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Method to obtain platelet-rich plasma from rabbits (*Oryctolagus cuniculus*)[1]

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ABSTRACT.- Pazzini J.M., De Nardi A.B., Huppes R.R., Gering A.P., Ferreira M.G.P.A., Silveira C.P.B., Luzzi M.C. & Santos R. 2016. **Method to obtain platelet-rich plasma from rabbits (*Oryctolagus cuniculus*).** *Pesquisa Veterinária Brasileira 36(1):39-44.* Departamento de Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Campus Jaboticabal, Via de Acesso Paulo Donatto Castellane s/n, Jaboticabal, SP 14884.900, Brazil. E-mail: josipazzini@hotmail.com

Platelet-rich plasma (PRP) is a product easy and inxpesnsive, and stands out to for its growth factors in tissue repair. To obtain PRP, centrifugation of whole blood is made with specific time and gravitational forces. Thus, the present work aimed to study a method of double centrifugation to obtain PRP in order to evaluate the effective increase of platelet concentration in the final product, the preparation of PRP gel, and to optimize preparation time of the final sample. Fifteen female White New Zealand rabbits underwent blood sampling for the preparation of PRP. Samples were separated in two sterile tubes containing sodium citrate. Tubes were submitted to the double centrifugation protocol, with lid closed and 1600 revolutions per minute (rpm) for 10 minutes, resulting in the separation of red blood cells, plasma with platelets and leucocytes. After were opened and plasma was pipetted and transferred into another sterile tube. Plasma was centrifuged again at 2000rpm for 10 minutes; as a result it was split into two parts: on the top, consisting of platelet-poor plasma (PPP) and at the bottom of the platelet button. Part of the PPP was discarded so that only 1ml remained in the tube along with the platelet button. This material was gently agitated to promote platelets resuspension and activated when added 0.3ml of calcium gluconate, resulting in PRP gel. Double centrifugation protocol was able to make platelet concentration 3 times higher in relation to the initial blood sample. The volume of calcium gluconate used for platelet activation was 0.3ml, and was sufficient to coagulate the sample. Coagulation time ranged from 8 to 20 minutes, with an average of 17.6 minutes. Therefore, time of blood centrifugation until to obtain PRP gel took only 40 minutes. It was concluded that PRP was successfully obtained by double centrifugation protocol, which is able to increase the platelet concentration in the sample compared with whole blood, allowing its use in surgical procedures. Furthermore, the preparation time is appropriate to obtain PRP in just 40 minutes, and calcium gluconate is able to promote the activation of platelets.

INDEX TERMS: Platelet-rich plasma, platelet concentration, revascularization, rabbits.

RESUMO.- [**Método de obtenção de plasma rico em plaquetas de coelhos (*Oryctolagus cuniculus*).**] O plasma rico em plaquetas (PRP) é um produto de fácil obtenção a baixo custo, destacando-se pelos seus fatores de crescimento na reparação tecidual. Para obtenção do PRP, a centrifugação do sangue total é realizada com tempos e forças gravitacionais específicas. Assim, o presente trabalho teve por objetivo estudar o método da dupla centrifugação para obtenção do PRP, a fim de avaliar a eficácia de aumento da

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concentração de plaquetas no produto final, a preparação de gel de PRP e otimizar o tempo de preparação da amostra final. Quinze coelhos Nova Zelândia Branco, fêmeas, foram submetidos à coleta de sangue para a preparação de PRP. As amostras foram separadas em dois tubos estéreis contendo citrato de sódio. Os tubos foram submetidos ao protoloco de dupla centrifugação, com a tampa fechada a 1600 revoluções por minuto (rpm) durante 10 minutos, resultando na separação dos glóbulos vermelhos, plaquetas e plasma contendo os leucócitos. Na sequência, foram destapados para pipetar o plasma e transferí-lo para outro tubo de estéril. O plasma foi novamente centrifugado a 2000pm durante 10 minutos, resultando em duas partes: a parte superior, que consistia em plasma pobre em plaquetas (PPP) e a parte inferior do botão de plaquetas. Parte PPP foi descartado de modo que apenas 1ml de PPP permaneceu no frasco juntamente com o botão de plaquetas. Este material foi agitado suavemente para promover a ressuspensão das plaquetas, o que resultou na produção de PRP. O protocolo de centrifugação dupla foi capaz de promover a concentração de plaquetas 3 vezes maior em relação à amostra de sangue inicial. O volume de gluconato de cálcio utilizado para a ativação das plaquetas foi de 0,3ml, e foi suficiente para coagular a amostra, e o tempo de coagulação variou de 8 a 20 minutos, com uma média de 17,6 minutos. O tempo da centrifugação do sangue até a obtenção do PRP gel levou apenas 40 minutos. Concluiu-se que o PRP foi obtido com sucesso pelo protocolo de centrifugação dupla, sendo capaz de aumentar a concentração de plaquetas na amostra em comparação com o sangue total, permitindo assim a utilização de PRP em procedimentos cirúrgicos. Além disso, o tempo de preparação foi adequado, permitindo a obtenção do PRP em apenas 40 minutos, e a utilização de gluconato de cálcio foi capaz de promover a ativação das plaquetas.

TERMOS DE INDEXAÇÃO: Plasma rico em plaquetas, concentração plaquetária, neovascularização, coelhos.

INTRODUCTION

Platelet-rich plasma (PRP) or platelet concentrate (PC) are products easy and inexpensive to obtain, and they reveal important growth factors involved in tissue repair. An example for a growth factor are alpha granules of activated platelets, which stimulate chemotaxis, fibroplasia and angiogenesis (Maia et al. 2009). Production of growth factors occur in many cells and tissues such as fibroblasts, macrophages, endothelial cells, osteoblasts, leukocytes, salivary and lacrimal glands, among others. However, in many studies the most interesting target are platelets, since they are relatively easy to obtain and have other important substances for regeneration processes and tissue repair (Santos 2007).

The growth factors produced by platelets are platelets derived growth factor (PDGF), transforming growth factor beta (TGFb), vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), fibroblasts growth factor (FGF), and insulin-like growth factor (IGF) (Bolta 2007). Among all growth factors, the most abundant are found in the alpha granules of platelets, the platelet derivatived growth factor (PDGF) and the transforming growth factor beta (TGFb). Both have specific activities such as angiogenesis, mitogenesis and activation of macrophages (Camargo et al. 2002). Functions performed by this factors are very important for body function and angiogenesis, for healing of surgical wounds and reduction of postoperative complications, especially tissue ischemia (Clifford et al. 2001).

To obtain the platelet-rich components is necessary to centrifuge the whole blood with specific times and gravitational forces. This component can be obtained through one (Macedo 2004, Messora et al. 2009) or two centrifugations (Carmona 2006, Vendramin et al. 2006, Barboza et al. 2008). According to Maia et al. (2009) platelet activation is performed near the application time and can be used for the purpose substances such as recombinant human thrombin, autologous thrombin, calcium gluconate and calcium chloride.

Gel platelet-rich plasma (PRP) is also described as tissue glues with further advantage over fibrin glue, as it has growth factors and cytokines, providing great benefit in wound healing, such as better re-epitelization, reduction of wound exudate and necrosis (Bhanot 2002, Marx 2004, Vendramin et al. 2010). Research related to improve platelet-rich plasma is very important, and numerous studies are being developed to make new discoveries about this blood by product. A recent study by Textor et al. (2011) tested different methods on how to obtain PRP comparing the effects of an automated method (Harvest Smart PReR2) and another with centrifugation of samples from horse blood, to evaluate the concentrations of growth factors in the different samples. However the results were not good, because the platelets were not activated with any activator as bovine thrombin or calcium gluconate.

Studies like these are important to improve the preparation of platelet-rich plasma, since this product has been the subject of much research because of its stimulating properties of angiogenesis. In reconstructive surgery many problems happen due to inappropriate angiogenesis, which causes necrosis of flaps and grafts, and failure of the surgical procedure. Therefore, the platelet rich plasma has been studied because is considered to be a product which stimulates angiogenesis and can be used in reconstructive surgery to reduce the rate of necrosis after surgical procedure and improve healing.

The present study aimed to investigate a method of double centrifugation to obtain platelet-rich plasma (PRP) in order to evaluate the effective increase of platelet concentration in the final product, preparation of PRP gel, and to optimize preparation time of the final sample.

MATERIALS AND METHODS

Patient selection. The procedure for preparation of platelet--rich plasma was performed at the Laboratory of Clinical Pathology, School of Agricultural and Veterinarian Sciences (FCAV), Universidade Estadual Paulista (Unesp), Campus Jaboticabal. This study was approved by Ethics Committee on the Use of Animals (CEUA), Unesp-Jaboticabal[3].

We used 15 White New Zealand rabbit females (170 days), weighing 3.45 kg, obtained from a rabbit breeder. They were kept

[3] Protocol number: 028406/12.

at the animal house of the Postgraduate Course in Veterinary Surgery in individual cages of 80cm x 50cm x 35cm dimensions. It was provided commercial feed for rabbits, vegetables and water *ad libitum*.

Method to obtain platelet-rich plasma. After the rabbits were sedated with chlorpromazine hydrochloride[4] (0.5mg/kg) and morphine[5] (0.5mg/kg) in the same syringe, given intramuscularly (IM), trichotomy of the neck region was performed to access the jugular vein, and antisepsis ws proceeded with 90% chlorhexidine and alcohol solution. The animals were positioned in sternal position with the neck in maximum dorsal extension to allow adequate visualization of the jugular vein.

Proceeded by venipuncture was collected 8ml of blood using a 10ml syringe attached to a needle 25 gauge x 7mm (Fig.1A). Sample was separated in two sterile tubes with capacity of 3.6ml containing sodium citrate[4] (anticoagulant) for the preparation of the PRP , and a third tube with a capacity of 1ml containing EDTA[5] (anticoagulant) was filled with 0.8ml blood which was intended to platelet count (Fig.1B). After determining the platelet count within the physiological parameters for the species has begun the preparation of PRP.

The tube with sodium citrate was employed in the production of platelet-rich plasma (PRP) by double centrifugation protocol in a laboratory centrifuge[6] (Fig.1C) as described by Morato (2013). Tubes were centrifuged with lid closed at 1600 revolutions per minute (rpm) for 10 minutes, resulting in the separation of red blood cells and plasma containing platelets and leucocytes.

In a laminar flow[7], tubes were uncapped and plasma was pipetted and transferred into another sterile tube (Fig.1D). Plasma was centrifuged again at 2000 rpm for 10 minutes (Fig.1E), and then resulted in two parts: on the top, consisting of platelet-poor plasma (PPP) and at the bottom of the platelet button. Part of the PPP was discarded so that only 1ml remained in the tube along with platelet button (Fig.1F). This material was gently agitated to promote platelets resuspension, resulting in the production of platelet-rich plasma (PRP).

After process of enrichment of the plasma, platelets concentration was counted in an automatic apparatus[8] with 80μL of the PRP sample, to verify that the platelet count was greater than 1.000.000/μL. After platelet count of the sample, PRP was activa-

[4] Clorpromazina® - Cristália Chemical and Pharmaceutical Products, Itapira, São Paulo.

[5] Dimorf , Cristália, Itapira, SP.

[4] Tube BD vacutaneir® sodium citrate, BD, São Paulo, SP.

[5] Tube pediatricBD vacutaneir® EDTA, BD, São Paul, SP.

[6] Modelo 206 I centrifuge, Fanem®, São Paulo, SP.

[7] Vecoflow Ltda - Modelo Bio 12, Campinas, SP.

[8] Poch - 100iV Diff , Medical Trade Representations and Imports Ltda, Recife, PE.

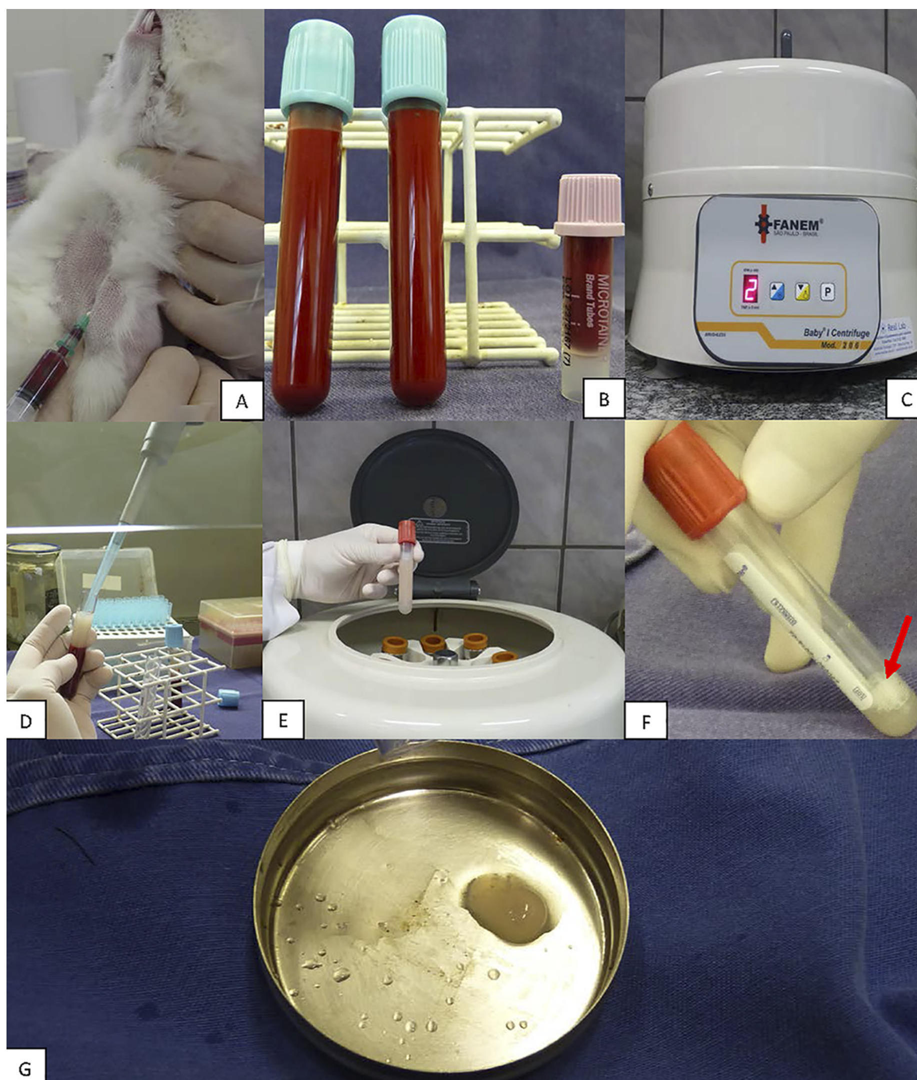

Fig.1. Images of the stages of obtaining PRP in rabbits at the Veterinary Hospital of the Faculty of Agricultural and Veterinary Sciences (FCAV) of Unesp, Jaboticabal Campus, 2013. (**A**) Positioning of the animal jugular vein puncture. (**B**) Blood samples for preparation of PRP, tube (blue cap) for PRP preparing and platelet count (purple cap). (**C**) Centrifuge used for preparation of PRP. (**D**) Blood samples after first centrifugation (**E**) Blood samples after second centrifugation, indicating the total volume of plasma. (**F**) Appearance of the second plasma after centrifugation and discarding the supernatant (arrow). (**G**) Sample after addition of 0.3ml calcium gluconate for platelet activation. PRP gel ready for use.

ted by addition of 0.3ml of calcium gluconat to form PRP gel. This process needs to be done close to the moment of their therapeutic use (Fig.1G).

Statistical analysis. The data (platelet count) were analyzed by paired t-test with significance level of 5%. Besides, data were analyzed descriptively by a table representing platelet count of the rabbits before and after enrichment of the platelet-rich plasma, and by a graph with mean platelet count pre and post enrichment.

RESULTS

Platelets count after enrichment of the platelet-rich plasma (PRP)

In the blood samples from rabbits the initial platelet count was within physiological standards for the specie, ranging from 290.000 to 678.000 platelets/uL. The mean platelet count obtained from 7.2ml of the blood samples after centrifugation was 1.176.933 platelets/uL (standard deviation = 980.546 plaletes/μL). The results obtained on rabbit platelet count before and after enrichment of the platelet-rich plasma (PRP) are shown in Table 1.

Table 1. Results of platelet count in blood samples of rabbits used for the preparation of PRP and number of platelets obtained after the enrichment, carried out at the Veterinary Hospital of the Faculty of Agricultural and Veterinary Sciences (FCAV) of Unesp, Jaboticabal Campus, 2013

Rabbit	Platelet sample (x 10^3plq/μL)	Count obtained in PRP (x 10^3plq/μL)	Difference between enrichments
1	470	1.172	702
2	352	4.420	4.068
3	347	1.238	891
4	304	1.123	819
5	502	1.810	1.308
6	339	640	301
7	403	1.380	977
8	216	640	424
9	318	523	205
10	352	605	253
11	440	1.429	989
12	287	471	184
13	396	644	248
14	434	754	320
15	417	805	388
Average	371,8	1.176, 933	805,13

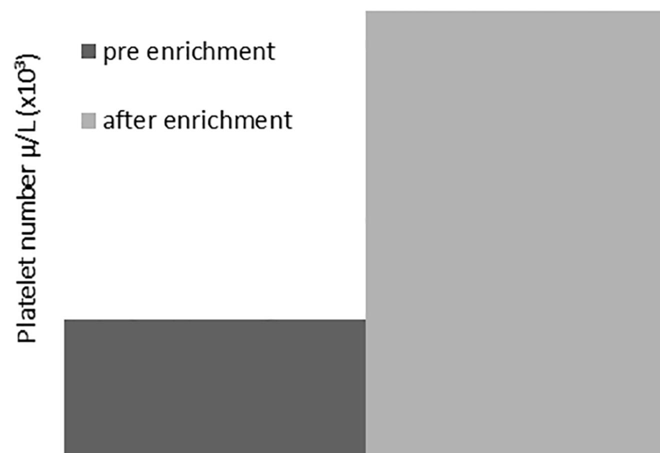

Fig.2. Result of the centrifugation to obtain PRP in rabbits which promotes an average platelet concentration three times higher than the initial amount of platelets in blood (p=0.006) at the Veterinary Hospital of the Faculty of Agricultural and Veterinary Sciences (FCAV) of Unesp, Jaboticabal Campus, 2013.

Double centrifugation protocol was able to obtain platelet concentration 3 times higher in relation to the initial blood sample. Figure 2 shows the mean values of platelet count pre- and post-enrichment of platelet rich plasma (PRP) (p=0,006).

Platelet activation

The volume of calcium gluconate used for platelet activation was 0.3ml, which was sufficient to coagulate the sample. Coagulation time ranged from 8 to 20 minutes, with an average of 17.6 minutes. Therefore, time of blood centrifugation until to obtain PRP gel took only 40 minutes.

DISCUSSION

The application of Platelet-rich plasma (PRP) for tissue repair is of great interest in veterinary medicine. However, due to the availability of several protocols for obtaining and the lack of standardization among them, it is difficult to decide which method to use. Thus, the information obtained in this study shows a low cost method for obtaining platelet-rich plasma, as well as ways to ensure success for the final product. Besides this, the results of the biological effects justify the application of PRP.

The blood collected for preparation of platelet-rich plasma was placed in sterile tubes containing sodium citrate (anticoagulant). White et al. (2000) describe that the use of other anticoagulants such as EDTA can cause structural and functional damage to platelets, and also may later cause damage to the treated tissues, suggesting that the choice of sodium citrate was the better option. On the other hand, use of other anticoagulants such as citrate dextrose solution (Lu et al. 2008) and heparin (Kasten et al. 2008) are described by other authors. Thus, this study followed the same protocol of Barboza et al. (2008) and Vanat et al. (2012) to using sodium citrate tubes. The choice of sodium citrate in this study was the ease to obtain it in a sterile form, reducing the risks of contamination at harvest, and not to cause structural and functional damage to platelets.

After the second centrifugation the concentration of platelets was evaluated, and 0.3ml of calcium gluconate were added to obtain PRP gel. According Efeoglu et al. (2004), through administration of a platelet activator, such as thrombin or calcium gluconate, which activates the coagulation system and results in formation of PRP gel, allowing its application in various surgeries. The platelet activator used in this study followed the same criteria recommended by the literature (Efeoglu et al. 2004, Morato 2013).

Platelet count obtained before the PRP preparation was 2.7 times lower compared with that obtained after. Data obtained in this study are similar to results found by Silva et al. (2011), since in their study the platelet count in the final sample, after obtaining PRP, was significantly higher than the platelet count of the initial blood sample. Evaluating the centrifugation protocol it was found that

the double centrifugation method, performed in this study and recommended by Camargo et al. (2002), was adequate; since the concentration of the platelets increased 3 times. Although in some samples the concentration of platelets has not increased as expected, it was still possible to obtain good results.

Platelet rich plasma is a product easy and inexpensive to obtain, however, when it began to be produced its attainment was more complex and costly, due to automated methods requiring specific equipment and kits, for higher volumes of blood, necessary to obtain higher concentration of platelets and larger volumes of PRP (Maia & Souza 2009). After conducting extensive research to bring the best collection method associated with reduced costs, as Macedo (2004), Barboza et al. (2008) and others, who developed protocols using laboratory centrifuges in the production of platelet rich plasma, this study was done to obtain simple and low cost methods to obtain platelet-rich plasma.

Although there is not yet a protocol standardized of centrifugation, some studies indicate a single centrifugation while others indicate double centrifugation (Macedo 2004, Camargo et al. 2002, Feres Junior 2004). As there is no agreement between single or double centrifugation, and there is no spin speed and time of obtaining established, this study standardized two cycles of centrifugation to obtain PRP; the first centrifugation speed 1600rpm (revolutions per minute) for 10 minutes, and the second 2000 rpm (rotation per minute) for 10 minutes. In this study, using the double centrifugation and applying the mentioned speeds, was possible to obtain the concentration of platelets in the final PRP in all animals, between 470.000 and 4.420.000/μL.

Although some authors have reported that the platelets count in PRP should present a higher concentration than 1.000.000/μL (Del Carlo et al. 2009) other authors had good therapeutic effects with a concentration greater than 300.000 platelets/uL (Carmona et al. 2009). In the present study we obtained the final platelet count between 470.000 and PRP 4.420.000/μL, what corroborates the literature (Carmona et al. 2009, Del Carlo et al. 2009).

The preparation time for platelet rich plasma in this study, from blood collection until to obtain the gel, was 40 minutes. Because to its applicability being in surgical procedures, time becomes an important factor. The time to obtain the PRP in this study showed similar results to that described by Garcez et al. (2013) and Morato (2013), who obtained the PRP preparation time between 30 and 50 minutes.

CONCLUSIONS

The results of this study indicate that the preparation of PRP gel by double centrifugation method is reproducible, easy to apply, and can be performed in veterinary clinics and hospitals, since they have laboratory equipment necessary to obtain the product.

Double centrifugation method increases platelet concentration in the PRP sample as compared with whole blood.

The preparation time is also appropriate and allows to obtain platelet rich plasma within 40 minutes.

The use of calcium gluconate promotes the activation of platelets (PRP).

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