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International Clinical Pathology Journal

Research Article

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Standardization and modification techniques of platelet-rich plasma (PRP) preparation in rabbit

Abstract

Background: Platelet-rich plasma (PRP) is used in several different fields of surgery to enhance natural healing by increasing concentrations of autologous platelets. However, there are controversial arguments in the literature considering the potential benefits of PRP, due to the deficiency of optimized and standardized preparation protocols.

Aim: The aim of the present study is to standardize a method of autologous PRP preparation in rabbits, and also compare this methodology with our new modified method.

Methods: 10 male New Zealand white rabbits were used in this study. After general anesthesia, 10ml of blood was collected from each animal via jugular vein, then the blood was divided into two equal amounts of 5ml. Blood samples were divided into two groups, and two different methods were implemented to prepare PRP. In group one, standardized technique with double centrifugation and in group two, our proposed modified protocol were used to prepare PRP with more than double centrifugation. Platelet count in PRP and whole blood samples with Neubauer slide.

Results: The amount of platelet in PRP samples was almost four times greater than the platelet amount in peripheral blood samples. More precisely, the average whole blood platelet count was 496, 073±51,273/µl. The average of platelet in PRP samples in group one and two were 1,877,157±604,783/µl and 2,086,945±856,423/µl respectively, which were significantly higher than whole blood.

Conclusion: It can be concluded that both of the methods used for producing autologous PRP show adequate platelet quantity and quality for rabbits.

Keywords: platelet rich plasma, platelet count, modification method, animal models

Volume 1 Issue 2 - 2015

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Received: June 15, 2015 | **Published:** September 02, 2015

Introduction

Platelet-rich plasma (PRP), a platelet concentrate made of autologous blood in a small volume of plasma.[1,2] PRP contains 4% RBCs, 95% platelets and 1% WBCs.[3,4] In reconstructive surgery, slow healing rate or low quality rate of tendons and ligaments as well as massive bone defects are major challenges for the patients.[5] Autologous bone grafting is regarded as the gold standard for filling bone defects. However, this method faces several problems such as donor-site morbidity and limited amount of donor bone.[6,7] To overcome this challenge, bone regeneration by means of tissue engineering has attracted increasing interest. The notion of tissue engineering is based on three basic parts: scaffolds, cells, and growth factors.[8] PRP has greatly attracted attention in scientific society and has been increasingly used for a variety of clinical applications such as orthopedics, sports medicine, dentistry, otolaryngology, neurosurgery, ophthalmology, urology, wound healing, cosmetic, cardiothoracic and maxillofacial surgery.[9] PRP therapy has gained broad popularity as a natural alternative to surgery since early 2009, because it is safe, nonsurgical and biological treatment.[3] Since PRP is an autologous blood product, it carries no risk of transmissible diseases.[8,10] Moreover, PRP can easily be obtained on the day of surgery by means of only two centrifugation steps and simple equipment.

Naturally, after an injury that causes bleeding, platelets are activated and aggregated together near the scar to release their granules contain growth factors which eventually stimulate the inflammatory phase and healing process. As PRP is rich of platelets, it accelerates endothelial, epithelial, and epidermal regeneration, stimulates angiogenesis, increases collagen synthesis, furthers soft tissue healing, reduces dermal scarring, enhances the homeostatic response to injury, and turns the inhibition of wound healing caused by glucocorticoids.[10] Moreover, PRP can be added to small bony defects, or to larger defects in combination with grafting material, to increase the rate of bone maturation and improve bone density.[10] PRP, contains high concentrations of growth factors like;[11] platelet-derived growth factor (PDGF)-AA, -BB,-AB, platelet-derived angiogenesis factor (PDAF), fibroblast growth factor(FGF), transforming growth factor (TGF)-b1 and b-2, insulin like growth factor-1 (IGF-1), connective tissue growth factor(CTGF), platelet derived epidermal growth factor (PDEGF), and platelet factor-4 (PF-4), which these factors have found in α-granules of platelets.[12–14] Such growth factors affect the chemotaxis, differentiation, proliferation and synthetic activity of bone cells, which regulates physiological remodeling and fracture healing.[10] In addition to growth factors, PRP also releases several bioactive proteins[2,10] that are responsible for attracting macrophages, mesenchymal stem cells and osteoblasts that promote removal of degenerated tissues and also enhance tissue regeneration and healing.[10] Several simplified PRP preparation protocols have been proposed to facilitate its clinical application.[15–20]

Marx et al.[2] suggested that a double-centrifugation method is essential to truly concentrate platelets from autologous blood. Indeed, several fundamental factors must be taken into account while preparing PRP to assure its quality, and consequently, its biological efficacy. It should be noted that the type of protocol used for PRP preparation greatly affects quantitative and qualitative characteristics of the PRP.[2] Some factors such as method of blood collection, type of anticoagulant, relative centrifugal force, method and time of centrifugation and time needed for platelet activation may affect biological quality and efficacy of PRP samples.[21,22] The aim of the present study was to standardize and modify a new technique for PRP preparation in rabbits. The quantity and quality of platelets in PRP samples of the conventional method, double centrifugation, in this study will be compared with our proposed modified method regarding PRP preparation.

Material and methods

Experimental model

In the present study, ten male New Zealand white rabbits, aged between 9 to 12months, weighing between 2.5-3kg were used, which all of them were primarily evaluated in term of CBC parameters. Our study on the experimental animals was conducted in total accordance with the internationally accepted principles for laboratory animal.

PRP preparation

In the first step, rabbits were anesthetized using intramuscular injection of xylazine (3mg/kg) and ketamine (25mg/kg). Blood samples were collected from the jugular vein using a 10ml syringe. Blood samples were transferred to anticoagulant tubes containing 0.35ml of 10% sodium citrate. After that, the blood sample of each animal was divided into two equal amounts of 5ml. Approximately 1ml of whole blood was separated for baseline whole blood analysis. Then, in the standard group blood was initially centrifuged at 160G, for 10minutes and in the modification group it was first centrifuged at 160G, for 6 minutes at room temperature.

After the first centrifugation, two fractions were observed in each sample: a red lower fraction that consists of packed red blood cells and an upper straw-yellow fraction that contains plasma component (Figure 1). The upper surface of packed red blood cells which called Buffy coat is rich in platelets and leukocytes. Plasma and buff coat were transferred to a new sterile centrifuge tubes. The retained component of blood samples of modification group were centrifuged for two times more at 160G for 2minutes to obtain more concentrated platelets. Then, in both groups the plasma and Buffy coat was centrifuged for the second round at 400G, for 15minutes (Figure 2), so two layers were eventually appeared: the upper two thirds of the sample was designated as PPP and was discarded, on the other hand, the lower third was PRP. After that, the platelets were activated by 0.05ml of 10% calcium chloride solution to each 1ml of PRP (Figure 3). It should be noted, in order to accelerate the preparation of PRP gel, it is essential, to put the calcium chloride solution in water bath at 37°C for 10minutes, just before adding the solution to PRP. From each prepared PRP, 200µL samples were taken to calculate platelet counts with Neubauer chamber (Figure 4). Also, smears were done on all samples in order to see the morphology of the platelets (Figure 5).

Statistical analysis

To compare the difference between the number of platelets in peripheral blood with those of groups one and two, student's T-test measure was used (P value<0/05).

Figure 1 The blood sample after first centrifugation.

Figure 2 The mixture of PPP and PRP before discarding the PPP.

Figure 3 PRP gel.

Citation: Maghsoudi O, Abarkar M, Anvar SA. Standardization and modification techniques of platelet-rich plasma (PRP) preparation in rabbit. *Int Clin Pathol J.* 2015;1(2):29–33. DOI: 10.15406/icpjl.2015.01.00007

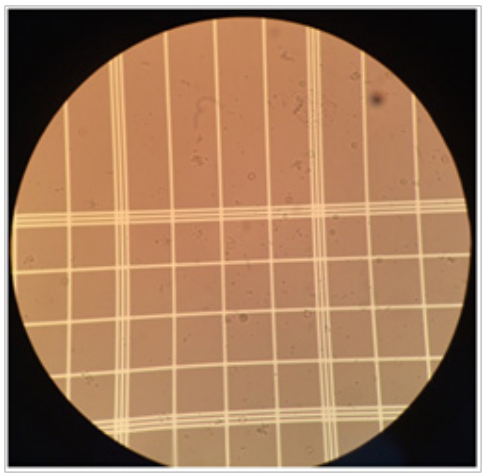

Figure 4 Using Neubauer chamber to calculate the number of platelets.

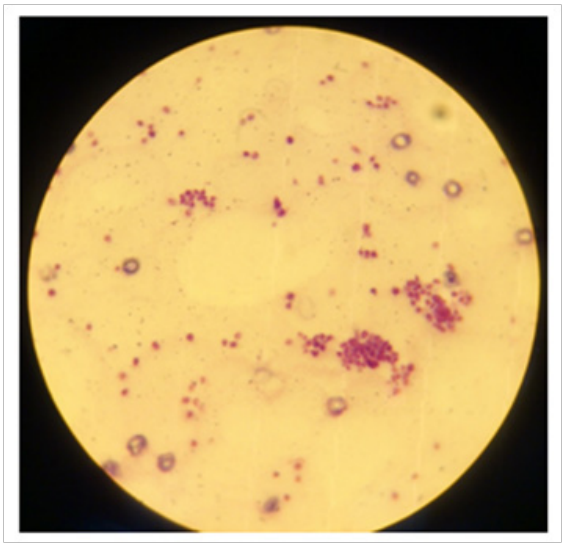

Figure 5 The PRP smear for demonstrating the normal morphology and aggregation of platelets.

Results

The PRP smears of both groups one and two showed significant higher concentrations of platelets compared to whole blood smears, and platelets demonstrated their original normal morphology and activity. However, the modified protocol caused few changes in platelet morphology. In both groups, a few numerous red blood cells and lymphocytes were seen around platelet aggregates. The average whole blood platelet count was 496,073±62,739/µl and the average of platelet in PRP samples in group one and two were 1,877,157±588,063/µl and 2,086,945±611,026/µl respectively, which were significantly higher than whole blood. It should be noted that number of platelets was counted with Neubauer chamber using 40x magnifications. The amount of platelet in PRP samples was almost four times greater than that of the peripheral blood samples. More precisely, platelet concentrations of PRP samples in the standard and the modification groups were 380%±10% and 420%±10% respectively which were significantly higher than platelet concentrations of the peripheral blood samples; whereas, there was no significant difference between the number of platelets in the standard group and the modification group.

Discussion

As stated by Marx et al.,[2] platelets damaged by the protocol used for preparing PRP will release premature growth factors. Consequently, the resulting outcome may be not satisfying. According to the two different protocols, the effects of the protocol to prepare PRP on the quality and quantity of platelets in PRP samples were evaluated in the present study through platelet smear analysis. Smears were officially accepted to analyze many parameters that are revelatory to platelet function, such as changes in morphology, size, staining specifications, degree of activation and clump formation.[23] A double-centrifugation technique should be applied to truly concentrate platelets from autologous blood.[24] On the other hand, low platelet concentration is caused by a single spin,[15] that it would produce a mixture of PRP and PPP which will not be the true PRP.[25,18]

Nagata et al.[17] attempted to determine the effectiveness of two protocols for PRP preparation in rabbits and argued that double-centrifugation cause higher platelets concentration in PRP. In our knowledge, several fundamental factors influence on both quantity and quality of PRP including the force of gravity (G), the type of the anticoagulant used, the type of clotting and activating agent, the number of platelets in donor's blood and PRP, and results of clinical applications.[17] In addition to the number of centrifugations the force of gravity (G) used in the centrifugation process is one of the important factors.[18] An increase in G may cause higher platelet concentrations.[26] However, it should be noticed that excessive increase the G in the first centrifugation may causes the number of platelets in the Buffy coat to get considerably close to red blood cells. Due to this phenomenon the quantity and efficacy of PRP reduce since during pipe ting the Buffy coat by sampler, a large number of red blood cells will be pipette, or a portion of platelets will not be pipette, also increasing the G for the second centrifugation may result in prematurely activate the platelets.[21] Efeoglu et al.[22] stated a standardized protocol for PRP preparation in rabbits; however the proposed protocol did not consider the effects of G as an important factor in the second centrifugation. The type of anticoagulant used during the process of preparing PRP can activate the premature platelets.[27] EDTA is potentially more damaging than citrate in the preparation of PRP gel since platelets appeared damaged by light microscopy.[22,28] In a flow cytometric analysis of platelet activation by Nishioka et al (2002), it was shown that sodium citrate and heparin have activated the minimum platelets, whereas EDTA causes significantly higher platelet activation but there is not any significant occurrence of platelet aggregation and release of intracellular constituents. Current knowledge suggests that citrate should be the anticoagulant of choice to obtain PRP for in vivo applications.[29–34]

As the growth factors have short half-lives in order to obtain the greatest effectiveness they should be activated just prior to injection.[3] Also, variable half-lives of growth factors cause effectiveness of the PRP to be dependent on how quickly it is used after activation. Thrombin and $CaCl_2$ are used as clotting agents to activate the platelets of PRP.[3,10] It has been shown that thrombin can result in rapid activation of platelets and ultimately a mass release of growth factors. As the result shows 70% of these growth factors are

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released within 10minutes and nearly 100% within 1hour. Also, the use of bovine thrombin has unfortunately been associated with the development of antibodies.[35,10] Whereas, platelet gel is formed by using $CaCl_2$, the growth factors release can be slowed and released over 7days.[35] According to results, in the ant coagulated state and placed on a sterile surgical table, PRP can be remained sterile and the concentrated platelets viable for up to 8hours.[6] It is not a valid idea that higher number of platelets necessarily accelerates tissue healing because an excessively concentrated PRP has negative effects on wound healing.[36,37] It is necessary that a "therapeutic PRP" show approximately one million platelets per micro liter in humans. Thus, in order to consider a PRP as "therapeutic PRP," it should have a platelet concentration of at least 300 to 400% greater than that of the whole blood.[6] Platelet concentrations of less than 10^6/µl was not reliable for enhancing wound healing.[38] Discarding the upper two thirds of the sample after second centrifugation may result in more highly concentrated platelets of PRP.[39] As suggested by Efeoglu et al.[22] the platelet concentration may be higher in a small volume of plasma than in a greater volume of plasma. PRP has acidic pH.[2] and also is rich in leukocyte. Furthermore, PRP results in chemotaxis and migration of cells such as WBCs. Accordingly, it might be claimed that PRP actually inhibits bacterial growth.

Conclusion

Whereas the average of platelets in PRP samples of both groups was approximately four times more than the average of platelets in whole blood, there was no significant difference between the number of platelets in the standard group and the modification group. Therefore it can be concluded that both of the methods used for producing autologous PRP show adequate platelet quantity and quality for rabbits. However, the modified protocol caused partly changes in platelet morphology.

Acknowledgements

None.

Conflict of interest

The author declares no conflict of interest.

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Citation: Maghsoudi O, Abarkar M, Anvar SA. Standardization and modification techniques of platelet-rich plasma (PRP) preparation in rabbit. *Int Clin Pathol J*. 2015;1(2):29–33. DOI: 10.15406/icpjl.2015.01.00007