



Full Length Research Paper

Standardization of the method to obtain therapeutic-quality platelet-rich plasma

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Platelet-rich plasma (PRP) is a reliable source for obtaining cells to regenerate tissues, with ease of availability in order to implement and standardize the ideal methodology in centrifugation strength and time for obtaining therapeutic-quality PRP, allowing its application to provide better and rapid recovery of muscular injuries, tendinitis, bone and ligament lesions. To evaluate PRP therapy, 150 patients with muscular lesions, tendinitis, shoulder, knee, ankle, hand and elbow injuries were treated. On application of PRP, we obtained 100% clinically significant symptomatic improvement in all 150 patients treated, who had musculoskeletal and ligament injuries, with a marked reduction of pain and inflammation. We concluded that the ideal concentration for obtaining PRP is at 1000 rpm with a time of 5 min; in addition, under these conditions the plasma lacks leukocytes and erythrocytes. The results were reproducible because the experiment was repeated at two institutions under the same conditions and similar results were obtained. The regeneration obtained in the affected patients is due to the fact that growth factors were released from the activated platelets; these initiate and modulate cicatrization in the tissues, which is a recent innovation to promote cicatrization, accelerating the power of tissue regeneration, with a platelet concentrate suspended in plasma.

Keywords: Growth factors, platelet activation, application, tissue regeneration, therapeutic quality.

INTRODUCTION

Platelet-rich plasma (PRP) is a reliable source for obtaining cells to regenerate tissues, with ease of availability. In short term clinical practice, it is utilized to concentrate growth factor-rich plasma (GFRP) by up to 388% above values found in normal plasma, for later application

in tissues, in a search to enhance the osteo-induction biological cascade. The pharmaceutical way in which PRP is utilized clinically is obtained by means of its gelling on adding thrombin and CaCl₂ to it. PRP gel is a compound of fibrinogen and activated platelets (by the addition of

thrombin), which determines the release of a cascade of the growth factors (GF) of platelet granules within a fibrin mesh (Camargo et al., 2012).

PRP is an innocuous, 100%-compatible material with minimal possibility of rejection. It acts as a bio-stimulator and as a bio-mediator against cellular aging; it restores and repairs tissue with properties indistinguishable from the original tissue and is easy to apply through minimally invasive therapeutic methods. Additionally, it allows the conservation of the harmony and physiology of the patient's own tissue structure that was unable to be improved by synthetic substances (De Bouille, 2007). Given its unsubstitutable source of benefits for humans, its use has been extended beyond curative medicine for further use in esthetic, cosmetic and sports medicine etc., in which the donor and the recipient is the patient him/herself, thus constituting an autologous material (Rosenthal et al., 2011). PRP is a new technology that is centered on improving the curative response after an injury. PRP is obtained from the peripheral blood of patients and is centrifuged to obtain a sample that is highly concentrated in platelets; the blood sample is submitted to degranulation to release GF with curative properties (Lopez-Vidriero et al., 2010).

Platelet activation in response to tissue and vascular damage causes the formation of a platelet tamponade and a blood clot whose functions are the consequence of hemostasis and the secretion of biologically active proteins involved in the tissue regeneration process. These proteins, the GF proteins, are secreted basically by the platelet structure, but not exclusively, and are also able to be produced by fibroblasts. PRP contains a small number of leukocyte cells that also contribute to cell defense by means of synthesis of interleukins that intervene in the unspecific immune response, contains cytokines, thrombin and other GF that are implicated in the cicatrization of wounds, possess biological properties and inherent adhesives (Rodriguez-Flores et al., 2012).

The concentrated preparation (the concentrate) is at present, injected into the patients at the site of injury. This can be effected intralesionally, intra-articularly or surrounding the involved tissue (Lopez-Vidriero et al., 2010). This technique provides novel concepts because it was thought that platelets only acted in tissue hemostatic and currently it is known that platelets can also exert an influence on wound repair, on vascular ulcer implantology, with good clinical evolution and promising results in the fields of sports medicine and arthroscopy (Jovani, 2009). Reconstruction of the anterior cruciate ligament in particular has demonstrated improved maturation of the autograft, diminution of donor-zone mortality and the pain threshold, in addition to improved allograft incorporation. Due to acceleration of the biological integration of the graft through the use of PRP, patients can initiate more intensive rehabilitation programs and can return to sports faster. Due to its autologous origin, its

easy preparation and excellent safety profile, PRP has opened up another therapeutic portal (Freymiller and Aghabo, 2004). The hematological products contribute to saving millions of lives annually, spectacularly improving the life quality and expectancy with potentially fatal disorders and making it possible to carry out complex medical and surgical procedures. Numerous clinical applications have been described for PRP, for ulcers, maxillofacial surgery and spinal surgery (Rodriguez-Flores et al., 2012). Applications in orthopedics include treatment for tendon and bone injuries, joint replacements, fractures in patients with diabetes, wound cicatrization etc.

PRP allows for better and more rapid recovery from muscle, tendon, bone and ligament injuries because it has GF α and GF β , which impede the proliferation, migration, differentiation and regeneration of the damaged tissue, favoring the activation of molecular processes in diapausing cells and resulting in angiogenesis, mitogenesis, chemotaxis and the synthesis of collagen and the extracellular matrix (ECM). The ruptures of ligaments involve serious injuries that affect different population groups, such as persons engaging in sports; thus, the rupture of ligaments is more frequent in young adults. Sports that entertain a greater risk of this injury type are those that imply change of direction and of rhythm (acceleration), such as skiing, basketball, or tennis. Other risk factors are muscular weakness, discoordination or lack of joint flexibility.

According to the World Health Organization (WHO, 2009), hematology services worldwide face the immense challenge of obtaining supplies of blood products sufficient for attending to the needs of patients and at the same time guaranteeing their quality and safety in the face of old and new threats to public health. Only if great attention is paid to the availability, safety and quality of blood products can the health-related objectives of the development of the millennium be reached. Therefore, the WHO has cited the demand to guarantee the availability, safety and quality of all blood-derived products; thus, developed countries have put into practice policies, strategies and procedures that have made possible widespread access to a complete gamma of safe blood products.

Consequently, the need arises to implement in the clinical laboratory an efficient methodology for obtaining PRP because the strength of centrifugation as well as the time of same exert an influence on the variability in the platelet concentrate and consequently on clinical efficacy, in that these factors vary in the different centrifuges utilized in the different laboratories on applying mechanical force incorrectly, with which the attributes of the platelets can be harmed. We in Mexico comply with the demand of the WHO for regimented surveillance of the quality of blood products. Our aim was to implement and standardize the optimal method in centrifugation strength and

No. 23
 Date 21/05/2011
 Time 10:45
 Mode WB

WBC ! 0.0x10³/μL
 RBC ! 0.01x10⁶/μL
 HGB ! 0.0g/dL
 HCT ! 0.1%
 MCV ---.-fL
 MCH ---.-Pg
 MCHC ---.-g/dL
 PLT PL* 33x10³/μL

WBC

LYM% ---.-%
 MXD% ---.-%
 NEUT% ---.-%
 LYM# ---.-x10³/μL
 MXD# ---.-x10³/μL
 NEUT# ---.-x10³/μL

RBC

RDW_SD ---.-fL
 RDW_LCV ---.-%

PLT

MPV PL* 5.7fL

time for obtaining therapeutic-quality PRP, allowing with its application better and more rapid recovery of muscle, tendon, bone and ligament injuries.

METHODOLOGY

To evaluate therapy with PRP, we treated 150 patients with muscular and tendon lesions in shoulder, knee, hand and elbow, to which PRP were applied as part of the protocol utilized in surgery, as well as part of the patients' treatment. We proceeded to carry out asepsis in the antecubital region near the puncture site with ethyl alcohol at 70%; this is mainly to avoid any type of contamination at the time of sample-taking. We took 6 to 8 vacuum tubes of peripheral blood with sodium citrate 0.109 M. Later, the tubes were placed in automated Sismex KX-21 Hematology analyzer (ROCHE®) equipment and we centrifuged these at 3200, 3000, 2800, 1800 and 1000 rpm for 5 and 10 min. We performed the preparation of PRP for its application at the injury site, mixing 0.5 ml of calcium gluconate at 10% with 1.0 ml de PRP for each cm³ of the lesion's surface. For example, in the meniscus region, infiltration is carried out on the medial meniscus in the medial portal region and in the mid-collateral and meniscus capsule zone, with a 22 x 32 and a 10 to 20 ml needle (black needle) syringe. Infiltration with local anesthesia, ropivacaine, is performed in the area of the injury; with the calcium gluconate-PRP mixture applied in the previously anesthetized region. Informed consent was obtained from the 150 patients.

RESULTS

The experiment was repeated five times at different speeds and at times of 5 and 10 min. From the respective repetitions, the mean was obtained, the result of which was that at 10 min of centrifugation at 3200, 3000, 2800, 1800, and 1000 rpm, concentrates of 0, 0, 0, 2 and 33 platelets/10³, respectively were obtained. At a time of 5 min at 3200, 3000, 2800, 1800 and 1000 rpm, concentrates of 20, 50, 135, 186 and 223 platelets/10³ were obtained, respectively (Figures 1 and 2).

It was determined that at 1000 rpm with a time of 5 min, an ideal concentrate was obtained for PRP; the results showed the total absence of leukocytes, erythrocytes and 223.000 platelets/mm³; thus, speed and time are adequate parameters for obtaining PRP. The results were reproducible because the experiment was repeated at two institutions under the same conditions and similar results were obtained. Later, once the plasma fraction was separated under aseptic conditions, we proceeded to application of the PRP, within a lapse of 5 to 10 min. On evaluating patients after application of the PRP, 100% significant symptomatic clinical improvement was obtained in the 150 treated patients with musculoskeletal and ligament injuries, the patients referring with less pain and observing tissue regeneration, as well as little or null inflammation.

DISCUSSION

Administration of PRP is always safe because patients

Figure 1. Hemogram at 1800 rpm for 10 min, the results show total absence of leukocytes, erythrocytes and 33,000 platelets/μL, therefore, this speed and time parameters are inconvenient for the preparation of a PRP.

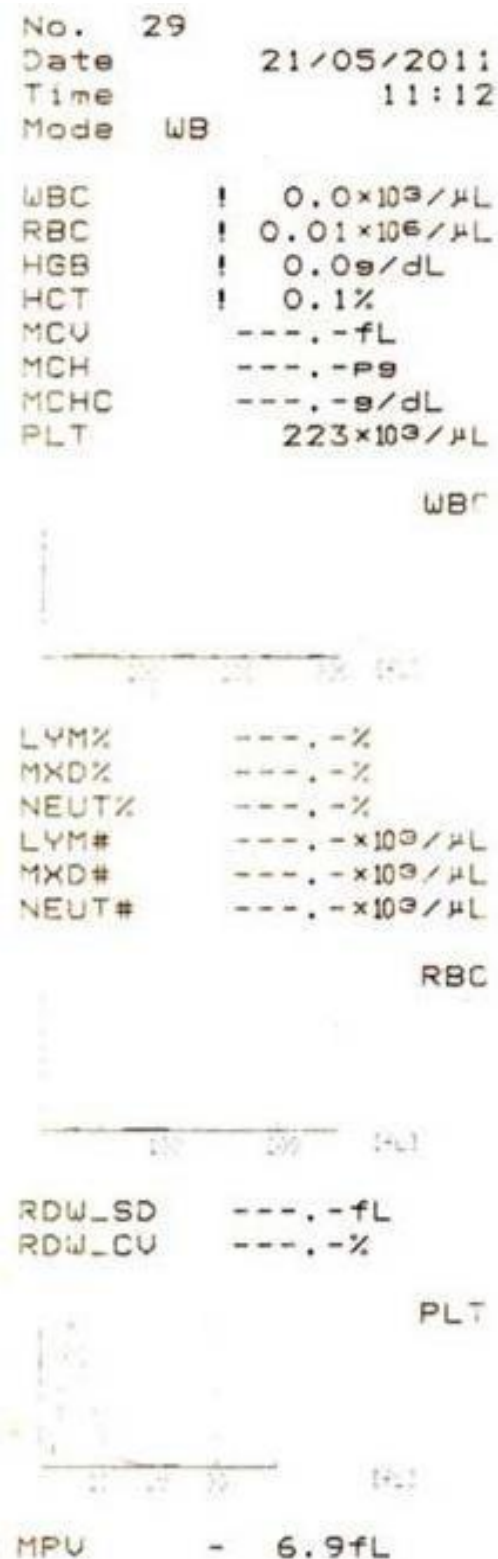


Figure 2. Hemogram at 1000 rpm for 5 min. The results show total absence of leukocytes, erythrocytes and 223,000 platelets/ μ L. Therefore, this speed and time parameters are suitable for the preparation of a PRP.

are not at risk of the transmission of diseases such as human immunodeficiency virus (HIV), hepatitis, among others; similarly, it can be employed in persons with resistance to being transfused without any problem (Rivera-Tocancipa and Rivera-Ortiz, 2011). In this respect, it is reported that PRP does not cause any negative effect on patients because these are autologous preparations. The procedure consists of processing the patient's own blood to obtain the PRP. However, there are counter indications in patients who suffer from coagulation disorders (thrombocytopenia, hypofibrinogenia, or who are receiving anticoagulant therapy). This technique will reduce recovery time by one half in patients with muscle lesions, tendon lesions or bone fractures (Schwartz et al, 2011; Garcia-Gimenez and Gonzalez-Nicolas, 2005).

With reference to the latter, the use of platelet preparations is subjected to numerous variables, which will be indicative of their clinical efficiency. Some of these variables are: platelet concentrate, technique for obtaining the platelets, concentration of secreted protein, manipulation and clinical application. In order to obtain optimal results requires concentrating the platelets between 3 and 5 times above the baseline value. Bearing in mind that a normal individual has in the area of 200,000 platelets/ μ l, an optimal centrifuge would be 1,000,000 platelets/ μ l; in our results, we were able to obtain 223,000 platelets/ mm^3 of plasma, maintaining stability. In this respect, it has been demonstrated that higher concentrations do not increase the effect with regard to wound cicatrization (Schwartz et al., 2011). Five percent of PRP is sufficient in a clot to promote the cicatrization (Hernandez and Rossani, 2007).

In the majority of studies carried out to date with PRP, selected protocols under empirical bases have been utilized and for this, PRP has been obtained by the procedures described but with great variables, for example, centrifugal strength ranges from 1000 to 6000 rpm and time of centrifugation from 2 to 15 and 20 min. But in practice, the number of leukocytes has not been controlled in the preparations, which impedes discerning between the usefulness of one or another preparation. In this investigation, we opted for utilizing 1000, 1800, 2800 and 3000 rpm and solely times of 5 and 10 min.

Controversially, it is mentioned that PRP contamination by leukocytes exerts an influence on the final number of GF and that greater risk contamination is obtained in preparations obtained by means of conventional centrifuges. However, in the present research, we were able to prove that it is indeed possible to utilize conventional centrifuges to obtain PRP, although the process requires pulchritude in the manipulation. There has been commentary on the fact that collecting PRP with a pipette renders the technique very imprecise because nearly non-intentionally, red blood cells and leukocytes are included (Jovani, 2009); this datum is in agreement with

another study, although it is difficult to maintain sterility, it is not impossible, thus the unavoidability of protocolizing the procedure and being careful to procure correct obtaining of PRP (Rodríguez-Flores et al., 2012). In this sense, it is reported that the platelets can be damaged, thus becoming activated above all if the same sample is centrifuged various times (Jovani, 2009).

It was possible to prove the latter with the results of this project, in which it was determined that speed and time directly influence the platelet concentration in citrated plasma, because at a greater time and speed, the concentration of platelets diminishes. There are other methods, such as electrophoresis, but these require more time and adequate equipment. In this respect, analysis was performed in which regional anticoagulation with trisodium citrate proved to be safe and effective strategy that helps in reducing costs in patients requiring continuous kidney replacement therapy due to renal injury in pediatric intensive care (Fernandez et al., 2012).

It has been determined that the participants' gender and age do not exert an influence on platelet count, because no statistically significant difference was observed (Jovani, 2009), with which we are in agreement, because we did not find differences in terms of the patients and the final platelet count number.

In Hematology, the term PC is employed to denominate a concentration >1 ml/ μ l and it is synonymous with PRP (Lopez-Vidriero et al., 2010). It has been indicated that when anticoagulated blood is centrifuged, it forms three layers according to the density. The middle layer is formed of white globules and the upper layer, by the plasma. The plasma layer is divided into upper with platelet-poor plasma, middle with platelets and lower with PRP (Rodríguez-Flores et al., 2012), which can be proven on centrifuging the samples. In our research, we chose to use sodium citrate, as did Jovani (2009), who cites that sodium citrate is a salt that uptakes the calcium ions found in the blood and that neutralizes these, forming a chemical compound called a chelate, thus impeding coagulation of the blood. In addition, sodium citrate does not alter the platelet membrane receptors and will permit reversibility of the process on adding calcium in the form of CaCl_2 . Sodium citrate has been used as an anticoagulant by diverse authors (Freymiller and Aghabo, 2004; Schwartz et al., 2011; Hernandez and Rossani, 2007; Martínez-Gonzalez et al., 2002; Perez-Sierra, 2010; Sanchez-Perez and Diez-Quijano, 2008; Reyes et al., 2002; Restituto, 2010; Chen et al., 2009; Ruiz-Macarilla et al., 2012; Fierro-Serna et al., 2011).

However, additionally citric acid and dextrose (CAD) has been employed as an anticoagulant; this is the oldest and most utilized anticoagulant, but it diminishes the pH of plasmas and delays and interferes with the processes of aggregation and platelet secretion (Jovani, 2009; Alanis-Blancas et al., 2010; BIOMET, 2011). In another study, no anticoagulant was utilized due to the urgency of

its use in the operating room (Roby, 2011). It is noteworthy that although the anticoagulant is very important in obtaining PRP, there are some authors who fail to mention it (García-Giménez and González-Nicolas, 2005; Perez-Sierra, 2010; Saenz-Torres et al., 2007; González-Ossa and Ortiz-Orrego, 2004; Orozco-Delclos, 2007).

On performing an analysis on the rpm utilized in each research, great variability was found as follows: 1,200 rpm (Rosenthal et al., 2011); 1,300 rpm (González-Ossa and Ortiz-Orrego, 2004); 1,400 rpm (Martínez-González et al., 2002; Acosta and Potdevin, 2011; Beca et al., 2007); 1,500 rpm (Roby, 2011; Torres, 2006); 1,800 rpm (Freymiller and Aghabo, 2004; Reyes et al., 2002; Bedon-Rodríguez and Villota-González, 2012); 2,400 to 3,600 rpm (Hernández and Rossani, 2007); 3,000 rpm (Orozco-Delclos, 2007); 3,200 rpm (Beca et al., 2007; BIOMET, 2011); 3,650 rpm (Martínez-González et al., 2002); 5,400 to 5,600 rpm (Hernández and Rossani, 2007; Reyes et al., 2009), and 6000 rpm (Chen et al., 2009). With respect to time of centrifugation, the review was carried out on the time utilized as follows: 2 min (Perez-Sierra, 2010); 3 min (Hernández and Rossani, 2007); 7 min (Jovani, 2009; Perez-Sierra, 2010; Sanchez-Perez and Diez-Quijano, 2008; Acosta and Potdevin, 2011; Beca et al., 2007; Torres, 2006); 8 min (Freymiller and Aghabo, 2004; Schwartz et al., 2011; Reyes et al., 2002; Bedon-Rodríguez and Villota-González, 2012); 12 min (Ruiz-Macarilla et al., 2012); 15 min (BIOMET, 2011), and 20 min (González-Ossa and Ortiz-Orrego, 2004). In previously performed works, this research coincides with the time utilized of 5 min (Chen et al., 2009; Roby, 2011) and 10 min (Jovani, 2009; Saenz-Torres et al., 2007). The technique employed in this research was of only a sole centrifugation, which coincides with that applied by other authors (Jovani, 2009; Freymiller and Aghabo, 2004; García-Giménez and González-Nicolas, 2005; Sanchez-Perez and Diez-Quijano, 2008; Reyes et al., 2002; Restituto, 2010; Chen et al., 2009; Fierro-Serna et al., 2011; Alanis-Blancas et al., 2010; BIOMET, 2011; González-Ossa and Ortiz-Orrego, 2004; Orozco-Delclos, 2007). In addition, the double-centrifugation technique was utilized (Schwartz et al., 2011; Hernández and Rossani, 2007; Martínez-González et al., 2002; Perez-Sierra, 2010; Reyes et al., 2009; Acosta and Potdevin, 2011; Saenz-Torres et al., 2007; Beca et al., 2007). In this regard, the blood was centrifuged for 3 min at 2,400 to 3,600 rpm in two cycles and it was observed that in the first cycle, PRP is extracted and in the second, "platelet-exquisite" PRP is obtained (Hernández and Rossani, 2007).

On the other hand, on performing application of PRP, there was a satisfactory response in patients in 3 to 10 days; this fact is in agreement with Fierro-Serna and collaborators (2011), who found out that the use of growth factor-rich platelets (GFRP) can benefit the post-

operatory period of patients after the surgical removal of the lower third molars, with soft tissue regeneration and less pain and inflammation. In another experiment, cicatrization and gum retraction was observed 39 days after PRP treatment (Chen et al., 2009). In this work, PRP was applied within a lapse of 5 to 10 min, a lesser amount of time than that employed in other procedures, in which the transfer was 12 to 14 min, achieving overcorrection with PRP-enriched lipografts. As can be observed, in each research analyzed, this coincides with some authors in centrifugation time or speed, but none coincide in terms of the parameters standardized in the present investigation. It is timely to point out that a study was performed in which it is considered that the intrinsic factors such as race, gender and age influence the final concentration of cells and GF in pure PRP in horses (Giraldo et al., 2013).

Finally, the benefit was observed of GLRP in the closing of recurrent palatine fistula in the treatment of cleft lip and palate (Bedon-Rodriguez and Villota-Gonzalez, 2012). Also recently, the antibacterial effect was evaluated *in vitro* of pure PRP against microorganisms that colonize the oral cavity, finding activity in the following: *Enterococcus faecalis*; *Candida albicans*, *Streptococcus agalactiae* and *Streptococcus oralis* (Drago et al., 2013), in which it is suggested that platelet concentrates can be used against postoperative infections and that they would represent the missing link between osteoinductive and antimicrobial activity. Likewise, Sánchez and collaborators affirm that certain molecules implied in tissue repair, such as GF, stimulate cell receptors; thus, the biological response depends not only on the amount of GF, but also on the cell receptors of each patient (Sanchez, 2010), because GF are autogenous endomodulators of PRP that naturally mold the treated zone as a "zone memory code" (Hernandez and Rossani, 2007). With the present research, it is proposed that for obtaining PRP, the following should be considered: the type of anticoagulant utilized for preparing the PRP; the technique to employ and the time and speed of centrifugation; in addition, it is probable that PRP obtained with these results aids in improving therapeutic behavior in tissue regeneration.

Conclusion

Centrifugation speed and time exert an influence directly on the platelet concentration in citrated plasma; the best parameters for obtaining PRP in our experiment were at 1,000 rpm for 5 min. This procedure, which is standardized in research can be carried out in every clinical laboratory to obtain therapeutic-quality PRP. The experiment was conducted in different laboratories and with different equipment and no significant differences was found, but it is necessary for each laboratory to

determine the most adequate parameters for obtaining good quality PRP. On administering PRP, a better and rapid recovery was achieved for muscle, tendon, bone and ligament injuries because PRP contains GF which are tissue and tendon regenerators for application in Traumatology.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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