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Influencing factors for preparation of platelet-rich plasma in horses

Fatores que influenciam o preparo do plasma rico em plaquetas na espécie equina

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Highlights

Both protocols tested were able to sufficiently concentrate platelets.
The protocol with the higher rotational force and the longer time produced better PRP.
Other variables interfere directly with the replicability of the technique.

Abstract

There is no consensus in the literature as to the best platelet-rich plasma (PRP) acquisition method or the ideal platelet concentration to stimulate tissue repair. Besides that, most studies do not provide a clear and replicable description of the methods used, which makes standardization and result comparison difficult. Thus, this study aimed to accurately describe the method and evaluate factors influencing PRP preparation in equines. In this regard, two protocols were used, P1 and P2, based on two centrifugation methods, which differed in blood volume, speed and time, and platelet pipetting and resuspension. In conclusion, factors such as centrifugation strength and time are essential to obtain PRP with adequate platelet count. However, other factors such as tube type and plasma pipetting and resuspension methods directly interfere with the replicability of the technique, and hence influencing PRP applicability.

Key words: Platelet concentration. Double centrifugation. Standardization.

Resumo

Não há consenso na literatura quanto ao melhor método de obtenção do plasma rico em plaquetas (PRP) ou à concentração plaquetária ideal com o objetivo de estimular o reparo tecidual. Além disso, grande parte

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dos trabalhos não faz descrição clara e reprodutível dos métodos utilizados, o que dificulta a padronização e a comparação dos resultados. Dessa forma, este trabalho teve por objetivo descrever detalhadamente a metodologia e avaliar fatores que influenciam o preparo do PRP em equinos. Para isso, foram utilizados dois protocolos, P1 e P2, baseados em duas centrifugações, que diferiram quanto ao volume de sangue colhido, velocidade e tempo das centrifugações e método de pipetagem e ressuspensão das plaquetas. Conclui-se que fatores como força e tempo de centrifugação são essenciais à obtenção de um PRP com concentração plaquetária adequada. Contudo, fatores adicionais como tipo de tubo e métodos de pipetagem e ressuspensão do plasma interferem diretamente na repetibilidade da técnica, influenciando a aplicabilidade prática do PRP.
Palavras-chave: Concentração plaquetária. Dupla centrifugação. Padronização.

Introduction

Platelets are small discoid blood cells formed from megakaryocytes in the bone marrow and have two types of intracellular granules. Dense granules store adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and calcium, while α-granules store coagulation factors, growth factors and other proteins (Zucker-Franklin, 1996). At latency, platelets are not thrombogenic and require stimulation for activation, which can occur from their contact with collagen. Once activated, they morphologically alter and develop pseudopods, which promote platelet aggregation and granule content release (Everts et al., 2006). These cells play a primordial role in haemostasis, in addition to being considered sentinel immune cells that assist initiation and organization of inflammatory events (Herter, Rossaint, & Zarbock, 2014).

Plasma rich in platelets (PRP) is an autologous plasma preparation, enriched with a higher platelet concentration than that of the whole blood (Malanga & Goldin, 2014). Most PRP acquisition methods are based in one or two centrifugations of autologous blood collected with anticoagulant (Davis et al., 2014). Its therapeutic potential relies on platelet capability of releasing and supplying supraphysiological amounts of growth factors and cytokines that, in turn, promote regenerative stimulus and help in tissue repair by scarring (Peter, Wu, Diaz, & Borg-Stein, 2016).

The first reports of PRP clinical use date from the 1980s, in cardiothoracic and maxillofacial surgeries (Ferrari et al., 1987). Currently, it has been reported in other fields such as plastic surgery, orthopaedics, and sports medicine (Nguyen, Borg-Stein, & McInnis, 2011). In animal models, PRP has been used in studies involving cornea neovascularization, conjunctive tissue regeneration, and surgical wounds reepitalization (Lima, 2009). In equines, PRP has been researched since the beginning of 2000 in wound treatments, tendinitis, desmitis, osteoarthritis, and fracture consolidation (Pereira et al., 2013).

PRP acquisition methods consist of whole blood centrifugation, aiming to concentrate platelets and reduce red blood cells and leukocytes (Sampson, Gerhardt, & Mandelbaum, 2008). There is vast literature about PRP; however, studies are distinct

regarding the applied method in its acquisition. Thus, there is difficulty in creating technical standards in new studies and result comparison (DeLong, Russell, & Mazzocca, 2012). Two main challenges for PRP standardization are the individual peripheral blood variation concerning platelet concentration and growth factors, and the several preparation stages (Dragoo, Wasterlain, Braun, & Nead, 2014).

Most studies describe PRP acquisition method only in relation to the number and time of centrifugations, while the g-force, which is the force exerted on spinner rotor, is calculated based on its radius and spin speed. Details regarding blood collection, sample conditioning, and plasma pipetting after centrifugation are almost always omitted. In a review of 105 clinical studies on PRP use in musculoskeletal disorders treatment, only 11 (10%) provided a clear and replicable protocol description, and 17 (16%) provided quantitative measurements of the final product composition (Chahla et al., 2017). Thus, given the inconsistency in PRP preparation methods, there is no consensus on the best protocol. In this context, this study aimed to describe the method in detail and to evaluate factors influencing PRP preparation in equines.

Material and Methods

All procedures involving animals in this study were approved by the Animal Use Ethics Committee of the Federal University of Goiás, CEUA/UFG (protocol number 074/2016). Twenty-one healthy horses, of different breeds, aged between three and 18 years old, and with an average weight of 350 kg were used. Two protocols (P1 and P2) were tested to obtain PRP, both adapted from Carmona (2006).

For P1, samples of blood from eight animals were aseptically collected by jugular puncture with a 21G needle and vacutainer glass tubes containing 3.2% sodium citrate (BD®). Five 4.5-mL tubes were filled, totalling 22.5 mL blood from each animal. The tubes were homogenized and, after 10 minutes, a period for natural red blood cell sedimentation, were centrifuged for one minute, at low rotation (900 rpm). Plasma remaining in the upper portion of tubes was transferred to a single 10-mL non-anticoagulant glass tube. The transfer was performed with the aid of an automatic pipette with a minimum capacity of 100 µL and a maximum of 1000 µL and a plastic tip with equivalent capacity, being careful not to capture buffy coat zone cells, whitish layer between red blood cells and plasma. Pipetting was always performed by the same operator. The plasma containing tube was centrifuged again for five minutes at 1800 rpm. Then, about 60% of the upper plasma, defined as platelet-poor plasma (PPP), was pipetted and discarded. The remaining portion of the PRP tube was protected from light and kept for 30 minutes in an automatic haematological homogenizer (Nihon Kohden Celltac Alpha®) for platelet counting.

For protocol P2, samples of blood from thirteen animals were collected aseptically by jugular puncture with 21G needle and vacutainer glass tubes containing 3.2% sodium citrate (BD®). Twenty-four 4.5-mL tubes were filled, totalling 108 mL blood from each animal. Tubes were homogenized manually until their first centrifugation, performed at 1000 rpm for 10 minutes. In a laminar flow fume hood, with the aid of a 14G peripheral intravenous catheter and 10 mL disposable syringe, the supernatant plasma was collected. At this stage, the tube wall

was gently scraped with the catheter needle while aspirating with the syringe to collect platelets that might have adhered there after the first centrifugation. Supernatant plasma was collected and transferred to 10-mL non-anticoagulant plastic tubes (Biocon®), being careful not to capture cells from the buffy coat zone. Plasma transfer was always performed by the same operator. Then, the new tubes were centrifuged at 4000 rpm for 10 minutes. After the second centrifugation, about 80% of plasma was discarded (PPP) and the remaining fraction (PRP) was homogenized for 30 seconds in a vortex shaker and transferred to a sterile syringe with an 18G spinal needle. A fraction of the final product was reserved for platelet counting.

In both protocols, a fraction of the whole blood was separated to obtain baseline platelet values, red blood cells, and leukocytes. Whole blood and PRP platelet counts obtained in P1 and P2 were performed on an automated haematology analyser (Nihon Kohden Celltac Alpha®).

Statistical analysis was conducted in a quantitative form, calculating means and standard deviations of the platelet concentrations acquired in P1 and P2. Thereafter, the t-Student test was performed to compare means between groups. Statistical difference was considered when $p<0.05$. Data were analysed using the Excel software.

Results and discussion

PRP production is based on two principles, including the formation of a density gradient of blood constituents by centrifugation, and creation of a product with altered blood component concentrations by selectively collecting a fraction of the density gradient previously formed (Delong et al., 2011). Although most studies describe PRP acquisition as of easy execution and replicability (Pereira et al., 2013), our experience in this study is in accordance with Michelson (2007), who stated that the preparation of this product seems to be considerably delicate and dependent on a series of variables.

Both protocols were able to sufficiently concentrate platelets. However, in P1, this result was achieved in only one of eight animals in the first preparation attempt. In five animals, two attempts were necessary, and in the other two animals, three attempts were necessary to recover a minimum platelet increment of two times. In protocol P2, platelet concentration was recovered in the first attempt in all animals.

Whole blood and PRP platelet count from protocols P1 and P2 are described in Table 1. Platelet increment was calculated by the ratio between PRP platelet count and the whole blood platelet count of the same animal. Thus, the number of times the PRP platelet concentration was higher than that of whole blood was obtained.

Table 1
Platelet counts (x 10^3/µl) of whole blood (WB) and platelet-rich plasma (PRP) recovered by protocols P1 and P2, with their respective platelet increments in relation to the basal value of each animal

Protocol 1				Protocol 1			
Animal	WB	PRP	Platelet increment	Animal	WB	PRP	Platelet increment
P1A1	103	326	3,17	P2A1	178	713	4,01
P1A2	120	325	2,71	P2A2	94	501	5,33
P1A3	125	411	3,29	P2A3	186	1241	6,67
P1A4	132	564	4,27	P2A4	89	1171	13,16
P1A5	161	514	3,19	P2A5	133	931	7,00
P1A6	100	412	4,12	P2A6	143	389	2,72
P1A7	114	349	3,06	P2A7	167	569	3,41
P1A8	132	270	2,05	P2A8	200	410	2,05
				P2A9	196	380	1,94
				P2A10	104	1033	9,93
				P2A11	139	579	4,17
				P2A12	107	309	2,89
				P2A13	108	409	3,79

P1 and P2 protocols recovered plasma with concentrated platelets, on average 3.24- and 5.16-times above baseline, respectively. P1 resulted in greater uniformity between the products recovered from different animals (standard deviation = 0.67), while P2 recovered PRP with great variation between individuals (standard deviation = 3.17). P2 protocol recovered, on average, a higher platelet concentration, although both protocols are statistically similar (Table 2).

Tube material and needle calibre used in blood collection seem to influence PRP. According to some authors, glass tubes coated with silicone are better than polypropylene tubes. On the other hand, when not coated with silicone, glass tubes can prematurely activate platelets, influencing the final platelet count (Michelson, 2007). However, in this study, there was no change in final platelet count when using glass tubes not coated with silicone, which might have been due to the scraping of tube wall while plasma pipetting after the first centrifugation. Yet, in previous experiments, we did not obtain satisfactory results with polypropylene tubes, presenting platelet aggregation and decrease in platelet numbers in the final counting. Regarding needle calibre, the use of needles with calibres above 22G (Dhurat & Sukesh, 2014) has been prescribed in the literature, which was performed with success in both tested protocols in our study.

Table 2
Mean, variance, standard deviation, and PRP platelet increment statistical analysis recovered from protocols P1 and P2, in relation to the baseline value

	P1	P2
Mean	3.24a	5.16a
Variance	0.44	10.90
Standard deviation	0.67	3.17

Different letters within the same line differ from each other by the Student's t-test (P <0. 05).

The number and speed of centrifugations directly influence final platelet concentration and can result in morphological changes in platelets, which leads to their aggregation (Michelson, 2007). Researchers compared seven protocols for PRP acquisition in equine species and concluded that the higher the g-force and centrifugation time, the higher the final product platelet concentration (Pereira et al., 2013). This information corroborates the results of the current study, in which the protocol with the higher rotational force for a longer time, in the two centrifugations (P2), acquired PRP with higher platelet concentrations, on average.

According to the literature, changes in basal blood platelet counts of each individual also influences the final platelet concentration in PRP (Michelson, 2007; Vendruscolo et al., 2012). However, our results show a lack of positive correlation between basal platelet counts and PRP platelet counts, since even individuals with lower baseline values produced PRP with high platelet concentrations when using P1 and P2 protocols.

A variable that seems extremely important but overlooked in PRP protocols is plasma pipetting process after the first blood centrifugation. Most protocols recommend total plasma volume transfer immediately above the buffy coat zone to a new tube (Vendruscolo et al., 2012; Pereira et al., 2013); however, a more detailed description is still required. In this context, this study found that plasma transfer using an automatic pipette and plastic tip, as performed in P1, does not allow total plasma removal without contamination by leukocyte and red blood cells. On the other hand, in P2, when the transfer was performed with the aid of a 14G peripheral intravenous catheter needle and a 10-mL syringe, plasma withdrawal was of higher precision and lower contamination index. Besides, some platelet aggregates were deposited on the tube wall after the first centrifugation, and by scraping with the catheter needle, these aggregates were again diluted in plasma.

The expected therapeutic effect of PRP is based on the release of growth factors by platelets and their binding to their respective receptors without saturation, triggering proliferative tissue response (Delong et al., 2011). Based on the studies available in the literature, it is inferred that a two to fourfold increase in platelet numbers in PRP relative to whole blood is suitable (Marx et al., 1998; Marx, 2001; Anitua et al., 2005; Graziani et al., 2006; Sánchez et al., 2007; Anitua et al., 2008; Sánchez et al., 2010; Redler, Thompson, Hsu, Ahmad, & Levine, 2011; Torricelli et al.,

2011). Conversely, increases of less than two times do not allow expected cellular response (Haynesworth, Kadiyala, Liang, & Bruder, 2002), while increases of four to six times produce controversial results (Haynesworth et al., 2002; Schlegel, Kloss, Schultze-Mosgau, Neukam, & Wiltfang, 2003; Giusti et al., 2009; Broggini et al., 2011). However, increases over six times can lead to apoptosis, reduction in growth factor receptors and their desensitization (Gruber, Varga, Fischer, & Watzek, 2002; Haynesworth et al., 2002; Weibrich, Hansen, Kleis, Buch, & Hitzler, 2004; Choi et al., 2005).

In this regard, the two protocols tested in this study were able to concentrate platelets in the final product. In P1, however, the concentration was obtained in most animals after two to three attempts, and in P2, it was obtained always at first. Moreover, the mean platelet count obtained in P2 was higher than in P1, and even within the controversial range (5.16 times above baseline), there is dilution possibility, final platelet concentration adjustment, and PRP use from platelet-poor plasma (PPP) (Fusegawa, Goto, Handa, Kawada, & Ando, 1999; Cattaneo, Lecchi, Zighetti, & Lussana, 2007; Ljungkvist, Olofsson, Funding, Berntorp, & Zetterberg, 2019), while non-concentrated P1 samples had to be discarded.

Changes in platelet count methods also represent a problem for standardization of PRP acquisition method and comparison between studies. Most studies use automatic haematology counters to perform blood component counts; however, it should be taken into account that when there are platelet aggregates, the equipment considers the aggregate as a single platelet. Thus, it is recommended that the sample be agitated in a haematological tube homogenizer for at least five minutes, immediately before counting, so that platelets are suspended and disaggregated (Woodell-May, Ridderman, Swift, & Higgins, 2005). In this study, P2 samples were homogenized in a vortex shaker for 30 seconds and P1 samples in a haematological homogenizer for 30 minutes, which might have improved resuspension in a shorter time and reflected higher P2 platelet concentration than that of P1, having the highest homogenizing power of the vortex stirrer.

Conclusion

Factors such as centrifugation force and time are essential to obtain PRP with a suitable platelet concentration. However, other factors such as tube type and plasma pipetting and resuspension methods interfere directly with the replicability of the technique, influencing PRP applicability.

Bioethics and biossecurity committee approval

All procedures, treatments and animal care were in compliance with the Ethics Committee on Animal Use of the Federal University of Goiás (CEUA/UFG) (process 074/2016).

Declaration of conflicting interests

We have no conflict of interest to declare.

Authors' Contributions

Ana Carolina Barros da Rosa Pedroso: responsible for conducting the experiment,

obtaining and analyzing the data and writing the article. Andréia da Costa Peixoto: responsible for conducting the experiment, obtaining and analyzing the data. Evelyn de Oliveira: responsible for conducting the laboratorial analysis of the experiment. Helena Tavares Dutra: responsible for conducting the experiment, obtaining and analyzing the data. Roberta Carvalho Basile: responsible for the elaboration of methodology and review of the article. Luciana Ramos Gaston Brandstetter: responsible for guidance during experiment and review of article. Veridiana Maria Brianezi Dignani de Moura: responsible for guidance during experiment, analysis of the microscopic variables and review of article.

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