Effect of intra-articular administration of autologous PRP and activated PRP on inflammatory mediators in dogs with osteoarthritis

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Abstract: The aim of this study was to investigate the effects of the intra-articular use of platelet rich plasma (PRP) and bio-physically activated PRP (BPRP) on the inflammatory mediators for the treatment of osteoarthritis in dogs. The animals included in this study were 36 mix breed dogs diagnosed with osteoarthritis in the stifle as a result of the clinical and radiological examinations. The dogs were randomly divided into three groups: PRP (platelet-rich plasma), BPRP (biophysically activated platelet-rich plasma) and control (given 0.9% isotonic saline). These three main groups were each further divided into two groups as single and double according to the number of intraarticular administrations. Joint fluid analyses, a clinical examination (Hudson Visual Analog Scale and Canine Brief Pain Inventory Tests) radiographic and ultrasonographic examinations were performed on days 1, 15, 30, 60, and 90 for each group. Genesis System 2 branded and BPRP preparation kits were used in this study. An ELISA method was used to measure the cytokines in charge of the inflammatory mediation (IL-1β, IL-6, IL-10, TNF-α) in the synovial fluid samples. The records obtained from the walking and pain rating tests were subjected to a statistical analysis program and a Mann-Whitney *U* test was performed. The results of the ELISA were evaluated by a Tukey test. There was a significant difference between the single and double groups of the PRP administration on days 60 and 90 ($P < 0.05$) in the walking and pain scores. The double groups of the PRP had better results than the single groups. There was a significant difference between the single groups of the PRP and BPRP for the IL-10 on the 30th day ($P < 0.05$). In the single application groups, the BPRP was better than the PRP on day 30 in the IL-10 measurements. In the comparison of the single and double administration groups, there was significant difference between the single and double groups of the BPRP on day 90 ($P < 0.05$). The double groups of the BPRP had better results than the single groups. In addition, the biophysically activated PRP was found to be superior to the PRP for the IL-10 content. In conclusion, the efficacy of the PRP and BPRP was related to the degree of the osteoarthritis (OA). Especially the success rate in the acute OA patients was higher due to the anti-inflammatory activity of the BPRP. Moreover, the double administration groups gave more positive results than the single administration groups.

Keywords: anti-inflammatory effect; degenerative joint disease, IL-1β; IL-6; IL-10; TNF-α

Osteoarthritis (OA) is defined as a heterogeneous disease characterised by the slow progressive degeneration in the cartilage of the synovial joints in addition to changes in the bone and soft tissue (Tamura et al. 2002; Arican 2014).

Modified treatment modalities for OA are much more effective than ordinary treatments. These modified methods include: training, walking therapy and bandages, physiotherapy (ultrasound, thermotherapy, cryotherapy, low level laser, subcu-

taneous electrical stimulation), acupuncture, medications (nonsteroidal drugs, glycosaminoglycan administration), regenerative treatment (intraarticular corticosteroid, hyaluronic acid, platelet rich plasma – PRP), stem cell administration, autologous protein solution, abrasion, arthroplasty, osteotomy, and eventually joint resection (Lohmander 1994; Aragon et al. 2007; Sanderson et al. 2009).

Recent OA treatments are intended not only to cure joint pain and inflammation, but also to improve the anabolic activity of the chondrocytes and increase the tissue repair by halting tissue degeneration. The intra-articular administration of the PRP is thought to have the potential to slow down the progression of OA by stimulating cartilage anabolism, due to the action of the growth factors it contains (Stief et al. 2011; Knop et al. 2016).

PRP is a rich platelet concentrate containing higher amounts of growth factors than normal blood and is obtained by centrifuging the patient's own blood (Pietrzak and Eppley 2005; Anitua et al. 2009). Fahie et al. (2013) and Arican et al. (2015) reported that the concentration of thrombocytes used in the healing process should be at least 4–8 times higher than their usual concentration.

The release of the recovery and growth factors from the thrombocytes before the administration of the PRP is called the activation of platelets. The biophysical activation of the PRP (BPRP) is the process of the physical release of the growth factors in the platelets. Activated platelets are transformed into a gel form. The advantage of the gel matrix form is that the PRP shows better adhesion to tissue and acts as a scaffold for cell migration and extracellular matrix formation on the healing site especially with intra-articular administration. Platelet activation is possible by chemical and physical methods. Special equipment is needed for the physical activation: the PRP is passed through this equipment to release the growth factors. The advantage of the physical activation is that the 100% autologous system is protected and it does not have any side effects (Yilmaz and Kesikburun 2013; Knop et al. 2016).

Many biological variables may affect the clinical outcomes following the intra-articular PRP administration. Given this fact, the aim of this study was to investigate the effect of the intra-articular PRP and BPRP administration on the clinical findings and the joint fluid analysis (inflammatory mediators) for the treatment of OA in dogs.

Cytokines especially have anabolic and catabolic effects on the cartilage metabolism. The cytokine IL-6 plays a role in the destruction of the cartilage (Brenn et al. 2007); TNF-α increases the amount of the collagenase enzyme, which causes cartilage damage and stimulates the IL-1β synthesis; IL-10 plays a role in the suppression of the IL-1 and TNF-α synthesis. In recent studies, IL-10 has been shown to be a target for the treatment of OA (Fernandes et al. 2002). In addition, Arican et al. (2015) showed that inflammatory mediators were suppressed by following the injection in dogs treated with PRP. However, it has been shown that the enzyme and inflammatory mediator concentrations begin to increase again in 20–30 days. Therefore, in this study, we aimed at comparing and evaluating the results of the single and double administrations of PRP and BPRP, for their anti-inflammatory effects.

MATERIAL AND METHODS

This study took place under the Ethics Committee Guidelines and the 2015/42 decision number of the Selcuk University, Faculty of Veterinary Medicine, Experimental Animal Production and Research Center.

The animals under study were 36 mixed breed dogs (6 females, 30 males, mean weight 30 ± 1 kg, mean age 5 ± 1 years) diagnosed with osteoarthritis in the stifle joint as a result of the clinical and radiological examination. The dogs with the OA were randomly divided into three groups: PRP, bio-physical activated PRP and control (0.9% isotonic saline). These three main groups were further divided into two subgroups, single and double, according to the number of intraarticular administrations. The dogs included in the study did not undergo any surgical procedure in the last six months, any intraarticular injection in the last three months, and no parenteral steroid anti-inflammatory drugs in the last month. The joint fluid analysis, clinical examination, radiographical and ultrasonographical examinations were performed in each group on days 0, 15, 30, 60, and 90.

Clinical examination

For the routine clinical examination of the dogs, tibial compression and cranial drawer sign tests

were performed on the affected joint after sitting, walking, climbing a ladder, going down a ladder, running and jumping activities. Pain rating tests (Canine Brief Pain Inventory, CBPI) and walking rating tests (Hudson Visual Analog Scale, HVAS) were performed (Hudson et al. 2004; Brown et al. 2008). The repeated walking and pain rating tests were performed and evaluated by the same veterinarian.

Radiographic examination

The radiographic examinations (Regius 110; Konica Minolta, Tokyo, Japan) (standing Latero-Medial (L-M), Cranio-Caudal, Tibial Compression L-M) of the dogs were performed on the specified days. The radiographs were evaluated by the same veterinarian. The radiographic interpretation was evaluated according to the Kellgren-Lawrence scoring (Kellgren and Lawrence 1957) (0th Degree: No radiographic findings; 1st degree: Narrowing of the joint cavity, possible osteophytic proliferation; 2nd degree: Significant osteophytic proliferation, severe narrowing of the joint cavity; 3rd degree: Severe narrowing in the joint cavity, sclerosis, severe osteophytic proliferation, deformity in the subchondral bone; 4th degree: Severe sclerosis, wide osteophytic proliferation, marked subchondral deformity).

Ultrasonographic examination

An ultrasound examination (Esaote Piemedical, Maastricht, The Netherlands) of the stifle was performed with a 5–7.5 MHz convex probe. In the ultrasonographic evaluation, the scoring system developed by Muzzi et al. (2009) was used. According to this system, increases in the synovial fluid (the presence of anechoic and hypoechoic areas due to the increased fluid between the tibial and femoral intercondylar fossae are scored (0: none; 1: mild; 2: moderate; 3: severe; 4: more severe). The intra-articular tissue reaction (the presence of the fibrous tissue at the connection points of the cranial intermeniscal ligament and/or cranial cruciate ligament) (0: none; 1: mild; 2: moderate; 3: severe; 4: more severe) and the subchondral cartilage line (condylar area) (0: anechoic; 1: hypoechoic; 2: hyperechoic; 3: heterogeneous) are also scored.

Preparation of platelet-rich plasma

Genesis Autologous Cell System 2 (NeoGenesis, Seoul, Republic of Korea) branded PRP (30 ml) preparation kits were used in the study to obtain the standardised 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 $1\,700 \times g$ for 5 minutes. 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.

Preparation of biophysically activated platelet-rich plasma

The prepared PRP was injected into one end of the Genesis Biophysical activator instrument and an empty Luer lock injector was assembled at the other end of the instrument. The activation process involved injecting the platelets from one injector into the other injector over the activator for a total of 30 times. The prepared PRPs and BPRPs were injected into the joint within 30 minutes.

The platelet concentrate (PRP and BPRP) was injected into the joint until sufficient resistance was obtained to push the syringe plunger back.

Sampling of joint fluid

The joint fluid samples taken from each dog in the study groups on the specified days were centrifuged

at 448 *g* for 5 min and then kept frozen at −80 °C until examination. The joint fluid samples were dissolved shortly before the examination. Various cytokines that work as inflammatory mediators (IL-1β, IL-6, IL-10, TNF-α) were measured by ELISA.

Measurement of inflammatory mediators by the ELISA

The following canine commercial ELISA kits based on the dual antibody sandwich ELISA principle were used: Canine TNF-α ELISA Kit (Catalogue No: ECA0020), Canine IL-1β ELISA Kit (No: ECA0042), Canine IL-6 ELISA Kit (No: ECA0013), Canine IL-10 ELISA Kit (No: ECA0012) from Wuhan Fine Biological Technology Co. Ltd. (Wuhan, China). The ELISA kits were analysed using an ELISA reader device) (MWGt Lambda Scan 200; Bio-Tek Instruments, Winooski, USA).

Statistical analysis

The records obtained from the HVAS and the CBPI tests used in the clinical examination were subjected to analysis using SPSS 20.0 (IBM, USA). The Mann-Whitney *U* test was used to evaluate the data.

The results of the joint fluid ELISA tests run on the six groups were evaluated by Tukey's test, comparing the single and double administration groups, and also comparing the results within the single and double administration groups.

RESULTS

In the HVAS evaluation, a significant difference ($P < 0.05$) was found on day 60 and 90 between the double and the single PRP administration groups (overall, mood, attitude, activity, playfulness, exercise, arising stiff, bedding stiff). However, no significant difference was found in the CBPI test.

According to the Kellgren-Lawrence score; the 1st degree was found in 26 cases, the 2nd degree in 7 cases, the 3rd degree in 1 case and the 4th degree osteoarthritis in 1 case. There were no differences in the radiographic scores on day 1, 15, 30, 60 and 90.

Various degrees of effusion were encountered in the joint space of all the dogs. The echogenicity was increased in the synovial membranes due to the increase in the amount of the synovial fluid in the affected stifle joint with OA and the sharp line was lost in the articular cartilage. Cartilage degeneration was not fully observed. The ultrasound scoring of the 36 dogs with OA are estimated with increasing synovial fluid, intraarticular tissue reaction and subchondral cartilage line was 2–3.

TNF-α: There was no significant difference between the groups mentioned in the measurement results of the TNF-α with the ELISA in the joint fluid analyses (Figure 1).

IL-10: There was a significant difference between the single PRP administration and the single BPRP administration on day 30 ($P < 0.05$). When comparing the single and double administration groups, there was a significant difference between
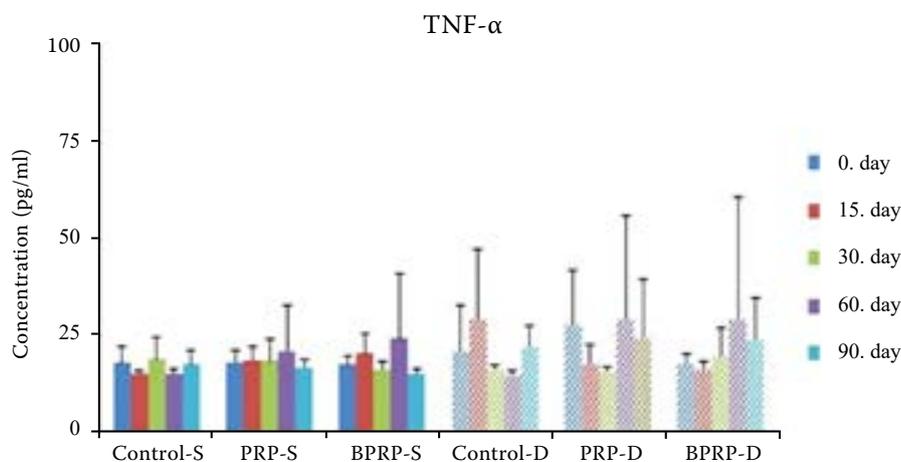

Figure 1. Statistical evaluation of the TNF-α by ELISA

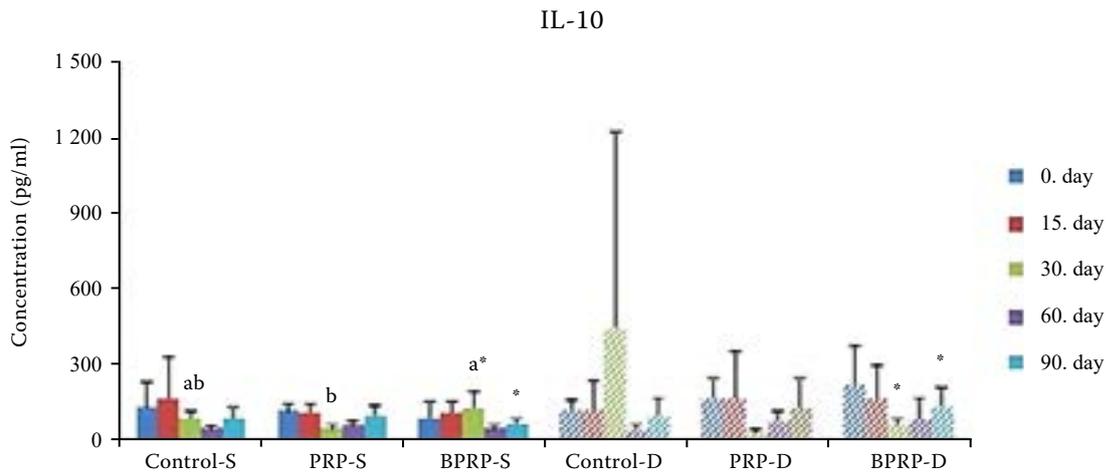BPRP = bio-physically activated PRP; PRP = platelet rich plasma

Figure 2. Statistical evaluation of the IL-10 by ELISA

BPRP = bio-physically activated PRP; PRP = platelet rich plasma
*There is a difference between the BPRP single and BPRP double on the 30th and 90th days ($P < 0.05$)
a,b,abThere was a statistically significant difference between the single application groups (Control Single, PRP Single, BPRP Single) on day 30 ($P < 0.05$)

the single and double BPRP administration groups on day 90 ($P < 0.05$) (Figure 2).

IL-6: There was a significant difference between the single administration group of the control and BPRP groups on day 30 ($P < 0.05$). There was a significant difference between the control group of the PRP and the BPRP groups on day 90 ($P < 0.05$). Although there was not any significant difference between the single and double administration groups on day 60 and 90, the PRP and the BPRP double administration were much more positive than the single administration (Figure 3).
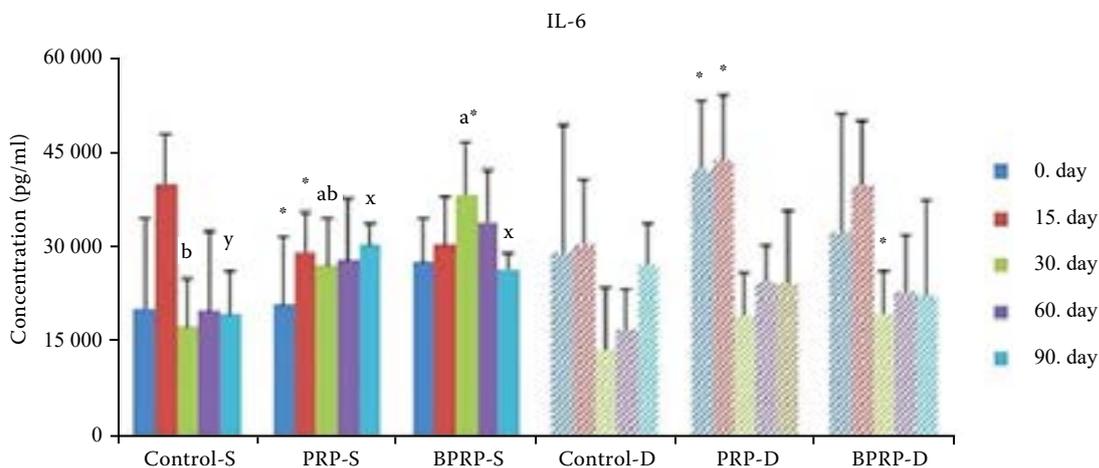

Figure 3. Statistical evaluation of the IL-6 by ELISA

BPRP = bio-physically activated PRP; PRP = platelet rich plasma
*There is a difference between the PRP single and the PRP double on the 0th and 15th days ($P < 0.05$); there is a difference between the BPRP Single and the BPRP double on the 30th day ($P < 0.05$)
a,b,abThere was a statistically significant difference between the single application groups (Control Single, PRP Single, BPRP Single) on day 30 ($P < 0.05$)
x,yThere was a statistically significant difference between the single application groups (Control single, PRP single, BPRP single) on day 90 ($P < 0.05$)
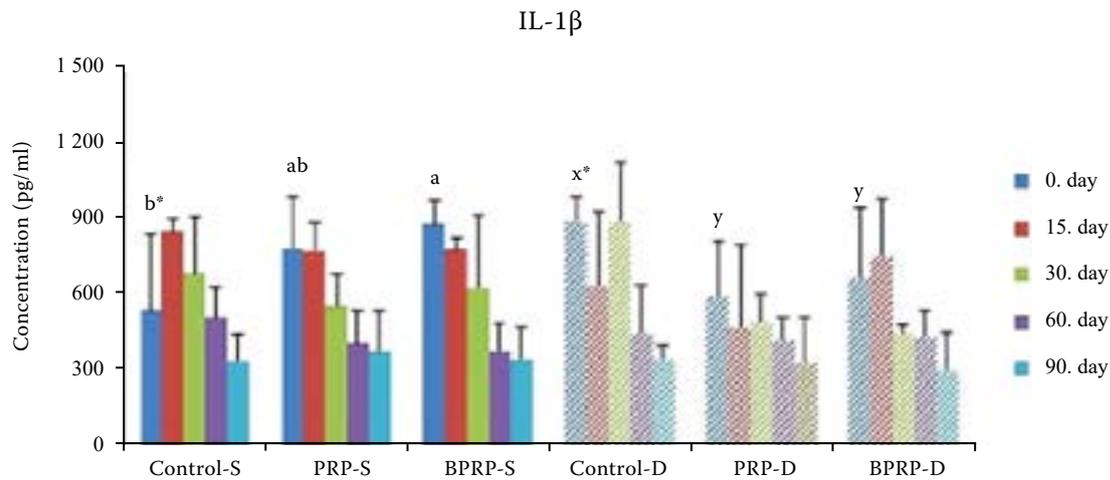
Figure 4. Statistical evaluation of the IL-1β by ELISA

BPRP = bio-physically activated PRP; PRP = platelet rich plasma

*There is a difference between the Control Single and the Control Double on the 0th day ($P < 0.05$)

[a,b,ab]There was a statistically significant difference between the single application groups (Control Single, PRP Single, BPRP Single) on day 0 ($P < 0.05$)

[x,y]There was a statistically significant difference between the double application groups (Control Double, PRP Double, BPRP Double) on day 0 ($P < 0.05$)

IL-1β: There was a significant positive difference between the double administration groups of the PRP and the BPRP and the control groups on day 30th ($P < 0.05$) (Figure 4).

DISCUSSION

In this study, Genesis Autologous Cell System 2 was used to achieve and maintain the standards for the platelets. This kit enabled us to obtain 4–8 times more platelets than the normal PRP preparation method on healthy dogs (Fahie et al. 2013; Arican et al. 2015).

In this study, the behaviour of the dogs was observed and noted by the walking (HVAS) and pain (CBPI) scoring tests for the clinical effects of PRP and BPRP. These walking and pain tests have been used in previous studies and have been subjectively accepted (Hudson et al. 2004; Brown et al. 2008). Hudson et al. (2004) developed a questionnaire for the assessment of pain and lameness in dogs. Their study, which lasted for 3 years, showed that it would be valid in the evaluation of a mild-moderate degree of lameness in dogs. It is acceptable that the dogs should be checked by the same veterinarian. We performed the subjective HVAS and CBPI tests for the 36 dogs in our study. Based

on the evaluation, we determined that the double administration groups were positive compared to the single administration groups for the subjective HVAS and CBPI tests. Fahie et al. (2013) evaluated their patients two times (1st week and 12th week) in the same study. In our study, it is thought that there are no significant differences due to the proximity of the evaluation times to each other (the evaluations were performed on each one of the following days: 1, 15, 30, 60 and 90).

Radiographic evaluations: there was no significant difference between day 1 and 90 in the cases treated by Innes et al. (2004) who evaluated 58 articulation genus affected OA in dogs. However, there was a radiographic change at the examination in the 7th and 13th months. Similarly, Fahie et al. (2013) did not find a difference between the radiographic examination on day 1 and 90. In our study, there was no radiographic change even in the control group. It was concluded when we followed up with a short period of three months. Therefore, we suggest that the follow-up time should be more than three months in similar studies.

It is generally preferred to use a high frequency (≥ 10 MHz) linear probe for the ultrasonographic examination of the anatomical structures of the knee joint. However, images up to 4 cm in the knee joint can be examined with 5–10 MHz probes

(Muller and Kramer 2003). Grassi and Cervini (1998) showed that the increased echogenicity in the synovial membranes and the sharp line was lost in the articular cartilage due to the increased synovial fluid in the knee joint that was affected by the OA. The ultrasonographic examinations of the dogs were performed with a 5–7.5 MHz convex probe. The synovial effusion, subchondral structures and intra-articular fibrotic tissue were examined according to the scoring system. There were various degrees of effusion in the joint spaces of all the dogs. There was increased echogenicity in the synovial membranes and the sharp line was lost in the articular cartilage due to the increased synovial fluid in the knee joint that was affected by the OA. However, there was difficulty in diagnosing the cartilage lesions by ultrasound. This is due to the fact that the ultrasound probe that we used was 7.5 MHz. It was shown that an ultrasonographic diagnosis would be useful as an auxiliary diagnosis.

The TNF-α levels were not significantly different in the single and double administration groups when compared with each other. However, there was neither a positive nor a negative relationship between the groups. Hay et al. (1997) compared the joint fluids of affected dogs by a naturally occurring OA and an experimental OA with healthy dogs. The TNF-α levels were higher in the healthy joint fluids. Venn et al. (1993), in their study on 12 dogs, cut the cruciate ligament of one knee while they left the other knee intact; after three months, synovial fluid samples were taken from both joints and the level of the TNF-α was measured. At the end of the study, the TNF-α levels were higher in the experimental fluid samples than in the control group. As a result; TNF-α (pro-inflammatory mediator) especially increases in the acute phase and triggers the activation of other inflammatory mediators. Therefore, there was no statistical change in the TNF-α levels due to fact that all the dogs did not have acute OA.

Maccoux et al. (2007) examined the cytokine levels (IL-1β, IL-6, IL-10 and IL-8, IL-17) in four different joint tissues of both healthy and affected joints. As a result of the study, it was determined that the IL-1β, IL-6, IL-10 levels increased in the OA joint tissue compared to the control group. IL-10 inhibits the synthesis of type II collagen and the aggregate, significantly reducing the secretion of the TNF-α and IL-1β. IL-10 has a chondro-

protective effect (Jansen et al. 2008). They found that when IL-10 was added *in vitro* to the synovial samples from the patients with OA, the TNF-α and IL-1β levels were suppressed (Jansen et al. 2008). It is thought that the PRP and BPRP administration to the OA joints can cause an increase in the IL-10 level. In our study, a statistically significant difference was found between the single and double administration of the BPRP on the 90th day. Also, a positive result was obtained in the comparison of the PRP double administration and the single administration groups on the 90th day. According to this information, it can be concluded that double administration is better than the single application. Furthermore, there was a statistically significant difference in the single administration between the PRP and BPRP on the 30th day. A single administration of the PRP decreases the level of IL-10 more than the BPRP on the 30th day. According to this, the BPRP is better organised and more effective in the joint. Also, this can be seen in the 30th day measurements of the double administration groups, even if it is not statistically significant.

Ley et al. (2007) compared the OA in affected and healthy animals. The IL-6 level was higher in the OA affected animals. IL-6 has been reported to inhibit the type II collagen production in the animal models. Also, Maccoux et al. (2007) showed that the IL-6 level was higher in the OA affected joint tissues and the joint fluid. In our study, a negative relationship was found between the single administration BPRP and the control groups on the 30th day. A negative relationship was found between the BPRP, the PRP and the control groups on the 90th day. However, there was a non-statistical positive decrease in the IL-6 levels in the double administration groups on the 1st and 30th days in the BPRP and PRP groups. These contrasts obtained in the study can be attributed to the random grouping of OA in the dogs and the different degrees of OA.

One of the most important pro-inflammatory cytokines involved in the pathophysiology of OA is IL-1β. This plays a role in the suppression of collagen and aggregate synthesis. It has also been reported that it stimulates the production of free radicals that directly damage the articular cartilage (Shakibaei et al. 2005). It has been shown that IL-1β was higher in the OA affected joints.

In our study, there was a statistically positive result in the BPRP and the PRP as compared to the control group for the double administration

on the 30th day. Although it was not statistically significant, in the both groups. However, a positive change in the IL-1β level was seen with the PRP and the BPRP. This can be attributed to the growth factors. The IL-1β could be a better predictor for the treatment follow-up in patients with OA.

Sundman et al. (2011) reported that platelets increses the anabolic signaling and leukocytes increases the catabolic signaling as a result of investigation of the effects of leucocytes and inflammatory mediators in PRP. In addition, Pochini et al. (2016) evaluated cytokine amounts in PRP preparations obtained by 3 different methods. As a result, they thought that the use of PRP in cartilage tissue might cause a strong proinflammatory effect.

In our study, on day 15 in single and double PRP and BTZP groups and 60 day TZP and BTZP double application groups in the negative increase in negative meaning in the IL-6 level, the double application of PRP and BTZP suggests a decrease in the level of TNF-α at days 60 and 90. This is considered to be evidence of that PRP in cartilage tissue might cause a strong proinflammatory effect.

As a result, the efficacy of the PRP and BPRP is related to the degree of the OA. The success rate of acute OA is especially higher due to its anti-inflammatory activity. When the walking and pain scores and the IL-10 levels were compared, it was seen that the double administration groups gave more positive results than the single administration groups. In addition, the BPRP was better than the PRP for the IL-10 levels. However, since the other parameters do not support these results, additional studies on this subject are considered necessary.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest.

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