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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

The Impact of Duration and Force of Centrifugation on Platelet Content and Mass in the Preparation of Platelet-Rich Plasma

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Abstract

Purpose Platelet-rich plasma (PRP) is an autologous prepared plasma enriched with platelets and obtained after a centrifugal separation and aggregation procedure. However, the optimized preparation protocol for PRP is still controversial and there are no standardized preparation protocols. The aim of this study is to show the effect of time and force of the centrifugation on the concentrations of platelets and to optimize the effective PRP preparation protocol.

Methods For the study, whole blood was drawn into 24 different 6-ml standard tubes containing 0.6 ml anticoagulant citrate dextrose solution—formula A. The samples were centrifuged separately at forces of $45\times g$, $180\times g$, $400\times g$, $725\times g$, $1130\times g$ and $1630\times g$ for 5, 10, 15 and 20 min. Every sample was analyzed, and a comparison was made between all groups.

Results No significant difference was observed in terms of platelet concentration, mean platelet volume or platelet mass between all groups ($p > 0.05$). The mean ± SD of platelet mass in baseline is $1890 \pm 134 \times 10^3$ fL/μL. The mean ± SD of platelet mass in the high centrifugal force of 1630×g was $3395 \pm 564 \times 10^3$ fL/μL, $2638 \pm 425 \times 10^3$ fL/μL, $2355 \pm 449 \times 10^3$ fL/μL and $2109 \pm 41 \times 10^3$ fL/μL over times of 5, 10, 15 and 20 min, respectively. The mean ± SD of platelet mass in the low centrifugal force of $45\times g$ was $2002 \pm 1623 \times 10^3$ fL/μL, $2491 \pm 1591 \times 10^3$ fL/μL, $2611 \pm 876 \times 10^3$ fL/μL and $3003 \pm 511 \times 10^3$/μL over times of 5, 10, 15 and 20 min, respectively.

Conclusions Platelets should be evaluated with platelet mass not including platelet concentrations alone, but also with mean platelet volume, which symbolizes the size of platelets while comparing platelet-rich plasma preparation protocols and kits. This could be a new starting point for comparison of PRP for all applications in the literature. All centrifugation forces and times could produce biologically reactive PRP. It may be only suggested that if high acceleration force is used, low durations should be selected, or if low acceleration force is used, long time of centrifugation should be selected.

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Keywords Centrifugation · Concentration · Mean platelet volume · Platelet mass · Platelet-rich plasma

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Introduction

Platelet-rich plasma (PRP) is defined as a volume of plasma having platelet content higher than peripheral blood. The purpose of centrifugation, which is the first step

during PRP preparation, is to accumulate higher amounts of platelets in a certain volume. Thus, the conditions used in the preparation steps, such as centrifugal acceleration and time, the number of centrifugation steps and the type of anticoagulant, were all studied basically in the literature by comparing the platelet concentrations [1, 2].

As is known, platelets are the smallest and most heterogeneous structures in all blood components. Platelets are simple anucleate cytoplasmic particles fragmented from the cell body of megakaryocytes [3]. Thus, platelets vary more in cellular volume than any other circulating blood element in mammals. It is also reported that larger platelets which contain more dense granules are enzymatically and metabolically more reactive than their smaller counterparts [1, 4]. Mean platelet volume (MPV) has the potential to serve as an easily measured in vivo marker of platelet activation [5]. Evidence indicates that an increase in mean platelet volume can potentially overwhelm current platelet-based biological therapeutics. Therefore, in our previous study, it was suggested that it would be appropriate to evaluate platelets with their concentration and mean platelet volume (MPV)—a parameter of platelet size—together while comparing platelet-rich plasma preparation protocols and kits [1].

Autologous PRP has less safety concerns than other cell-based regenerative therapies [6]. When cell therapies are involved in the clinic, it is of utmost importance to assure the quality of the cellular component [7]. In addition, high costs and the need to have specialized equipment to prepare PRP have critically reduced the use of autologous platelets [7]. Although commercial systems claim that the activities and the effectiveness of their ready-to-use kits are measured by the numerical highness of the platelet concentrations; obviously, what is important is how many times the basal values. So, the centrifugation protocols may have a critical impact on PRP quality and have been highly variable with respect to the force and time of centrifugations.

The aim of this study was to emphasize the aspects of the centrifugation step in preparing PRP without using any commercial kits and to discuss the factors involved in obtaining the final suitable composition. It is believed that different acceleration forces and durations may affect the yielded platelet concentration and the clinical efficacy of PRP. Furthermore, according to our current knowledge, this study can be seen as the first study comparing the various centrifugal forces and times in which platelet volume is used together with platelet concentration.

Materials and Methods

All experimental protocols used in this study were conducted according to the ethical guidelines of the Declaration of Helsinki and international regulations. After approval by the ethics committee of our institute (No 0565/4677), the informed consents of volunteer subjects were taken. Peripheral blood from five healthy male volunteer donors (18–25 years old) was collected using closed blood collection systems (Vacutainer®, Ref: 301746; BD Diagnostics). The volunteers had no relevant diseases. All donors were not on any medication known to affect platelet functions for 7 days before the study.

Blood Collection and Centrifugation Method

Prior to the blood collection for the experimental study, the whole blood samples from all volunteers were collected separately into ethylenediaminetetraacetic acid disodium (EDTA K3) washed standard tubes (Vacuette®, Ref.454041; Greiner Bio-One) for baseline cell counts.

For the experimental study, the whole blood was drawn by venipuncture and collected into 24 different 6-ml standard tubes (Vacuette®, Ref.456055; Greiner Bio-One) containing 0.6 ml anticoagulant citrate dextrose solution—formula A (ACD-A, at the ratio of 9:1) which consists of sodium citrate, citric acid and dextrose. Centrifugation is accomplished with a multi-purpose centrifuge (NF 800®, NUVE Industrial Materials Manufacturing and Trading Co., Turkey) at room temperature.

To isolate plasma, those standard tubes containing 5.4 ml whole blood and 0.6 ml ACD-A were centrifuged at six various centrifugation forces including low, middle and high spins (45, 180, 400, 725, 1130 and $1630\times g$) and four different times (5, 10, 15 and 20 min). The relative expression of acceleration to the gravitational acceleration (g) is traditionally referred to as relative centrifugal force (RCF). The revolutions per minute (RPM, rpm, r/min) gives the frequency amount of a rotation. In our study, RCF values of 45, 180, 400, 725, 1130 and $1630\times g$ corresponding to RPM values of 500, 1000, 1500, 2000, 2500 and 3000 rpm were used.

Hematological Analysis

A small portion of each sample (1 ml) after the centrifugation was used for hematological analyses. All specimens were taken carefully from the lower one-third of plasma portion immediately above the buffy coat layer without ever mixing it by the same person. The reason not to take samples including the buffy coat layer is that this region already has an intense platelet content which may not reflect the change in plasma [1]. Concentrations of red blood cells (RBCs), white blood cells (WBCs) and platelets were measured with a multi-automatic blood corpuscle analyzer. Hematological routine parameters such as mean platelet volume (MPV) and hematocrit were also analyzed.

Concentrations were expressed in counts/μL, and mean platelet volume was expressed in femtoliter (fL).

Platelet mass was defined as total platelet volume of sample obtained which was calculated as total platelet count in the obtained sample multiplied by mean platelet volume (fL) observed in the same region.

Statistical Analysis

SPSS (Statistical Package for Social Sciences, USA) 15.0 Data Analysis System was used for data analysis. Before statistical significance assessment, first a Kolmogorov–Smirnov test for normality and Levene's test for homogeneity were performed to determine whether to use parametric or nonparametric tests. Results were expressed as mean ± SD for the groups. All the groups were analyzed against each other using the Kruskal–Wallis H test, whereas Mann–Whitney U test was used for the comparison of independent groups. p values under 0.05 were accepted as significant.

Results

The baseline blood counts of all the volunteers were comparable ($p > 0.05$). The mean ± SD of WBC count was 6.92 ± 2 × 10^3/μL, RBC count 5.44 ± 0.6 × 10^6/μL and platelet counts 218 ± 16.8 × 10^3/μL.

The changes in platelet concentrations according to the centrifugal forces (g) and times (min) are shown in Fig. 1, but it was significant only in all time parameters of the high acceleration group of 1630×g in comparison with baseline ($p < 0.05$). The mean ± SD of platelet concentrations in the high centrifugal force of 1630×g was 442 ± 89.3 × 10^3/μL, 344 ± 48.9 × 10^3/μL, 310 ± 67.5 × 10^3/μL and 265 ± 44.2 × 10^3/μL over times of 5, 10, 15 and 20 min, respectively. The mean ± SD of platelet concentrations in the low centrifugal force of 45×g was 269 ± 213.7 × 10^3/μL, 339 ± 215.5 × 10^3/μL, 351 ± 127.8 × 10^3/μL and 410 ± 71.5 × 10^3/μL over times of 5, 10, 15 and 20 min, respectively.

The change in mean platelet volume is shown in Fig. 2 according to the centrifugal force and time parameters in comparison with baseline, but it was significant only in all time parameters of the low acceleration group of 45×g and high acceleration group of 1630×g in comparison with baseline ($p < 0.05$).

Figure 3 shows platelet mass among centrifugal force and time parameters, but it was significant only in all time parameters of the high acceleration group of 1630×g in comparison with baseline ($p < 0.05$). The mean ± SD of platelet mass in baseline is 1890 ± 134 × 10^3 fL/μL. The mean ± SD of platelet masses in the high centrifugal force of 1630×g was 3395 ± 564 × 10^3 fL/μL, 2638 ± 425 × 10^3 fL/μL, 2355 ± 449 × 10^3 fL/μL and 2109 ± 41 × 10^3 fL/μL over times of 5, 10, 15 and 20 min, respectively. The mean ± SD of platelet masses in the low centrifugal force of 45×g was 2002 ± 1623 × 10^3 fL/μL, 2491 ± 1591 × 10^3 fL/μL, 2611 ± 876 × 10^3 fL/μL and 3003 ± 511 × 10^3/μL over times of 5, 10, 15 and 20 min, respectively.

Discussion

PRP treatment has recently been recognized as a safe, nonsurgical, relatively inexpensive and easily prepared biological treatment. However, the wide usage in many fields turned this new advantageous biotechnology to a disadvantageous manner. Currently, there are more than 16 available platelet separation systems which have available disposable kits with a price from $US 175–1150 per kit, whereas the cost of a reliable manual PRP preparation could be less than 4 USD [7–9]. General use in all fields of medicine had enabled the production of a wide variety of commercial systems worldwide, and each system created their own protocols to show itself advantageous. As a result, a wide variety of protocols have been developed within a range of studies with manually prepared PRP to the studies that suggest the commercial kits as a necessity. Each study compared their results with different methods leading to confusion about the protocols, and thus, questions of the accuracy of the protocols have arisen. However, on the other hand, some studies were published investigating the centrifugation forces and time to optimize the preparation of PRP. The question of whether the PRP samples obtained from all those different protocols may yield the appropriate platelet concentration remains controversial.

Graziani et al. compared the effects of different concentrations of PRP (2.5 ×, 3.5× and 4.2–5.5×) on fibroblast–osteoblast cell cultures, and they showed that rational biological activity was maximized at the 72nd hour of application [10]. They reported that the maximum effect of PRP was present at a concentration of 2.5-fold [10]. In addition, the authors stated that high concentrations of PRP resulted in decreased cell proliferation [10]. Rappl et al. [11] emphasized that PRP which has a concentration up to threefold of baseline platelet concentration resulted in faster granulation tissue and better outcomes than higher concentrations. In a bone regeneration study of a rabbit model, it was shown that low platelet concentrations (0.5–1.5×) produced only a small positive effect and that high concentrations (6–11×) had inhibitory and cytotoxic effects on osteoblastic activity, whereas the moderate platelet concentration group (2–6×) produced

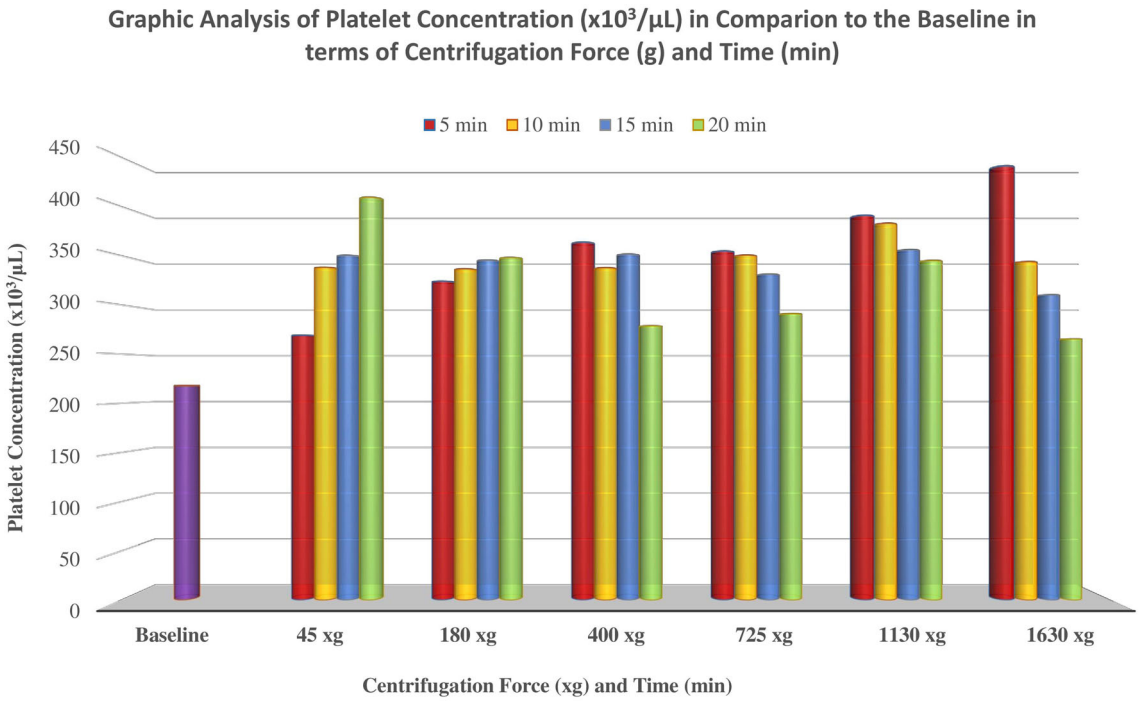

Fig. 1 Graphic analysis of platelet concentration ($\times 10^3/\mu L$) in comparison with the baseline in terms of centrifugation force (g) and time (min). There is no significant difference compared to all groups ($p > 0.05$) but it was significant only in all time parameters of the high acceleration group of $1630 \times g$ in comparison with baseline ($p < 0.05$). As seen in the graphic, the concentration tends to decrease with time in high acceleration forces, and the concentration increases with increasing time in low acceleration forces

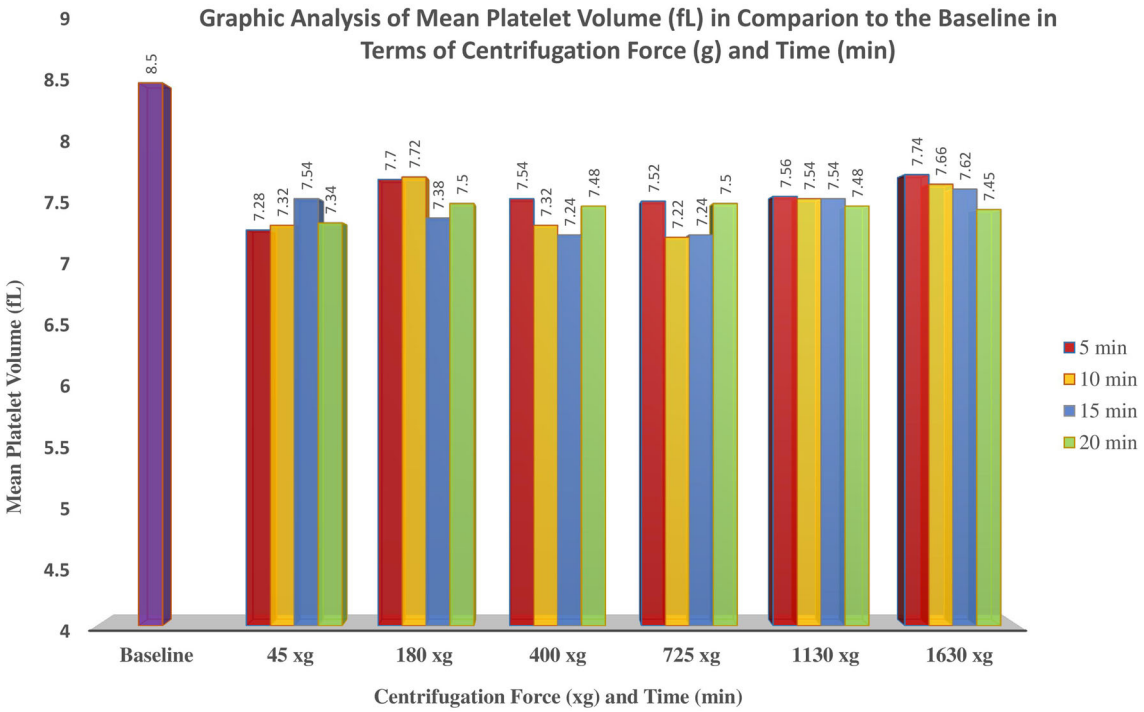

Fig. 2 Graphic analysis of mean platelet volume (fL) in comparison with the baseline in terms of centrifugation force (g) and time (min). The numbers mapped over the columns indicate the mean MPV values of the group as femtoliter. It was significant only in all time parameters of the low acceleration group of $45 \times g$ and high acceleration group of $1630 \times g$ in comparison with baseline ($p < 0.05$)

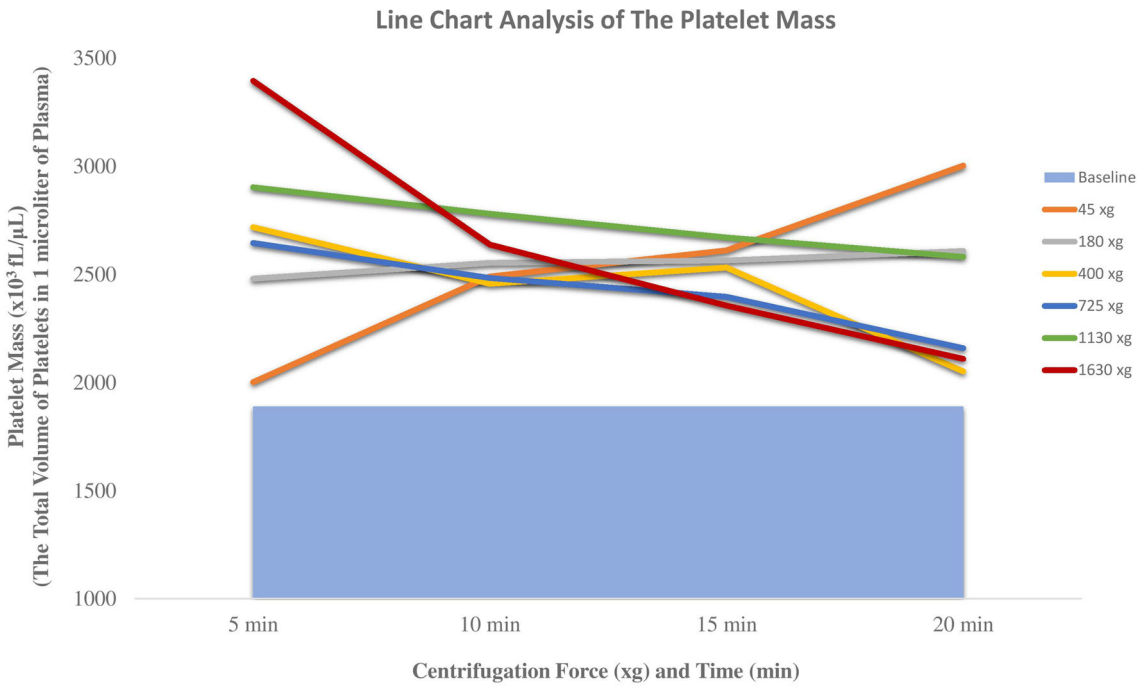

Fig. 3 Line chart analysis of the platelet mass (× 10^3 fL/µL) which refers to the total volume of platelets in 1 Âµl (µL). The blue rectangle at the base represents the baseline platelet mass. As seen in all groups, the yielded specimens were above baseline. The platelet mass is more accurate to make a comparison between groups

positive biological effect [12]. Anitua et al. [13] studied the effect of increasing amounts of platelets in fibroblast cultures and showed that the maximum proliferation rate was obtained by growth factors with a twofold or fourfold platelet concentration. Akhundov et al. [7] noted that the overall goal in PRP treatment was to achieve a concentrating factor of 2–3 times in whole blood for any given patient. Briefly, it is seen that PRP with higher concentrations creates an inhibitory effect rather than a positive effect. It is generally mentioned that the intermediate platelet concentrations (minimum 1.3 ×–2 × and maximum 4 ×–6 × of concentration) should be achieved for optimum results [10–14]. It should be underlined that for concentrations below this level, the effect may be suboptimal, while beyond this range, there may be a paradoxically inhibitory effect [10–14]. Weibrich et al. [12] concluded that the platelet concentration of PRP is important to get a clinically positive effect and to determine its biological outcome. Our study shows that optimum concentrations can be achieved at any centrifugation force and time. However, it may be noted that the time should be kept short if a high centrifugal force is selected, and if low centrifugal forces are performed, the centrifugation time should be kept longer.

Another point of discussion is the importance of mean platelet volume in such studies. It is a fact that it would be more appropriate to use the platelet volume in conjunction with platelet concentration. Mammalian anucleic platelets develop from the cytoplasm of megakaryocytes, the only polyploid hematopoietic cell [15]. Since they are cytoplasmic particles fragmented from megakaryocytes, platelets differ in cellular volume within a wide range. Kuter noted that the body conserves the mass, not the number, of platelets [16]. Whitfeld and Martin reported that platelet numbers and size appeared to be accounted for by genetic and nonshared environmental influences only [17]. Besides that, they noted that a significant negative correlation exists between platelet numbers and platelet size [17]. It could be speculated that there is a roughly constant circulating platelet mass in the human body [18]. The body wants to keep the total mass of platelets in a normal and unchanged level, not the platelet count [16]. The existing total mass of the platelets and the effect produced by this mass should be the main focus rather than the numerical values of the platelets. According to the published reports, larger platelets are enzymatically and metabolically more reactive than their smaller counterparts due to containing more dense granules [4, 19]. Notably, mean platelet volume (MPV), the most commonly used measure of platelet size, can be seen as a potential marker of platelet reactivity [4, 5]. In light of that evidence, it is necessary to compare platelet-based biologic suspensions not only by the platelet concentrations but also with the platelet mass parameter, which includes the platelet volume.

The data we obtained are in parallel with the study reported by Perez et al. [2]. According to the analysis of the

graph of platelet masses, it could be speculated that in low acceleration forces, the separation increases in parallel with time. On the contrary, the platelet mass decreases with time in an acceleration force of 1630 xg. In a study by Lee et al., the inactivated PRP and activated PRP with thrombin and calcium chloride were compared and the authors reported that no statistical relationships exist among any of PRP experimental groups through the analysis of the change in growth factors according to the activation [20]. It was inferred that the platelet membranes were damaged by high-speed centrifugation in the absence of PRP activation, influencing the activation of platelets, and as such, the growth factors are released [20]. As the reason for this, it could be speculated that the separation is completed early in high acceleration forces and the platelets may go into disintegration with increasing time periods. Results of our study may suggest a high acceleration force of $1630 \times g$ and low duration of 5 min. If low acceleration forces are selected, a long time is required to ensure complete accumulation of platelet mass.

There are some limitations in the current study. One of the major limitations of our study was that the study had a very small sample size. Another limitation could be that this study requires additional clinical experiments to determine the clinical reflection of the study on cellular processes. Since a growing number of articles have been published about PRP applications by either commercial kits or manual methods, our study may start to raise new studies to clarify a controversial issue about the preparation of these products. Larger studies are necessary to further characterize the in vivo effect of the different platelet masses including the parameters of MPV and concentration of the PRP.

As a conclusion, all the evidence in the literature indicates that platelets should be evaluated with platelet mass not including platelet concentrations alone, but also with MPV, which reflects the size of platelets while comparing platelet-rich plasma preparation protocols and kits. This could be a new starting point for comparison of the results of the PRP for all applications in the literature. These relationships may provide a better understanding of these platelet parameters and may contribute to their use as helpful diagnostic aids. It was observed that there was no significant difference between the centrifugation forces and time in our study. All centrifugation forces and time could produce a biologically reactive PRP. It may be only suggested that if high acceleration force is used, low durations should be selected, or if low acceleration force is used, long time of centrifugation should be selected.

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Compliance with Ethical Standards

Conflict of interest All the authors (KO, YK, OC, UK) confirmed no funding supporting the work and no statement of financial interest and no conflict of interest. All authors bear the responsibility of this letter.

Ethical Approval All experimental protocols used in this study were conducted according to the ethical guidelines of the Declaration of Helsinki and international regulations. Approval by the ethics committee of our institute (No: 0565/4677) was obtained.

Informed Consent Informed consents of volunteer subjects were taken.

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