Platelet concentration of plateletrich plasma from dogs, obtained through three centrifugation speeds

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Abstract

The platelets release at least 4 growth factors (Platelet Derived Growth Factor. â$_1$ and â$_2$ Transforming Growth Factors and Insulin-like Growth Factor) which are responsible for the migration and activation of cells that will start the reparation of soft tissues and bones. The Platelet Rich Plasma is an autogenous source for Growth Factors, obtained by platelet concentration by centrifuging total blood. This study aimed the comparison of platelet concentrations in plasma centrifuged in three different centrifugation speeds (1300, 1600 e 3200rpm), for the production of platelet rich plasma. Blood was drowned from 15 dogs, 40ml of each, and these were divided into four groups and centrifuged at 800rpm. Then the first group was centrifuged at 1300rpm, the second at 1600rpm, the third at 3200rpm and the last was used as control, named plasma. The mean percentage increase in the platelet concentration for each technique was: 1300 – 183%, 1600 – 210% and 3200 – 222%. But in centrifugation at 3200 rpm, platelets presented altered morphology and different sizes in every sample studied, which was understood as severe cell damage. It was concluded that the best technique for the preparation of the platelet rich plasma in dogs consisted of the previous centrifugation of the blood at 800rpm for ten minutes, and then the plasma should be separated. This plasma is then submitted to a second centrifugation of 1600rpm for 10 minutes, and the platelet poor plasma is separated and discharged.

Key words:
Platelet rich plasma.
Centrifugation speed.
Dogs.

Introduction

The PDCF, platelet derived coagulation factors, stimulate mitosis of medullar cells that will produce FTC- â, the stimulation of endothelial cells starts the angiogenesis and macrophages are attracted to the lesion site. [1,2] They stimulate granulation tissue formation, DNA synthesis, it is a potent fibroblast and osteoblast mitogenous agent and it also raises the synthesis of prostaglandins and bone reabsorption. [3,4] The platelet growth factors act for approximately 8 days. [5]

According to Marx [5], the PRP is an autologous plasma volume that contains a platelet concentration above what is usually found in total blood. The normal ratio of platelets in the human blood ranges around 150000/ìl to 350000/ ìl, the median being 200000/ ìl. He infers that the concentration of 1000000 platelets/ ìl, in 5ml of plasma should be the defined measure called PRP, because it was scientifically proved to ameliorate bone and soft reparation.

Obarrio [6] believes that the platelet concentration in human PRP oscillates between 500.000 a 1.000.000 cells per microliter. Marx et al.´s double centrifugations were at 5600rpm and 2400rpm, and he demonstrated a 338% increase in platelet concentration in the PRP, compared to total blood. [1]

Associated to the bone graft, PRP acts as a support to the bone formation, improving new bone quality and decreasing bone healing time. [7]

The process for the obtention of PRP

should not cause harm to the platelets and it should be done in a sterile and free of pathogens way. [5]

According to Marx[5], to concentrate the platelets of autologous blood, it should be done two centrifugations: the first one intends to separate the red cells from plasma, which contains leukocytes, platelets and coagulation factors, also called, rich plasma. This separation is accomplished because of the different densities of each component. [4] The red cells being the biggest among them, will be at the lower portion, while the plasma will be at the top portion, and it is composed of platelet rich plasma, in the central portion, and platelet poor plasma in the top portion of the plasma. [8] The second centrifugation will cause total separation of the platelets with leukocytes and some erythrocytes from the platelet poor plasma (PPP). [5]

The young platelets are larger, mixing with the top 1mm layer, therefore that layer should be included in the production of PRP. [1]

The separation has to be done in two centrifugations, because the erythrocytes obstruct the separation between PRP and PPP, therefore it does not matter the time or speed of only one centrifugation. [5]

Harvest Company has a cellular separator called "SmartPrep" that uses 45 to 55ml of venous blood. [2] However the equipment used is very expensive, therefore preventing the use of PRP by most of the clinicians. [9]

Kim et al. [10] made an experiment with dogs using PRP. For that he collected 10ml of blood from each animal, separating it in two tubes with 10% trisodium citrate. His first centrifugation was at 1000rpm for 10min, separating the blood in three layers. One milliliter of poor plasma from each tube was discharged and the rest centrifuged again for 1500rpm for 10min. His platelet count in an automatic counter determined 443.000/mm^3 in total blood, and 1.735.000/mm^3 in PRP, an increase of 392% in the concentration.

Rossi Jr et al. [9] proposed a protocol for obtaining PRP using a conventional centrifuge. They collected 4,5ml blood samples from dogs, which were centrifuged at 750rpm for 10min. About 1ml of the top layer was collected. In every PRP sample they found approximately 1.000.000 to 1.200.000 platelets for micro liter.

This study intended to compare platelet concentrations in plasma, submitted to three different speeds of centrifugation (1300, 1600 e 3200rpm), for the production of Platelet Rich Plasma.

Material and Method

Experimental Delineation

Fifteen dogs weighing from 10 to 30 kg, males and females, between 2 and 8 years of age were used. Forty ml of blood were extracted from each dog, in four assay pipes of 10 ml, containing sodium citrate, forming four groups in the experiment. The samples were first centrifuged at 800rpm for 10minutes. After the plasma was separated from the red cells, the first group was used as control, and the others were centrifuged at 1300rpm, 1600rpm and 3200rpm, all for 10minutes. After the second centrifugation, 80% of the top plasma was removed and the rest was mixed with the platelets, forming the PRP.

Procedings for the platelet count in blood and in PRP

Each blood sample was diluted in ammonium oxalate: for the PRP samples, 10 ìl of prp were diluted in 990 ìl of solution, and for the plasma samples, 50 ìl of plasma were diluted in 950 ìl of solution, remaining at rest for 10min, when it was mixed again. Then, 10ìl of the solute was put in each side of a Neubauer Chamber, remaining in a moist chamber for 15 min, before the counting in optic microscope.

Statistical Evaluation and Analysis

The statistical analysis used for this experiment was the t Student that verified possible significant differences between the blood groups of each animal. For each animal, only the mean of two counts was considered.

Results

After counting with the use of optic microscope, the platelet numbers per il of blood were calculated, and are demonstrated in figure 1. In centrifugations 1300 and 1600 rpm the platelets presented expected size and morphology. In centrifugation 3200 rpm, platelets presented altered morphology and different sizes in every sample studied, which was understood as severe cell damage. For a better analysis of the data, it was calculated the difference percentile between platelet levels for the plasma and for platelet rich plasma in different centrifugations, as shown in table 1.

The mean proportions of 1300,1600, 3200 are different in relation to the control, because in the t Student test the statistics were significant, rejecting the hypothesis of equality of the means:

- % mean of group 1300 is different from the % of plasma: Statistics t = 2,12; Probability 0,02;
- % mean of group 1600 is different from the % of plasma: Statistics t = 2,13; Probability 0,02;
- % mean of group 3200 is different from the % of plasma: Statistics t = 3,38; Probability 0,002.

Testing the difference between the concentration percentile means, it was concluded that:

- % mean of group 1300 is different then the % of 1600, Statistics t = -1,40; Probability = 9,1%;

Table 1 - Mean between two countings of the platelet concentration obtained through different centrifugationns: 1300, 1600, 3200 rpm, plasma concentration, and percentual difference between each technique and plasma concentrations. (The plasma being 100%)

Animals	1300 RPM	% of 1300 RPM	1600 RPM	% of 1600 RPM	3200 RPM	% of 3200 RPM	Plasma
1	267.500	158%	305.000	180%	306.250	181%	169.000
2	440.000	190%	437.500	189%	428.750	185%	231.500
3	467.500	240%	491.250	253%	742.500	382%	194.500
4	581.250	123%	1.301.250	275%	733.750	155%	472.500
5	688.750	228%	862.500	286%	843.750	279%	302.000
6	497.500	108%	590.000	128%	640.000	139%	460.000
7	457.500	120%	493.750	129%	570.000	149%	382.000
8	1.076.250	251%	586.250	136%	510.000	119%	429.500
9	707.500	173%	593.750	146%	692.500	170%	408.000
10	831.250	107%	901.250	116%	1.023.750	132%	776.500
11	332.500	686%	430.000	887%	222.500	459%	48.500
12	342.500	99%	256.250	74%	467.500	135%	347.000
13	120.000	45%	218.750	81%	345.000	128%	269.000
14	308.750	157%	382.500	195%	1.123.750	572%	196.500
15	261.250	63%	288.750	70%	575.000	139%	412.500
Mean	492.000	183%	542.583,3	210%	615.000	222%	339.933,3

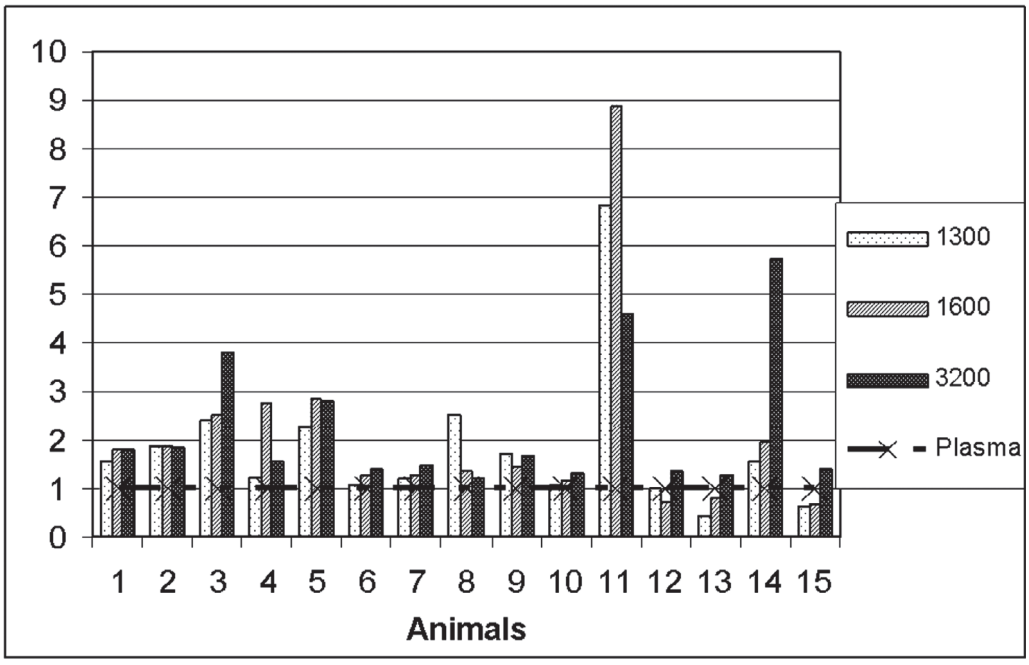

Figure 1 - Proportional values of platelets obtained from the concentrations of different rotation speeds (1300, 1600 and 3200 rpm) compared to the platelet concentration in plasma of each animal. Plasma is equal to 1

- % mean of 1300 is not different then the % of 3200 Statistics t = -1,09; Probability = 14,6%;

- % mean of 1600 is not different then the % of 3200 Statistics t = -0,28; Probability = 38,9%.

As the sample is restricted, we can calculate the trust level at 10%, when it was found a good statistical significance, meaning the mean percentage of 1300 rpm is different then the mean percentage of 1600 rpm, since the probability is 9,1%.

Discussion and Conclusion

Marx [5] defined that the human physiologic platelet count varies between 100.000 and 300.000 per microliter, and a 430.000 per microliter platelet count was observed in dogs, being reasonably larger then what is expected for people. Marx[5] also determined a 1.000.000 platelet count is defined as platelet rich plasma in humans and Obarrio et al. [6] consider this number varies between 500.000 and 1.000.000, but the mean for the different concentrations in dogs was approximately 550.000 platelets for microliter, and in our experiment, this number only exceeded 1.000.000 platelets in four dogs.

Marx [1] demonstrated that the concentration of PRP is 338% larger than normal plasma in humans. We observed an increase of approximately 204% in the concentration in relation to plasma, and these increases were of 180% for 1300rpm, 210% for 1600rpm and 220% for 3200rpm.

The preparation time for the PRP was approximately 25 minutes, which is about the same Marx [1] found for humans: 20 to 30 minutes.

As in Marx [1] papers, for a better analysis of the data obtained, we considered, for statistical purposes, the proportional values of each studied group, being the platelet concentration value in a given PRP was calculated in relation to the platelet concentration of the plasma in the same dog, assuring a more valid analysis of the expected variations for each centrifugation, and not using the absolute value for each animal.

The analysis demonstrated a bigger

difference in the concentration obtained at 3200rpm, in relation to the plasma, but it should be noted that all the samples prepared by this technique showed medium to severe platelet damage, and Marx [5] inferred the process for the obtention of PRP should not cause harm to the platelets. This damage could lead to an apparently high count, but these cells are not capable of releasing the growth factors necessary for tissue repair, so this technique was not considered good, since the use of the PRP obtained was doubtful. The use of an automatic counter was considered inappropriate for this procedure, because the machine cannot differentiate healthy cells from damaged cells, so we chose to count it manually.

Between the two other centrifugations, 1300 and 1600rpm, no severe morphologic changes were noted in the cell, which supposes their competency to release the growth factors. Comparing the results between these two centrifugations, the 1600rpm one had a better concentration in 11 of the 15 samples, also with a trust level of 10%, maintaining a good statistical trust.

The percentage of 1300rpm is different then the percentage of 1600rpm, since the probability is 9.1%, being the 1600rpm centrifugation technique the best one between these two.

It was concluded that the best technique for the preparation of the platelet rich plasma in dogs consists of the previous centrifugation of the blood at 800rpm for ten minutes, and then the plasma is separated and submitted to a second centrifugation of 1600rpm for 10 minutes, and the poor plasma is separated and discharged.

Concentração plaquetária no plasma rico em plaquetas de cães, obtida por três velocidades de centrifugação

Resumo

Plaquetas liberam ao menos quatro fatores de crescimento (Fator de Crescimento derivado de Plaquetas, Fatores de transformação de crescimento â$_1$ and â$_2$ e Fator de crescimento semelhante a insulina) responsáveis pela migração e ativação de células que iniciarão os processos de reparação de tecidos moles e ossos. O Plasma Rico em Plaquetas é fonte autógena de fatores de crescimento, obtida pela concentração das plaquetas através de centrifugação de sangue total. Este estudo visa a comparação das concentrações plaquetárias no plasma obtidas por três diferentes velocidades de centrifugação (1300, 1600 e 3200 rpm), para produção de Plasma Rico em Plaquetas. 40 ml de sangue total foram retirados de cada animal, divididos em quarto grupos, e centrifugados inicialmente a 800 rpm. A seguir, as amostras do primeiro grupo foram centrifugadas a 1300 rpm, as do Segundo a 1600 rpm, as do terceiro a 3200 rpm e as do quarto grupo foram usadas como controle, denominadas plasma. O aumento médio da porcentagem na concentração de plaquetas para cada técnica foi: 1300 - 183%, 1600 - 210% e 3200 - 222%. No entanto, a centrifugação a 3200 rpm, as plaquetas apresentaram a morfologia alterada e tamanhos diferentes em cada amostra estudada, que foram compreendidas como danos celulares severos. Como conclusão deste estudo, obteve-se que a melhor técnica para a preparação do plasma rico em plaquetas de cães consiste na centrifugação precedente do sangue em 800 rpm por dez minutos, separando o plasma, sendo este submetido a uma segunda centrifugação de 1600 rpm por 10 minutos, separando e desprezando o plasma pobre em plaquetas.

Palavras-chave:
Plasma rico em plaquetas.
Velocidade de centrifugação.
Cães.

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