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 concentração de plaquetas na amostra final.

INDEX TERMS: Platelet-rich plasma, platelet concentration, revascularization, rabbits.
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 the different samples. However the results were not good, platelet rich plasma has been studied because is considered to be a product which causes necrosis of flaps and grafts, and failure of the surgical procedure. Therefore, the platelet rich plasma 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. We used 15 White New Zealand rabbit females (170 days), weighing 3.45 kg, obtained from a rabbit breeder. They were kept

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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 hydrochloride⁴ (0.5mg/kg) and morphine⁵ (0.5mg/kg) in the same syringe, given intramuscularly (IM), trichotomy of the neck region was performed to access the jugular vein, and antisepsis was 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 to two sterile tubes with capacity of 3.6ml containing sodium citrate⁴ (anticoagulant) for the preparation of the PRP, and a third tube with a capacity of 1ml containing EDTA⁵ (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 centrifuge⁶ (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 flow⁷, 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 apparatus⁸ 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 activated.

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⁴Clorpromazina® - Cristália Chemical and Pharmaceutical Products, Itapira, São Paulo.
⁵Dimorf, Cristália, Itapira, SP.
⁶Tube BD vacutainer® sodium citrate, BD, São Paulo, SP.
⁷Tube pediatricBD vacutainer® EDTA, BD, São Paulo, SP.
⁸Modelo 206 I centrifuge, Fanem®, São Paulo, SP.
⁹Vecoflow Ltda - Modelo Bio 12, Campinas, SP.
¹⁰Poch - 100IV Diff., Medical Trade Representations and Imports Ltda, Recife, PE.
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/µL. The mean platelet count obtained from 7.2 ml of the blood samples after centrifugation was 1,176.933 platelets/µL (standard deviation = 980.546 platelets/µL). The results obtained on rabbit platelet count before and after enrichment of the platelet-rich plasma (PRP) are shown in Table 1.

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 0.3 ml, 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 0.3 ml 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 1600 rpm (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/µL (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).

Acknowledgments.- To Dr. João Ademir Oliveira for the contribution in statistical analyzes, to the Faculty of Agricultural and Veterinary Sciences, Unesp-Jaboticabal, and to the Coordination of Improvement of Higher Education Personnel (CAPES) for granting a graduate student stipend.

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