Research article

EFFECTS OF BREED/SPECIES AND GENDER ON PLATELET CONCENTRATION IN AUTOLOGOUS PLATELET RICH PLASMA

MIRANDA Stephania[1], DE MELLO COSTA Maria Fernanda[2,3,*], JEUNON SENNA Juliana[4], FRAPOINT João Castañon[4], DE ALENCAR Nayro Xavier[1], BARROSO LESSA Daniel Augusto[1]

[1]Departamento de Patologia e Clínica Veterinária, Faculdade de Veterinária, Niterói, Brazil; [2]CHASP, Waikato Institute of Technology, Hamilton, New Zealand; [3]Faculdade de Medicina Veterinária, Universidade de Vassouras, Vassouras, Brazil; [4]Curso de Medicina Veterinária, Faculdade de Veterinária, Niterói, Brazil

Platelet rich plasma (PRP) is an autologous biological product harvested by consecutive centrifugations of whole blood and separation of plasma in a stepwise protocol. PRP has been successfully used to stimulate healing in orthopedic and dermatological conditions, both in humans and animals. The principle is the fact that α- granules inside platelets contain a high concentration of growth factors, that once released can interfere with cellular communication and speed up healing. Standardization of PRP requires establishing a gold standard for the preparation and evaluation of the product, especially considering that platelet concentration and, therefore, growth factor concentration, might vary due to a number of variables. Factors such as age, gender, race or breed, and immune status of the patient might interfere with PRP quality and with treatment results, although little is known about such interferences. This research investigated the effect of breed/species and gender in platelet concentration in autologous PRP from horses and mules. The results demonstrate that Quarter Horses provided PRP with the greatest amount of platelets, although mules had a higher concentration percentages in relation to the initial platelet counts.

Key words: biological products, blood platelets, horses, methods

INTRODUCTION

Several innovative treatments based on autologous products such as cells or blood products have been instituted in the past twenty years, especially in the fields of dentistry [1], plastic surgery [2] and sports medicine [3]. One of such products is Platelet Rich Plasma (PRP), a biological by-product of whole blood centrifugation, rich in growth factors contained in platelets' α- granules, capable of improving and expediting healing [4].

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*Corresponding author: e-mail: Fernanda.Costa@wintec.ac.nz or mariafernanda.costa@adedevassouras.edu.br

Once blood from a patient is harvested, consecutive centrifugations leading to a 3 to 5-fold platelet concentration generate PRP [5]. Platelet activation followed by release of 95% of growth factors contained in α- granules is believed to occur within the first hour after clot activation [6,7] and is reported to be essential for treatment success [8].

PRP has been frequently used in equine medicine, mostly to repair bone [9], joint [10] and soft tissue [11] lesions.

Extrinsic factors such as preparation protocol and technique can interfere with PRP quality [12-14], adding a source of variation in treatment outcomes. Intrinsic factors such as gender, age and breed can also provide sources of variation, although these have not been investigated yet.

In order to propose a gold standard protocol for PRP preparation these factors need to be studied so recommendations can be made for possible adjustments and effects on growth factor concentration and viability. This preliminary study investigated the effect of breed/species and gender in PRP from Thoroughbred racehorses, barrel racing Quarter Horses, Mangalarga Marchador exhibition pacers and exhibition mules.

MATERIAL AND METHODS

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals (UFF Animal Ethics Committee, 822/UFF, 2016). No animals were harmed or were subjected to unnecessary suffering during the course of this research.

Three high performance breeds were selected to be included in this research: Quarter Horses (QH), Thoroughbreds (TB) and Mangalarga Marchador (MM) Pacers. Twenty four healthy horses were registered to participate based on informed consent from owners. Inclusion criteria were absence of clinical abnormalities and lameness, and normal hematology.

Eight Mangalarga Marchador (MM) horses (4 females and 4 males) from a farm at Iguaba Grande, eight Quarter Horses (QH) from a training farm at Araruama (3 females and 5 males), and eleven Thoroughbred (TB) racehorses (3 females and 8 males) from a racing Stud in Rio de Janeiro City, participated in the study. All locations were at sea level in the state of Rio de Janeiro, Brazil. All horses were kept in individual stables, fed commercial feed, *ad libitum* water, and either grass (MM), grass hay (QH) or alfalfa hay (TB). All animals were between 4 and 10 years of age.

Eleven mules (MU, 7 females and 4 males) were also engaged in the study in order to offer comparison between species. All mules were kept at a farm in Vassouras, Rio de Janeiro state, Brazil, at an altitude of 434 meters above sea level. These animals were kept on pasture (native, mixed) with *ad libitum* water and had between 5 and 7 years of age.

Blood samples were collected into 6 sodium citrate vacuum tubes from the jugular veins of each animal, were homogenized through gentle inversion of the tubes for 10 times and immediately sent for processing in an adjacent portable lab. Each animal had a total of 24 mL of whole blood collected.

Processing consisted of separation of 20 μL of blood which was diluted in 1980 μL of ammonium oxalate for initial platelet count in a hemocytometer. The six tubes with whole blood were rested at room temperature for two hours and then centrifuged in a bench top centrifuge (Centribio™) for 10 minutes at 120 g for plasma separation. This process was followed by aspiration of the top two-thirds of the supernatant for discard. The bottom third (platelet poor plasma, PPP) was aspirated and deposited in conic falcon tubes for a second centrifugation, this time at 240g for 10 minutes. The aspiration process was repeated and the bottom third resulting from the second centrifugation consisted of PRP. Final platelet count in the hemocytometer was conducted by diluting 200 μL of PRP into 19800 μL of ammonium oxalate.

Percent concentration was calculated using the formula: platelet count in PRP/ initial platelet count*100.

Plasma protein with a manual refractometer was measured at each step of the protocol to ensure platelet concentration was absolute and not relative to volume.

Vascular endothelial growth factor (VEGF) was measured for the investigation of the degree of platelet activation. Aliquots (1mL) from plasma samples from each processing step (after the 2 hour resting period - PR), from PPP and PRP) were placed into cryotubes, immediately frozen in liquid nitrogen, and transported in dry ice to a -80º freezer were they were kept for approximately 2 weeks. Aliquots were thawed a single time for measurement of VEGF concentration by ELISA (Kingfisher, DIY0705E-003), according to manufacturer's instructions. Dilution of samples to buffer was 1:1 and all samples were analyzed in duplicate. The standard curve for concentration calculations was analyzed with CurveExpert Professional® software.

All statistical analysis was conducted with the software Minitab 18®. Normality of data was investigated using Shapiro-Wilk test and average $\pm$ standard deviation (SD) or median $\pm$ first quartile were used to present initial and final platelet counts, as well as VEGF concentrations in PR, PPP, and PRP.

Pearson's Correlation test was used to investigate the relationship between initial and final platelet counts and a Student t-test for paired samples was used to analyze the differences between initial and final platelet counts and in each of the groups (MM, QH, TB, MU).

ANOVA followed by Tukey test was used to investigate the source of variation of platelet concentration among groups and gender effect on platelet and VEGF concentrations. MANOVA was used to investigate the source of variation in VEGF among groups (MU, MM, QH, TB) and stages of PRP preparation (PR, PPP and PRP).

Informed consent: Informed consent has been obtained for client-owned animals included in this study.

RESULTS

Average ± SD for platelet counts are shown in Table 1 and demonstrate that the protocol used for PRP preparation concentrated samples between 307.42 to 441.35%.

Table 1. Average ± SD initial and final platelet counts from equids and percent concentration

Breed/Species	Initial platelet concentration (PI) (x 10^3/μL)	PRP (final) platelet concentration (x 10^3/μL)	Percent concentration (%)
MM	183.4 ± 41.7[ab]	741.4 ± 112.7[c]	404.25
MU	154.9 ± 27.3[b]	683.7 ± 89.1[d]	441.35
QH	249.3 ± 35.1[a]	766.4 ± 55.7[c]	307.42
TB	159.6 ± 85.3[b]	567.7 ± 224.9[e]	355.70

Legend: MM = Mangalarga Marchador pacers; MU = mules; QH = Quarter Horses; TB = Thoroughbreds. Different letters on columns mean significantly different platelet counts (p < 0.05). The last column shows percent concentration calculated using the formula PRP/PI*100.

The final platelet concentration was significantly higher than the initial platelet concentration in all groups (MM p < 0.001, T = -17.08, 95% CI -653.3 to -480.7; MU p < 0.001, T = -17.89, 95% CI -594.7 to -463.0; QM p < 0.001, T = -31.99, 95% CI -555.4 to -478.9; TB p < 0.001, T = -7.05, 95% CI -537.1 to -279.1).

ANOVA demonstrated that breed/species significantly influenced variation in initial (p = 0,006, r^2 = 21.2%) and final (p < 0.0001, r^2 = 50.6%) platelet counts.

Pearson's correlation showed a positive, albeit weak, association between initial and final platelet counts (r = 0.32; p = 0.02) which was confirmed through linear regression (p = 0.02, adjusted r^2 = 8.7%).

There was no difference in mean platelet concentration at any stage of PRP preparation between males and females (p > 0.05 for all stages and groups).

Average plasma protein ± SD for each breed/species is shown in Table 2. The Student t-test for paired samples showed no differences between initial and final plasma protein (p > 0.05).

Median and first quartile VEGF concentrations for each group by PRP preparation stage is shown in Table 3. MANOVA demonstrated that VEGF concentration did not vary significantly with breed/species (p = 0.122) nor stage of PRP preparation (p = 0.420) and ANOVA demonstrated that gender also did not influence VEGF concentrations (p = 0.262).

Table 2. Average ± SD plasma protein equid samples before and after processing for PRP harvesting

Breed/ Species	Initial plasma protein (g/dL)	Final plasma protein (g/dL)
MM	7.0 ± 0.3	7.0 ± 0.4
MU	7.0 ± 0.4	7.0 ± 0.4
QH	5.8 ± 0.3	6.0 ± 0.4
TB	6.8 ± 0.3	6.8 ± 0.4

Legend: MM = Mangalarga Marchador pacers; MU = mules; QH = Quarter Horses; TB = Thoroughbreds.

Table 3. Median and dispersion measurements of VEGF concentrations from equids at each stage of PRP preparation

Breed/ Species	Stage of PRP preparation	Median VEGF (ng/mL)	Q1 VEGF (ng/mL)
MM	PR	0.955	0.954
MU	PR	0.954	0.953
QH	PR	0.827	0.798
TB	PR	0.943	0.789
MM	PPP	0.955	0.953
MU	PPP	0.953	0.953
QH	PPP	0.812	0.787
TB	PPP	0.904	0.795
MM	PRP	0.954	0.953
MU	PRP	0.952	0.952
QH	PRP	0.886	0.845
TB	PRP	0.855	0.831

Legend: MM = Mangalarga Marchador pacers; MU = mules; QH = Quarter Horses; TB = Thoroughbreds. CI = confidence interval. Q1 = first interquartile. PR = after resting stage of PRP preparation, PPP = after first centrifugation, PRP = after second centrifugation.

DISCUSSION

Final platelet concentration was significantly higher than initial platelet concentration in all groups of equids, indicating that the protocol used for PRP preparation efficiently concentrated platelets more than 3-fold. This degree of concentration is one of the prerequisites for PRP harvesting to be considered successful [15] since there is an association between platelet numbers and mesenchymal stem cell proliferation [5] and growth factor concentration [16]. The concentration of platelets observed in this study was considered to be absolute and not relative to volume, since protein content was preserved during the process of PRP extraction.

Breed and species but not gender significantly influenced both initial and final platelet counts, which might indicate different immunological status in each cohort or larger production of platelets. In any case all horses included in the study had similar vaccination protocols, had not been vaccinated or exposed to antigens or toxins in the last three months previous to the study, and were considered healthy at the time of collection based on absence of abnormal results on hematology and clinical examination. Previous studies in humans have demonstrated that gender, age, and genetic background influence platelet counts [17], as well as platelet function, in conjunction to diet and race [18]. In the case of the present study, platelet function was not evaluated.

Previous studies in South American Argentinian and Colombian Creole horses [19] and in humans have demonstrated that gender affected growth factor concentration in human PRP [20] but this was not confirmed in this study, where VEGF concentrations did not vary significantly between breeds nor in relation to gender. One reason for this difference might be the assay used for VEGF measurement since in the present study an equine VEGF ELISA kit was used, rather than human VEGF ELISA. Studies have demonstrated a correlation between VEGF and platelet concentration in whole blood and serum, but not in plasma [21], as is the case here. The lack of variation in VEGF concentration through each step of the PRP preparation protocol indicates no significant degranulation during preparation, preserving growth factors within the α-granules which favor PRP viability for application [16]. PRP prepared in this fashion can be activated immediately prior to administration, theoretically allowing maximum release of granule contents at the time of treatment. Further studies comparing growth-factor concentrations in activated PRP versus non-activated PRP in equids are required.

The lack of standardized protocols in the preparation of PRP for clinical use is one of the major obstacles in comparing results from clinical trials and no golden standard protocol has been suggested for either horses or mules. This study demonstrates that PRP preparation protocol does influence final platelet output, potentially influencing clinical outcomes. From a clinical perspective, treatment results can only be evaluated scientifically once the range of platelets and growth factor concentrations obtained in PRP are established, and despite individual variations, a standardized protocol is paramount for this goal.

Some evidence of positive results in the treatment of orthopedic conditions with application of PRP in tendons [11] and joints [10] of horses has been described but it is hard to obtain a consensus through lack of reference ranges for this biological product. The literature suggests that the minimum platelet concentration for PRP is, at least, three times the initial platelet concentration [8]. It has been suggested that, in order for wound healing to be stimulated, platelet concentration in PRP should exceed 1,000,000/mL [22].

There are no prescribed reference ranges for VEGF in PRP of plasma reported for horses or mules, to the best of our knowledge. In humans, physiological concentration of VEGF is reported as 0.74 ± 0.37 pg/10^6 platelets in non-concentrated platelets [23] and as 0.56 ± 0.36 pg/10^6 platelets in PRP [24], highlighting the lack of agreement between publications due to sample origin and method variation.

There is also a lack of consensus regarding effects of previous activation of platelets on clinical outcomes [25] and effects of freeze-thawing cycles on platelet activation and growth factor release [26]. The gold standard for verification of platelet activation in PRP is aggregometry [27], an elegant method not accessible for most practitioners. In this experiment, one cycle of freeze-thawing did not appear to induce platelet activation, as indicated by lack of significant variation in VEGF concentrations.

Results in the present study indicate variations in PRP platelet concentrations are dependent on factors such as breed/species, further emphasizing the need for clear recommendations regarding origin of sample, method of preparation, reference ranges for platelet numbers and growth factors in PRP of horses and mules. The lack of a consensus in the available literature concerning these variables make it extremely hard for therapeutic outcomes in clinical conditions to be evaluated and defended consistently.

Platelet content in PRP varied according to breed and species of equid but not gender and in this study involving three horse breeds and mules. Quarter Horses provided greatest platelet numbers in PRP but mules achieved a higher percentage concentration in comparison to whole blood. VEGF concentrations did not change significantly during the preparation protocol indicating preservation of α-granules within the platelets.

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Authors' contributions:

SM designed the PRP protocols, collected and processed the samples, was involved in the drafting of the manuscript. MFMC was involved in the conception and design of the experiment, collected samples, conducted statistical analysis and interpretation, was involved in the drafting of the manuscript. MFMC, JJS and JF collected samples, has given final approval of the version to be published and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. SM, NXA and DABL helped with concept and design, has given final approval of the version to be

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UTICAJ RASE/VRSTE I POLA NA KONCENTRACIJU TROMBOCITA U AUTOLOGNOJ PLAZMI BOGATOJ TROMBOCITIMA

MIRANDA Stephania, DE MELLO COSTA Maria Fernanda, JEUNON SENNA Juliana, FRAPOINT João Castañon, DE ALENCAR Nayro Xavier, BARROSO LESSA Daniel Augusto

Plazma bogata trombocitima (PBT) je autologni biološki proizvod koji se dobija centrifugovanjem pune krvi i odvajanjem plazme u protokolu koji podrazumeva više faza. PBT se usešno koristi u stimulaciji zarastanja u ortopediji i dermatologiji, kako kod ljudi tako i životinja. Princip koji se koristi podrazumeva da α-granule u trombocitima sadrže visoke koncentracije faktora rasta koji kada se oslobode, mogu da utiču na međućelijsku komunikaciju i ubrzavanje zarastanja. Standardizacija PBT preparata podrazumeva uspostavljanje „zlatnog standarda" i evaluaciju proizvoda, naročito u odnosu na koncentraciju trombocita, a samim tim i koncentraciju faktora rasta koji mogu da variraju kao posledica većeg broja promenljivih faktora. Neki od faktora kao što su starost, pol, rasa/vrsta kao i imunski status pacijenta mogu da utiču na kvalitet PBT pa samim tim i na ishod tretmana ovim preparatom. Ipak, malo se zna o tačnom delovanju i interferenciji pomenutih faktora. U studiji se ispituju efekti rase/vrste kao i pola na koncentraciju trombocita autolognog PBT kod konja i mula. Rezultati ukazuju da PBT dobijen od „Quarter" rase konja, bio sa velikom koncentracijom trombocita uprkos činjenici da je kod mula uočen veći procenat koncentracije u odnosu na početni broj trombocita.