Int J Clin Exp Med 2020;13(7):5040-5049
www.ijcem.com /ISSN:1940-5901/IJCEM0109759

Original Article

Effect of platelet-rich plasma on serum and urine biomarkers in patients with knee osteoarthritis

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Received January 29, 2020; Accepted May 7, 2020; Epub July 15, 2020; Published July 30, 2020

Abstract: Background: Biomarkers may reflect changes in cartilage metabolism and could be used in diagnosis and evaluation of treatment response in knee osteoarthritis. The aim of this study is to determine the effect of platelet rich plasma (PRP) injections on serum and urine biomarkers in patients with knee osteoarthritis (OA). Methods: The study included 26 patients who were diagnosed as grade III knee OA according to ACR criteria and Kellgren-Lawrence classification. One cc of PRP was obtained from 20 cc of venous blood after double centrifugations at 400 g for 10 minutes. The patients received 3 injections of PRP at 3-weeks intervals. Serum Procollagen II N-terminal propeptide (PIINP), osteocalcin (OC), cartilage oligomeric matrix protein (COMP) and urine collagen-II telopeptide (U-CTX-II) were examined in all patients before treatment and at 3 and 6 months. Clinical outcome was evaluated using Western Ontario and McMaster Universities Arthritis Index (WOMAC) questionnaire, SF-36 and visual analog scale (VAS) in all patients before injection and at 3 and 6 months follow-up visits. Results: There was no statistically significant difference in cartilage Type II collagen (CII) degradation markers (CTX-II, COMP) and biosynthesis marker of PIINP ($P>0.05$). Significant increase was determined in serum OC levels ($P<0.05$) which reflects CII biosynthesis. Statistically significant improvement in all WOMAC parameters ($P<0.05$) and subscores of SF-36 ($P<0.05$) was noted. Conclusion: We found significant clinical improvements in knee OA patients treated with PRP injections. Our results suggest that PRP injection does not affect cartilage breakdown but effects CII biosynthesis, which is reflected by increased OC levels. However further studies are needed.

Keywords: Biomarker, knee osteoarthritis, osteoarthritis, platelet rich plasma

Introduction

Osteoarthritis (OA) is known to be a maladaptive proinflammatory response of joints to micro- and macro-trauma, which is characterized by intracellular stress and extracellular matrix degradation [1, 2]. Considered to start with molecular changes and inflammation, OA results in joint degeneration that has recently been accepted as multiple organ failure [3, 4].

Recent studies have evaluated knee osteoarthritis as the most common cause of disability in elderly patients [5]. There are some conservative treatment methods such as disease-modifying osteoarthritis drugs (DMOADs), physical therapy, intra-articular injections, braces, and regenerative methods (e.g. stem cell and platelet-rich plasma (PRP)) [6]; however, these methods have failed to be effective in a group of patients. Early diagnosis, prognosis prediction and treatment response are important for such patients. Otherwise, the disease results in joint damage and disability [7, 8].

PRP is a fragment of plasma that contains standard high levels of platelet obtained from the patient's own blood by appropriate centrifugation methods [9]. It has been thought to increase cartilage regeneration. There are several studies measuring change of cartilage volume [10, 11]. Also according to recent studies PRP application has been thought to be clinically effective in patients with early stage knee osteoarthritis [12, 13]; but there is not enough objective evidence that supports efficacy of PRP application in cartilage regeneration.

Table 1. Demographic features of the patients

	PRP Group (n=26)
Age (mean ± SD) (years)	57.12±9.369
Body Mass Index (kg/m^2)	32.47±5.57
Gender ratio (Female %; Male %)	84.6; 15.4

Biomarkers reflect changes in chondrocyte metabolism and joint damage in OA. Recently, the BIPED (burden of disease, investigative, prognostic, efficacy of intervention and diagnostic) classification has been in use for classifying the markers [14]. These markers are also used for early diagnosis and treatment response monitoring [15, 16]. The aim of the present study was to determine the clinical effect of PRP injection in patients diagnosed with grade 3 knee osteoarthritis and the change in levels of blood and urine biomarkers reflecting the cartilage turnover.

Materials and methods

This study was designed as a single-center, prospective clinical trial. The study included patients suffering from knee pain, who presented at the Physical Medicine Rehabilitation Department of Katip Celebi University Atatürk Training and Research Hospital between January 06, 2015 and December 31, 2015. The study protocol was approved by the Katip Celebi University Ethics Committee. A written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient selection and sampling

The sample size was based on differences in final CTXII levels, revealing a large effect size (d type value) of 0.8, a power of 80%, and a false-positive rate of 5%. Each treatment arm of this study required 16 patients [17]. As a result, 92 patients were evaluated in total. The study inclusion and exclusion criteria are presented below.

Inclusion criteria

●Age between 40-75 years.

●Moderate-severe knee pain scored at least 4 over 10 points on a VAS or loss of joint range of motion.

●Based on the diagnostic criteria of ACR as knee osteoarthritis.

●Radiologically had grade -3 knee osteoarthritis (including large osteophytes, marked joint, severe sclerosis and definite bony deformity) according to Kellgren-Lawrence classification.

Exclusion criteria

●Uncontrolled systemic disorder.

●History of rheumatic disease.

●Patients with another symptomatic joint or those with asymptomatic OA in >3 joints.

●History of acute trauma, acute meniscopathy, anterior-posterior cruciate ligaments or collateral ligament injury or tear in the effected knee.

●History of surgery, manipulation, mobilization or arthroscopy in the effected knee.

●History of medication use over a period of 10 days before and after treatment.

●History of steroid, local anesthetics or hyaluronic acid injection, kinesiotaping, prolotherapy or neural therapy over the last 3 months.

●Anemia or thrombocytopenia (Hemoglobin <12 g/dl, platelet <150,000/uL), bleeding disorders, patients using anticoagulant or antiaggregant medications.

●Active malignancy.

●Infection or suspicious of infection Reflex sympathetic dystrophy or neurodeficit of the effected extremity.

After clinical and radiological evaluation, 26 patients were included in the study. The demographic features of the patients included in the study are provided in **Table 1**.

PRP preparation protocol and PRP administration to patients

The PRP preparation protocol was based on the preliminary laboratory study of previous research investigating the efficacy of PRP in partial and total layer supraspinatus tears and the efficacy of platelet-rich plasma administration in patients with severe knee osteoarthritis [18, 19]. According to the preliminary laboratory study, mono and double centrifugations

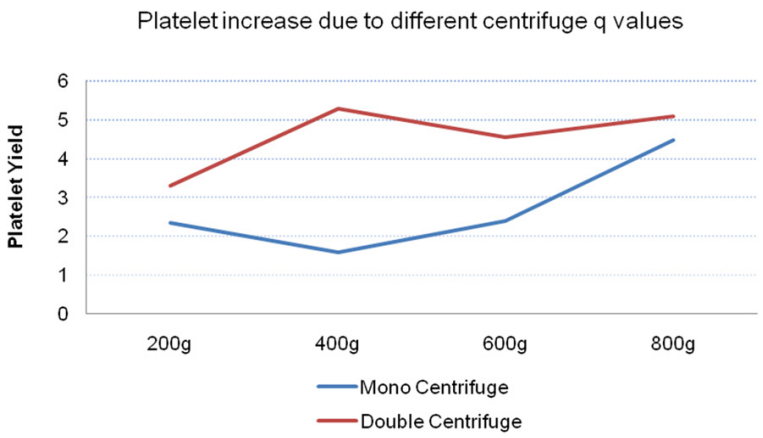

Figure 1. Platelet yields following mono and double centrifugation at different g levels [13].

of fresh whole blood obtained from the blood bank were performed at 200, 400, 600, and 800 g for four times and a total of 32 samples were obtained [20]. One cc of PRP was obtained from each sample, the platelet counts were analyzed, and then the samples were stored in -80°C and activated with 10% calcium chloride ($CaCl_2$) for 30 minutes [20]. Activation of platelets was determined via P-selectin analysis. Growth factor analysis was performed via the analysis of platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and insulin-like growth factor (IGF) using ELISA method [21]. As platelet fragmentation rates are known to increase at forces above 800 g [22, 23], double centrifugation at 400 g for 10 minutes was selected for the PRP preparation protocol [18]. Platelet yields following mono and double centrifugation at different g levels are provided in **Figure 1**. The levels of P-selectin and growth factors at different g levels after mono and double centrifugation are presented in **Table 2** [18]. The process and injection of PRP, blood and urine sampling and clinical examination were all performed by the same physician. Venous blood samples of 20 cc were obtained from the patients and transferred into 10 cc sterile sodium citrate tubes. After double centrifugation at 400 g for 10 minutes, approximately one cc of PRP was obtained. In order to ensure pathogen-free PRP, all transfers were performed under laminar flow. The levels of platelets and growth factors were not analyzed prior to injection since such levels were already analyzed in the previous laboratory study. Additionally, the PRP products were not activated prior to the injection as the platelets are known to be activated when they connect with collagen tissues. Three PRP injections at intervals of three weeks were performed for each patient. The PRP products were injected intra-articularly with a 21G needle using a superolateral approach. Sterile conditions were supplied during the process. The patients were not allowed to use nonsteroidal anti-inflammatory drugs or any other drug with a potential effect on outcomes for 10 days before and after the PRP injection. The patients were allowed to use only cold packs and paracetamol. All patients were given a home exercise program including knee range of motion and quadriceps strengthening exercises. Patients were also informed about avoiding overuse of the extremity for 48 hours.

Blood & urine sampling, biological procedures and clinical follow-up

Blood and urine samples were collected from the patients before the treatment and at months 3 and 6, within 4 hours of getting out of bed and not later than 10:00 a.m. prior to breakfast in order to prevent diurnal variation [24]. Peripheral venous blood samples from the patient and control groups were collected into heparin-containing tubes and gel-included serum separator tubes. Tubes were centrifuged at 3000 rpm for 10 minutes, and serum and plasma samples were separated. Serum, plasma and urine samples were stored at -80°C until the assay. Quantitative determination of serum PIINP (Procollagen II N-Terminal Propeptide Elisa kit, Cloud-Clone Corp, Wuhan, PRC), serum COMP (Human cartilage oligomeric matrix protein Elisa kit, Bioassay Technology Laboratory, Korea), plasma osteocalcin (Human osteocalcin instant Elisa Kit, eBioscience, Austria) and urine CTX-II (Cross-linked C-terminal telopeptides of type II collagen Elisa kit, Bioassay Technology Laboratory, Korea) were performed by the same biochemistry

Table 2. The P-selectin and growth factor levels at different g levels after mono and double centrifugation (Mean ± SD) [13]

		P-selectin [ng/ml]	VEGF [pg/ml]	EGF [pg/ml]	IGF [pg/ml]	PDGF [pg/ml]	TGF-β [pg/ml]
200gi	MC	2.87±1.1	1336.06±198.9	67.57±12.5	18.70±1.6	27528.45±3662.8	15394.04±1217.7
	DC	2.26±0.1	6664.57±502.4	379.27±14.5	19.74±1.9	91446.50±5808.8	16175.49±7406.3
400gi	MC	2.04±0.5	2502.59±377.7	110.60±19.6	16.43±2.4	38179.85±11618.2	2988.78±191.3
	DC	2.57±0.3	10381.33±413.1	388.91±9.7	19.96±2.8	190927.96±36619.3	8386.72±1540.9
600gi	MC	3.07±0.9	5031.69±485.8	212.70±21.7	14.90±1.7	134090.24±89834.9	15357.10±5817.6
	DC	2.64±0.4	4510.33±960.0	198.76±53.3	22.39±1.5	354313.56±117672.6	15199.06±7761.4
800gi	MC	3.46±1.1	5727.53±398	191.91±38.6	16.12±4.3	59618.94±13366.0	11276.95±4210.8
	DC	2.18±0.1	9245.67±5195.2	276.19±139.2	16.03±0.6	211616.01±7331.9	10437.75±2472.1

MC: Mono centrifugation; DC: Double centrifugation.

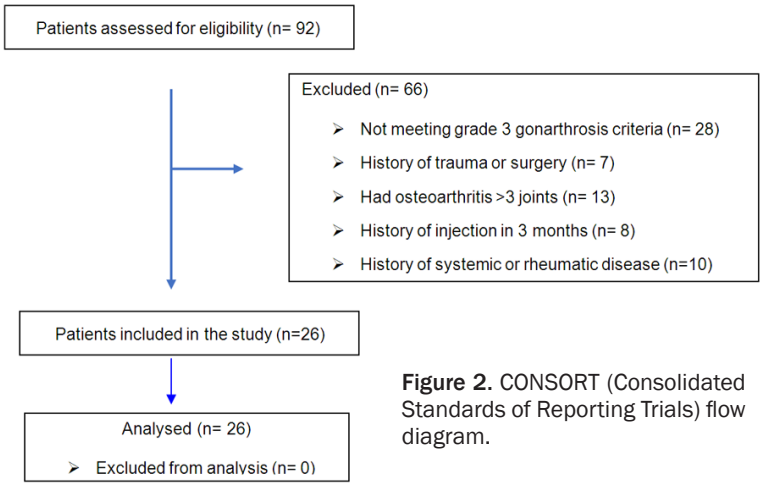

Figure 2. CONSORT (Consolidated Standards of Reporting Trials) flow diagram.

Table 3. Change in VAS score after platelet-rich plasma injection

		0-3 month	3-6 month	0-6 month
VAS exercise	median# Min# Max#	9.00, 4, 10	5.00, 0, 10	3.50, 0, 10
	p value×	0.001**	0.007**	0.001**
VAS at rest	median# Min# Max#	5.00, 3, 10	2.00, 0, 9	0, 0, 9
	p value×	0.001**	0.011**	0.001**
Night pain	*p* value××	0.016**	0.063	0.001**

*Significant increase; **significant decrease; #Friedman test; ×Wilcoxon signed-rank test; ××Cochran test.

specialist using commercial enzyme-linked immunosorbent assays according to the manufacturer's instructions. The sensitivity of the assays was 39.17 pg/ml, 15.66 pg/ml, 0.2 ng/ml, and 0.021 ng/ml, respectively.

Clinical examination was performed before PRP injection, and at third- and sixth-month controls. For all patients, pain, stiffness and function parameters were evaluated using the Western Ontario and McMaster Universities Arthritis (WOMAC) score. Quality of life of the patients was assessed using the Short-Form (SF-36) and subscores (physical function, physical role, social function, pain, emotional role, mental health, general health and vitality), and change in the pain score was determined using the Visual Analogue Scale (VAS) with a numerical rating between 0 and 10.

Statistical analysis

Statistical analysis was performed using SPSS version 25.0 statistical software (IBM Corp., Armonk, NY, USA). Consolidated standards of reporting trials (CONSORT) flow diagram is presented in **Figure 2**. Analyses were performed at an 80% power, a 95% confidence interval and a 5% error rate. The Shapiro-Wilk test was used to assess the normality of the distribution of the data. The results of the data analysis are expressed in descriptive statistics for continuous data; in mean and standard deviation values for normally distributed parameters, and in median (minimum-maximum) values for non-normally distributed parameters. Changes over time in numeric variables that were normally distributed were tested using an analysis of variance, and then the Bonferroni test was used as a post hoc method. Numeric and ordinal variables that were not normally distributed were tested using the Friedman test. Following the Friedman test, Bonferroni and Wilcoxon signed rank tests were used to compare the results. The binary categorical variables at

Table 4. Change in WOMAC score after platelet-rich plasma injection

WOMAC		Months 0-3	Months 3-6	Months 0-6
Pain	median## Min## Max##	6.000, 2.5, 8.0	3.750, 0.5, 8.0	1.500, 0.0, 8.0
	P value*	0.001**	0.001**	0.001**
Stiffness	median## Min## Max##	4.3750, 0.0, 10.0	2.5000, 0.0, 8.75	1.2500, 0.0, 7.50
	P value*	0.031**	0.008**	0.001**
Function	median## Min## Max##	6.3200, 1.91, 10.0	5.0000, 0.0, 10.0	2.1350, 0.0, 7.64
	P value*	0.001*	0.001*	0.001*

*Significant increase; **significant decrease; ##Friedman test; *Wilcoxon signed-rank test.

Table 5. Change in subscores of SF-36 after platelet-rich plasma injection

SF-36		Months 0-3	Months 3-6	Months 0-6
Physical function##	Median	14.00	16.00	19.50
	Min	10	10	11
	Max	25	30	32
	P value*	0.007*	0.004*	0.001*
Physical role**	Median	4.00	6.00	8.00
	Min	4	4	4
	Max	4	8	8
	P valueμ	0.001*	0.125	0.001*
Social function##	Median	4.00	6.00	7.00
	Min	3.0	3.0	3.0
	Max	10.0	10.0	10.0
	P value*	0.007*	0.040*	0.002*
Pain#	Mean ± SD	4.842±1.76	6.465±2.53	7.462±2.41
	P value	0.002**	0.005**	0.001**
Emotional role**	Median	3.00	3.00	6.00
	Min	3	3	3
	Max	6	6	6
	P valueμ	0.001*	0.063	0.001*
Mental health#	Mean ± SD	16.038±4.97	17.962±6.30	19.500±6.96
	P value	0.074	0.126	0.014*
General health#	Mean ± SD	14.077±3.98	15.808±3.85	16.423±4.41
	P value	0.008*	0.0276	0.001*
Vitality#	Mean ± SD	12.769±4.61	14.538±5.48	16.962±5.38
	P value	0.084	0.091	0.002*

*Significant increase; **significant decrease; #Bonferroni test; ##Friedman test; *Wilcoxon signed-rank test; **Cochran test; μMcNemar test.

months 0, 3 and 6 were compared using the Chocran test, which was followed by dual comparisons using the Bonferroni and McNemar tests. A *p* value of <0.05 was considered statistically significant.

Results

After PRP injection of three sessions, there was a statistically significant decrease in exercise VAS (P=0.001) and VAS at rest (P=0.001) and night pain (P=0.016) at month 3, and the decrease was continued during the six-month follow-up (P= 0.001). Change in VAS scores after PRP injection is presented in **Table 3**.

Clinical improvement was observed in WOMAC total score and subscores of pain, stiffness and function. At the third month visits, a significant improvement was identified in pain (P=0.001), stiffness (P=0.031) and function (P=0.001), and the improvement in WOMAC scores was sustained at the sixth month visits (P= 0.001). Change in WOMAC scores after PRP injection is presented in **Table 4**.

At the six month follow-up, significant improvements were achieved in SF-36 score (P<0.05) and subscores of physical function (P=0.001), physical role (P=0.001), social function (P=0.002), pain (P=0.001), emotional role (P=0.001), mental health (P=0.014), general health (P=0.001) and vitality (P=0.002). Statistically significant improvements were determined in emotional role and general health at the third month; however, there was no significant difference between third and sixth months. The scores were significantly better at the sixth month control (P=0.001). Although mental health and vitality scores were not significantly

Table 6. Biomarker levels after platelet-rich plasma injection

		Before treatment	Month 3	Month 6	*P* value
CTXII*	Mean ± SD	6.18±1.02	6.09±0.94	5.9±0.94	0.458*
	Median	1080.78	1401.82	1158.26	
COMP**	Min	312.082	339.946	303.414	0.155**
	Max	6400.505	6159.328	6228.196	
	Median	11062.5	12146.06	12969.7	
PIINP**	Min	8379.44	8121.47	5984.27	0.482**
	Max	25092.35	19227.71	27400.04	
	Median	1.48150	2.52400	2.07750	
Osteocalcin**	Min	0.477	0.471	0.532	0.001#
	Max	7.229	6.047	7.644	

*Bonferroni test; **Friedman test; #Wilcoxon signed-rank test.

different at the third month visits (P=0.084), the scores were significantly better after the six month follow-up (P<0.05). Change in subscores of SF-36 after PRP injection is presented in **Table 5**.

At six month follow-up, there was no significant change both in cartilage degradation markers of urine CTXII (P=0.458) and in blood COMP (P=0.155) levels. Additionally, there was no change in cartilage formation marker of blood PIINP levels (P=0.482), but there was a statistically significant increase in blood OC levels (P=0.001) at the sixth month follow-up. Change in biomarker levels after PRP injection is presented in **Table 6**.

Discussion

Clinical consequences of knee OA have brought up the question of how to prevent progression of the disease. Therefore, several studies have been conducted about the use of regenerative therapies in patients with knee OA. Although there is no consensus about the efficacy of PRP application, recent studies have found it clinically effective in early stage knee OA [25, 28]. The present study observed improvement of VAS, WOMAC and SF-36 scores at the six month follow-up, suggesting a significant clinical improvement.

It is possible to use biomarkers for early diagnosis, prediction of prognosis and treatment response in knee OA. Most of the researches about effect of intra-articular injections on biomarkers were designed based on hyaluronic acid (HA) application; however, they produced conflicting results. The literature does not contain any study investigating the effect of PRP injection on biomarkers. Henrotin et al. followed-up 45 patients after HA injection and found the type II collagen degradation (coll2) and NO2 levels higher in patients with severe knee OA and the CTXII levels decreased much more in patients responding to HA treatment at the third month controls [29]. Conrozier at al. detected a decrease in levels of urine CTXII, but no significant change in serum COMP and PIINP levels in HA-injected patients after a three-month follow-up [17]. Gonzales et al., in turn, demonstrated a significant increase in CTXII levels in urine samples of HA-injected patients at the sixth month control [30]. Gabriel et al. determined an increase in COMP and OC levels and no significant change in MMP and pyridinium crosslink creatinine (pyr/Cr) levels in blood and urine samples of HA-injected patients [31].

OARSI identified biomarkers using the 'BIPED' classification and concluded that the best indicators of cartilage destruction were CTXII and COMP levels [14]. For this reason, CTXII and COMP were included in the present study as cartilage degradation markers. Bruyere et al. clinically and radiologically followed-up 62 patients with knee OA for one year and found that the increase in blood HA and urine CTXII levels was associated with progression of OA [32]. Furthermore, recent studies found CTXII and COMP levels associated with radiological progression [33, 34]. Lawalley et al. followed-up 533 patients by measuring the knee joint gaps at 36th and 48th month controls and identified CTXII as one of the most important three biomarkers with a predictive value for knee OA [34]. In the present study, there was

no significant change in CTXII levels after three sessions of PRP injection at the third and sixth month controls. This may suggest that PRP application is unlikely to be effective in cartilage degradation. Yet, the results indicate that the level of CTXII did not increase during the six-month follow-up and there was no control group in this study, it is not possible to answer whether PRP application prevented increase in CTXII levels or not.

COMP is another biomarker that has been studied and believed to reflect cartilage breakdown. A meta-analysis study indicated that CRP and COMP could be used to diagnose knee OA. Moreover, high levels of CRP and COMP were found to be associated with the incidence of knee OA and high risk of developing knee OA [35]. Hosnijeh et al. followed-up blood levels of C1M, CRPM, COMP, urine levels of CTXII, and radiological progression in knee OA patients for five years. The authors stated that COMP and CTXII levels were associated with radiological progression; however, high levels of CRP and COMP were associated with synovial inflammation [36]. A meta-analysis by Hosnijeh et al. suggested that the CTXII level was correlated with the incidence of knee and hip OA [37]. Likewise, another meta-analysis established that the COMP level was associated with the risk of knee OA. There was no significant difference in blood levels of COMP between Kellgren grade-1 and grade-3 patients, but the levels were significantly higher in patients with grade-2 knee OA. Therefore, there was no significant correlation between the COMP levels and the grade of knee OA [38]. In the present study, a clinical improvement was determined in patients administered three PRP injections; however, no significant change was detected in blood levels of COMP. This finding may indicate that PRP injection has no effect on cartilage degradation.

Besides cartilage degradation, cartilage formation, repair and synovial inflammation play an important role in pathogenesis of OA. In OA pathogenesis, cartilage formation markers are not as clear as cartilage breakdown markers. OC and PIINP are markers suggested as formation markers by OARSI in BIPED classification. Previous studies about these markers determined that CS846 and PIINP might be used for diagnosis in future [14]. Another formation marker is OC, as it is the most common non-collagen protein secreted by osteoblasts in the bone [39]. Prior studies stated that OC could not be used for disease prediction [40]. Even though OC was not found useful for prediction of the disease, it has been concluded that high levels of OC might be protective for knee OA [41]. Wang et al. followed-up 28 healthy men via blood OC, urinary pyridinoline and deoxypyridinoline levels and magnetic resonance imaging for two years. Although the authors could not found any correlation between blood levels of OC and cartilage volume, they found an association between high levels of OC and low risk of OA [42]. In an animal experiment performed by Huang et al., 24 rabbits were followed-up for 15 months after surgically induced OA. It was shown that CTXII levels increased in rabbits with knee OA, while OC levels decreased [40]. The study by Bruyere et al. suggested that radiological progression could be predicted with increased OC or decreased HA levels in the three-year follow up of knee OA patients [43]. In the present study, no significant change was found in PIINP levels, but there was a statistically significant increase in OC levels of patients with grade-3 knee OA after the six-month follow-up. Considering that CTXII, COMP and PIINP levels did not change significantly, can an increase in level of OC alone reflect cartilage formation or repair? Can clinical improvement beside the increased OC levels be considered in favor of cartilage formation or repair? Or are there extra mechanisms with a role in clinical improvement? Comprehensive studies with a large group of patients are needed.

One of the most important limitations of the present study is the heterogeneity of autologous PRP application, although platelet yields and growth factor levels were shown in the previous study [18]. There have been several studies about biomarkers, but there is still no consensus about which biomarker should be used for diagnosis, prognosis, prediction or treatment response [44]. For this reason, the present study evaluated the markers that have been studied more and included in OARSI's BIPED classification [14]. Physical activity, diurnal rhythm, hunger and satiety may also affect the biomarker levels. In order to prevent this limitation and provide standardization, the samples were collected before 10 am, as soon as the patients get out of the bed and before breakfast. The other important limitation is the

lack of control group. There is need for further investigations for new studies performed for large group of patients including control group.

Conclusion

After three sessions of PRP injection in patients with grade 3 knee OA, a statistically significant improvement was determined in VAS, WOMAC and SF-36 scores at the third month control. This clinical improvement continued during the six-month follow-up. Although no significant difference was found in cartilage degradation markers of CTXII, COMP and formation marker of PIINP, there was a significant increase in OC levels at the six-month follow-up. These results may indicate that PRP injection is clinically effective, has no efficacy in cartilage degradation, yet it may increase cartilage formation by increasing the OC levels in patients with grade 3 knee OA.

Disclosure of conflict of interest

None.

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