

Original Research Article

A standardized research protocol for platelet-rich plasma (PRP) preparation in rats

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Abstract

Introduction: The urgent need for studies using standardized protocols to evaluate the real biological effects of PRP has been emphasized by several authors. **Objective:** The purpose of this study was to standardize a methodology for autologous Platelet-Rich Plasma (PRP) preparation in rats. **Material and methods:** Twenty-four, 5 to 6-month-old, male rats, weighing 450 to 500 g were used. After general anesthesia, 3.15 ml of blood was collected from each animal, via cannulation of the jugular vein. A standardized technique of double centrifugation was used to prepare PRP. PRP samples and peripheral blood platelets were then manually counted using a Neubauer chamber. Student's *t*-test was used to compare the differences between the number of platelets in peripheral blood and PRP samples ($p < 0.05$). In addition, PRP and peripheral blood smears were stained to see platelets' morphology. **Results:** All surgical procedures were well tolerated by the animals and they

were healthy during the entire experimental period. PRP samples showed higher significantly platelet concentrations than peripheral blood samples (2,677,583 and 683,680 respectively). **Conclusion:** Within the limits of this study, it can be concluded that the method used produced autologous PRP with appropriated platelet quantity and quality, in rats.

Introduction

Reconstruction of facial skeletal hard tissue defects is a field in markedly evolution. The focus of researches on healing in the 1980s was the collection, handling, and transplant of bone-competent cells while in 21st century, the focus has been the use of growth factors capable of stimulating and supporting those cells [4].

Researchers on the field of bucomaxillofacial surgery has continuously searched for improving bone grafting techniques and tried to find ways to obtain the regeneration of the existing defects through a higher bone density and as quickly as possible [25]. In 1998, Marx *et al.* [21] proposed the local use of platelet-rich plasma (PRP) to accelerate autologous bone grafting maturation. According to these authors, autologous bone grafts with PRP healed more quickly and presented a greater bone density than grafts without PRP.

Dentistry study on PRP has been characterized by a peculiar scientific path, firstly initiating on humans [3, 18, 21, 27] then, followed by animal models [1, 8, 12, 32], and now, by *in vitro* studies [13, 16, 17, 30]. These later, mainly, have demonstrated that controversial outcomes of the *in vivo* studies had possibly occurred due to the use of inappropriate techniques for PRP preparation. Indeed, several fundamental factors must be considered during PRP preparation to assure its quality, and consequently, its biological effect.

PRP acts by accelerating the process of tissue healing through releasing growth factors inside platelet α -granules [21]. Therefore, qualitative and/or quantitative alterations in platelets may potentially affect PRP regenerative capacity. Therefore, the choice of the anticoagulant and coagulant, rotation force and number of centrifugations, time elapsed between the sample activation and its clinical use, and the method of blood collection are some factors affecting PRP biological effect [6, 7, 20].

The necessity of studies on the assessment of PRP biological effects with standardized methodologies was emphasized by Grageda (2004)

[10]. This author highlighted that additionally to the careful observation of all technical details during PRP preparation, the selection of an adequate animal model is important.

The aim of this study was to develop a standardized protocol for autologous PRP in rats.

Material and methods

Experimental model

Twenty-four male rates (*Rattus norvegicus*, albino, Wistar), aged between 5 and 6 months, weighing between 450 and 500 g (Sao Paulo State University, Vivarium of the School of Dentistry of Araçatuba) were used. The animals were kept in an environment with 12 hour cycles of light per day and temperature between 22 and 24°C. The experimental protocol was approved by the Ethical Committee in Animal Experimentation of the Sao Paulo State University, *Campus* of Araçatuba. During all the experimental time period the animals ate selected solid food and water *ad libitum*.

PRP preparation

To execute the experimental procedures, the rats were anesthetized through intramuscular injection of xylazine (6 mg/kg) and ketamine (70 mg/kg). The animals were submitted to cannulation via jugular vein, by adapting the technique of Harms and Ojeda (1974) [11]. By using a 5 ml disposable syringe containing 0.35 ml of 10% sodium citrate, 3.15 ml of each animal blood was collected (figure 1). The blood was kept in 5 ml silicone vacuum tubes (Vacuum II®, Vitfend Corporation Indústria e Comércio Ltda., Itupeva, SP, Brazil). The same blood amount of each animal was immediately replaced through the injection of sterile saline. The canula placed in the jugular vein was removed and a hemostatic solution was locally applied (figure 2A). The tissues were repositioned and sutured (figure 2B).

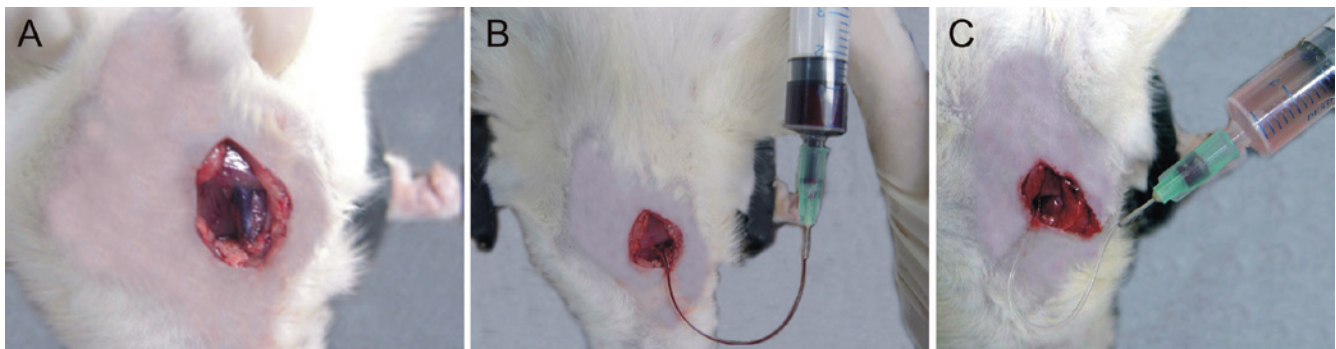


Figure 1 - (A) Jugular vein exposure to blood collection; (B) cannulation of jugular vein and collection of 3.5 ml of blood; (C) replacement of the blood volume collected by saline injection

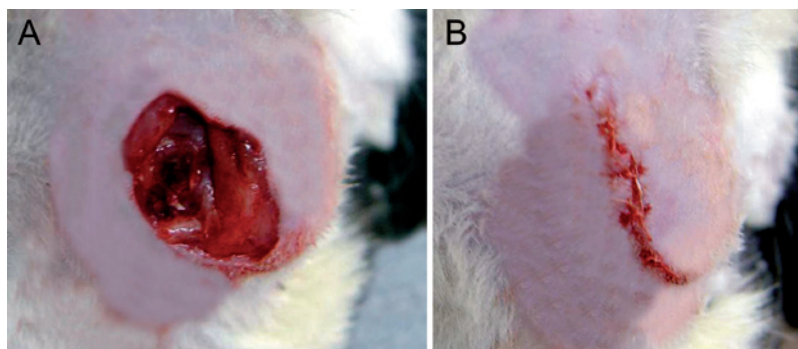


Figure 2 - (A) hemostatic solution applied in jugular vein after canula removal; (B) tissues repositioned and sutured

PRP preparation was carried out by adapting the protocol proposed by Sonnleitner *et al.* (2000) [28], using a refrigerated laboratorial centrifuge (Beckman J-6M Induction Drive Centrifuge, Beckman Instruments Inc., Palo Alto, CA, EUA) and a vertical laminar flow cabinet (Veco®, Veco do Brasil Indústria e Comércio de Equipamentos Ltda., Campinas, SP, Brazil) for manipulating the biological samples. The collected blood was firstly centrifuged at 160 G, for 20 minutes, at environmental temperature (22°C). Then, a red lower fraction (red cell component) and an upper straw-yellow turbid fraction (serum component) were observed. A point was marked at 1.4 mm below the line dividing the two fractions. All the content above this point was pipetted and transferred to other 5 ml vacuum tube (figure 3), in which a line corresponding to 0.35 ml was drawn from the tube's bottom [24]. The sample was then submitted to a new centrifugation at 400 G, for 15 minutes, resulting in two components: one above the line drawn on the tube (platelet-poor plasma

- PPP) and other below the line (PRP) (figure 4) [24]. Similar amounts of PRP and PPP (0.35 ml) were pipetted and transferred to different sterile dappen dishes (figure 5). Following, they were activated by 0.05 ml of 10% calcium chloride solution (ScienceLab.com Inc., Houston, TX, EUA) to each 1 ml of PRP or PPP.

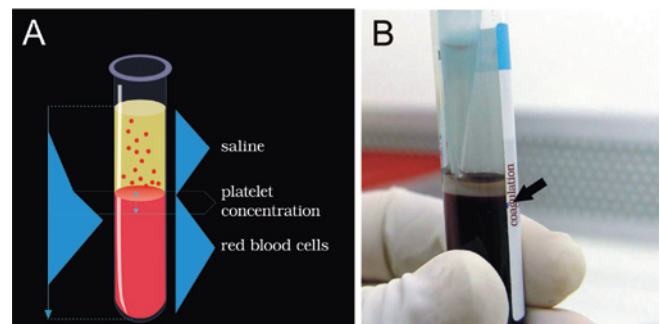


Figure 3 - (A) Schematization of the obtained result after the first centrifugation; (B) pipetting of the content localized above the marked line (arrow) performed in the vacuum tube after the first centrifugation

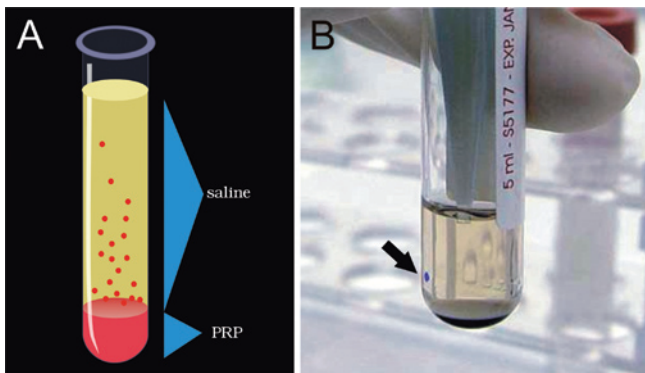


Figure 4 - (A) Schematization of the obtained result after the second centrifugation; (B) Result obtained after the second centrifugation. The marked line on the tube (arrow) separates PPP from PRP

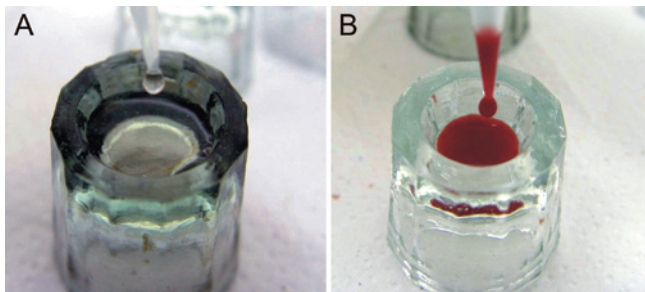


Figure 5 - (A) PPP and (B) PRP separated and ready to be activated

Platelet counting

The platelets within animal's peripheral blood and PRP samples were counted manually in a Neubauer chamber, through binocular optical microscope, at 40X magnification objective lens. For this purpose, each blood sample was separated, diluted and homogenized in Brecher liquid. Moreover, peripheral blood and PRP sample smears were performed and stained with quick panoptic (LB, LaborClin, Pinhais, PR, Brazil).

Statistical analysis

The significance level of the differences between the platelet amount present in peripheral blood and PRP samples was determined through Student's *t* test ($p < 0.05$).

Results

Clinical follow-up

All surgical procedures were well tolerated by the animals and they were healthy during the entire experimental period. Postoperative period was uneventful.

Study on platelet counting

Platelets presented their normal morphology. PRP smears exhibited a greater platelet amount than peripheral blood smears. Mean platelet amount within animal's peripheral blood was $683,680 \pm 186,229 \times 10^3$ platelets/ μl , while PRP samples presented a mean of $2,677,583 \pm 1,201,418 \times 10^3$ platelets/ μl (figure 6). Therefore, mean PRP platelet amount was about four times greater than that observed within peripheral blood samples.

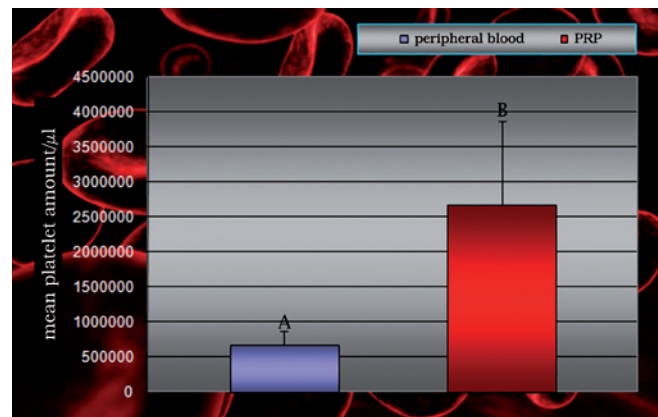


Figure 6 - Mean platelet amount/ μl and standard deviations of peripheral blood and PRP samples. Different letters indicate statistically significant differences between groups ($p < 0.05$)

Discussion and conclusion

The use of inappropriate experimental models and the lack of standardization of a PRP preparation protocol make difficult the establishment of definitive conclusions on its real biological effect. The aim of this study was to develop a standardized PRP preparation protocol in an experimental model of easy use.

This study was planned taking into account the amount and quality of the platelets within PRP samples. Concerning to the platelet amount, the protocol of double centrifugation used in this study, as recommended by Marx (2001) [19], enabled a mean percentage increase of about 390% in PRP platelet amount in comparison with peripheral blood platelet amount. According to Marx (2004) [20], this platelet concentration within PRP would be considered as therapeutical, and capable of hard and soft tissue regeneration. Animal studies demonstrated that the use of therapeutic PRP samples accelerated bone healing [14, 31].

Regarding to platelet amount, PRP samples smears showed that the used PRP preparation protocol did not promote any alteration in platelet

morphology. According to Marx *et al.* (2004) [20], any damage to platelet membrane during PRP preparation will result in secretion of growth factors in a non bioactive state, which would lead to unfavorable clinical outcomes. To assure concentrated platelets' quality, it is necessary to consider the anticoagulant and the velocity used for centrifugation, among other factors. We employed sodium citrate as anticoagulant. Sodium citrate does not alter the platelets' membrane, allowing that the sample's anticoagulated state be changed to a coagulated state by adding calcium chloride solution [2, 15]. Also, platelets' activation during blood collection procedures with sodium citrate is smaller than in samples collected with EDTA [7]. Concerning to centrifugation velocity, the maximum force used in this present study was 400 G. This centrifugation velocity does not lead to early platelets' activation during PRP preparation, which would happen when greater forces are employed, resulting in growth factors loss within supernatant plasma [6].

At PRP clinical application moment, it is necessary that the anticoagulated sample be changed to a coagulated state. The activator used for coagulating PRP may interfere in growth factor sequence of releasing and, consequently, in the tissue healing process. Our study used only 10% calcium chloride solution for PRP activation. A positive effect of PRP activated by only this solution was also demonstrated by previous studies that evaluated either the healing of tooth extraction sites [2] or osteoblasts proliferation *in vitro* [9]. Additionally, it is important highlighting that PRP activation by only calcium chloride preserves its autologous nature, avoiding the use of bovine thrombin and its risks of coagulopathy development [5, 29, 15]. In studies conducted by our team, PRP activation only by calcium chloride potentializes the healing of critical-size defects at rat calvaria [22, 23].

In addition to all caution regarding to the possible factors compromising PRP biological effect, it is important to consider the experimental model used. According to Marx (2004) [20], some studies evaluate homogenous PRP samples and, therefore, the observed biological responses could have been influenced by the occurrence of immunological reactions. In this present study, it was possible to obtain autologous PRP samples, although the rat is a small animal and has a reduced blood volume. Among the advantages presented by this experimental model animal, the low cost and possibility of using in large amount are emphasized [26].

Every study should involve, fundamentally, the production of a high quality PRP for then evaluate

the biological effects produced by it [20]. Within the limits of this study, it can be concluded that the used method enabled the production of an autologous PRP with appropriate quantity and quality of platelets, in rats.

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