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Original Article

How can clinically-safe and effective Platelet Rich Plasma (PRP) be obtained in a laboratory?

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Abstract

Aim: To compare open and closed systems in the preparation of Platelet Rich Plasma (PRP) in terms of feasibility, cost, and safety.
Materials and Methods: In this prospective study 10 patients who were undergoing controlled ovarian hyperstimulation and intrauterine insemination, were included. Open system PRP prepeared from these patients collected blood which have the highest number of platelets. Open system PRP's platelet concentration and cost were compared with closed system kit.
Results: It was found that 778g (2000rpm) was the best centrifugal force, and 5 minutes was the optimum duration for having the highest level of platelets. The mean platelet concentrations in the open system (425 ± 91.2 × 10^3/ml) were statistically significantly higher than the closed system (298 ± 88.32 × 10^3/ml) (p = 0.021). No growth was observed in the culture inoculated with PRPs obtained using open system. In the cost analysis, the open system was significantly more economic than the closed system.
Conclusions: Higher platelet concentrations can be achieved using a low-cost sterile open system centrifugation method than the closed system kit in the laboratory setting effectively.

Keywords: Centrifuge; open system; platelet rich plasma; preperation; PRP

INTRODUCTION

Platelet-rich plasma (PRP) is the autologous plasma fraction obtained from whole blood. It is an enriched suspension of growth factors that can be prepared easily and utilized clinically. Many growth factors, such as PDGF (platelet-derived growth factor), TGF-β (transforming growth factor-beta), VEGF (vascular epidermal growth factor), are all secreted from the alpha granules upon activation of platelets. Due to its promoting effects on cell proliferation, apoptosis, chemotaxis, cell differentiation and angiogenesis, therapeutic use of PRP began in 1998 for wound healing and tissue repair purposes (1). It is used in ocular surgery, maxillofacial surgery, orthopedics, plastic surgery, sports medicine and dermatology, as well as cardiac surgery (2-6). In recent years, there have been pieces of evidence on the positive effect of PRP treatment (via endometrial and follicular development) on pregnancy rates among patients using assisted reproductive techniques (7-9).

MATERIALS and METHODS

A total of 10 female patients who were admitted to Gazi University, Center of Assisted-Reproduction Treatment between 2018 and 2019 were included in this study after obtaining their informed consent. The study was approved 'Ministry of Health Stem Cell Transplantation Scientific Advisory Council' with Council number 56733164/203. Patients were between 20 and 45 years old, and were unable to conceive for over a year, and were going to undergo controlled ovarian hyperstimulation and intrauterine insemination. Inclusion criteria were i) to have a hematocrit level between 30 - 35 and ii) to have a platelet level between 150.000 - 400.000. Patients who had any bleeding disorders or blood disorders, have undergone blood transfusion within the last three months, have used anticoagulants or NSAID within the two weeks before the operation, use medication which alter the platelet levels, were in a diet, were smoking, were splenectomized, had IgA deficiency, have undergone major lower abdominal surgery, have a history of malignancy or psychiatric disorder were excluded.

This study was planned as a two-phased study. In the first phase, the objective was to answer the following questions: "Should the centrifugation be performed in a single step or in two steps?" and "What is the centrifugal force and duration to obtain the maximum amount of platelets?". The first phase was completed with the determination of

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the centrifuge method, centrifugal force, and duration by which the maximum amount of platelets were obtained. In the second phase, using the outcome of the first phase, open system PRP was prepared under sterile laboratory conditions at a low cost. The number of platelets and leukocytes in the PRP obtained via the open system were compared with the PRP obtained via the closed system using a commercial kit.

In the first phase, hematological values (hematocrit, platelet, leukocyte counts) of the 2 ml blood collected from 9 patients who fill the inclusion criteria were measured using Sysmex XN-1000 (Sysmex Corporation, Japan). Without traumatizing venous walls, using a 20 ml injector that contained 2 ml citrate (3% citrate-phosphate-dextrose (CPD)) as an anticoagulant, 18 ml blood was collected from the antecubital area via a branule. Shaken very slowly for 1 minute 20 ml blood transferred equally into two sterile 10 ml tubes. For every patient, each of the two 10 ml tubes were named as the 1st group and the 2nd group. Within 2 minutes after the collection of blood for each group, two-step centrifugation was performed at 22°C. Centrifugal forces and durations used in the first step of the first phase are given in Table 1.

Table 1. Centrifugal forces and durations used in the first step centrifugation in the first phase

Patients no / Centrifugal force g / rpm†	1st group	2nd group
Patient #1 / 157 / 900	15 minutes	10 minutes
Patient #2 / 280 / 1200	15 minutes	10 minutes
Patient #3 / 381 / 1400	15 minutes	10 minutes
Patient #4 / 562 / 1700	5 minutes	10 minutes
Patient #5 / 778 / 2000	5 minutes	10 minutes
Patient #6 / 857 / 2100	5 minutes	10 minutes
Patient #7 / 1029 / 2300	5 minutes	10 minutes
Patients #8 / 1215 / 2500	5 minutes	10 minutes
Patients #9 / 1418 / 2700	5 minutes	10 minutes

rpm†: Revolutions per minute

In the first step of the first phase, the centrifuge was performed for 10 and 15 minutes at forces below 500 g, and for 5 and 10 minutes at forces over 500 g. For patients #1, #2 and #3 (157 – 280 - 381 g, respectively), the 1st group samples were centrifuged for 15 minutes and the 2nd group samples for 10 minutes. For patients #4, #5, #6, #7, #8 and #9 (562 – 778 – 857 – 1029 – 1215 - 1418 g, respectively), the 1st group samples were centrifuged for 5 minutes and the 2nd group samples for 10 minutes. All centrifugations were performed in 15 ml flat bottom sterile tubes using Heraeus Labofuge 400 (Thermo Scientific™ Heraeus™ Labofuge™ 400 Centrifuges). Immediately after the centrifugation, platelet-enriched 1.5 ml plasma, which is found at the intermediate fraction above the erythrocyte fraction that precipitated at the bottom of the tubes and at the lower 1/3rd portion of the top fraction, was gently collected with an insulin injector and transferred into a separate sterile tube. Of the collected plasma, 0.25 ml was taken with an insulin injector and transferred into a separate tube and submitted to hematological analysis. The remaining 1 ml of PRP and approximately 3 - 4 ml of platelet-poor plasma (PPP) found at the 2/3rd portion of the top part of the tube were collected into a separate 15 ml flat bottom sterile tube using a sterile insulin injector. To concentrate high volume PPP and PRPs obtained from 18 patients, the second step of the first phase was initiated. In the second step, PRPs obtained from the donors were centrifuged at 2383 g (3500 rpm) for 5 minutes at 22°C. After the centrifugation, 1 ml of Platelet-concentrated plasma (PCP) that precipitated at the bottom 1/3rd portion of the tube was transferred to a separate sterile tube using a sterile insulin injector. Of the collected PCP, 0.25 ml was transferred to a separate sterile tube with insulin injector and submitted to hematological analysis. Whole blood hematologic values were compared with the hematologic values of both PRP after single-step centrifugation and PCP after two-step centrifugation.

In the second phase of the study, the centrifugal force and duration determined in the first phase were used in the open system. Then, the platelet, leukocyte, erythrocyte and immunoglobulin counts in the PRPs obtained using open system and closed system (T-Lab PRP Kit) were compared. Hematological analysis of the 2 ml blood samples collected from 10 female patients were performed using Sysmex XN-1000 (Sysmex Corporation, Japan). Without injuring venous walls, using a sterile injector, 18 ml blood was collected from the antecubital area via branule and 9 ml of it was transferred into a 15 ml flat bottom tube that contained 1 ml citrate (3% CPD) as an anticoagulant, to obtain PRP using the open system. To obtain PRP using the closed system, the remaining 9 ml was transferred to the T-Lab PRP kit. Within 2 minutes after the collection of blood for each group, the tubes were shaken very slowly for 1 minute and single-step centrifugation was performed for both groups. Open system group (samples in the tube containing citrate) were centrifuged at 778 g for 5 minutes at 22°C, while the group transferred to T-Lab PRP kit were centrifuged at 830 g for 10 minutes at 22°C, based on the recommendations of the manufacturer, using Heraeus Labofuge 400 (Thermo Scientific™ Heraeus™ Labofuge™ 400 Centrifuges). In both groups, platelet-enriched 1 ml plasma, which is found at the intermediate fraction above the erythrocyte fraction and at the lower 1/3rd portion of the top fraction, was gently collected with an insulin injector. Afterwards, 0.25 ml of this sample was submitted to hematological analysis. The remaining 0.75 ml PRP and the whole plasma (approximately 4 ml) was inoculated into 30 ml of BacT/ALERT® FA Plus - Ref. 410851 using a sterile injector in the microbiology laboratory, in order to identify whether there are any microbiological contaminations. Costs of obtaining PRP using various closed system commercial kits and the open system method in the laboratory setting were compared.

Statsitical anayses

Statistical analyses were performed using SPSS 22.0.0.0 software (Version 22; IBM Corp, Amonk, NY). For continuous variables, median ± standard deviation values were used. Leukocyte, platelet, erythrocyte, and immunoglobulin values of whole blood and PRP products were compared using paired student t-test.

RESULTS

In the first phase of the study, it was found that the volume of the PRP obtained after the first spin was between 2.5 and 4.8 ml, and increased progressively as the centrifugal force increased. Centrifugal forces, durations, platelet, and leukocyte concentrations of the patients are shown in Table 2. It was detected that the maximum platelet concentration (1.4-fold higher than the concentration in whole blood) was achieved in patient number 5, when single-step centrifugation method, at 778 g for 5 minutes, was used.

In the first centrifugation step, platelet count was higher when the samples were centrifuged at under 500 g for 15 minutes than for 10 minutes. Higher platelet count was detected when the samples were centrifuged at more than 500 g for 5 minutes than for 10 minutes. When the centrifugal force was increased from 280 g to 778 g for the samples centrifuged for 10 minutes, it was found that the rate of platelet concentration increased, peaked at 778 g, and 857 g, and decreased again (Figure 1). It was found that platelets can be concentrated at these two centrifugal forces, but not for the remaining 7 patients. At the end of the second centrifugation step, which was performed at 2383 g for 5 minutes, in all donors, the majority of the platelets formed a pellet at the bottom of the tube. The platelet counts in PCPs were lower than the counts in whole blood (average of 0.34-fold), and platelets could not be concentrated from PCPs.

Table 2. Centrifugal force used for 5, 10 and 15 minutes in the first step centrifugation in the first phase and whole blood leukocyte and platelet counts

Open System First Phase Centrifuge

Patient no.	Centrifuge force (g)		Whole blood (× 10^3/ml)	Values after 5 minutes of centrifugation / Whole blood values (× 10^3/ml)	Values after 10 minutes of centrifugation / Whole blood values (× 10^3/ml)	Values after 15 minutes of centrifugation / Whole blood values (× 10^3/ml)
1	157 g	Platelet	321	X†	129 / 0.4	162 / 0.5
		Leukocyte	7.4	X†	16.9 / 2.2	15.6 / 2.1
2	280 g	Platelet	261	X†	115 / 0.4	159 / 0.6
		Leukocyte	3.6	X†	7.01 / 1.9	6.09 / 1.6
3	381 g	Platelet	234	X†	147 / 0.6	175 / 0.7
		Leukocyte	6.5	X†	11.9 / 1.8	10.6 / 1.6
4	562 g	Platelet	188	170 / 0.9	125 / 0.6	X†
		Leukocyte	8.9	14.7 / 1.6	13.6 / 1.6	X†
5	**778 g**	**Platelet**	**255**	**361 / 1.4**	**287 / 1.1**	X†
		Leukocyte	**4.56**	**5.0 / 1.1**	**3.4 / 1.1**	X†
6	857 g	Platelet	274	359 / 1.3	305 / 1.1	X†
		Leukocyte	6.8	5.5 / 0.8	3.9 / 0.8	X†
7	1029 g	Platelet	298	242 / 0.8	219 / 0.7	X†
		Leukocyte	10.3	5.3 / 0.5	3.4 / 0.5	X†
8	1215 g	Platelet	215	131 / 0.6	101 / 0.4	X†
		Leukocyte	9.6	2.9 / 0.3	1.3 / 0.3	X†
9	1418 g	Platelet	304	185 / 0.6	139 / 0.4	X†
		Leukocyte	5.8	1.2 / 0.2	0.7 / 0.2	X†

X†: No centrifugation performed

In the first centrifugation step, when the ratio of the leukocyte count in the PRP obtained via 10 minute-centrifugation to the leukocyte count in whole blood was analyzed, it was found that the maximum concentration of leukocytes (2.2-fold higher than the amount in whole blood) was achieved in patient number 1 (at the lowest centrifugal force). As the centrifugal force increased, leukocyte count in PRP progressively decreased and reached to a minimum (0.2-fold lower than whole blood) in patient number 9. When the centrifugal force exceeded 562 g, leukocyte count in PRP decreased significantly (Figure 2).

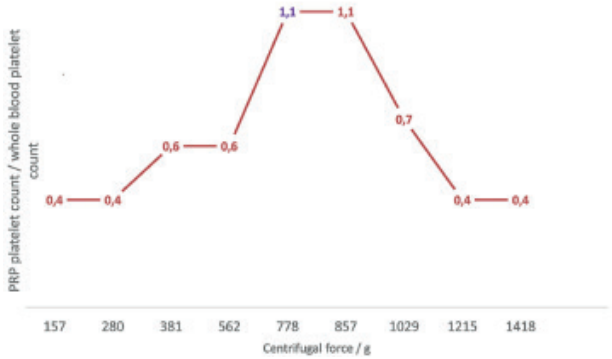

Figure 1. The effect of increase in the centrifugal force on the ratio of platelet count in PRP to whole blood platelet count in the first phase study (PRP platelet count/whole blood platelet count per patient)

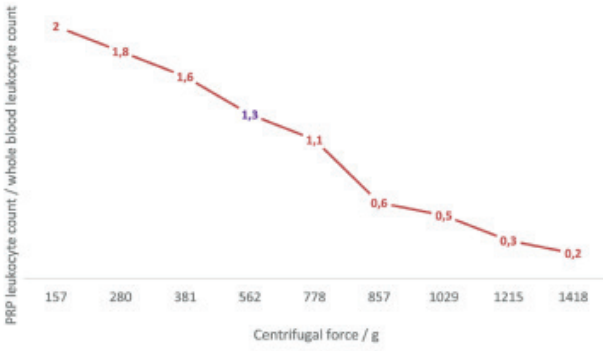

Figure 2. The effect of increase in the centrifugal force within 10 minutes of centrifugation on the ratio of leukocyte count in PRP to whole blood leukocyte count in the first phase study (PRP leukocyte count/whole blood leukocyte count per patient)

Table 3. Results of the Second Phase Study

	A.Whole blood	B. Open System	C. Close System	P Value
Erythrocyte (mean) (× 10^3/ml)	4.7±0.3	0.18±0.10	0.05±0.03	AB P = 0.645 AC P = 0.389 BC P = 0.223
Leukocyte (mean) (× 10^3/ml)	6.9±1.0	8.05±1.18	2.76±2.04	**AB P = 0.008** AC P = 0.134 BC P = 0.632
Platelet (mean) (× 10^3/ml)	264.3±54	425.3±91.2†	298.9±88.3¶	**AB P = 0.020** **AC P = 0.011** **BC P = 0.021**
IG (immunoglobulin G) (mean) (× 10^3/ml)	0.02±0.01	0.37±0.42	0.33±0.24	AB P = 0.211 AC P = 0.146 BC P = 0.146
PRP Volume (mean) (ml)	10	3.7 ± 0.12	4.01 ± 0.7	

† The mean platelet count in the open system group is 1.61-fold (1.21 - 2.16) higher than the platelet count in whole blood
¶ The mean platelet count in the closed system group is 1.1-fold (0.76 - 1.42) higher than the platelet count in whole blood

After the second centrifugation step, performed at a high centrifugal force, leukocyte count in PRPs obtained from all donors was less than or equal to 0.05 ×10^3/ml. It was observed that as the duration of centrifugation increased, leukocyte count decreased.

In the second phase of the study, the group from the open system PRP at a centrifugal force of 778 g and a duration of 5 minutes was compared with the group from the closed system PRP. The results of the second phase are given in Table 3. The volume of PRP was between 3.5 - 3.9 ml (3.73 ± 0.12) in the open system group, and between 3.7-4.2 ml (4.01 ± 0.17) in the closed system group. The mean platelet count was 264 ± 54.4 × 10^3/ml in whole blood, 425 ± 91.2 × 103/ml in open system, 298 ± 88.3 × 10^3/ml in the closed system. A significantly higher amount of platelet concentration was detected in the open system than the closed system (p=0.002). When compared with the whole blood platelet concentration, the amount of platelet concentrated using the open system is approximately 1.61-fold (1.21 - 2.16) higher than the whole blood platelet count. However, the amount of platelet concentrated in the closed system group was 1.1-fold (0.76 - 1.42) higher than the whole blood platelet count. When whole blood leukocyte concentrations (6.9 (5.73 - 8,60) × 10^3/ml) were compared with the open system leukocyte concentrations (8.05 (5.7 - 9.81) × 10^3/ml), it was found that significantly higher amount of leukocytes can be concentrated using open system (p = 0.008). However, no statistically significant differences were detected between whole blood leukocyte concentrations and closed system leukocyte concentrations.

Table 4. Total cost of materials used in open system PRP preparation

Item	Price per unit/ml solution	Number of ıtems used	Cost (Turkish Lira)
Branule	0.5 ₺	1 unit	0.5 ₺
10 ml tube	0.4 ₺	1 unit	0.4 ₺
Citrate (Solution 3% CPD)	1.75 ₺	1ml	1.75 ₺
15 ml flat botom tube	0.9 ₺	1 unit	0.9 ₺
Insulin syringe	1.75 ₺	1 unit	1.75 ₺
5 ml syringe	0.7 ₺	1 unit	0.7 ₺
Total			6 ₺

The duration between collecting blood from patients and obtaining PRP was around 15-20 minutes, both in open and closed systems. No growth was observed in neither of the culture media inoculated with the PRPs obtained from 10 patients using the sterile open system, as of the 6th day. The total cost of the materials used in the laboratory to obtain PRP using open system was less than 10₺ (Table 4). Costs of some of the closed system kits that are available in Turkey are given in Table 5. When compared with various closed system commercial kits, it was seen that the cost of PRP prepared using the open system in the laboratory setting was significantly lower.

Table 5. Platelet concentration efficiency of some of the PRP kits in Turkey, their costs, and the volume of blood collected from the patients to obtain PRP

PRP kit	PRP platelet / Whole blood platelet	The volume of blood collected from the patient	Cost (Turkish Lira)
T-lab PRP†	2 - 5 hold	10 ml	150 ₺
Y-cell bio PRP kit‡	7 - 9 hold	15 ml	375 ₺
Truecell§	4 - 7 hold	8 ml	110 ₺
Prepcell¶	4 - 7 hold	10 ml	100 ₺
DPG PRP£	6 hold	9 ml	295 ₺

† T-Lab PRP Kiti, Turkey, tlabprpkit.com
‡ Ycellbio™, USA, www.ycellbio.com
§ Truecell CGF, Turkey, www.truecellcgf.com
¶ PREP CELL PRP, Turkey, medikalone.com
£ DPG PRP BioCell Plus, Italy, www.dpgprp.om

DISCUSSION

In the first phase of this study, with using open system PRP preparation, a centrifugal force of 778 g (2,000 rpm) was the best centrifugal force and 5 minutes was the optimum duration. In the second phase, when closed and open system PRP preparation were compared, it is found that a significantly higher amount ($p = 0.021$) of platelets concentrated with open system PRP preparation than closed system PRP preparation, which was 1.6-fold higher than the platelet count in whole blood.

The primary aim in PRP preparations using the open system is to obtain the maximum amount of platelets. The amount of platelets obtained depends on the combinations of many parameters such as the volume of collected blood, hematocrit level, shape of the tube the blood is centrifuged in, centrifuge machine used, centrifugal speed and duration, the type of anticoagulant used and the type of the purchased PRP kit. In the past decade, as the clinical use of PRP became widespread, PRPs with different contents were obtained using various methods. Multiple classification systems were developed to identify the efficiency of open system and closed system PRPs (14-16). These classifications indicate that higher platelet concentration increases the quality of PRP. However, when the platelet concentration is lower than that of the whole blood, an efficient cellular response cannot be achieved and when the platelet concentration is 2.5-fold higher than that of the whole blood, an inhibitory effect on recovery can be observed (17). Thus, classification systems regarding the methods of PRP preparation have not been efficient and feasible.

When preparing PRP, after the centrifugation of whole blood, blood cells are arranged into fractions depending on their density. Since the density of blood cells are close, after centrifugation, contamination with blood cells between the fractions is inevitable. To reduce this contamination to a minimum, centrifugation must be performed at high centrifugal powers (500 g or more) and for a short duration (5 - 10 minutes). At lower centrifugal forces (150 - 300 - 450 g), longer duration (10 - 15 minutes) is required (18). By using different centrifugal powers and durations, PRP products with different ingredients can be obtained. According to the concentration of leukocytes being higher or lower than the number of leukocytes in whole blood, PRP is named leukocyte-rich or leukocyte-poor and are used in different areas of medicine (13). The clinical significance of the concentration of leukocytes or erythrocytes in PRP is not yet clear, but Dohan Ehrenfest and Joseph Alsousou argue that higher amount of mononuclear cells in PRP play a major role in the secretion of proteins and in the positive effects of the cytokines secreted during tissue repair (13,19). In the first phase of this study, for the centrifuge duration of 10 minutes, the total amount of leukocytes in PRP decreased as the centrifugal force increased. Additionally, leukocyte count significantly decreased when the force exceeded 562 g. However, as the primary aim of the first phase, the present study was to obtain the maximum platelet count in PRP by centrifuging whole blood, 778 g is identified as the optimum centrifugal force. In the second phase of this study, when the ingredients of the open system PRP was compared to the closed system commercial kit, the leukocyte count in the open system PRP was determined to be significantly higher than the closed system. Thus, by using the open system PRP preparation method in the laboratory setting, PRP that contained more leukocytes and more platelets than the closed system were obtained.

Piao et al. argue that the shape of the tube in which the blood is centrifuged during PRP preparation is one of the factors that affect the amount of platelets in PRP. In order to obtain the maximum amount of platelets in PRP, they recommend the use of 15 ml flat bottom sterile tubes instead of the conical bottom tubes (18). In the present study, in open system PRP preparation, the centrifugation steps were performed in 15 ml flat bottom tubes to obtain a higher number of platelets. Since clinical feasibility in the standardization of open system PRP preparation protocol is the primary objective of this study, the fact that these tubes were the most frequently used tubes in clinics enabled its practicality. Moreover, since it provides a more controlled transport than the pipette when collecting PRP from the tubes after the centrifugation, insulin injectors, which are low-cost, readily available, and sterile, were preferred in this study.

The volume of blood collected from the patients and the type of anticoagulant used are among the other factors that affect the platelet concentration in PRP. As the collected blood volume increases, platelet count in the total volume will increase, thereby increasing the number of platelets that can be concentrated. In the literature, high volumes of blood collection from the donors, varying between 30-450 ml, and obtaining a higher volume of PRP using two-step protocols, are reported (20-21). However, to reach such a high total blood volume, blood samples were needed to

be collected from patients numerous times. Hence, this study determined the volume of blood collected from the patients as 10 ml, which is low and acceptable. Thus, an increase in patient compliance and clinical feasibility was targetted in this study. In addition, low volume enabled us to use a low amount of citrate to prevent the aggregation of platelets. Aggregation of platelets causes the platelet count in the solution to appear low and the inhibition of the secretion of growth factors from their granules. Citrate is an anti-aggregating agent that acts as an anticoagulant for 2 -4 hours by maintaining the structural and physiological characteristics of platelets and is frequently used in the clinics (22). The study also used sterile citrate as it was readily available and low-cost.

When performing open system PRP preparation, the most important point is to use single-use sterile materials and to perform the transport of the products according to the principles of sterility. In the present study, PRPs obtained using open system method under sterile conditions were inoculated into culture media and observed that there were no bacterial growth in any of them. The primary requirement to inject PRP obtained in the laboratory setting into the organism is to ensure the product's sterility. Thus, although more costly, it seems more appropriate to use closed-system kits in clinics where sterility principles cannot be met.

CONCLUSION

In recent years, PRP treatment is widely used in infertile patient groups to increase endometrial receptivity. With repeated PRP applications, treatment cost increases significantly. Since neither the government nor the private insurance companies cover the PRP treatment, increased cost can be a barrier for the patients who are expected to respond to this treatment. The open system PRP preparation is established as more economical than the closed system in the present study. In our opinion, easily applied, standardized, sterile, and low-cost open system PRP protocols will increase the patients' access to treatment and encourage many clinicians to use PRP.

Conflict of interest: The authors declare that they have no competing interest.

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Ethical approval: The study was approved 'Ministry of Health Stem Cell Transplantation Scientific Advisory Council' with Council number 56733164/203.

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