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Release of transforming growth factor beta 1 and platelet derived growth factor type AB from canine platelet gels obtained by the tube method and activated with calcium salts

Liberación de factor de crecimiento transformador beta 1 y factor de crecimiento derivado de las plaquetas tipo AB a partir de geles de plaquetas caninos obtenidos mediante el método del tubo y activados con sales de calcio

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RESUMEN

Los objetivos de este estudio fueron: 1) medir las concentraciones del factor de crecimiento transformante beta 1 (TGF- β_1) y factor de crecimiento derivado de las plaquetas tipo AB (PDGF-AB) en plasma y gel de plaquetas (PG) activados con sales de calcio (gluconato o cloruro) en caninos, y 2) determinar las correlaciones entre los resultados celulares y la concentración de factores de crecimiento (GF). Se colectaron muestras de sangre de catorce perros Fila brasileiro. Para obtener sangre entera y plasma se utilizó EDTA y para preparar los concentrados de plaquetas (PC) se utilizó solución ACD-A. Los PC fueron sometidos a gelificación por la adición de sales de calcio. Antes de la activación de los PC se realizó conteo de plaquetas y leucocitos. La concentración de los factores de crecimiento fue determinada mediante ELISA en los sobrenadantes de los PG y plasma. Se observó diferencias estadísticamente significativas ($P < 0,01$) entre los valores de plaquetas y leucocitos en sangre entera y los PC. No se encontraron diferencias estadísticamente significativas entre las concentraciones de TGF- β_1 y PDGF-AB en los PC y plasma en función de la sal de calcio empleada para la activación de los PC. La concentración de TGF- β_1 estuvo altamente correlacionada con el número de plaquetas presente en el PC. La metodología descrita permite producir PG con potencial terapéutico en medicina regenerativa canina.

Palabras clave: gel de plaquetas, plasma rico en plaquetas, perro, medicina regenerativa, factores de crecimiento, sales de calcio.

SUMMARY

The objectives of this study were: 1) to measure the concentrations of transforming growth factor beta 1 (TGF- β_1) and platelet-derived growth factor type AB (PDGF-AB) in plasma and platelet gel (PG) activated with calcium salts (gluconate or chloride) in dogs, and 2) to determine correlations between cell results and growth factors (GF) concentrations. Blood samples were collected from fourteen Brazilian Fila dogs. EDTA was used to obtain whole blood and plasma while ACD-A solution was used to prepare platelet concentrates (PC). Calcium salts were added to PC to induce their gelification. Platelet and leukocyte count was performed before PC activation. The concentration of growth factors in PG supernatants and plasma was determined by ELISA. Statistically significant differences ($P < 0.01$) between platelet and leukocyte count were observed when comparing whole blood and PC. No statistically significant differences were found between the concentrations of TGF- β_1 and PDGF-AB in PC and plasma according to the calcium salt used for the activation of PC. The TGF- β_1 concentration was highly correlated with the number of platelets concentrated in the PC. This methodology was useful for producing PG with therapeutic potential for canine regenerative medicine.

Key words: platelet gel, platelet rich-plasma, dog, regenerative medicine, growth factors, calcium salts.

INTRODUCTION

Under physiological conditions, platelets (after activated) release substantial amounts of growth factors (GF), such as transforming growth factor beta 1 (TGF- β_1), platelet-derived growth factor (PDGF) and, other proteins that modulate inflammation and wound repair (Sutter *et al* 2004, Argüelles *et al* 2006). Platelet concentrates (PC)

(also known as platelet-rich plasma - PRP) are an autologous source of GF and therefore have been widely used in human medicine for procedures such as alveolar-maxillary reconstruction (Thor *et al* 2007), plastic (Cho *et al* 2011) and orthopedic surgery (Hakimi *et al* 2010), sports medicine (treatment of tendon injuries in athletes) (Sánchez *et al* 2003), treatment of chronic skin ulcers (Greppi *et al* 2011) and corneal ulcers (Alio *et al* 2007), among others.

There is an increased use of PC in horses to treat chronic musculoskeletal diseases (Carmona *et al* 2007, Argüelles *et al* 2008, Waselau *et al* 2008). However, there are few reports on the clinical use of PC in dogs

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(Hauschild *et al* 2005) although this animal has been widely used as experimental model to evaluate the effect of PRP as an adjuvant biomaterial (single or combined with other substances) in maxillary bone regeneration (Dutra *et al* 2008) or appendicular bone regeneration models (You *et al* 2007), and in osseo-integration tests (Casati *et al* 2007).

Platelet concentrates has been clinically used in a liquid form for local injection in tendons, ligaments or joints (Carmona *et al* 2011). However, clinicians in some circumstances induce the activation of the PC by adding an activating substance, such as thrombin or a calcium salt (Mazzucco *et al* 2008). This procedure induces platelet activation with externalization of alpha granules and release of growth factors, polymerization of fibrin and subsequent formation of a platelet gel (PG) (Dohan Ehrenfest *et al* 2006). PG are used as biomaterials in orthopedic, ocular, nervous or skin surgical procedures (Anitua *et al* 2007).

Recently, we described a simple manual tube method procedure for producing PC from canine blood intended for regenerative medicine porpoises in this species (Silva *et al* 2011). In this manuscript we present an analysis of the release of two GF (TGF- β_1 and PDGF-AB) stored in canine platelets from PG activated with two calcium salts, gluconate and chloride. This is a novel study, since there is little information about the release of these growth factors from canine PG.

The objectives in this study were 1) to measure and to compare the concentration of growth factors TGF- β_1 and PDGF-AB in plasma and PG supernatants obtained from canine PC that were activated either with calcium gluconate 10% or calcium chloride 10% and 2) to determine correlations between cell results and concentrations of the growth factors studied. The hypothesis of this study was that the release of TGF- β_1 and PDGF-AB from canine PG is the same independent of the calcium salt used for platelet activation.

MATERIAL AND METHODS

ANIMALS

Fourteen Brazilian Fila dogs from local breeders were used, seven males and seven females with a median age of 36 months (range 18 to 108 months), clinically healthy at time of blood collection and serologically negative for leishmaniasis and ehrlichiosis. Dogs with platelet counts less than 175×10^3 PLT/ μ L were not included (Jain 1993). This study was approved by the local ethics committee, protocol number 125/2009.

PREPARATION OF PLATELET CONCENTRATES

We used the tube method described by Silva *et al* (2011) for producing canine PC. Briefly, blood collection

was performed by saphenous vein puncture with a butterfly catheter 21 G (Shandong Weigao Group, Weihai, China). Two blood samples of each animal were placed in an 8.5 mL tube with 1.5 mL ACD solution A (ACD-A) (trisodium citrate 22 g/L, citric acid 8 g/L and dextrose 24.5 g/L) (Becton Dickinson and Company, New Jersey, USA). The samples were left on ice for 30 minutes before starting the experiments. A further blood sample was placed in a 4.5 mL tube with k_3 EDTA (Ethylenediaminetetraacetic acid) (Shandong Weigao Group, Weihai, China) for complete blood count (CBC). To obtain the PC, the ACD-A blood tube was centrifuged at 191 g for 6 minutes at 4 °C (SIGMA 3K30, Osterade, Germany). Next, a micropipette of a fixed volume of 1000 μ L was used to collect (approximately), the first 100 μ L of the red portion and the first 900 μ L of plasma, below and above the interface blood-plasma, respectively.

CELL EVALUATION

Samples were analyzed using automated counting device by volumetric impedance (Abacus Junior Vet, Budapest, Hungary). Each sample was analyzed by triplicate. The hematological parameters tested were PCV, platelet count (PLT/ μ L), leukocytes (WBC/ μ L), relative (% cells) and absolute (cells/ μ L) values of LYM, MID, GRA and mean platelet volume (MPV fL) (Silva *et al* 2011).

ACTIVATION OF PLATELET CONCENTRATES AND FORMATION OF THE PLATELET GELS

One mL of PC from each animal was activated with 0.1 mL of calcium gluconate 10% or 0.1 mL of calcium chloride 10%. After activated, the samples were kept in incubation with calcium salts at room temperature (22-26 °C) for two hours (Argüelles *et al* 2006). Platelet gels were released from the tube walls and centrifuged at 1500 g for 10 minutes. Plasma was obtained from blood with EDTA by the centrifugation protocol described above. The PG supernatants and plasma samples were aliquoted and frozen at -80 °C for later determination of TGF- β_1 and PDGF-AB.

DETERMINATION OF THE CONCENTRATION OF TRANSFORMING GROWTH FACTOR BETA 1 AND PLATELET-DERIVED GROWTH FACTOR TYPE AB

The concentration of both GF in plasma and PG supernatants were determined by ELISA sandwich with specific antibodies against canine TGF- β_1 (Mouse/Rat/Porcine/Canine TGF- β_1 , MB100B, R&D Systems, Minneapolis, USA) and human PDGF-AB (Human PDGF-AB, DHD00B, R&D Systems, Minneapolis, USA), with a mean detection sensitivity of 4.6 and 1.7 pg/mL, respectively. ELISA was performed by duplicate for each sample according to the manufacturer. Reading (Bio-

chrom, Anthos 2010, Cambridge, UK) was performed at 450 nm and 540 nm for TGF- β_1 and PDGF-AB, respectively. GF concentration only were measured in samples from 12 dogs.

STATISTICAL ANALYSIS

Data was evaluated for normality by using the Shapiro-Wilk test. Cell results and TGF- β_1 concentrations were presented as mean and mean standard error. The concentrations of PDGF-AB were presented as median and interquartile range. Cell data from whole blood and PC were compared by using the student's test (t) for paired samples. A one-way ANOVA was performed to compare TGF- β_1 concentration in plasma and PG supernatants. The PDGF-AB concentrations were analyzed by a Friedman test. PDGF-AB only was detected in the 45% of the samples, (left-censored data), and for the statistical analysis these values were matched to the ELISA's detection threshold test (1.7 pg/mL). Both GF were tested for correlation with cell counts by using Pearson and Spearman tests. The accepted statis-

tical difference was $P \leq 0.05$ for all tests. The statistical program SPSS 16.0 (SPSS Inc, Chicago, IL, USA) was used for this analysis.

RESULTS

CELL EVALUATION

Cell counts in whole blood and PC presented statistically significant differences ($P < 0.01$) with respect to the values of PLT, WBC, relative and absolute values of GRA, absolute of MID, relatives of PCV and LYM. However, there were no statistically significant differences in absolute values of LYM and relatives values of MID (table 1).

TRANSFORMING GROWTH FACTOR BETA 1 (TGF- β_1)

There were no statistically significant differences between concentrations of plasma and the PG supernatants activated with calcium gluconate or calcium chloride (table 2).

Table 1. Overall results of the cellular variables (data presented as mean (standard error)).

Resultados generales de las variables celulares (datos presentados como media (error estándar)).

Variable	Whole Blood	Platelet Concentrate	P value
PLT (fragments $\times 10^3 / \mu\text{L}$)	366.65 (30.25)	570.82 (42.42)	<0.001
PCV %	44.30 (1.39)	4.33 (0.73)	<0.001
WBC (cells $\times 10^3 / \mu\text{L}$)	14.11 (1.44)	7.05 (0.44)	<0.001
LYM (cells $\times 10^3 / \mu\text{L}$)	3.63 (0.95)	3.40 (0.34)	NS
LYM%	22.03 (2.96)	50.77 (4.74)	<0.001
MID (cells $\times 10^3 / \mu\text{L}$)	0.63 (0.14)	0.23 (0.03)	<0.001
MID%	4.07 (0.39)	3.26 (0.39)	NS
GRA (cells $\times 10^3 / \mu\text{L}$)	9.85 (0.66) a	3.41 (0.40)	<0.001
GRA%	73.92 (3.03) a	44.32 (4.20)	<0.001
MPV (fL)	9.1 (0.27)	9.1 (0.28)	NS

Statistically significant differences by t test. NS = Non significant.

Diferencias estadísticamente significativas mediante la prueba t. NS = no significativo.

Table 2. TGF- β_1 concentration in plasma and supernatants of platelet concentrates (PC) activated with calcium gluconate or calcium chloride (data presented as mean (standard error)).

Concentración de TGF- β_1 en plasma y en sobrenadante de concentrados autólogos de plaquetas (PC) activados con gluconato o cloruro de calcio (datos presentados como media (error estándar)).

Blood component	TGF- β_1 ng/mL	P value
Plasma (n:12)*	22.08 (3.79)	NS
Platelet concentrate activated with calcium Gluconate**	19.55 (2.69)	NS
Platelet concentrate activated with calcium Chloride**	19.98 (1.58)	NS

* EDTA was use as anticoagulant. ** ACD-A was used as anticoagulant. NS = Non significant.

* EDTA fue usado como anticoagulante. ** ACD-A fue usado como anticoagulante. NS = no significativo.

PLATELET-DERIVED GROWTH FACTOR AB (PDGF-AB)

The PDGF-AB concentrations were not detected in some samples. This GF was detected in 66% in the plasma samples obtained with EDTA. PDGF-AB was detected in 41.6 % of the PG supernatants, however this GF was not detected always in the same samples (table 3). There were no statistically significant differences between plasma concentrations and the concentrations obtained for this GF in the PG supernatants, either activated with calcium gluconate or calcium chloride.

CORRELATIONS

No correlation between plasma concentrations of both growth factors and the number of platelets in whole blood counts with EDTA was found. However, positive Pearson's correlations (ρ) were found between PLT/ μ L count in PC and TGF- β_1 concentrations in the PG supernatants activated either with calcium gluconate ($\rho = 0.746$ $P = 0.05$) or calcium chloride ($\rho = 0.836$ $P = 0.01$). There was no correlation between the PDGF-AB concentrations and cell counts.

DISCUSSION

There are some controversies about the classification of PC intended for regenerative medicine purposes in humans. There is some consensus for classifying PC in function of the cellular content and the quality of the fibrin mesh formed after PC activation. In general, PC with platelet count 1.3 to 3 fold and WBC count similar or slightly inferior to the whole blood values are con-

sidered as pure-platelet rich plasma (P-PRP) (Dohan Ehrenfest *et al* 2011). On the other hand, when PC present a platelet count 4 fold or more and WBC count 2 fold or more compared to the basal count of these cells in whole blood, these PC are considered as leukocyte-platelet rich plasma (L-PRP) (Dohan Ehrenfest *et al* 2011). When both PRP are activated with calcium salts or thrombin a PG is formed. The PG obtained from P-PRP is termed pure-platelet rich gel (P-PRG) and the gel obtained from L-PRP is known as L-PRG (Dohan Ehrenfest *et al* 2011). The PC obtained with the method evaluated in this study is thus considered as P-PRP and the gel obtained after activation should be considered as P-PRG. This is the first study describing the initial release of TGF- β_1 and PDGF-AB from canine P-PRG.

An effective method for concentrating platelets should firstly focus on obtaining live and non-activated platelets instead of concentrating a large number of them (Marx 2004). The technical procedures used in this study for preventing platelet activation (and consequently producing release of platelet GF) (Ferrer *et al* 2001, Egidi *et al* 2010) during PC preparation, were: 1) storage of blood samples in ice during 30 minutes and 2) centrifugation at low temperature (4 °C). This methodology has been used to determine plasma TGF- β_1 concentration without interference of platelet activation (Heinemeier *et al* 2003). However, it is very important to consider that when cold storage or centrifugation time is prolonged platelets are activated and could develop cold storage-related lesions (Ayukawa *et al* 2009, Shrivastava 2009).

The MVP values in our study did not differ between whole blood and PC, and were within the normal range described for dogs (Boudreaux and Ebbe 1998). EDTA

Table 3. Median (interquartile range) of the concentration (pg/mL) of platelet derived growth factor AB in plasma and platelet concentrate (PC) supernatants activated with either calcium gluconate or calcium chloride.

Mediana (rango intercuartil) de la concentración (pg/mL) de factor de crecimiento derivado de plaquetas AB en plasma y sobrenadante de concentrado autólogo de plaquetas (PC) activados con gluconato de calcio o cloruro de calcio.

Dog #	Plasma	PC activated with calcium Gluconate	PC activated with calcium chloride
1	140.46	222.3	167.64
2	305.19	167.64	59.26
3	86.31	-	-
4	140.46	194.92	113.36
5	167.64	-	-
6	222.3	-	86.31
7	113.36	59.26	194.92
8	-	-	-
9	-	-	-
10	-	-	-
11	59.26	249.8	-
12	-	-	-
Median	99.84 (159.15)	1.70 (214.62)	1.70 (120.88)

was used in this research because this substance is considered the anticoagulant of choice for counting blood cells (Wayne 2003), as well as for preserving cell morphology and avoiding the effect of to dilute the sample (Meinkoth and Allison 2007). However, we believe that the anticoagulant (EDTA) used for both basal hemogram and for collecting plasma, affected the TGF- β_1 and PDGF-AB concentration, since this anticoagulant produce platelet damage and consequently release of platelet related GF. In this sense, the plasma concentration of the GF evaluated in this study presented an artifactual increase of the GF related with functional and structural damage of the platelets possibly without inducing platelet activation. This fact limited our study since EDTA is not an ideal anticoagulant for measuring platelet activation (Macey *et al* 2002). This technical condition prevents the comparison between the plasma and PG supernatants of these GF and further studies are necessary to know the real plasma concentration of these proteins by using ACD-A as anticoagulant.

Vieweg *et al* (1999) reported the serum TGF- β_1 concentration for two dogs (16.5 and 19.9 ng/mL) using a human ELISA kit. Although they were not representative, the average values of plasma and supernatants obtained from PG were closer to the data obtained in our study. Moreover, another study (Neumann *et al* 2008) described plasma TGF- β_1 concentrations between 0.193 y 0.598 ng/mL in 29 canine blood samples collected with EDTA that were obtained by double centrifugation at 1000 g x 15 minutes followed by 10000 g x 10 minutes at an temperature of 4 °C. These values were substantially lower than those obtained in our study in plasma by using a single centrifugation episode and the same anticoagulant and temperature during centrifugation.

In this study, no difference was found between the TGF- β_1 and PDGF-AB concentrations in P-PRG supernatants activated with calcium gluconate or with calcium chloride at the same concentration and dose, which may indicate that the canine PC could be activated by any of these substances. Stief *et al* (2011) obtained mean TGF- β_1 concentrations of 1.2399 ± 0.5908 ng/mL in autologous conditioned plasma (ACP) obtained with ACD-A in 10 dogs. The concentrations were lower than the TGF- β_1 concentrations in the P-PRG supernatants of our study. The protocol used in this study allowed us to concentrate a higher average number of platelets ($570.82 \times 10^3/\mu\text{L}$) when compared to the reports of Stief *et al* (2011), who did not report a correlation between platelet count and TGF- β_1 concentration in the ACP supernatants. It is important to bear in mind that the ACP and our PC could be technically classified as P-PRP, but the cellular concentration of both substances dramatically influenced in their own profile of growth factors.

On the other hand, the differences found for the TGF- β_1 concentration measured in our study, when compared to other studies (Vercelli *et al* 2003, Neumann *et*

al 2008, Stief *et al* 2011), could be due to the methodology used for collecting and processing the samples, and potentially due to the fact that in our study we used a canine-specific ELISA kit. This last situation was not considered in the aforementioned studies where human-specific ELISA kits were used (Vercelli *et al* 2003, Neumann *et al* 2008, Stief *et al* 2011).

Using an ELISA kit for determination of human PDGF-AB, Vieweg *et al* (1999) reported a mean serum PDGF-AB concentration of 410.77 ± 177.56 pg/mL in seven dogs. However, other study (Stief *et al* 2011) using the same kit was unable to detect the concentration of this GF in plasma samples of 10 dogs. In our study, which used an ELISA kit similar to that used in previously mentioned studies, no reading was obtained for all samples in both plasma and P-PRG supernatants. In general, PDGF-AB concentrations in this study were about half the concentrations reported for Vieweg *et al* (1999). These differences could be due to the methodology for collecting and processing the samples. The failure to obtain readings by using this ELISA kit could be caused by a low activity of the PDGF present in dog serum and plasma (Bowen-Pope *et al* 1989) and to low homology between human and dog PDFG-AB.

The methodology used in our study is useful for producing PG and consequently release growth factors that might be acceptable for clinical or experimental use as a regenerative therapy in dogs. The relevance of the method reported here is that platelets can be concentrated by using ACD-A as anticoagulant, at single and soft centrifugation during short time. Our hypothesis was confirmed, since the GF concentration in P-PRG supernatants was similar when both calcium salts were used (gluconate or chloride) for PC activation. However TGF- β_1 concentration was highly correlated with the number of platelets concentrated in the PC (P-PRP).

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