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In House Double Centrifugation Method for Preparation of Homologous Platelet-Rich Plasma (Prp) in Dogs

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Abstract

The production of inexpensive autologous canine platelet rich plasma (PRP) with no requirement for specific equipment may provide a relatively easy source of growth factors and cell-rich fractions for stimulating healing processes and regeneration. In dogs many different manual or semi-automated methods for PRP production are described achieving wide ranges of platelet concentration, but few studies provide information about platelet activation and platelet-derived-growth-factor (PDGF) concentration. The aim of this study was to evaluate the in-house manual double centrifugation method that would produce small amounts of autologous canine PRP from a relatively small volume of whole blood. Thus making it a practical technique for autologous PRP production in the clinic. A double centrifugation protocol was followed for all samples. Platelet counts (PLT/µL) leucocyte counts (WBC/µL) and PCV (packed cell volume), were calculated on the WB and PRP Blood smears were performed from all whole blood and PRP samples to evaluate the percentage of activated platelets. PDGF were measured on PRP samples by ELISA. The mean platelet concentration was 906900 platelets/µL ± 166259 in a mean of 1.01 ml PRP obtained from 8 ml of whole blood. There was a significant difference ($p < 0.0001$) between the percentage of activated platelets in WB (22.9 ± 14.2) and in PRP (39.05 ± 19.4). The mean concentration of PDGF in PRP was 1562.23 ± 684.72 ng/L. Poor correlation was found between PDGF concentration and activated platelet percentages in PRP. This method appears to produce adequate concentration of platelets and PDGF and may be appropriate for clinical use.

Keywords: *Canine; Platelet rich plasma; Platelet-Derived-Grow-Factor; Platelet morphology; Regenerative medicine*

Abbreviations: PRP: platelet rich plasma; PDGF: platelet-derived-growth-factor; ELISA: Enzyme-Linked ImmunoSorbent Assay; PLT: platelet; WB: whole blood; WBC: leucocyte count; PCV: packed cell volume; REV: Transfusion Unit; BCC: blood cell component; SEC: sine erythrocytes component; PPP: platelet poor plasma; ILGF: Insulin-Like Grow Factor; IGFBP-3: Insulin-Like Grow Factor Binding Protein -3

Introduction

Platelet-rich plasma (PRP) is a product derived from whole blood, characterized by platelet (PLT) concentrations above baseline in a small volume of plasma [1]. As a result it contains increased concentrations of platelet-derived growth factors [2]. The high concentration of growth factors in the platelet alpha granules (Platelet Derived Growth Factor, Epidermal Growth Factor, Insulin Growth Factor, Vascular Growth Factor Endothelium etc.) can stimulate cell proliferation, angiogenesis, wound healing, production of fibroblasts, collagen, osteoblasts, and decrease the inflammatory reaction and accelerate the healing process [3].

In human medicine the regenerative features of autologous PRP are used predominantly in orthopaedic surgery, maxillofacial surgery, dentistry, medicine, cosmetic surgery and in dermatology [4,5]. Most studies of the use of PRP in animals report animal treatment as a model for human medicine [6-10].

Studies of the use of PRP in veterinary medicine have concentrated on the therapeutic use of PRP in musculoskeletal, tendon and soft tissue injuries in horses [11-13]. There are few reports of the therapeutic use of PRP in dogs [2,14-16].

The production of inexpensive autologous canine PRP with no requirement for specific equipment may provide a relatively easy source of growth factors and cell-rich fractions for stimulating healing processes and regeneration [5].

In dogs many different manual or semi-automated methods [17] for PRP production are described [14,18-20] achieving wide ranges of PLT concentration, but few studies provide information about platelet activation and the values of Platelet-Derived-Growth-Factor (PDGF) concentration [2,17,19].

The aim of this study was to evaluate a manual double centrifugation method that would produce small amounts of autologous canine PRP from a relatively small volume of whole blood. Thus making it a practical technique for autologous PRP production in the clinic.

Materials and methods

Dogs

Blood samples were collected from 20 healthy adult dogs (PLT count within the reference range), weighing between 20 and 45 kg and from 2 to 7 years of age. The dogs included a variety of different breeds admitted to the Transfusion Unit (REV) of the Department of Animal Science and Food Safety. All dogs were fasted for 12 hours before blood sample collection. Owner consent was obtained for additional blood sample collection during routine screening for heartworm in potential blood donors.

Sample Collection

Eight ml of blood was collected from the cephalic vein using a 10 ml syringe (Sterile syringe 10 ml PIC, Italy) and a large gauge (21 gauge) needle (Hypodermic needle, 21G x 1 1/2" PIC, Italy) in order to minimize platelet activation. The blood was immediately placed into a test tube with a conical bottom (CELLSTAR® Centrifuge Tubes, Polypropylene, Sterile, 15 ml, graduated conical bottom, blue screw cap Greiner Bio-One, Germany) (Tube A) containing 1 ml of sodium citrate 3.8 %. All samples were maintained at room temperature (18-25°C), on a laboratory blood rocker and processed and analysed within 30minutesof collection.

Protocol of PRP preparation

The same double centrifugation protocol was followed for all samples. All Tube A samples were centrifuged at 610g for 10 minutes at room temperature using a laboratory centrifuge (EBA 20, Hettich, Germany) to produce a blood cell component (BCC) in the bottom of the tube and sine erythrocyte components (SEC) in the upper fraction of the tube. The entire SEC, comprising buffy coat (rich in young large platelets), was transferred to another 10 ml graduated conical tube without anticoagulant (Tube B) (CELLSTAR® Centrifuge Tubes, Polypropylene, Sterile, 15 ml, graduated conical bottom, blue screw cap Greiner Bio-One, Germany) and centrifuged at 1600g for 15 minutes. This centrifugation resulted in two new components: platelet poor plasma (PPP) in the upper fraction and a platelet pellet in the lower fraction (visible as a red button on the bottom of the tube). After removing PPP, the platelet pellet was re suspended in approximately 25% of the PPP volume to obtain PRP [21] for use in the study. The time and relative force of centrifugation was chosen based on preliminary studies (data not shown).

Platelet and leukocyte counts

Platelet counts (PLT/µL) leucocyte counts (WBC/µL) and PCV (packed cell volume), were calculated on the WB, SEC, PPP and PRP by an automatic analyser using optical and volumetric impedance measurements (CELL-DYN 3500 Plus Abbott, Weisbaden, Germany). All samples were stored on a laboratory blood rocker for a minimum of 5 minutes before platelet count was performed.

To establish the accuracy of the Cell-Dyn 3500 when counting platelets in canine blood samples with PLT counts above the normal range a pooled PRP sample with high platelet concentration (1 ml volume; 858,000 PLT/µL) obtained from a unit of fresh canine whole blood, was assayed at different dilutions.

To establish the intra-assay variation of the procedure 10 x 8 ml samples from one unit of canine fresh whole blood (CPDA) obtained from Department Transfusion Unit (REV) were subject to the protocol of PRP preparation on the same day. The coefficient of variation (CV) was calculated as SD/mean X 100.

Morphological evaluation of platelets

To evaluate the degree of platelet activation two blood smears stained with May Grunwald Giemsa were prepared from each sodium citrate WB sample and PRP. Morphological signs of activation were the appearance of pseudopods (spider leg-like projections) on the surface of the platelet (Halmay., et al. 2005). Stained blood smears were scanned at 400 - fold magnification to evaluate platelet count and platelet morphology. Detailed examination of morphology was performed at 1000 - fold magnification with an oil-immersion objective. Two hundred platelets were evaluated in duplicate on each glass slide.

Platelet derived growth factors

The concentration of PDGF (ng/L) in all PRP samples was determined using an ELISA sandwich kit specifically developed with monoclonal antibodies against canine PDGF (Sun Red Biological Technology Co. Ltd. Shanghai). According to the manufacturer the assay has a measurement range of 8 to 2000 ng/L and a sensitivity of 7.176 ng/L. The correlation coefficient R is 0.95, and intra and inter-assay coefficient of variation (CV) 8% and 10% respectively. An ELISA test was performed in duplicate for each sample and readings were performed at 450 nm according to the manufacturer's instructions.

Statistical analysis and Platelet counting

All quantitative measurements were described using summary statistics (mean, standard deviation, medium minimum, maximum).

Mean, standard deviation, median of PCV, WBC and PLT count from WB and PRP and percentage platelet activation in WB and PRP and DPGF concentration on PRP were calculated after calculating the normal distribution of parametric data using the D'Agostino-Pearson test. The differences between mean values of PCV, WBC and PLT calculated on WB and PRP and between mean values of percentage of activated platelet in WB and PRP were compared using the Mann-Whitney test for independent samples.

Spearman's coefficient of rank correlation (rho) was used to evaluate the relationship between PCV value, leucocyte and platelet counts in WB and in PRP.

Paired samples T test was used to evaluate the statistical difference between mean percentages of activated platelets in WB and PRP. Correlations between concentrations of PLT (µL) and PDGF (ng/L) and between concentrations of activated PLT (%) and PDGF (ng/L) in PRP were performed using Pearson (ρ) test.

The increase in platelet concentration over whole blood baseline values was calculated using the following equation: Platelet Count PRP/ Platelet count in whole blood [37].

For all tests significance was set at $P < 0.05$. Statistical analyses were performed using commercial software (MedCalc Software v.11.5.1 Mariakerke, Belgium).

Results

Double centrifugation was performed on 20 samples and resulted in an increase in platelet concentration in all samples. PRP was prepared in approximately 1 hour.

Mean ± standard deviation of platelet concentration (µL) was 228800 ± 39598 in WB and 906900 ± 176776 in PRP. The mean final volume of PRP was 1.01 ± 0.31 ml.

Platelet concentration in PRP was increased by an average 399 % compared to baseline concentrations in WB and the leucocyte count was increased by an average 260 % compared to baseline level in WB.

Table 1 reports mean and standard deviation of WBC and PLT count in WB and PRP, and the statistical analyses of differences.

The mean PDGF concentration in PRP samples was 1562.23 ± 684.72 ng/L. There was a significant difference ($p < 0.0001$) between the percentage of activated platelets in WB (22.9 ± 14.2) and in PRP (39.05 ± 19.4). Correlation coefficients between concentrations of PLT (103/µL) and PDGF (ng/L) in PRP and between activate platelet percentage (%) and PDGF (ng/L) concentration in PRP were $\rho = 0.23$ and $\rho = 0.19$ respectively.

The CV of platelet counts performed on 10 samples of PRP obtained from the same dog was 8.43.The mean recovery of the PLT count on pooled PRP sample with high platelet concentration was 16.4%.

Variable	Sample	Mean ± SD	Median	P Mann-Whitney	Spearman's coefficient (rho)
WBC (/µL)	Whole blood	9267 ± 3151	8425	< 0.0001	rho = 0.596 p = 0.0055
	PRP	24365 ± 1212	21150		
PLT (µL)	Whole blood	228800 ± 59705.06	218000	< 0.0001	rho = 0.598 p = 0.0053
	PRP	906900 ± 166259.1	907500		

Table 1: *Mean, standard deviation and median values of WBC and PLT in whole blood and PRP and Spearman's coefficient of rank correlation (rho) results, on 20 samples undergoing manual double centrifugation for canine PRP production.*

Dilution	Expected (x 10^3/uL)	Observed (x 10^3/uL)	Recovery
1:1	858	858	100
1:0.75	643.5	753	117
1:0.5	429	500	116.5
1:0.25	214.5	248	115.6

Table 2: *Platelet concentration and percentage recovery of platelets using the CellDyn 3500 Analyser on a diluted PRP pooled sample.*

Discussion

This study describes a reliable method for producing PRP from a small amount of canine whole blood and evaluates both quantity and quality of platelets in PRP. In all samples this in house double centrifugation tube protocol achieved the target platelet concentration of 1000 x 10^3 platelet/µL ± 20% as used in the majority of human medicine studies [1,3,5] and/or three to six fold increases in platelet concentration with respect to whole blood, as has been the target used in recent veterinary studies [2]. A mean of 1.01 ml of PRP was obtained from a mean of 8 ml of whole blood in this study, similar to the results of Jansen., *et al*. (2004) in which 0.6 ml of PRP was isolated from each of 8 samples of 9 ml of canine whole blood. The mean platelet concentration in PRP in this study was 906900 platelets/µL ± 166259 which is within the values obtained in other canine studies [2,6,16,18,23] using single or double centrifugation, with values ranging from 2070780 PLT/mL [24] to 435000 PLT/mL [25]. The majority of human studies take the definition of PRP from a study by Marx (2001) that report that a good PRP should have a platelet concentration of 1 million/µl in 5 ml plasma or a 338% increase in platelet concentration as compared to whole blood. The same target is used to define "good PRP" in veterinary medicine despite the fact that there are differences between platelets from man and other animals in terms of platelet size and reactivity [20]. Although the biological rationale for the use of PRP is theoretically sound, both in human and veterinary medicine, the precise characteristics of the optimal PRP is yet to be elucidated. In particular the ideal canine PRP platelet concentrations are yet to be determined and there is no consensus on the platelet concentration required to have therapeutic effects in PRP [25-27].

There are numerous contradictory results for the most effective platelet concentration for PRP in animal studies and, in particular, in the dog [25,27]. Jansen (2004) found no activity in PRP with platelet concentrations of 1 million/μL and Rai (2007) demonstrated activity in PRP with a platelet concentration of 450,000/μL.

In our samples there was a significant correlation between platelet counts in WB and in PRP ($p < 0.0001$) in accordance with the finding of Eby (2002).

The platelet count is one of the key factors by which PRP quality can be evaluated, but the qualitative modification of platelets can have a big impact on the regenerative efficacy of PRP [29,30]. Smears are a valid way to evaluate changes in platelet morphology that are indicative of platelet function. In the present study small numbers of activated platelets were found in WB. This finding suggests possible early platelet activation at the moment of blood collection. A further significant increase in activated platelets was found between WB and PRP smears ($P = 0.0001$). Platelets are extremely sensitive to any kind of trauma, from blood extraction to PRP production. In this study, platelet concentration was achieved using a double centrifugation protocol during which cellular friction, may have contributed to platelet activation [19]. The amount of PDGF available at the end of PRP production process depends on the particular technique used to obtain the PRP (from blood collection to last sample centrifugation) [32]. This study shows a poor correlation ($\rho = 0.19$) between PDGF concentration and activated platelet percentages in PRP and also between PDGF and PLT concentration in PRP ($\rho = 0.23$). The relationship between platelet and PDGF concentrations is far from clear. Some authors report correlation between platelet and PDGF concentrations [4,19] and others do not [11,31,32]. Several factors might contribute to this lack of correlation, for example manipulation–induced platelet stress and variable susceptibility of platelets to stress. Furthermore the biological variability of growth factor concentration among individuals must be taken into account [31]. Acceptable biological variations for Insulin-Like Grow Factor (ILGF) and Insulin-Like Grow Factor Binding Protein - 3 (IGFBP - 3) in man, are 14.6 and 10.1 within subject and 45.4 and 63.9 between subjects respectively [33]. Although the comparison of ranges of biological variation between dogs and man may be erroneous since the isoforms being considered are different, in the absence of specific data for dogs it is conceivable the variability that exists in people also exists in the dog.

In our study the PDGF concentration in PRP is similar to that referred to by Stief (2011). However, this study evaluated the concentration of PDGF-BB isoform using a human ELISA kit. The comparison of platelet growth factor concentrations in canine PRP [19,34] could be influenced by methodological aspects such as the type of antibody used for measurement and the specific isoform evaluated. In our study PDGF concentrations were measured with a specific antibody for canine PDGF whereas others studies [19,34] used human antibodies, making a direct comparison between results difficult. Further studies using a greater number of samples are necessary to extend our understanding of factors affecting the concentration of PDGF in canine PRP.

A limitation of our study is that we did not evaluate PDGF concentration after PRP activation. We could speculate that the concentration of PDGF might increase after PRP activation. Further studies will be needed to investigate this possibility.

The significant difference between PLTs in WB (median 218000/μL) and PLTs in PRP (median 907500/μL) ($P < 0.0001$) and the significant and positive correlations between platelet count in WB and platelet count in PRP ($rho = 0.596$, $p = 0.0051$) show that the method described can concentrate platelets in a small amount of plasma. Furthermore, the significant difference between WBC in WB (median 8,425/μL) and WBC in PRP (median 21,150/μL), ($p < 0.0001$) and the significant correlation between WBC in WB and WBC in PRP ($rho = 0.596$, $p = 0.005$) with WBC in PRP being higher that WBC in WB suggests good buffy coat inclusion during the preparation method. This is important for inclusion of giant and young platelets.

It has been reported that leukocyte concentrations in PRP should be controlled to minimize inflammation after PRP injection [35], but high WBC counts are acceptable in PRP preparations used for autologous topical application [36] and other authors believe that WBC are important regulatory cells contained in PRP and necessary for wound healing [37]. One study placed a limit on WBC concentration in PRP of no more than twice the concentration of the baseline value [20].Thus the desirable WBC count is still a matter of speculation [38] and to date there are no studies that definitely elucidate the significance of leukocytes in canine PRP.

Conclusion

In conclusion, this in house double centrifugation method is repeatable, inexpensive and does not require special equipment. It allows final product to be produced in 1 hour and achieves an adequate platelet and PDGF concentration in the PRP. The limitation of this manual method is that it is operator dependent and requires experienced technicians. Despite the relatively small amount of whole blood initially collected in this study a sufficient volume of autologous PRP for clinical use was obtained. Additional *in vitro* and *in vivo* studies are necessary to assess if the induction of activation could further increase PDGF concentrations and to assess the therapeutic efficacy of this PRP in dogs.

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