

# AVOIDING LEUKOCYTE CONTAMINATION AND EARLY PLATELET ACTIVATION IN PLATELET-RICH PLASMA

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The objective of this study was to describe a new platelet-rich plasma (PRP) protocol with a reduced concentration of leukocytes and intact platelets. We collected 8 mL of venous blood (VB) from marginal ear veins of 10 male New Zealand white rabbits in acid dextrose citrate Vacutainer tubes. Tubes were centrifuged at 302g for 10 minutes. All plasma was collected in plastic tubes to avoid buffy-coat contamination and centrifuged at 2862g for 5 minutes. A 10% calcium chloride activator (10 PRP:2 CaCl<sub>2</sub>) was added to the lower third of this plasma (PRP), and the PRP gel was obtained. Mean platelet count was  $317.7 \times 10^3 \pm 39.9/\mu\text{L}$  in VB and  $1344.9 \times 10^3 \pm 347.5/\mu\text{L}$  in PRP. Leukocyte counts were  $3.96 \times 10^3 \pm 2.01/\mu\text{L}$  and  $0.46 \times 10^3 \pm 0.45/\mu\text{L}$  in VB and PRP, respectively. Mean platelet enrichment was  $327.4 \pm 97.8\%$ . All differences were statistically significant ( $P > .05$ ). This protocol is practical and reproducible, resulting in a high concentration of intact platelets to help tissue repair and low levels of leukocytes.

**Key Words:** platelet-rich plasma; leukocyte; protocol

## INTRODUCTION

**P**latelets are cells originating from megakaryocytes in the bone marrow; together with erythrocytes and leukocytes they form the blood tissue. Some important platelet functions are hemostasis induction by clot formation and growth factor availability, which helps the healing process.<sup>1,2</sup>

Platelet-rich plasma (PRP), first introduced to the

oral surgery community by Whitman et al,<sup>3</sup> is a cell concentrate obtained by an autologous blood centrifugation process and has been widely used in medicine and dentistry since then. PRP use is based on the premise that it contains a larger number of platelets than whole blood, thereby releasing a significantly higher amount of growth factors at the surgical site compared with clotting. These growth factors contribute to increased vascularity and bone maturation.<sup>4-6</sup>

Platelet-derived growth factor, transforming growth factor, vascular endothelial growth factor, platelet-derived endothelial cell growth factor, basic fibroblast growth factor, and platelet activating factor are present at platelet alpha-granule; they regulate mitosis, cellular differentiation, metabolism, collagen production, and vascular in-growth,<sup>7,8</sup> enhancing bone formation qualitatively.<sup>3</sup>

As cited in the literature, the main clinical benefits of using PRP are faster radiographic maturation and a histomorphometrically denser bone,<sup>1</sup> earlier implant placement, earlier function, and greater predictability.<sup>9</sup> According to some studies considered to be refer-

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ences in PRP obtention in rabbits, there are several protocols and methodologies for preparing PRP. These studies describe different centrifugation speeds, different sites for blood collection, different volumes of blood required, different proportions of plasma considered to be PRP, and different substances for gelling PRP.<sup>10–12</sup>

Aghaloo et al<sup>10</sup> collected 10 mL of autologous venous blood (VB) from New Zealand white rabbits in a Vacutainer tube (Becton Dickinson, Franklin, NJ) containing 1.1 mL of adenine citrate dextrose (ACD) anticoagulant. Samples were centrifuged twice, first at 215g for 10 minutes and then, after plasma separation, at 863g for 10 minutes. The top half was discarded (platelet-poor plasma = 10 000 to 50 000 platelets/mm<sup>3</sup>), and the lower half resulted in PRP (625 000 to 1 495 000 platelets/mm<sup>3</sup>). Then, 5000 units of topical bovine thrombin was reconstituted with 5 mL of 10% calcium chloride (CaCl<sub>2</sub>) and added to PRP. The ratio of PRP to CaCl<sub>2</sub> activator was 10:1.

Efeoglu et al<sup>11</sup> described 2 protocols. In the first protocol, 9 mL of VB was collected in ethylene diamine tetraacetic acid (EDTA) tubes. The whole blood was centrifuged at 300g for 10 minutes, and the pink plasma (plasma with a few erythrocytes) was centrifuged again at 5000g for 5 minutes. Only the lower half of the plasma was called PRP (1000 × 10<sup>3</sup>/μL). In the second protocol, after pink plasma centrifugation, the upper two thirds of the plasma was discarded and only the lower third was considered PRP (3134 × 10<sup>3</sup>/μL).

Butterfield et al<sup>12</sup> prepared PRP by acquiring 21 mL of rabbit blood and using different centrifugation speeds. For the initial spin, the authors centrifuged the whole blood at 150g for 20 minutes; for the second spin, the plasma was centrifuged at 400g for 10 minutes. Afterward, bovine topical thrombin and 10% CaCl<sub>2</sub> was mixed with PRP. The average PRP platelet count was 2.061 × 10<sup>3</sup>/mm<sup>3</sup>.

Considering the many differences among the methodologies used to prepare PRP, including platelet counts, gelling substances used, and especially the lack of care regarding leukocyte contamination, this study describes a new protocol for PRP isolation. This protocol takes into consideration the amount of leukocytes in PRP, which chemotactically attracts inflammatory cells to the surgical site, and platelet integrity maintenance until PRP placement at the surgical site once the early platelet activation decreases growth factor kinetics.

#### MATERIAL AND METHODS

This study was approved by the Ethics Review Committee for Animal Research at the São Paulo

State University of Araraquara. The study was divided into 2 steps. In the pilot phase, a total of 12 tubes containing VB collected from 6 New Zealand white male rabbits weighing approximately 4 kg were submitted to different protocols in order to establish a new one. In the second step, 10 tubes from 10 New Zealand white male rabbits with the same weight had 8 mL of VB collected and the new protocol was tested. Animals were not anesthetized, although they were manually restrained while the sample was drawn.

As seen in Table 1, different protocols were tested using different anticoagulants, centrifugation speeds, and CaCl<sub>2</sub> solution volumes. A methodology was standardized (Table 2), and in this study, 8 mL of VB was drawn from the marginal ear vein of each rabbit with a scalpel and a 10-mL syringe. Immediately, to prevent clot formation, VB was combined inside Vacutainer tubes containing 1 mL of acid ACD anticoagulant.

The PRP was prepared via a 2-step protocol. First, VB was centrifuged (centrifuga Hermle Z380; Labnet Co, Woodbridge, NJ) at 300g for 10 minutes. The plasma, separated from the red blood cells (RBCs) and most of the white blood cells (WBCs), was set apart in a sterile plastic tube and centrifuged again at 2860g for 5 minutes. The upper two thirds of the preparation was designated PPP, and the lower third was designated PRP, as described by Efeoglu et al.<sup>11</sup> The platelet pellet that adhered to tube walls in PRP was resuspended before its clinical use. Blood cell counts for each sample were determined electronically (Coulter T-890, Coulter Electronics, Illinois) before and after centrifugation to confirm the obtention of a platelet concentrate with a low leukocyte value.

After this step, 10% CaCl<sub>2</sub> was added to it in a ratio of 10 PRP to 2 CaCl<sub>2</sub> vol/vol for gelling PRP. Bovine topical thrombin was not used to avoid contamination and infection by a xenogenous material.<sup>13</sup> Platelet enrichment percentage as well as WBC, RBC, and platelet counts were determined before and after centrifugation, in the pilot study, and after the new protocol was established, just as proposed by Marx et al.<sup>1</sup>

#### RESULTS

The volume of PRP obtained was approximately 2 mL, including the volume of CaCl<sub>2</sub> used (approximately 0.35 mL). The time spent was 2 minutes for PRP gelling and 30 minutes for PRP preparation.

In the pilot study, the average platelet counts in VB, in PPP, and in PRP of each tube were 402.4 × 10<sup>3</sup> ± 121, 26.2 × 10<sup>3</sup> ± 44.5 and 740.8 × 10<sup>3</sup> ± 472.8

TABLE 1

Protocols tested, platelet counts in venous blood, platelet-poor plasma, platelet-rich plasma and enrichment percentage in the pilot study\*

Tube	Protocols						Platelet counts ( $\times 10^3/\mu\text{L}$ )			
	Blood volume	Anticoagulant	1st spin	2nd spin	CaCl <sub>2</sub> /PRP	PRP	VB	PPP	PRP	EP
1	4 mL	0.25 mL sodium citrate	-	-	-	-	-	-	-	-
2	4 mL	0.25 mL sodium citrate	-	-	-	-	-	-	-	-
3	4 mL	0.25 mL sodium citrate	-	-	-	-	-	-	-	-
4	4 mL	0.5 mL sodium citrate	120g 10 min	410g 10 min	1/10	Lower half	383	68	377	-1.5
5	4 mL	0.5 mL sodium citrate	160g 10 min	2500g 5 min	1/10	Lower third	435	4	921	111
6	4 mL	0.5 mL sodium citrate	160g 10 min	2500g 5 min	1/10	Lower half	616	13	135	-78
7	4 mL	0.5 mL sodium citrate	160g 10 min	2500g 5 min	1/10	Lower third	401	5	1520	279
8	4 mL	1.0 mL CPD-A	160g 10 min	2500g 5 min	4/10	Lower third	384	131	995	159
9	4 mL	0.5 mL ACD	160g 10 min	2500g 5 min	2/10	Lower third	268	6	766	185
10	4 mL	0.8 mL ACD	160g 10 min	2500g 5 min	2/10	Lower third	256	2	713	178
11	4 mL	0.5 mL ACD	160g 10 min	2500g 5 min	2/10	Lower third	557	7	94	-83
12	4 mL	0.5 mL ACD	302g 10 min	2862g 5 min	2/10	Lower third	322	-	1147	256
Mean $\pm$ SD							402.4 $\pm$ 121	26.2 $\pm$ 44.5	740.8 $\pm$ 472.8	111.7 $\pm$ 135.7

\*VB indicates venous blood; PPP, platelet-poor plasma; PRP, platelet-rich plasma; EP, enrichment percentage; CaCl<sub>2</sub>, calcium chloride; ACD, adenine citrate dextrose; CPD-A, citrate phosphate dextrose-adenine. A dash indicates that no data were collected.

platelets/ $\mu\text{L}$ . The average enrichment percentage of platelet count in PRP related to VB in the pilot study was  $111.7 \pm 135.7$ . The counts for figured elements, platelet enrichment percentage of each tube in the pilot study, and mean counts are shown in Table 1.

Table 3 shows WBC, RBC, and platelet counts in VB and in PRP for each animal after the new protocol was established. The average counts of each type of cell in VB were  $3.96 \times 10^3 \pm 2.00$ ;  $5.64 \times 10^6 \pm 0.44$ , and  $317.7 \times 10^3 \pm 39.90/\mu\text{L}$ , respectively. The average counts for each type of cell in PRP were  $0.46 \times 10^3 \pm 0.45$ ;  $0.02 \times 10^6 \pm 0.01$  and  $1344.9 \times 10^3 \pm 347.50/\mu\text{L}$ , respectively. The average enrichment percentage of platelets in PRP in relation to VB in the new protocol was  $327.4\% \pm 97.8$ . Table 3 also illustrates the platelet enrichment percentage for each rabbit in the new protocol.

Differences between groups were analyzed using the Student *t* test for a level of significance of 5%.

## DISCUSSION

Nowadays, the use of PRP in combination with autogenous or artificial bone grafts presents an alternative treatment in oral and maxillofacial surgery.

Some PRP benefits include the absence of concerns regarding antigenicity or infectious diseases and the presence of large amounts of growth factors that promote the proliferation and differentiation of mesenchymal cells into osteoblasts, enhancing wound healing and bone formation.

Although many studies applying PRP have been extensively developed and the results deeply investigated,<sup>1,2,4,7,14-26</sup> many aspects are still uncertain. Some of these aspects include side effects,<sup>27</sup> quantification

TABLE 2

Platelet-rich plasma protocol in rabbits\*

Step	Procedure
1	Collect 8 mL of VB in glass tube containing 1 mL of ACD
2	Gently mix the blood
3	1st centrifugation: 302g for 10 minutes
4	Collect plasma, avoiding red and white cells
5	Place plasma into a plastic tube
6	2nd centrifugation: 2862g for 5 minutes
7	PRP = lower third (discard top two thirds)
8	Count PRP cells
9	Add CaCl <sub>2</sub> to PRP (ratio 2:10)
10	Wait 2 minutes for PRP gel

\*PRP indicates platelet-rich plasma; ACD, adenine citrate dextrose; VB, venous blood; CaCl<sub>2</sub>, calcium chloride.

TABLE 3

Elements in venous blood and platelet-rich plasma and enrichment percentage of platelets in the new protocol\*

Animal	White cells ( $\times 10^3/\mu\text{L}$ )		Red cells ( $\times 10^6/\mu\text{L}$ )		Platelets ( $\times 10^3/\mu\text{L}$ )		
	VB	PRP	VB	PRP	VB	PRP	EP (%)
1	6.6	0.7	5.38	0.02	286	1166	342
2	3.6	0.3	5.08	0.03	291	1629	459
3	4.0	0.3	6.66	0.01	357	988	176
4	7.5	-	5.69	0.02	349	1426	308
5	3.5	1.3	5.87	0.02	255	1031	304
6	-	-	5.90	0.05	299	1602	435
7	3.8	0.6	5.78	0.02	288	909	215
8	3.7	1.1	5.36	0.04	365	1297	255
9	3.0	0.2	5.34	0.01	317	1361	329
10	3.9	0.1	5.35	0.01	370	2040	451
Mean $\pm$ SD	3.96 $\pm$ 2	0.46 $\pm$ 0.45	5.64 $\pm$ 0.44	0.02 $\pm$ 0.01	317.7 $\pm$ 39.9	1344.9 $\pm$ 347.5	327.4 $\pm$ 97.8

\*VB indicates venous blood; PRP, platelet-rich plasma; EP, enrichment percentage. A dash indicates that no data were collected.

of the ideal amount of platelets and growth factors to be used at the surgical site, the best protocol for obtaining PRP, and especially the fact that few controlled studies support the real benefits of PRP.

The presence of deleterious habits, systemic diseases, and standardization of gender and body weight that could interfere with the results, and the difficulty in obtaining frequent radiographs or biopsies, make studies in animals an important alternative for in vivo controlled studies.

Butterfield et al<sup>12</sup> stated that rabbits are an appropriate model for PRP investigations because they have equal amounts of coagulation factors compared with humans and offer a sufficient volume of blood for preparing PRP. According to Efeoglu et al,<sup>11</sup> the quantity of blood that can be collected from an adult rabbit is 15 mL. In our pilot study, it was determined that 8 mL of blood would be a sufficient volume to obtain PRP (2 mL) without the need for transfusion or saline infusion.

Anesthesia during blood collection was not used to avoid peripheral vasoconstriction caused by the anesthetic, a fact that could complicate the procedure. Moreover, Vacutainer tubes were not directly used because the negative pressure closes the ear vein. After the blood was collected with the help of a syringe and placed inside the tube, it was gently manipulated allowing the blood to mix entirely with the anticoagulant, preserving platelet integrity and avoiding clot formation.

Considering the fact that anticoagulants based on citrate are considered to be the choice for PRP preparation in human surgeries,<sup>1,2,5,11,28</sup> the ACD anticoagulant was used in this study instead of EDTA, as Efeoglu et al<sup>11</sup> recommended in their experiment. According to Landesberg et al,<sup>5</sup> citrate is better for

preserving platelet membrane integrity than EDTA. This way, platelet growth factors are only released at the surgical site and not during PRP preparation.

In the present study, no bovine thrombin was used to avoid the risk of contamination and infection by a xenogenous material, as suggested by Christgau et al,<sup>13</sup> even though Marx<sup>29</sup> has stated that bovine topical thrombin is a safe initiator of clotting with no risk of infection or inflammation enhancement. Actually, bovine thrombin was not necessary in this new protocol because PRP gelling occurred within 2 minutes.

Concerning the methods for preparing PRP, different centers have different protocols. Most of the variations are found in the volume of blood collected, the number and speed of centrifugation, and the type of anticoagulant used. The data obtained by Aghaloo et al<sup>10</sup> and Efeoglu et al<sup>11</sup> were used as the starting point to establish this new protocol. As seen in Table 1, some tests were made and, according to the difficulties found, modifications were implemented.

In the pilot study, upon visual inspection the first 3 tubes revealed small amounts of clot. Probably the volume of sodium citrate was not sufficient to avoid blood coagulation. Because of this, the volume of citrate was doubled (0.5 mL) in tube 4, and no clot formation was observed. A slow rotation protocol was also designed for tube 4. The first rotation used was 120g for 10 minutes and then a new plasma centrifugation was performed at 410g for 10 minutes. However, platelets were not separated from erythrocytes and leukocytes as observed at the PRP negative enrichment percentage.

Rotation speed was increased to 160g for 10 minutes and then to 2500g for 5 minutes. These

modifications showed better results for the enrichment percentage, except in tubes 6 and 11, which also had negative results.

The citrate phosphate dextrose-adenine (CPD-A), an anticoagulant based on citrate as well as ACD, is commonly used in blood banks for platelet conservation. However, it is not commercially available in Vacutainer tubes. Moreover, ACD is part of CPD-A and had already been used by Aghaloo et al.<sup>10</sup> For this reason, ACD was present in tubes 9, 10, 11, and 12 and showed good results in all except sample 11.

According to data from Efeoglu et al,<sup>11</sup> in this study the lower third of the total volume of plasma after the second centrifugation was named PRP. Also, CaCl<sub>2</sub> was used at 2:10 proportion with gelling around 2 minutes. No differences were found from tube 10, which contained 0.8mL of ACD in relation to tubes 9, 11, and 12, which had only 0.5 mL of anticoagulant. The low amount of anticoagulant was chosen as pattern.

The difficulty in releasing the platelet pellet from the tube wall after the second centrifugation was considered. It was easier to release the pellet using the plastic tube than the glass tube, especially when the second speed of centrifugation had to be increased to more than 600g. This may be attributable to the fact that the glass can damage the platelets causing their aggregation and activation.

Because platelet counts in the pilot study were still beyond the counts cited in the literature, it was decided to increase the first and the second spins to achieve better results, as seen in Table 1.

According to Efeoglu et al,<sup>11</sup> platelet counts in VB of New Zealand white rabbits varied from 250 to 750 × 10<sup>3</sup>/μL. Our results were within normal limits, with mean counts of 317.7 × 10<sup>3</sup> platelets/μL ± 39.9. The number of platelets in PRP in this study was 1344.9 × 10<sup>3</sup>/μL ± 347.5 while platelet counts in other studies were approximately 3134 × 10<sup>3</sup>/μL,<sup>11</sup> 1050 × 10<sup>3</sup>/mm<sup>3</sup>,<sup>10</sup> and 2061 × 10<sup>3</sup>/μL.<sup>12</sup> Platelet enrichment percentage in our study was 327.4% ± 97.8. In Efeoglu et al<sup>11</sup> it was approximately 429%.

Marx,<sup>29</sup> discussing the ideal concentration of platelets to enhance bone and soft tissue regeneration, indicated that a sufficient cellular response to platelet concentrations first begins when a 4-fold to 5-fold increase over baseline platelet numbers is achieved. In this study we were able to achieve a 4-fold to 5-fold increase in 7 rabbits. In rabbits 3, 7, and 8 the increase was around 3 times.

A study investigating the real benefits of the association of PRP and autogenous bone in in vivo surgeries is being developed and is the aim of future research.

## CONCLUSION

This protocol is a practical and low-cost method to obtain a gel with high amount of platelets and few leukocytes, ready to be used in surgical sites.

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