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Comparative Study of Four Methods of Preparation of Autologous Plasma-Rich Platelet in Sheep

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Abstract: The therapeutic evaluation of the biological effect of platelet-rich plasma (PRP) is the subject of several studies in regenerative medicine. The aim of this study was to compare four different methods of preparation of autologous platelet concentrates in sheep. Various specimens were collected from six adult healthy male sheep of the local breed. Blood smears were also carried out on all the samples, in order to see the morphology of the platelets. The data obtained in the manual platelet count were submitted to statistical analysis (repeated measures ANOVA, Tukey HSD test, $P<0.05$). The result was that the total mean number of platelets in whole blood was $521.56 \pm 97.01 \times 10^3/\mu l$. The PRP samples in Groups I and III showed significantly higher mean platelet counts than Group II and IV ($1438.25 \pm 221.72 \times 10^3$ /µl and $1430.75 \pm 293.63 \times 10^3/\mu l$, respectively). In conclusion, within the limit of this study, it can be concluded that the best technique of platelet harvesting in sheep is obtained with the double centrifugation protocol used for animals of group I.

Key words: Platelet Counts · Platelet-Rich Plasma · Centrifugation · Blood · Sheep

INTRODUCTION

The search for protocols favoring haemostasis and healing is a recurring problem in all surgical disciplines. Platelet-rich plasma (PRP), as a biological adjuvant, offers innovative new pathways [1]. It is defined according to Marx [2] as a restricted plasma volume containing a high platelet contingent rich with growth factors. Therapeutic administration of PRP as a treatment decreases regenerative disorders and accelerates the healing of a wide range of tissues. However, there are controversies in the literature regarding the potential benefits of using this procedure and also controversies regarding the protocols of its preparation. Anitua [3] and Aghaloo *et al.* [4] reported that these controversies are probably related to the lack of appropriate standardization of different preparations and a formal definition of PRP. Several simplified protocols for the preparation of PRP have been developed to facilitate its clinical application.

Basically, centrifugation is considered as a basic process of preparing different PRP. Various methods for the preparation of PRP have been reported in the literature. These include commercial systems such as Curasan®, Plateltex® and manual methods using the laboratory centrifuge reported by Marx [2], Landesberg *et al.* [5] and Okuda *et al*. [6]. The clinical efficacy of each of these methods remains uncertain and depends on several variables. The role of centrifugation's speed and time on the platelet concentration achieved in each of the methods is fully evaluated [7]. According to Marx [8] and Carneiro *et al.* [9] the PRP obtained by double centrifugation must have a platelet concentration of 300 to 400%, higher than that of whole blood, in order to consider it as a "therapeutic PRP". However, Anitua [3] uses only one centrifugation although the platelet concentrations obtained by this procedure have not been reported.

The aim of this study was to determine the effects of the regime presented by the speed and the time of centrifugation on the number of platelets in platelet concentrates obtained using four different methods of preparing PRP in sheep.

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MATERIAL AND METHODS

Experimental Model: Six clinically healthy adult male sheep weighing 25-30 kg were used in this study. The experimental protocol was carried out at the Biotechnology Laboratory of the Bioactive Molecules and the Cellular Physiopathology, Department of Biology, University of Batna 2, Algeria.

PRP Preparation: A volume of 85 ml of blood was drawn from each animal by jugular puncture. This sample requires the use of 06 tubes of 15 ml of capacity containing 02.25 ml of citrate dextrose (ACD) [8]. As soon as the blood was collected, it was immediately distributed in sterile dry tubes of 05ml of capacity; in total we obtained 21 tubes containing 04 ml of blood for each one. A single tube used for the count of platelets in the initial blood whereas the rest of the tubes (20 tubes) underwent centrifugation at different rates established as presented in table 01.

Table 1: Summary table of the various protocols used for the preparation of platelet concentrates.

Method	Group I	Group II	Group III	Group IV
1st centrifugation	1800 r.p.m for 08 min	1800 r.p.m for 08 min	2500 r.p.m for 05 min	2400 r.p.m for 03 min
2nd centrifugation	1000 r.p.m for 08 min	_	_	3000 r.p.m for 12 min

•GGroup I: Method proposed by Carneiro *et al*. [9].
•GGroup II: Method proposed by Hernandez-Fernandez *et al*. [10].
•GGroup III: Method proposed by Nair *et al*. [11].
•GGroup IV: Method proposed by Milano *et al*. [12].

All these protocols were carried out with respect to the various centrifuging regimes established by the authors. The modification concerns only the initial blood volume (85ml of blood for each method).

Protocol for the Preparation of PRP by Single Centrifugation (Group II and III): Separation of the cellular elements from Group II and III blood was carried out using simple centrifugation (Laboratory centrifuge; Hettich zentrtrifugen D-78532 tuttlingen) The blood samples were centrifuged at the rates established by each method (Speed and appropriate duration of each technique). The process took place at an ambient temperature of 22 ° C. accordingly, we obtained three basic components; Red blood cells (Tube bottom), PRP (Middle of the tube) and platelet-poor plasma (PPP) (At the top of the tube). A mark was made on the line separating the globular pellet from the supernatant. All the content above this mark (About 08 to 09 ml) was pipetted which corresponds to the volume of PRP.

Protocol for the Preparation of PRP by Double Centrifugation (Groups I and IV): The first centrifugation: the blood cell separation was carried out using a laboratory centrifuge, the Group I tubes were centrifuged at 1800 r.p.m for 08 minutes and the Group IV tubes centrifuged at 2400 r.p.m for 3 minutes in an ambient temperature of 22 °c. we obtained two basic components: blood cell components in the lower fraction and serum which corresponds to the upper fraction.

The second centrifugation: all the contents above the globular pellet were pipetted and transferred to another 5 ml dry tube without anticoagulant. The sample was then centrifuged again at 1000 r.p.m for 08 minutes for group I tubes and 3000 r.p.m for 03 minutes for group IV tubes. There resulted a small red fraction at the bottom of each tube and a supernatant at the top. The fraction that will be pipetted is 0.5 ml of each tube above each red fraction. The total amount of PRP collected for each samples is 02.5ml.

The Number of Platelets: The number of platelets was determined on whole blood and on PRP samples under a microscope with a haemocytometer (Neubauer improved chamber) after 1/200 dilution with a Formol 0.5 ml, acetone 2.5 ml, physiological saline 20 ml and distilled water (QSP per 100 ml).

Realization of Smears: The realization of the whole-blood and PRP smears (The staining with the May-Grunewald Geimsa: M.G.G) aims to analyze the platelet richness and the presence or absence of the aggregates. This is done on the basis of each count.

Statistical Analysis: Statistical analysis was performed; the results of counts obtained in each of the methods used were expressed as mean plus standard deviations and medians. The comparisons between the different methods are carried out using ANOVA with the statistical software SPSS version 23. This analysis of variance followed by a comparison between groups using Tukey HSD test with a statistical significance fixed at $P < 0.05$.

RESULTS AND DISCUSSIONS

From the results presented in table 2, we note that the number of platelets in the whole blood of all individuals considered in this study corroborates with that provided by the literature [13] with a variation of 521.56 ± 97.01x10^3/µl. Concerning the number of PRP platelets of each protocol (FIG. 01), our results are similar to those

Table 2: Results of platelet count averages of the four groups according to each established rate (ANOVA).

Groups	Mean platelet concentration ± SD (X 10^3/µl)	N	95 % Confidence Interval Lower Limit	Upper Limit	F	SignificanceP-value
Whole blood	521.56±97.01	6	419.75	623.37	17.24	p < 0,0010.000 (***)
Group I	1438.25±221.72 c	4	1085.43	1791.06		
Group II	995.33±48.95 bc	3	873.72	1116.93		
Group III	1430.75±293.63 c	4	963.51	1897.98		
Group IV	958.66±289.75 ab	3	238.86	1678.46		

The presented means which are followed by different letters are significantly different (ANOVA: Tukey HSD test, P <0.05was considered statistically significant).

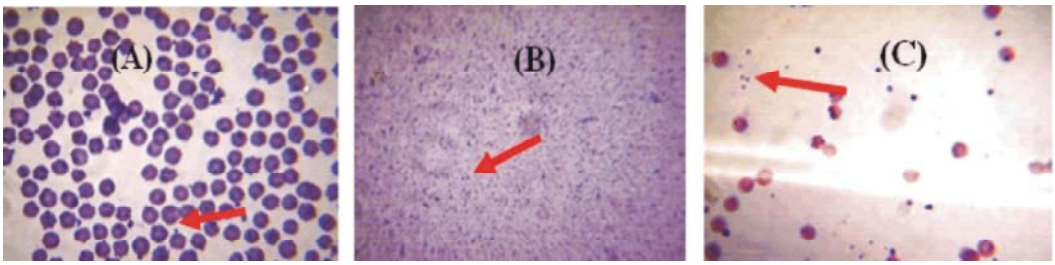

Fig. 1: Smears showing platelets (Arrows) of whole blood (A). PRP smears showing platelets (Arrows) of group I: (B) and group II: (C) (Staining with M.G.Gx100).

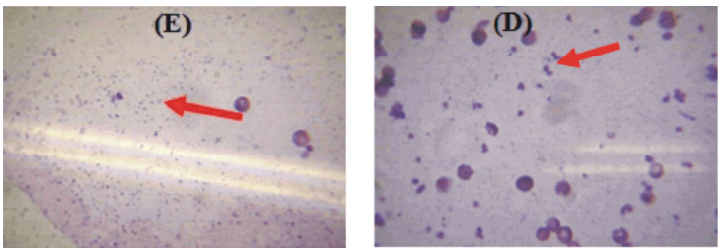

Fig. 2: PRP smears showing platelets (Arrows) of group III: (D) and group IV: (E) (Staining with M.G.Gx100).

reported by the authors. The methods proposed by Carneiro *et al*. [9] and Nair et al. [11] have a higher platelet count with a variation of 1438.25 ± 221.72×10^3/µl and 1430.75 ± 293.63×10^3/µl respectively. The methods proposed by Hernandez-Fernandez *et al*. [10] and Milano *et al.* [12] reveal lower levels of thrombocytes (995.33 ± 48.95×10^3/µl and 958.66 ± 289.75 × 10^3/µl).

Platelet counting by the manual or automated method is widely discussed in the literature; the majority prefers the use of automated methods. Several works which have been published in this context, Woodell-may *et al*. [14] and Briggs *et al*. [15] confirm that only automated hematology analyzers can accurately count the platelets in the PRP. Some others advocate the manual method [16-18] whose simple reason is to avoid all individuals with prior thrombocytopenia.

Careful examination of PRP smears confirms all of our results, Figure (B):1 and (D):2 show enormous thrombocytes. We also note the absence of aggregation phenomena for all smears. It appears that dextrose is the best anticoagulant for different preparations of autologous PRP in sheep.

Analysis of variance ANOVA (Table.1), revealed a significant difference at the threshold of significance á = 5% of thrombocytes count between the different methods used compared to whole blood. Likewise, the comparison between the different methods (Intergroup comparison) using the Tukey HSD test confirm this hypothesis with a highly significant difference between groups I and III (***) and significant for groups II and VI (*). Our results are supported by some authors who reported that the clinical effectiveness of each of these methods remains uncertain and optimized in relation to the different variables of the PRP preparation process as well as the dose used. According to Marx [8], Nagat *et al*. [18], Dugrillon *et al*. [19] and Weibrich *et al*. [20] total treated blood volume,

sampling, number of turns, time of centrifugation and centrifuge rotational speed, all of these parameters concur and control the quality of PRP. Concerning the therapeutic dose, analytical gaps in published PRP works sometimes lead to contradictions and confirm our doubts about the biological identity of these preparations.

Some authors have also reported that moderate centrifugation regimes favor separation of platelets while superior speed and longer time centrifugations are no desired. This fact may be due to agglutination or disintegration of platelets with premature release of the contents of the platelets granules. This hypothesis was also confirmed by Sabarish *et al*. [21] who reported that several variables affect the quantity and quality of PRP. Furthermore, concerning the fraction of white blood cells, Marx [8] confirmed that the use of the double centrifugation process is essential wherein the PRP obtained is practically free from all cells of the white line. These may have a detrimental effect on epithelialization following premature release of pro-inflammatory factors potentially responsible for the degradation of the extracellular matrix.

CONCLUSION

Methods and protocols for the preparation of platelet-rich plasma in sheep are very diverse and widely controversial in the literature including their associated biological effects. On the basis of the results obtained, it appears that all the protocols used enable us to have platelet concentrates with very varied platelet contents. The ANOVA statistical study reveals that the protocols proposed for the animals of group I and III collect the highest platelet concentration. Because there is no official consensus to optimize and standardize a specific method, the main relevant aspects to be controlled during the preparation of PRP for specific in vivo applications are the initial volume of blood taken, the nature of the anticoagulant used, the speed and time of centrifugation.

At the limit of this study and due to the small number of animals used, it can be concluded that the best technique of platelet harvesting in sheep is obtained with the protocol used for animals of group I.

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