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Effects of breed, age and gender on equine platelet rich plasma and correlation of platelet count with its physical aspect

[*Efeitos de raça, idade e gênero sobre o plasma rico em plaquetas em equinos e correlação da contagem plaquetária com seu aspecto físico*]

L.B. *Paz*[1], A.A. *Beck Júnior*[1], A.M. *Engelmann*[1], J.V.G. *Mucha*[1], M.I. *Frank*[2], R.C.F. *Pereira*[3], A. *Krause*[3], F.D. *De La Côrte*[3]

[1]Graduate, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Undergraduate, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[3]Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

ABSTRACT

Platelet rich plasma samples from 50 healthy horses of five different breeds (Thoroughbreds – TB, Brazilian Criollo Horses – BCH, Brazilian Sport Horses – BSH, Miniature Horses – MH and Crossbred Horses - CB), were investigated as to breed, age, and gender effect for platelet concentration. Moreover, a score for physical analysis was established to correlate PRP physical aspect with platelet count. Platelet count was performed by an automatic hematology analyzer and by manual count. PRP physical analysis was based on color, aspect, and capacity to separate blood components. MH showed significant higher platelet concentration than BSH ($p<0.05$), while the other breed comparisons showed no significant difference. There was no significant difference for gender but there was a weak correlation of age with PRP platelet concentrations ($r_s = -0.24$). Most of the PRP presented yellow color, the separation of blood components showed no correlation, but the aspect showed a moderate correlation ($r_s = 0.30$) with platelet count. Results suggest that PRP platelet concentration can be influenced by intrinsic factors such as breed. Additionally, the analysis of PRP aspect can help to evaluate the quality of the product when there is no access to platelet counts.

Keywords: PRP, horses, intrinsic factors, physical analysis

RESUMO

Amostras de plasma rico em plaquetas de 50 cavalos saudáveis, de cinco raças diferentes (Puro Sangue de Corrida, Crioula, Brasileiro de Hipismo, Pônei Brasileiro e sem raça definida), foram investigadas quanto à concentração plaquetária em relação à raça, à idade e ao sexo. Além disso, um escore foi estabelecido para correlacionar o aspecto físico do PRP com a contagem plaquetária. A contagem celular foi realizada por um analisador hematológico automático e pelo método manual. A análise física baseou-se na cor, no aspecto e na capacidade de separar hemocomponentes. Pôneis tiveram concentração plaquetária significativamente maior que os Brasileiros de Hipismo ($P<0,05$), mas as demais comparações entre raças não apresentaram diferença quanto à contagem plaquetária. Não houve diferença para o gênero, mas a idade demonstrou correlação fraca com as concentrações plaquetárias do PRP ($r_s = -0,24$). A maioria dos PRPs apresentaram coloração amarela, e a separação dos hemocomponentes não mostrou correlação com a contagem plaquetária, mas o aspecto apresentou uma correlação moderada ($r_s = 0,30$). Os resultados deste estudo sugerem que a concentração plaquetária do PRP pode ser influenciada por fatores intrínsecos, como a raça. Além disso, a análise do aspecto do PRP pode ajudar na avaliação da qualidade do produto quando não há acesso à contagem plaquetária.

Palavras-chave: PRP, cavalos, fatores intrínsecos, análise física

Corresponding author: leticiabpaz@hotmail.com
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INTRODUCTION

The interest in Platelet rich plasma (PRP) therapy is currently growing due to the high counts of platelets and its bioactive growth factors that can achieve therapeutic effects such as reducing inflammation and enhancing tissue repair (Marx, 2004; Fortier *et al*., 2011). The growth factors are derived by platelet's α-granules (Nurden, 2011) and promote anabolic along with angiogenic effects, being essential in the healing process. Therefore, once known as a type of regenerative therapy with great potential and many clinical benefits, its use has been applicable to dentistry (Petrungaro, 2001), human (Anitua *et al*., 2004) and veterinary medicine areas (Carter *et al*., 2003; Tambella *et al*., 2018).

Among the several protocols of PRP preparation described for the equine species, the most reliable ones are composed by two centrifugations (Pereira *et al*., 2013) that separates the red blood cells and leukocytes from plasma followed by concentration of the platelets (Foster *et al*., 2009). Based on its well-known beneficial action on wound healing in horses (Rossi *et al*., 2009), the use of PRP in equine medicine has already been applied as support to healing fractures (Carmona and López, 2011), as treatment for chondral injuries (Yámada *et al*., 2012), skin burns (Maciel *et al*., 2012), laminitis (Carmona *et al*., 2012), endometritis (Reghini *et al*., 2014), tendon and ligament injuries (Pereira *et al*., 2018), osteoarthritis (Smit *et al*., 2019) and its homologous use in equine distal limb skin wounds (Pereira *et al*., 2019).

Despite its diverse clinical applications in equine medicine, there are still factors that differ between authors and can influence in the PRP quality. The several preparation methods (Pereira *et al*., 2013) and experience of the person processing the plasma are extrinsic aspects that can interfere in the composition of the final product. In addition, age, breed, and gender (Giraldo *et al*., 2013; Miranda *et al*., 2018) are considered intrinsic factors that can make PRP final components variable among different individuals. The aim of the study was to evaluate the effects of breed, age, and gender in PRP between five different breeds and to correlate its physical aspect with the platelet count.

MATERIALS AND METHODS

Based on the existing population of horses in the Southern Brazil, five different breeds were selected to be included in this research: Thoroughbreds (TB), Brazilian Criollo Horses (BCH), Brazilian Sport Horses (BSH), Miniature Horses (MH) and Crossbred Horses (CB). Fifty healthy horses with the mean age of 9.12 (±5.44) years old, were included in the study based on owners' consent, absence of hematologic and clinical abnormalities.

Ten TB horses (5 males and 5 females), ten BCH (7 males and 3 females), ten BSH (5 males and 5 females), ten MH (6 males and 4 females) and ten CBH (6 males and 4 females) participated in the study. All animals were in the state of Rio Grande do Sul, Brazil. The horses were kept in individual stables, fed commercial feed, alfalfa hay and ad libitum water.

The PRP preparation was based on the protocol previously described by Pereira *et al*. (2013). Initially, 450 ml of blood was collected aseptically from each horse by puncture of the external jugular vein using a commercial blood-transfusion bag containing citrate-phosphate-dextrose solution with adenine as an anticoagulant (CPDA). From each collection, 100 ml of whole blood was distributed in three Falcon type 50 ml polypropylene tubes (35ml of blood in two tubes and 30ml in the last one).

The tubes were centrifuged at 224g for 10 minutes to separate the blood components (red blood cells, buffy coat with leukocytes and plasma with platelets) and the supernatant plasma portion obtained was transferred to two new Falcon tubes and centrifuged at 440g for 10 minutes. After the second centrifugation step, the supernatant platelet-poor fractions were discarded and 10ml of platelet rich plasma were obtained.

PRP physical analysis was performed by a blind evaluator and was based on color, aspect, and capacity to separate blood components in the first centrifugation. This score was created according to our team's experience with PRP, and it was analyzed through pictures (Fig. 1). Color was graded in yellow, light yellow, strong yellow and reddish. Aspect was considered clear (1), nearly turbid (2) and turbid (3). The

separation of blood components was analyzed based on how the layers were presented in the Falcon tube after first centrifugation. The ones that did not separate the blood components after the first centrifugation, the separation was graded poor (I). The layers that had a short portion mixed was considered medium (II) and the ones that presented three layers perfectly distinguished, the separation was considered good (III).

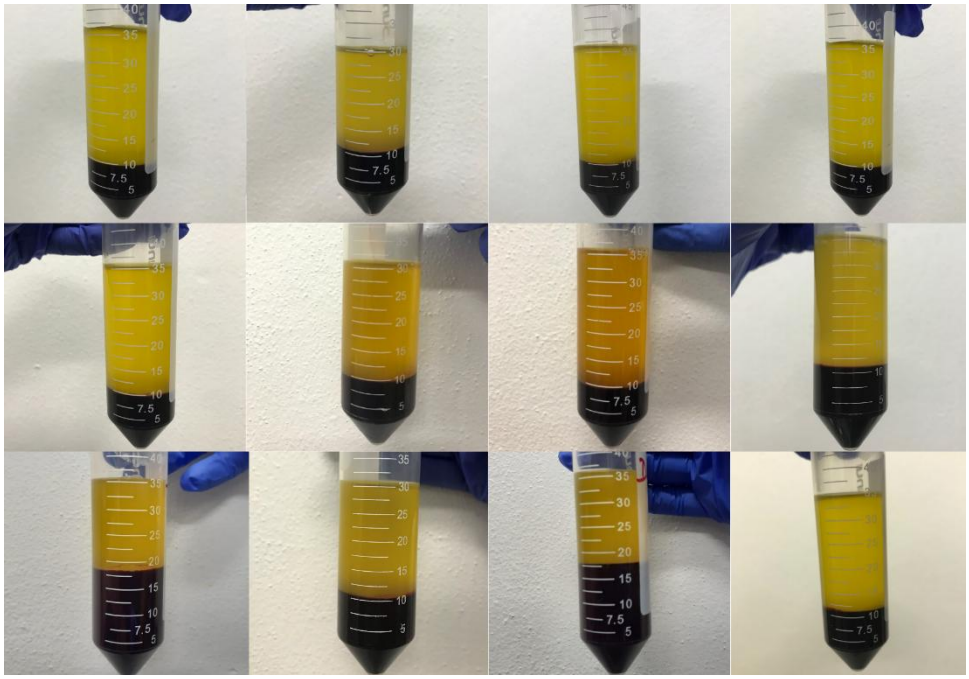

Figure 1 Twelve of the fifty pictures of PRP after first centrifugation, evaluated on color, aspect and capacity to separate blood components in the first centrifugation.

The platelet, mean platelet volume (MPV), red blood cell and leukocyte count was performed by an automatic hematology analyzer (Mindray BC-2800Vet) and the platelet count was also determined by the direct manual method, as described by Roshal and Gil (2019). It was performed in a Neubauer chamber, using a binocular optical microscope with a magnification of 400 x. Each PRP sample was diluted and homogenized in Brecher liquid with 1% ammonium oxalate to determine the number of platelets. The whole protocol of manually processing PRP was done in a laminar flow cabinet to prevent contamination.

All data were analyzed with the software GraphPad Prism 6.0. Normality of variables was investigated using the Shapiro-Wilk test. Nonparametric samples were evaluated by Wilcoxon or Kruskall-Wallis test as required. When necessary, adjustments for multiple comparisons with the Dunn's test were performed. Parametric samples were examined by the unpaired t-test and specific correlations by Spearman's test. The interpretation of the values followed Dancey and Reidy (2004) literature.

This study was approved by the Ethics Committee on Animal Use of the Federal University of Santa Maria (Protocol number CEUA 2850180621).

RESULTS AND DISCUSSION

There were significant ($p<0.0001$) differences for platelet, MPV (Fig. 2), red blood cell and white blood cell count evaluated between equine whole blood and platelet rich plasma. As expected, the PRP platelet concentration was significantly higher than the whole blood platelet count and the MPV was lower than whole blood in all horses included in the study (Table 1). It means that the PRP preparation method used was able to concentrate platelets successfully, inducing certain platelet damage due to the process of platelet aggregation. The platelet count in PRP is

dependent on the whole blood platelet concentration (Barbosa *et al*., 2008) and the method of preparation (Rinnovati *et al*., 2016) as well.

Whitlow *et al*. (2008) showed that platelet concentrations in PRP are commonly three to five-fold over whole blood concentration levels in humans and in horses, MPV ranges from 4.3 to 5.6 fL (Grondin and Dewitt, 2010). Although there is lack of information on platelet counts for each species in veterinary medicine, horses have the lowest reported platelet count of mammals (Boudreaux and Ebbe, 1998) and cannot be compared with human absolute values considering relevant species differences. In these cases, the fold change or percent concentration are used to evaluate the quality of platelet concentrates.

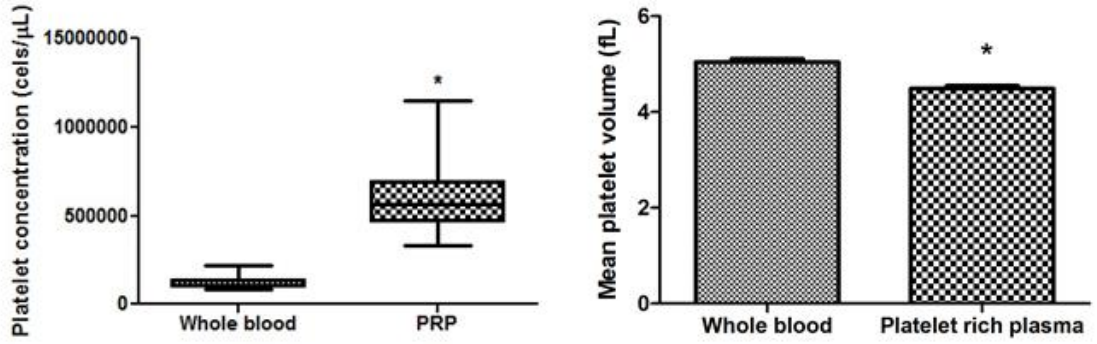

Figure 2 Different platelet concentration ($p < 0.0001$) and Mean platelet volume (MPV) were observed between samples from whole blood and PRP (*).

Table 1. General results for platelet, MPV, red blood cell and white blood cell count of whole blood and platelet rich plasma (PRP)

Variable	Whole blood (n=50)	PRP (n=50)
Platelet (10^3/ µL)	177.72 (±39.95)	596.13 (±238.77)
MPV (fL)	5.02 (±0.47)	4.48 (±0.41)
Red Blood Cell (10^6/ µL)	7.20 (±1.37)	0.00
White Blood Cell (10^3/ µL)	7.02 (±0.22)	1.11 (±0.17)

All horses presented significant lower ($p<0.0001$) red blood cells and white blood cells in the processed PRP when compared with whole blood (Fig. 3). The PRP is composed by plasma, platelets, and some white blood cells, depending on the method of preparation. According to Fontenot *et al*. (2012), there is an uncertain definition about the real composition of PRP final product in the literature. Some protocols of PRP preparation leaves a portion of red blood cells in the final product and that can be considered inappropriate (Pereira *et al*., 2013). The presence of white blood cells (WBC) in the PRP sample have beneficial effects such as immunomodulatory and antimicrobial function, however, leukocytes also have been correlated with expression of catabolic cytokines in human PRP (Sundman *et al*., 2011), consequently a reduced leukocyte count in the PRP is recommended.

Apart from the MH that showed significant higher platelet concentration than BSH ($p<0.05$), all the other breed comparisons showed no significant difference ($p>0.05$) due to similar PRP platelet values between these groups (Fig. 4). Furthermore, considering the platelet percent concentration (Table 2) based on formula described by Miranda *et al*. (2018), there was a trend for higher platelet concentrations in the Miniature Horse breed.

Giraldo *et al*. (2013) evaluated the effects of the breed on cellular content from equine PRP samples. The author reported higher platelet counts in Colombian Criollo Horses when compared to Argentinean Criollo Horses, representing a significant breed influence on PRP final components. Following the same research line, Miranda *et al*. (2018) compared platelet concentration between Quarter Horses,

Thoroughbreds, Mangalarga Marchador pacers and mules, identifying that the PRP platelet content varied according to breed and species of equid. These data in addition with the results of our research, improves the advance of homologous PRP new research.

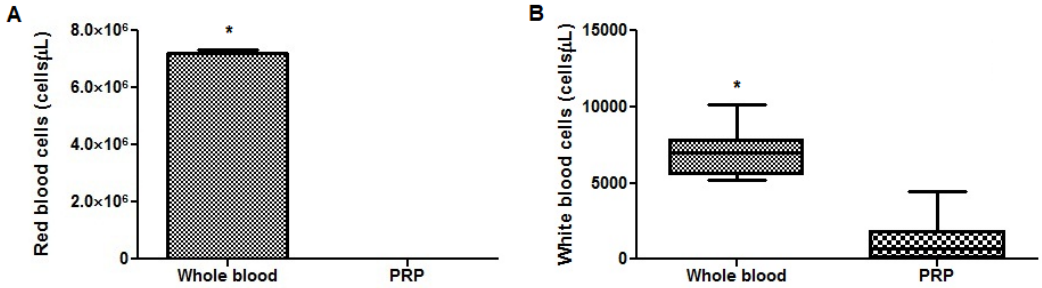

Figure 3 A) Different ($p < 0.0001$) red blood cell count between whole blood (*) and PRP. B) Different ($p < 0.0001$) white blood cell count between whole blood and PRP.

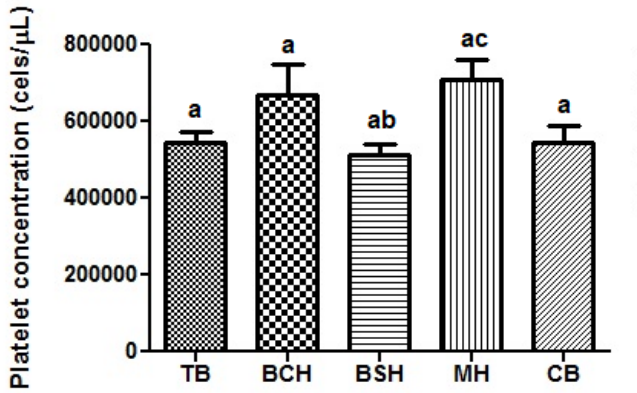

Figure 4 Platelet concentration comparison between Thoroughbreds (TB), Brazilian Criollo Horses (BCH), Brazilian Sport Horses (BSH), Miniature Horses (MH) and Crossbred horses (CB). Different letters indicate statistical difference between groups ($p < 0.05$).

Table 2. Whole blood and platelet rich plasma (PRP) platelet count between Thoroughbreds (TB), Brazilian Criollo Horses (BCH), Brazilian Sport Horses (BSH), Miniature Horses (MH) and Crossbred Horses (CB) with its percent concentration rate (PRP/PI*100)

Breed	Initial platelet concentration (PI) (10^3/µL)	PRP platelet concentration (10^3/ µL)	Percent concentration (%)
TB	120.80 (±41.05)	544.80 (±29.30)	450.99
BCH	129.90 (±32.93)	670.15 (±77.02)	515.89
BSH	104.20 (±17.03)	510.75 (±31.65)	490.16
MH	107.70 (±16.81)	710.07 (±48.95)	659.30
CB	126.60 (±20.24)	544.90 (±43.39)	430.41

There was a weak correlation of age with PRP platelet concentrations (Fig. 5), demonstrated by the Spearman's rank correlation coefficient ($r_s = -0.24$), as the negative value means that as the age increases, the platelet counts tend to decrease. Weibrich *et al*. (2002) investigated growth factor levels in human platelet-rich plasma and correlations with donor age and platelet count, but the author did not find significant influence on the outcome. However, Giraldo *et al*. (2013) reported that equine PRP cellular content and growth factors concentration was also influenced by age.

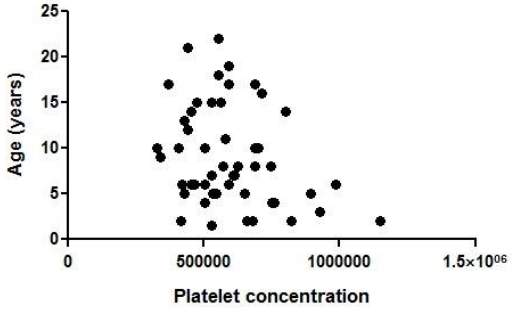

Figure 5 Correlation between age (years old) of the animals and platelet concentration in PRP.

The PRP platelet values analyzed in this research presented different statistical behavior for gender. It showed no significant difference ($p = 0.09$) between females in comparison with males. Platelet counts in human PRP, tends to be slightly higher in women than in men, as described by Weibrich *et al*. (2002). Similarly, Giraldo *et al*. (2013) reported that platelet counts for equine PRP were significantly higher in females when compared to males, therefore the numerical imbalance in their study between genders (30 male and 10 female) may have interfered on the final analysis of the authors.

Subsequently, the PRP physical analysis data revealed interesting results. According to the blind evaluation, most of the platelet rich plasma samples presented yellow colored (Table 3), that is considered the normal plasma presentation (Nin *et al*., 2009).

Table 3. Distribution of the platelet rich plasmas analyzed by its colors

Color scale	Percent rate
Yellow	48%
Light yellow	32%
Strong Yellow	14%
Reddish	6%

In addition, the scores for aspect and separation of blood components were analyzed in correlation with PRP platelet count. The gross aspect showed a moderate correlation with PRP platelet count due to the Spearman's rank correlation coefficient ($r_s = 0.302$) and the scale for separation of blood components indicated no correlation taking into consideration the Spearman's coefficient ($r_s = 0.070$). It means that the samples with nearly turbid (2) or turbid (3) aspect can probably have the best platelet concentration (Fig. 6).

There is limited information available regarding the platelet rich plasma physical presentation in human literature (Nin *et al*., 2009) and there are no described reference ranges of equids PRP physical appearance in veterinary reports, to the best of our knowledge. It is believed that this information can help veterinary practitioners in the daily routine to evaluate PRP quality when there is no possibility of access to a laboratory in the field to determine the real platelet counts.

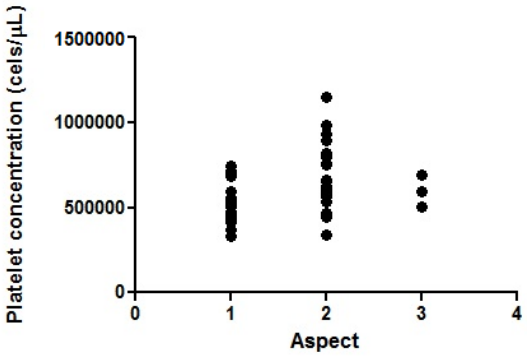

Figure 6 Correlation between PRP aspect (1 - Clear, 2 - Nearly turbid, 3 - Turbid) and platelet concentration.

CONCLUSIONS

Overall, the result in this study suggests that PRP platelet concentration can be influenced by intrinsic factors such as age and confirms that it can be influenced by breed. Methods of PRP preparation and sample handling should be standardized to reduce these effects. Moreover, with moderate results, physical aspect analysis should be a promising PRP quality evaluation method when there is no access to platelet counts. It does not replace laboratorial analysis, but considering field routine, a skilled practitioner would be able to correlate these aspects to the final product quality.

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