^{a*}
PRP	217.90±45.43	210.63±82.25^{ab}	223.02±57.79^{ab}	240.77±62.05^{ab}	189.62±58.77^{b}
BPRP	227.69±22.25	172.26±81.02^{b}	165.75±20.65^{ab*}	163.30±39.57^{bc*}	128.37±46.10^{b*}
MSC	182.96±71.41	173.88±26.06^{ab}	169.06±40.43^{ab}	121.97±58.78^{c}	143.75±32.65^{b}
PRP+MSC	166.02±61.56	119.11±74.72^{b}	138.41±64.43^{b}	74.59±33.28^{c*}	85.27±68.52^{b}
BPRP+MSC	150.64±58.15	139.99±43.02^{b}	242.70±38.50^{ab*}	161.78±52.78^{bc}	167.49±56.94^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

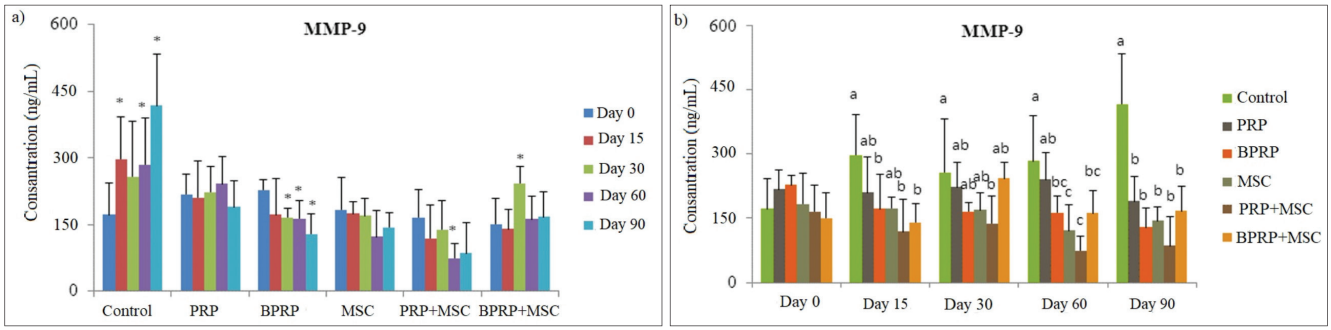

Fig 4. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-9 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

Table 5. *The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-13 (ng/mL) levels in dogs with osteoarthritis (Average ± SS)*

Group	Day 0	Day 15	Day 30	Day 60	Day 90
Control	15.98±9.34	26.83±11.31^{ab}	23.91±11.20	21.51±10.09	35.85±8.71^{a*}
PRP	24.49±11.96	24.44±11.15^{ab}	21.77±8.82	33.32±11.18	22.53±8.69^{ab}
BPRP	23.92±10.02	18.87±12.29^{ab}	20.00±10.61	18.20±8.73	15.46±13.17^{b}
MSC	19.71±10.76	32.10±15.00^{a}	30.69±15.14	19.18±14.36	23.64±11.19^{ab}
PRP+MSC	24.12±10.62	9.21±5.44^{b*}	17.07±6.41	13.79±13.08	7.95±6.10^{b*}
BPRP+MSC	22.16±7.32	25.25±14.25^{ab}	31.71±5.16*	22.87±11.53	16.49±3.58^{b}

*^{ab} Different letters in the same column are statistically significant (P<0.05); * Statistically significant compared to day 0 (P<0.05)*

MSCs on 90th day. MMP-8 levels also decreased in all groups at the end of the 90th day. MMP-8 level increased significantly in the control group on the 90th day. B-PRP + MSCs groups were significantly lower than the control

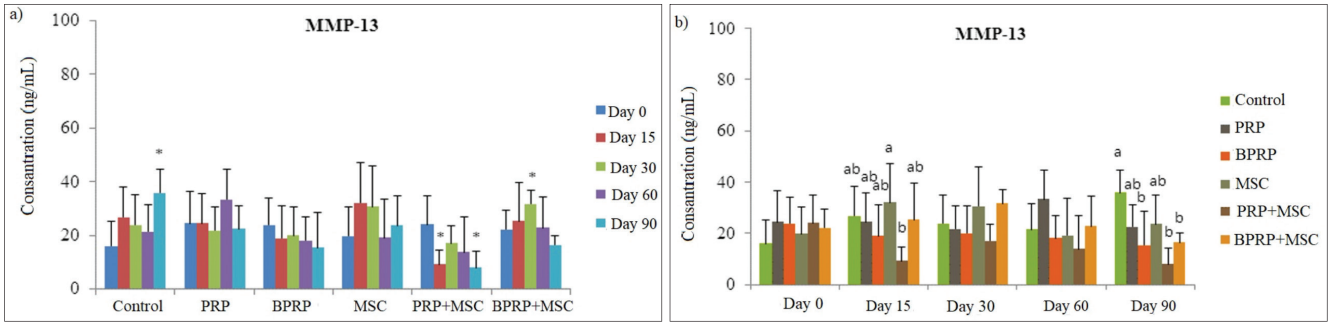

Fig 5. The effect of single and combined administration of PRP, PRP and MSC on joint fluid MMP-13 (ng/mL) levels in dogs with osteoarthritis. (a) In-group, (b) Shows differences between groups. ab- Different letters in the same column are statistically significant (P<0.05), * Statistically significant compared to day 0 (P<0.05)

group (P<0.05). MMP-9 level decreased significantly in the B-PRP group, PRP + MSCs group on the 60th and B-PRP + MSCs in 90th day. MMP-9 levels decreased after treatment in all groups. Especially, B-PRP+MSCs groups were significantly lower than the control group (P<0.05). Nevertheless, MMP-9 level increased significantly in the control group on the 90th day. And also, MMP-13 levels decreased after treatment in all groups.

Discussion

Treatment selections for dogs with osteoarthritis are varied. Despite this diversity, the clinical results of the treatment options are not satisfactory. However, studies conducted in recent years support different treatment alternatives such as weight control, NSAID, neutrocetic drugs and prosthesis applications [18]. The use of intraarticular, autologous PRP in the current treatment of osteoarthritis, which is common in dogs, has been demonstrated in our previous study [8,9,11,19]. Especially PRP has been shown to inhibit MMP-9 enzymes that are effective in the pathogenesis of osteoarthritis. Protease enzymes and cytokines play an important role in osteoarthritic pathogenesis. In particular, it causes the destruction of proteoglycan (PG) and collagen structure in cartilage tissue at the onset of the disease. In recent discussions, PRP and MSCs combination has been reported to provide faster recovery and positive results in clinical and gait analysis [19]. However, enzyme analyses are not available in any clinical studies presented. In addition, there is limited studies on the use of PRP with bioactivators [12]. In the current study, with a broad perspective on current treatment methods of osteoarthritis, clinical findings to be obtained as a result of joint fluid analysis for protease and collagenase enzymes (MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13) were evaluated.

Particularly, in the radiographic evaluation, 30 of 36 cases were diagnosed with 1 degree osteoarthritis, 4 cases moderate (2nd degree), and 2 cases severe (3rd degree) osteoarthritis. Evaluations were made based on Innes et al.[20]. In this study, it was considered that no examples could be provided in terms of breed, sex, weight and the degree of osteoarthritis. Therefore, it was not possible to ensure homogeneity between animal materials. Synovial fluid samples were taken from animals with osteoarthritis before and after treatment, in this way, it would provide the opportunity to be evaluated not only as a group but also individually. At the end of the treatment, it was observed that the cases generally improved. However, in some groups, the recovery process and a comfortable life started faster such as PRP + MSCs combination group.

The Hudson Visual Analog Scale-HVAS HVAS and Canine Brief Pain Inventory CBPI have been evaluated in previous studies [14,15,17] and are known as subjective methods of assessing pain and lameness. The results indicated that clinical efficacy of PRP + MSCs combination therapy. According to the subjective HVAS, statistical (P <0.05) changes were observed in the walking and pain tests of dogs on day 0 and 15th, 30th, 60th, and 90th (P<0.05) changes were observed in all groups. Lameness assessment was used with force plate analyzer in the study. The results were compared individually pre-op and post-op 90th day. According to the force plate findings, there is an improvement in (85%) cases. But force plate findings did not support clinical findings in 5 cases. As a result of the treatments, no difference was observed between the groups.

It has been shown that the amount of gelatinase enzymes (MMP-2 and MMP-9) increases in the joint fluid in osteoarthritis cases in dogs [4]. Increases in the amount of MMP-2 and MMP-9 enzymes have been demonstrated as an indicator of destruction in the joint cartilage and distinguishing the early stage of OA [20,21]. MMPs are catabolic mediators responsible for the degeneration of joint cartilage. An increase in the amount of MMP-2 and MMP-13 was determined especially in the advanced stages of the disease [21]. It has been reported that the levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 increase in case of joint damage [10,19,22]. Endogenous factors are effective in inhibiting MMP. α2 macroglubine is the most important regulator of collagenolysis to play active role in the inhibition of MMP-2, MMP-9 and MMP-

14 enzymes. The release of MMPs is controlled by TIMP. When MMP/TIMP is in equilibrium in normal tissue, the balance is disrupted in OA and MMP level increases [23]. It has been stated that MSCs and PRP application increase TIMP level. In the presented study, proMMP-9 (inactive form) has been demonstrated with different molecular weights on zymography gels. While 204 kDa was seen as the inactive form of MMP-9, 257 kDa was displayed as the active form of MMP-9. The destruction of the cartilage structure begins with the arrival of the enzyme in the active form [24]. ELISA results were also shown that, MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 levels increased in the control group. But MMP-2, MMP-8 and MMP-9 levels statistically significantly decreased in the B-PRP group and in addition MMP-13 levels decreased in the PRP-MSCs group. Furthermore, MMP-1 level also decreased in PRP-MSCs, MSCs and B-PRP groups on day 90th compared to day 0, but there was wide variation within the cases. No statistical difference was observed. The levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 decreased in all groups compared to the control group. One of the aims of the study here is to determine which group is more effective. According to the results obtained from the study, it has been shown that MMP activation is suppressed in the PRP group within one month. According to the zymography results, it was observed that enzyme activation continued in the control group. This situation has been determined as encouraging about the anti-inflammatory activity of PRP. In this study, MMP-13 level decreased in the 90th day of PRP, B-PRP, MSCs, MSCs + PRP, MSCs + B-PRP groups, while the most successful group was recorded as the MSCs + PRP combination group. MSCs + PRP tries to regulate the pathological effects of OA, for this purpose, it has been reported to have a role such as creating less inflammation, pain and providing lubrication in the joint. PRP reduces cartilage destruction in the formation of osteoarthritis by increasing endogenous HA production as well as its anti-inflammatory effect [25]. PRP is showing a similar effect to HA, inhibits the inflammatory mediator in the synovial membrane and articular cartilage structure [26]. In addition, PRP contains abundant stem cells, which enables the stem cell to be directed to the tissue to be treated [27]. In this respect, MSCs + PRP combination group showed statistically significant changes [18]. It has been also reported that PRP reduces MMP-13 expression, which has an important role in the development and destruction of OA, by almost 100% [5]. The combination use of PRP and MSCs causes more collagen II to proliferate, while reducing chondrocyte apoptosis [28]. Using a combination of PRP and MSC, both symptomatic and structural improvement was also noted [28,29]. Therefore, in the study, MSCs + PRP group was found to be the most successful in its activity against in terms of inhibition of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 enzymes. There is no study on the efficiency of autologous bioactivator platelet-poor plasma (B-PRP) in enzyme inhibition. In recent years, it is thought that bioactive proteins released from platelets and high concentrations of growth factors affect the regeneration of tissues such as ligaments and cartilage, which have limited healing capacity under normal conditions. It has been explained that uncovering the growth factors in the platelet without injecting it into the joint will further increase its effectiveness. However, MMP-1, MMP-2, MMP-8, MMP-13 were decreased in the B-PRP group, at the end of the 90th day after treatment. But there was no change in MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 levels between day 0 and day 90th in the stem cell combination of B-PRP. These results show us that the growth factors coming out of the platelet did not increase its effectiveness. Although the cases are seen well clinically, no change was observed between the pre-treatment and post-treatment radiological analyzes between the groups. When the radiological examination results were examined, it was determined that the treatments performed had no effect on the reduction of osteophytic growths too.

As a result, the results obtained from the PRP+MSCs combination group were more successful. It was noted that successful results could be obtained with PRP alone or in combination with MSCs, especially, repeated intraarticular injections are required in osteoarthritis. First, PRP has an anabolic effect on chondrocytes and synoviocytes, resulting in increased cell proliferation and hyaluronic acid release. Second, PRP could be acted as a bioactive cytoskeleton for cartilage destruction and to increase cartilage regeneration. Thirdly, PRP has potential for anti-inflammatory effect and alleviate OA symptoms. PRP has an anabolic effect on the proliferation of chondrocytes and MSC cells, as well as suppresses deregulation and development in the matrix. Although B-PRP is new, more studies need to be made to understand its effectiveness. Only B-PRP or MSCs combination was effective on many enzymes, but varying results were obtained between each case.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (M. Arican).

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Ethical Statement

This study was approved by the Selcuk University Animal Experiments Local Ethics Committee (Approval no: 2016/14).

Conflict of Interest

There was no conflict of interest in regards to authors reporting their findings.

Authors' Contributions

Study design, survey development, data analyses and manuscript preparation performed by all authors. MA: conception and design of the study, acquisition, analysis and interpretation of data, drafting and critical revision of the article; KÜ: analysis of the data and critical revision of the article. KP; acquisition of the data, EOU: acquisition of the data; GS: interpretation of data.

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