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Research Report

PLATELET RICH PLASMA PREPARATION PROTOCOLS: A PRELIMINARY STUDY

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ABSTRACT

Currently, therapy with Platelet Rich Plasma (PRP) has been widely used and continues to grow for various clinical applications. Along with its development, there are various options in the method of obtaining PRP, either automatic or manual, while one of the most reliable methods according to the literature is a double centrifugation method. The purpose of this research is to produce an optimization of the double centrifugation method. This study used experimental data obtained by conducting a research at the Clinical Pathology Laboratory of Dr. Soetomo Hospital, Surabaya. Experiments were conducted on stored blood obtained from the blood bag from Indonesian Red Crossand fresh blood from healthy donors with CPD anticoagulant. Results: PRP with optimum platelet count could be made with sufficient personal laboratory skills and amounted to 4.11 times with the platelet count of 1.152 million using 1300 rcf for 5 minutes for the first centrifugation, and 2300 rcf for 7 minutes for the second centrifugation.

Keywords: *Platelet Rich Plasma, Double Centrifugation Method, Stem Cell Therapy*

ABSTRAK

Latar Belakang: *Saat ini terapi dengan menggunakan Platelet Rich Plasma (PRP) telah banyak digunakan dan menjadi pilihan dalam dunia klinis. Adapun dalam perkembangannya terdapat beberapa pilihan metode, baik secara otomatis maupun manual, meskipun metode yang paling dipercaya adalah metode double centrifugation. Tujuan: Untuk menemukan optimalisasi yang paling tepat untuk metode tersebut. Penelitian ini menggunakan data eksperimental yang dilakukan oleh peneliti dari Laboratorium Patologi Klinik RSUD Dr. Soetomo Surabaya.* ***Metode:*** *Penelitian ini menggunakan plasma darah yang diperoleh dari Palang Merah, yang berasal dari donor dengan CPD anticoagulant.* ***Hasil:*** *PRP dengan perhitungan platelet optimum ditunjang dengan kemampuan laboratorium dapat dicapai hingga 4.11 kali dengan perhitungan 1.152 million, menggunakan 1300 rcf selama 5 menit pada putaran pertama dan 2300 rcf selama 7 menit pada putaran kedua.*

Kata kunci: *Platelet Rich Plasma, Double Centrifugation Method, Stem Cell Therapy*

INTRODUCTION

As a relatively new subject, stem cells can be said to have tremendous potential, starting early in life and growing up as a sort of internal improvement system, proliferate and differentiate without definite limits to later form the other cells as long as the relevant person or animal is still alive . As one of the internal improvement system, the proliferation of stem cells can be stimulated in the presence of growth factors. Growth factors are found in PRP (Platelet Rich Plasma) in large numbers.

In Indonesia, the current stem cell therapy is a field that currently emerged, and still not a lot of work of which the method is widely used and applicable. PRP was highly rated in terms of potential for use as a treatment of chronic tendinitis, wound healing, regeneration of cartilage or discs, as well as cardiac applications.

There are various methods used for the manufacture of this PRP, from sophisticated which can only be performed at hospitals using apheresis devices, up to a practical method that can be performed directly in clinics. From variously different methods and protocols, there are still no research in Indonesia on what the most optimal method is. Therefore, it is felt that there is a need to have a research and optimization on double centrifugation method. The method, according to the literature, is the most reliable one and relatively simple. So as to produce a protocol that PRP produces reliable and proven quality.

MATERIALS AND METHODS

This is a laboratory experiment which is intended to get the most reliable PRP making method with platelet count as the indicator. The materials and tools were sterile tubes, syringes, sterile long needle, CPD anticoagulant, centrifuge, and Sysmex automatic hematology analyzer. The experiment was conducted in the laboratory of clinical pathology department of Dr. Soetomo Hospital, Surabaya.

Sample Collection

The blood samples were obtained from a blood bag from a donor, and also from fresh blood from other donors. To get as much as 1 ml of PRP, we took blood samples of 10 ml with 9:1 CPD anticoagulant. For the optimization experiment of centrifugation phase and separation phase, we performed 30 tests, hence the total experiment required a sample size of 300 ml of blood.

PRP Isolation

Platelet count was conducted twice for a sample, one before, and another after the optimization process. The optimization process itself was a series of variation on velocity and time of centrifugation, twice on each sample. It varied from 200 to 2300 rcf, and 5 to 15 minutes on each centrifugation.

Separations were manually done with ± 1 mm of margin after each centrifugation, in order to purify as much as possible, the result was extracted from erythrocytes and Platelet Poor Plasma (PPP). The visible buffy coat from the first separation was essential to be maintained until the end of the optimization process. The first separation was done in order to separate erythrocytes as much as possible, while

Table 1. Platelet count result

Sample No.	1st Centrifugation		2nd Centrifugation		Platelet Count 1 (x 10^3/µl)	Platelet Count 2 (x 10^3/µl)	Rise
	Force (rcf)	Time (min.)	Force (rcf)	Time (min.)			
001	1200	5	2000	6	92	271	2.94 x
002	200	15	1500	15	87	194	2.23 x
003	300	10	1500	15	87	65	0.74 x
004	600	5	1500	15	87	106	1.22 x
005	900	5	1500	15	87	129	1.48 x
006	1200	5	1500	15	87	33	0.38 x
007	1500	5	1500	15	87	41	0.47 x
008	200	15	2000	6	87	90	1.03 x
009	300	10	2000	6	87	47	0.54 x
010	600	5	2000	6	87	23	0.26 x
011	900	5	2000	6	87	31	0.36 x
012	1200	5	2000	6	87	37	0.43 x
013	1500	5	2000	6	87	33	0.38 x
014	200	15	1500	15	170	62	0.37 x
015	200	15	600	15	104	109	1.05 x
016	600	30	1500	10	104	29	0.28 x
017	**1300**	**5**	**2300**	**7**	**280**	**1152**	**4.11 x**
018	900	5	2300	7	104	37	0.36 x
019	1300	5	2300	7	104	22	0.21 x
020	1200	5	2000	6	239	181	0.76 x
021	1300	5	2300	7	239	428	1.79 x
022	600	5	2000	6	234	140	0.59 x
023	1300	5	2300	7	234	186	0.79 x
024	600	5	2000	6	228	288	1.26 x
025	900	5	1500	15	228	292	1.28 x
026	1200	5	2000	6	228	431	1.89 x
027	1300	5	2300	7	228	898	3.94 x
028	600	5	2000	6	183	469	2.56 x
029	1200	5	2000	6	205	269	1.31 x
030	1300	5	2300	7	251	327	1.30 x

the second was to separate PPP leaving the bottom with 1 ml of liquid, which was considered to be the PRP.

Each set of variation was repeated at least 2 times to assure the reliability of the current method. The resulting data were analyzed by using simple analytical statistics. The thrombocyte count of the PRP obtained was measured by automated Sysmex XE-2100 Hematology Analyzer which had a unique fluorescent flow cytometry and hydrodynamic focusing technologies to minimize the review rates and produce accurate results even when interferences or artifacts were present.[1] Hence, the result presented in this study was made to be as accurate as possible, with very less likely to be false positive.

RESULTS AND DISCUSSION

Experiments of optimization of double centrifugation method in platelet rich plasma gave the following result:

Due to the limited budget towards the experiment, a PDGF BB test was performed only as an additional infromation, based on the highest result produced by the protocol, to ensure that it would still be a useful PRP after processed through the protocol. The result proved that it was still a useful PRP with PDGF BB 3401 pg/ml after processing through this protocol. This amount could still increase after activation, which was not included and tested further in this current preliminary study due to some fund limitation. But, as defined by Textor, the conventional human definition of PRP is plasma containing resting platelets at a concentration of at least 106/ul. The PRP may or may not be activated prior to use, hence the term "PRP" does not indicate that activation has occurred.[2] Alone, platelet count itself is considered to be one of the key factors used to standardize research studies for the regenerative capacity of PRP. Maximum platelet concentration of PRP with manual double centrifugation method by Kakudo is 7.9 x.[3] Too, qualitative changes in the platelet may also affect the regenerative potential of PRP. According to Marx, a damaged or nonviable platelet will not release bioactive growth factors, thus the resulting PRP may be disappointing. The PRP for clinical treatment should be sought about 1,000,000 platelets per microliter. Given that whole blood contains approximately 200,000 ± 75 000 platelets per microliter, then the therapeutic PRP must have an average percentage of increase of about 400% in platelet count.[4] Therefore, this experiment indicates that the optimum result from this experiment might be a standard for PRP making.

But as we can see in table 1, there were quite significant inconsistencies in the correlation between force and time of centrifugation to increase the platelet count after the double centrifugation method in PRP making. It seemed that it was very dependent on the fidelity of manual separation processes between the centrifugations. The later samples results were higher than the early ones since the researchers become more accustomed and skilled in the manual separation process. Errors might also be found because of the absence of PRP resuspension before the platelet was counted by the analyst. Resuspension could be important because there were trapped platelets in the buffy coat, so resuspension will show the tangible number of platelets in PRP.[5] Hence, it could be concluded that the factor of trained person in the whole process might be more significantly important rather than the centrifugation process itself. This might also explain the very wide spectrum and range in protocol in centrifugation force and time to this day, which ranges from 72 G for 15 minutes, 160 G for 20 minutes, 250 G for 10 minutes, 900 G for 5 min, 1300 G for 10 minutes, up to 1400 G for 4 minutes, for the first centrifugation. The second centrifugation itself has a variable speed from 400 G to 2000 G, and has a variable period of time from 6 minutes to 15 minutes. Similarly, with temperatures varying from 4° C, 15° C, up to room temperature.[5,6,7,8,9]

Anticoagulant Acid Citrate Dextrose (ACD) type A and low-speed centrifugation can be used to maintain the integrity of the platelet membrane.[5] But according to the study of Shimizu et al, PRP is created with the anticoagulant Citrate Phosphate Dextrose (CPD) had a 4% higher platelet count when using 5 ml of full blood, and 4.5% higher using 200 ml of full blood.[10] Hence, this experiment used CPD anticoagulant instead of ACD.

Overall, the highest increment in platelet count was obtained using 1300 rcf for 5 minutes for the first centrifugation, and 2300 rcf for 7 minutes for the second centrifugation as we can see in samples 017 and 027. This is quite different with the finding of Jo CH and colleagues from orthopedic surgery department of Seoul National University Boramae Hospital, which stated that the platelet count would be optimum with double centrifugation method with the use of 900 G in the first centrifugation for 5 min and 1500 Gin the second centrifugation for 15 minutes.[9]

Besides the double centrifugation method, there are already several automated methods developed independently by many institutions. Among these methods are Curasan PRP Kit by Curasan, Kleinostheim, Germany; method PCCS PRP System by 3i Implant Innovations, Palm Beach Gardens, Florida, United States; and methods SmartPrep by Harvest Technologies, Plymouth, Massachusetts United States. However, research by Castillo showed no significant differences in the platelet concentration, in the number of erythrocytes, the TGFβ1, or in the fibrinogen levels between the various automated methods.[11] And since there was no availability of any automated PRP machines in both the Medical Faculty Universitas Airlangga and Dr. Soetomo Hospital Surabaya, there was no difference in the method used in this experiment. But, based on Kurita et al experiment in 2008, double centrifugation method itself was described as the most accurate and rational choice for manual making experiment of PRP, conformed by Nagata in 2010, since it produced more platelets and more active substances.[7,12] Castillo also suggests that the usage of method and protocol should be based on associated rational choice instead of trying tremendous variability available

by automated machines which are described as "the jungle world of commercial proposals and products".

As the concentrated platelet, PRP rich in basic growth factors and has potential various clinical applications with no virtual risks. To initiate the process of wound healing, there are Platelet Derived Growth Factor (PDGF$\alpha\alpha$, PDGF$\beta\beta$, PDGF$\alpha\beta$), Transforming Growth Factors-β (TGF-β_1 and TGF-β_2), Vascular Endothelial Growth Factor (VEGF), Epithelial Growth Factor (EGF). In addition PRP also contains adhesion molecules necessary for bone grafting and the matrix of bone, such as osteocalcin, osteonectin, and bone morphogenic protein (BMP) -2 and BMP-4.[4,13] Platelets also have a Platelet Factor 4 (PF4), interleukin (IL) -1, Platelet-Derived Angiogenesis Factor (PDAF), Platelet-Derived Endothelial Growth Factor (PDEGF), Epithelial Cell Growth Factor (ECGF), Insulin-Like growth Factor (IGF), vitronectin, fibrinogen, fibronectin, and thrombospondin (TSP) -1.[14,15,16] PRP also shows some potential for treatment of myocardial infarction in experiments using the *musmusculus.*[17] In addition to the usefulness above, PRP is also potentially used in a variety of other clinical applications such as muscle healing,[18] peripheral nerve damages,[19] and maxillofacial surgeries.[20]

Suggestions

This project is a preliminary study about the manual making of for PRP and therefore its goal is to obtain a standard protocol to make PRP according to the present definition. For further study such as therapeutic effect and usage safety, further investigation is encouraged by using both experimental and clinical trials to follow up the therapeutic effects of the PRP in tissues or animal models by investigating the growth factors and the regeneration process from time to time there.

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