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Cellular components and TGF-β1 content of a closed Tube system for Platelet Rich Plasma acquisition in horse]

[*Componentes celulares e TGF-β1 do plasma rico em plaquetas obtido por sistema fechado em equinos*]

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ABSTRACT

Platelet-rich plasma (PRP) has been proposed as an agent to accelerate the healing process and stimulate the regenerative capacity of tissues due to its abundance of growth factors. A large variety of kits and protocols are available to obtain PRP by different cell-separation systems. However, the lack of standardization may lead to inconsistent results. The aim of this study was to characterize cellular composition, platelet parameters using the ADVIA 120 flow cytometer, and TGF-β1 concentration from the PRP product obtained through a closed system, using simple centrifugation. Six clinically healthy horses were used in this study. The protocol in the closed system resulted in approximately 1.6-fold higher platelet and approximately 2.0-fold lower white blood cell concentrations in comparison with whole blood values. The evaluated system was efficient in concentrating platelets and in retrieving a small number of leukocytes, using a protocol of single centrifugation at low speed.

Keywords: PRP, growth factors, platelet concentrate, concentration protocol

RESUMO

O plasma rico em plaquetas (PRP) tem sido proposto como um agente para acelerar o processo de cicatrização e estimular a capacidade regenerativa dos tecidos, devido à sua abundância de fatores de crescimento. Uma grande variedade de kits *e de protocolos está disponível para obtenção de PRP, utilizando-se diferentes sistemas de separação de células. No entanto, a falta de padronização pode levar a resultados inconsistentes. O objetivo deste estudo foi caracterizar a composição celular, os parâmetros plaquetários pelo citômetro de fluxo ADVIA 120 e a concentração de TGF-β1 do PRP obtido em sistema fechado, por centrifugação simples. Seis cavalos clinicamente saudáveis foram usados neste estudo. O protocolo no sistema fechado resultou em concentrações de plaquetas aproximadamente 1,6 vez maior e concentrações de leucócitos aproximadamente 2,0 vezes menores em comparação com os valores do sangue total. O sistema avaliado foi eficiente na concentração de plaquetas e na recuperação de um pequeno número de leucócitos, utilizando um protocolo de centrifugação única em baixa velocidade.*

Palavras-chave: PRP, fatores de crescimento, concentrado plaquetário, protocolo de centrifugação

INTRODUCTION

Platelet-rich plasma (PRP) is an autologous platelet concentrate proposed to accelerate healing process and to stimulate the regenerative capacity of tissues (Bosh *et al.*, 2010). PRP offers low-cost and easy acquisition of several components, such as fibrin, fibrinogen, leukocytes, mesenchymal cells, and growth factors (GF), most of them found in the platelets

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α-granules. GF are molecules of interest because of their properties, such as their ability in modulating inflammation and tissue repair, production of chemotaxis, cell proliferation and differentiation, angiogenesis, neovascularization, and extracellular matrix deposition (Arguelles *et al.*, 2006; Everts *et al.*, 2006). GF in the PRP include transforming growth factor beta-1 (TGF-β1), growth factor derived from platelets (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) (Weibrich *et al.*, 2002).

PRP has been reported in several different applications in veterinary medicine including osteoarthritis (Carmona *et al.*, 2007), skin lesions (Carter *et al.*, 2003) and tendinitis (Arguelles *et al.*, 2008; Maia *et al.*, 2009; Bosch *et al.*, 2010). However, the results are controversial because of the lack of standardization in the preparation processes, such as time and centrifugation velocity, as well as the ideal anticoagulant needed to maintain platelet parameters. All those factors lead to a PRP product of variable volume, molecular composition and different platelet, leukocyte and other cell type counts (Dohan and Choukroun, 2007; Dohan *et al.,* 2009; Lei *et al.*, 2009; Maia *et al.*, 2009).

Leukocyte function in platelet concentrates remains controversial, considered to influence the clinical outcome of the PRP therapy (Sundman *et al.*, 2011). The presence of leukocytes can provide a local environment at the site of PRP application with increased immunomodulatory capability that may aid in preventing or controlling infection. It is also possible that WBC enhances PRP growth factor concentration through its own release of GF or by stimulating platelets to do so (Castillo *et al.*, 2011). Carmona *et al.* (2011) stated that WBC can act directly in accelerating tissue repair on cartilage explants, due to its immunomodulatory function, thus optimizing GF action. According to Zimmermann *et al.* (2001), WBC concentration in PRP is accounted for one-third to one-half of the variance of GF concentration. Conversely, WBC release catabolic cytokines, matrix metalloproteinases (MMPs) and Interleukin-β (Clutterbuck *et al.*, 2010) that may negatively interfere with tissue recovery by increasing local inflammation. Therefore, WBC might not be a desired component in the PRP samples to me used (Castillo *et al.*, 2011). As an example, infiltration of neutrophils causes release of oxygen free radicals during inflammation and has been shown to play a central role in ischemic tissue injuries. Furthermore, there are studies that have shown their potential detrimental effects in tissue repair (Zimmermann *et al.*, 2003).

The wide size range of platelets, their rapid shape changes, and tendency to clump *in vitro* make their counting less reliable in comparison to other blood cells. Traditional cell counters which use impedance or light scattering contribute to relatively poor results. ADVIA 120® (Bayer Corporation) an automated hematology analyzer measures simultaneously the volume and refractive index or density of platelets, using a cell-by-cell with two angles of laser light scatter. This methodology is more reliable in obtaining accurate platelet counts and allows better discrimination between platelets of all sizes and other particles (Kunicka *et al.*, 2000). ADVIA 120® allows a more accurate platelet count than conventional impedance automated hematology analyzers and provides new parameters, such as mean platelet component (MPC), mean platelet mass (MPM), mean platelet volume (MPV), plateletcrit (PCT), platelet distribution width (PDW), platelet mass distribution width (PMDW), large platelets and aggregates.

MPC has been recently proposed as a valid screening parameter for assessing platelet activation. This parameter represents the average refractive platelet index and is quickly and easily measured by flow cytometry. A linear relationship has been shown between the density of platelets and their degree of activation. This relationship is reduced when platelets degranulate, indicating activation (Ifran *et al.*, 2005). The MPC has showed values inversely proportional to the expression of P-Selectin in dogs (Moritz *et al.*, 2003, 2005) and cats (Zelmanovic and Hetherington, 1998). In a study investigating morphological and phenotypic properties of the equine platelet activation, focused on the expression of CD62 (P-Selectin) and mediated by platelets-leucocytes interaction, was observed that 20% of the resting platelets had constitutive expression of P-Selectin. This might be due to earlier leukocyte stimuli and complicates the evaluation of the activation using this specific marker (Lalko *et al.*, 2003). Segura

et al. (2007), evaluating foals and adult horses with disseminated intravascular coagulation (pathologic condition that activates platelets), observed that MPC may be used to detect activated platelets in sick animals but stated that further studies are necessary.

In a human PRP study authors concluded that the quality of PRP is closely related to the type of anticoagulant used (Pignatelli *et al.*, 1996), because avoids spontaneous activation of platelets (Macey *et al.*, 2002). The microstructure of platelets collected with heparin, citrate, acid citrate dextrose (ACD) and citrate-theophylline-adenosine-dipyridamole (CTAD) was observed. ACD was proved to be better than heparin and sodium citrate (SC) to produce better quality PRP, maintaining the integrity of platelet structures and preventing the platelet spontaneous activation (Lei *et al.*, 2009). Giraldo *et al.* (2015), on the other hand, showed no difference between ACD-A, ACD-B and SC regarding platelet count and GF's release. Miranda *et al.* (2018), while studying the effect of SC and ACD on equids' PRP procurement, concluded that SC was better in concentrating platelets using a single-spin protocol when compared to ACD. No correlation between the numbers of platelet concentrates and regenerative effect was demonstrated thus far, but the key to successful PRP therapy is associated with the use of live platelets that are not activated during sampling and centrifugation processes (Marx, 2004; Carmona and Lopez, 2011).

In the present investigation, a new method and protocol to obtain PRP in horses using simple centrifugation was evaluated. Two anticoagulants (ACD and SC) were compared using the same speed centrifugation protocol. The hypothesis is that the ACD is better for equine platelets than SC because it has a higher source of carbon (dextrose), thus providing a substrate for platelet metabolism. It is also assumed that low speed and single spin centrifugation cause less platelet activation and platelet recovery can be of approximately twice the value of that in whole blood, recovering only a few leukocytes.

MATERIAL AND METHODS

This study was approved by the Ethical committee in animal research (CEUA/UFMG n. 033/11) and by the Institutional Committee for Care and Use of Animals of the Autonomous University of Barcelona, Spain. Blood analysis was performed in the Service of Hematology Department, at the School of Veterinary Medicine located in the *Universitat Autònoma de Barcelona*.

For this study, six clinically healthy horses of different breeds were used, five females and one male, with ages ranging from six to 14 years.

Whole blood was aseptically extracted by jugular venipuncture using a vacuum blood collection system with a 21G butterfly catheter (Venofix®, Melsungen, Germany) and were processed within 15 minutes after blood collection. To perform hemogram, blood was collected in an EDTA vacuum tube (Aquisel ®, Barcelona, Spain).

Blood samples were collected in four syringes of 10mL capacity, containing 1mL of an anticoagulant: two containing ACD (trisodium citrate – 22g/L, citric acid – 8g/L and dextrose 24.5g/L) (Venosafe, Leuven, Belgium) and two containing sodium citrate- SC (BD, Madrid, Spain). Once collected, samples were transferred to a 20 mL Proteal® tube according to manufacturer instructions. A 0.22 uM-filtered needle was first placed in one of the injection ports of the embolus (Fig. 1A), aiming to allow the internal-external pressure equilibrium. Then, the blood sample was introduced in the tube using a second needle port of the embolus (Fig. 1B).

One tube from each anticoagulant (ACD and CS) were centrifuged at 133g for 8 minutes and the other tube from each anticoagulant at 360g for 8 minutes. The centrifuge allows a slow deceleration for 2 minutes, to prevent re-mixed cells. After centrifugation, the lower 5 mL plasmatic fraction was recovered using the Push.Out® system, a closed mechanism that allows the selection of specific blood centrifuged fractions directly using luer-lock syringes (Fig. 1C).

Once the PRP was selected, a 0.5 mL aliquot was obtained for platelet analysis by flow cytometry (ADVIA 120® -Bayer Corporation). Another 1.0 mL sample was collected for platelet activation evaluation. The remaining PRP was centrifuged at 9000 rpm for 10 minutes to pellet the platelets. The supernatant was collected and immediately frozen for later determination of TGF-β1 in the *Servei di Cultius Cellulars, Producció d'Anticossos i Citometria (SCAC)* in *Instituti de Biotecnologia I de Biomedicina* of the *Universitat Autònoma de Barcelona*.

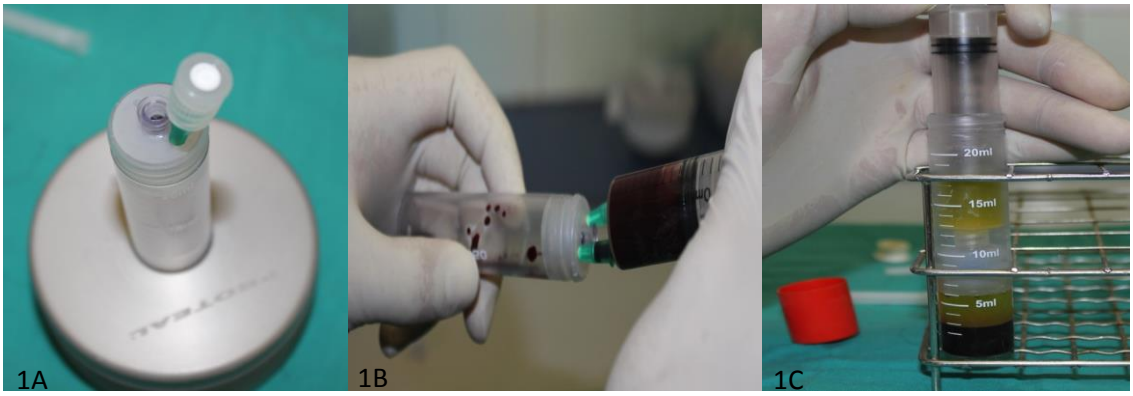

Figure 1. Closed system used in the preparation of platelet rich plasma. 1A: insertion of a 0.22uM filter needle in the embolus port; 1B: transference of blood into the Proteal® tube; 1C: extraction of PRP by coupling a syringe and using the Push.Out® system.

A complete blood count of each sample was performed using the ADVIA 120® analyzer (Bayer Lab, NY, USA). RBC, WBC, Platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), mean platelet component (MPC), platelet distribution width (PDW), mean platelet mass (MPM), platelet mass distribution width (PMDW) and large platelets and aggregates were measured. Calculation of centrifugation efficiency (Ce) was based on the formula: Ce = Platelet final concentration/platelet initial concentration.

The quantification of TGF-β1 in plasma was performed using a sandwich ELISA, using commercial antibodies from R & D Systems (DuoSet ref. DY240, R&D Systems). Mouse anti-human TGF-β1 was used as capture antibody and biotinylated chicken anti-human TGF-β1 as a detection antibody. Standards for human recombinant TGF-β1 were employed for the calibration curve as previous studies have demonstrated and validated the cross-reactivity of these antibodies with the equine specific antigen (Arguelles *et al.*, 2006).

Quantification of TGF-β1 by ELISA requires a previous release of the amino-terminal propeptides (latency-associated peptides) and activation of the binding protein joined by disulfide bonds, thus forming TGF-β1 complex inactive secreting mechanism. This activation of TGF-β1 was performed *in vitro* by adding 100µL of activation buffer [2.5N acetic acid (Ref. 010,520, Probus, Spain)], 10M urea (Ref. U-5128, Sigma, Barcelona, Spain) added to the same volume of plasma, and then mixed and incubated at room temperature for 10 min. The pH of the sample was neutralized with 100µL of neutralization buffer [2.7M NaOH (Ref. 141687.1211 Panreac, Spain)]/1M HEPES (ref. H3375, Sigma-Aldrich, Barcelona, Spain). For the test, different dilutions of the activated samples with dilution buffer (1.4% bovine serum albumin, Ref 10735078001, Roche, Spain), 0.05% Tween20 (Ref. 170-6531, Bio-Rad, Spain) in PBS were used.
Growth factor concentration efficiency (GFCe) was established by the formula: GFC= growth factor final concentration/growth factor initial concentration.

All data were analyzed by a commercial software (SPSS Inc., Illinois, USA). Data are expressed as mean ± standard deviation. Statistical comparisons were analyzed using analysis of variance (ANOVA) and *post-hoc* comparisons were performed using the Tukey test to

determine differences between media that present a normal distribution, including variables that showed normal distribution after log transformation (logX+1). For variables that were not normally distributed, even after log transformation, Kruskal-Wallis analysis and *post-hoc* comparisons between each groups using the Wilcoxon test were performed. Statistical significance was accepted as p values <0.05.

RESULTS

A higher platelet concentration was observed when using ACD at 133g for 8 minutes. Regardless of the anticoagulant employed in this protocol, its parameters were significantly different in comparison to the 360g protocol. It was observed an increase of 1.59 times baseline platelet count.

There was no significant difference (p>0.05) between the protocols for MPV, MPC and clumps, but both centrifugations' protocols differed (p<0.05) from whole blood (EDTA samples).

The concentration of leukocytes decreased as the centrifugation speed increased. PRP and whole blood cell count are presented in Table 1.

TGF-β1 analysis showed no significant differences between protocols (p>0.05). However, the lower speed protocols showed higher TGF-β1 concentration, as well as platelet count. TGF-β1 values are presented in Table 2.

Table 1. Average related values (X) and standard deviation (SD) of hematologic variables in samples of whole blood and PRP obtained from six healthy horses using different centrifugation protocols and anticoagulants

VARIABLES	EDTA		133g/8' CS		133g/8' ACD		360g/8' CS		360g/8' ACD	
	X	SD	X	SD	X	SD	X	SD	X	SD
PLT (10^3/µL)	165.50^{BC}	65.52	245.00^{AB}	45.27	262.50^{A}	44.84	131.67^{C}	48.88	155.83^{C}	54.91
MPV (fL)	8.10^{B}	0.94	12.27^{A}	1.88	11.47^{A}	0.98	11.55^{A}	1.79	11.62^{A}	1.342
MPC (g/dL)	23.53^{A}	3.47	18.05^{B}	2.34	18.15^{B}	1.25	17.62^{B}	2.41	17.47^{B}	1.73
PCDW (g/dL)	8.45	1.242	7.87	0.60	7.58	0.279	7.75	0.59	7.40	0.49
MPM (pg)	1.86^{A}	0.085	1.81^{AB}	0.082	1.80^{AB}	0.085	1.66^{C}	0.05	1.68^{BC}	0.05
PMDW (pg) (10^3/µL)	0.63^{A}	0.04	0.57^{AB}	0.034	0.56^{BC}	0.043	0.51^{C}	0.03	0.51^{C}	0.03
Large PLT (10^3/µL)	11.50^{B}	7.74	30.00^{A}	14.792	27.33^{AB}	12.64	12.33^{B}	5.43	14.17^{AB}	4.96
Clumps	1327.17^{A}	1620.87	38.0^{B}	14.46	41.67^{B}	8.48	115.33^{B}	196.65	41.0^{B}	23.07
RBC frag (10^6/µL)	7.71^{A}	0.89	0.04^{B}	0.01	0.04^{B}	0.01	0.02^{C}	0.01	0.02^{C}	0.01
WBC (10^3/µL)	7.77^{A}	2.19	0.09^{B}	0.09	0.07^{B}	0.04	0.01^{C}	0	0.02^{C}	0.01
Neut. (10^3/µL)	5.3^{A}	2.26	0.02^{B}	0.01	0.02^{B}	0.01	0.01^{C}	0	0.01^{BC}	0.01

EDTA- ethylenediaminetetraacetic acid; SC, sodium citrate, ACD, acid citrate dextrose, PLT, platelet; MPV, mean platelet volume, MPC; mean platelet component concentration; PCDW; platelet component distribution width; MPM mean platelet mass; PMDW platelet mass distribution width; RBC-frag, red blood cell fragments; WBC, white blood cell; NEUT, neutrophil; Means within rows with different letters are significant different (p<0.05).

Table 2. Average values and standard deviation of transforming growth factor (TGF) -β1 in platelet rich plasma from six healthy horses using different centrifugation protocols and anticoagulants.

Variable	133g/8' SC		133g/8' ACD		360g/8' SC		360g/8' ACD	
	X	SD	X	SD	X	SD	X	SD
PLT	245.0^{AB}	45.3	262.5^{A}	44.8	131.7^{BC}	48.9	155.8^{BC}	54.9
TGF-β1 (ng/mL)	3942.9	1762.3	3911.5	1810.9	2864.2	744.0	2372.4	1492.8
TP (mg/mL)	370.4	258.0	477.9	117.1	394.0	139.3	319.2	238.9
TGF-β1 (ng/mL TP)	9.7	4.4	14.0	4.7	13.8	3.2	10.9	5.6

SC, sodium citrate, ACD, acid citrate dextrose, PLT, platelet; TGF-β, transforming growth factor beta; TP, total protein; CFC: Growth factor concentration efficiency. Means within rows with different letters are significant different (p<0.05).

DISCUSSION

More than 16 platelet separation systems are currently available and produce different types of platelet-rich concentrates, significantly different in the relative amounts of platelets, leukocytes, erythrocytes, and anabolic and catabolic GF (Wasterlain *et al.*, 2012). A PRP containing less than a 2-fold increase in platelet concentration can be obtained by a simple centrifugation protocol which allows to collect all the supernatant plasma (Tamimi *et al.*, 2007). In the system evaluated in this study, approximately a 1.6-time platelet concentration - when compared

to whole blood - was achieved, using the 133g for 8 minutes protocol, thus supporting one of our hypothesis that single-spin centrifugation can concentrate a good platelets count. The main goal is to capture only platelets using a slower and shorter centrifugation spin regime. This yields platelet concentration typically around 2-3 times from the baseline whole blood levels (DeLong *et al.*, 2011).

According to Mazzocca *et al.* (2012) the "more is better" theory for the use of higher platelet concentration cannot be supported, since the optimal concentration depends on the cell type in which proliferation is desired. The protocols using double centrifugation can concentrate more platelets (Nagata *et al.*, 2010), however, with a simple centrifugation system we had equivalent results (Arguelles *et al.*, 2006). Simple centrifugation could concentrate platelets 1.4-time, whereas using double centrifugation, a 1.7-time concentration was achieved. Kisiday *et al.* (2012) evaluated the effect of PRP obtained with single and double spin centrifugation on anabolic and catabolic activity in meniscus cartilage explants and suggested a careful use of the double spin PRP protocol, because of the higher concentrations of white blood cells.

Since the regenerative effect of PRP depends on the level of GF released, the integrity of platelets is crucial. If the platelets are activated during the PRP production process, GF in platelets will be released into plasma (most of which is discarded during preparation), which will consequently decrease the local therapeutic efficacy of PRP (Arguelles *et al.*, 2006). Therefore, less platelet activation and more GF retention in platelets are the key factors to improve the potency of therapeutic PRP effects. Suggested parameters that may be related to evaluation of platelet activation (Mazzocca *et al.*, 2012), like MPC and MPV, did not differed between tested protocols on the present study, which can be explained by action of the anticoagulant used, although increase in large platelets, as activation evidence was most common seen in sodium citrate samples. Even if one can consider the small number of samples, clumps were more observed on EDTA samples. This information together evidences the superiority of ACD to SC as anticoagulant in the maintenance of platelet integrity.

In a study evaluating PRP anabolic and catabolic activities, with different PRP cellular compositions, it has been suggested that higher platelet concentrations (obtained from double centrifugation), caused a pro-inflammatory effect, resulting in a diminished biosynthesis of extracellular matrix (Kisiday *et al.*, 2012). Based on this data, the authors suggested that a simple centrifugation method is more appropriate to obtain PRP to be used in intra-articular therapies. The kit tested on this present study significantly decreased the WBC count, assuming that PRP is appropriate for intra-articular use, especially in ACD samples that rescued less WBC. Arguelles *et al.* (2006) also observed low levels of WBC, using simple centrifugation, and hypothesized that PRP's different compositions may lead to its use in different tissues, aiming their several characteristics on healing processes.

The ideal number of platelets to be injected in a lesion have not yet been defined and may vary according to the tissue type and number of receptors available for GF's - without hyper saturation - thus inducing a positive response on cellular proliferation, assured by absence of a negative feedback. However, only a few comparative studies on healing effects based on the PRP platelet concentration have been done, especially in veterinary medicine (DeLong *et al.*, 2011).

TGF-β1 values obtained by Arguelles *et al.* (2006), who performed prior platelet activation, were higher on both samples from single and dual spin centrifugation. There was no difference ($p>0.05$) in TGF-β obtained between the protocols here evaluated. However, Dugrillon *et al.* (2002) reported that the number of platelets is not always proportional to the amount of GF and more attention should be given to GF quality than to the concentration of platelet in the PRP. Platelets can be premature activated by excessive manipulation and pipetting, speed versus time of centrifugation, and use of glass instruments, which can lead to the prior release of GF and determine a less tissue effect (Schmitz *et al.*, 1998).

CONCLUSION

Simple closed centrifugation at lower speed protocol is more efficient in concentrating platelets as well as retrieving a small number of

leukocytes. ACD is better than SC in the platelet recover count, less platelet activation, and clumps formation. ACD and SC can be employed for the PRP- GF obtainment in lower speed centrifugation. MPC and MPV must be performed to best monitor platelet integrity assuming better therapeutical results.

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