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Evaluation of cytokine concentrations in a trehalose-stabilised lyophilised canine platelet product: a preliminary study

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ABSTRACT

Background Platelet transfusion is indicated for haemorrhage due to severe thrombocytopenia and for trauma associated coagulopathy. Febrile non-haemolytic transfusion reactions are a common complication of platelet transfusions in people and may be due to accumulated inflammatory cytokines. The present study aimed to determine the cytokine profile of a novel canine lyophilised platelet product following reconstitution, to assess the lyophilised platelets' activation response to physiological platelet agonists and to compare the cytokine profiles of basal and stimulated canine lyophilised platelets.

Methods Cell counts and biochemical analyses were conducted following reconstitution. Cytokine concentrations were measured with a canine-specific multiplex immunocapture assay and with an electrochemiluminescent ELISA. Aliquots of reconstituted product from three separate vials were activated for 10 minutes under non-stirred conditions using adenosine diphosphate, thrombin or convulxin and their cytokine concentrations compared with unactivated samples. Flow cytometry and light-transmission aggregometry were used to evaluate the product's ability to express a procoagulant surface, degranulate and aggregate. Fresh platelet-rich plasma was used as a positive control.

Results The product had a mean±SD particle count of $1.23\pm0.2\times10^9$/ml, contained platelets that expressed surface phosphatidylserine before agonist stimulation and was capable of aggregation in response to thrombin stimulation suggesting that the product may have haemostatic potential following in vivo administration. Cytokine concentrations measured by the immunocapture assay were generally low, while twofold to threefold increases relative to published intervals were noted for several cytokines using the ELISA. Concentrations of chemokine (C-X-C) motif ligand 8 and tumour necrosis factor-α were significantly increased as measured by the ELISA, but not by the immunocapture assay, while concentrations of KC-like were significantly increased as measured by the immunocapture assay. Stimulation with platelet agonists did not affect measured cytokine concentrations.

Conclusion Further study of the effects of administration of this lyophilised platelet product is warranted.

INTRODUCTION

Platelet transfusion is frequently used to control bleeding due to severe thrombocytopenia in people[1] and is now recognised as a crucial component of haemostatic resuscitation for traumatic haemorrhage.[2 3] The Pragmatic, Randomized Optimal Platelet and Plasma Ratios (PROPPR) trial in people indicated that a 1:1:1 ratio of plasma, platelets and packed red blood cells improved haemostasis and reduced deaths by exsanguination compared with a 1:1:2 ratio.[4] This emphasises the importance of platelets in the practice of haemostatic fluid resuscitation, but increases need for platelet products. The PROPPR study involved the use of fresh platelet concentrates that have a very short shelf-life, are difficult to store and hence are expensive to use.[5] Recent military conflicts have increased the need for multiple blood product components for management of major traumatic haemorrhage in resource-poor settings.[6–9] This has driven the development of platelet transfusion products that have longer shelf-lives and are easier to carry, store and use in conflict zones.[10] Lyophilised (freeze-dried) platelet products offer substantial benefits in this regard,[11 12] with one such product having successfully undergone phase I testing in people.[13] This product is derived from human platelets stabilised with trehalose before lyophilisation. Trehalose treatment is designed to protect platelet structure during the freezing part of the process and enhance the viability of platelets after reconstitution.[14–16]

A related canine product is commercially available for dogs and is undergoing evaluation in a randomised controlled trial in dogs with thrombocytopenic bleeding. This product contains lyophilised canine platelets and is labelled for the treatment of acute uncontrolled haemorrhage in dogs secondary to thrombocytopenia. The product is derived from leukoreduced canine platelets collected by apheresis or whole blood donation and contains platelets in a dehydrated buffer containing HEPES, NaCl, KCl, $NaHCO_3$, dextrose, trehalose, ethanol and polysucrose.

The platelets used to generate this product are derived from leukoreduced sources and hence should have minimal white blood cell contamination.

Residual leukocytes in blood products are believed to elaborate inflammatory cytokines during storage that mediate febrile non-haemolytic transfusion reactions (NHFTR), a common complication of platelet transfusions in people.[17–20] These accumulated cytokines represent an important part of the platelet storage lesion. It has been demonstrated in people that increased cytokine concentrations develop in stored platelet concentrates,[21] and that this can be prevented or diminished by leukoreduction using filtration of the platelet concentrates before storage.[22–25] The method of platelet recovery is also a potential factor in determining the cytokine profile of the resulting platelet product with different cytokine concentrations in products derived from platelet-rich plasma, buffy-coat extraction or collected by apheresis.[23–25] Compared with buffy coat sources, platelets derived from apheresis may generate lower cytokine concentrations during storage due to lower residual leucocyte counts.

Lyophilised products are therefore expected to have low cytokine concentrations reflective principally of the minimal plasma remaining within the final product. Stored platelets have the capacity to take up and also to generate certain cytokines (eg, RANTES) and the capacity for such release has been used to assess the maintenance of platelet secretory capacity in stored platelets.[26] The cytokine profile of this new canine lyophilised product has not been characterised and as such, the potential for this product to generate NHFTR is hard to predict. This study was undertaken to fill this knowledge-gap by measuring cytokines in the canine product.

The specific objectives of the present study were: to determine the cytokine profile of a reconstituted, unstimulated canine lyophilised platelet product (StablePlate RX); to assess the lyophilised platelets' activation response to physiological platelet agonists and to compare the cytokine profiles of basal and stimulated canine lyophilised platelets. It was hypothesised that unstimulated canine StablePlate RX has a cytokine profile consistent with healthy normal canine plasma and that stimulation of canine StablePlate RX induces release of proinflammatory and anti-inflammatory cytokines.

MATERIALS AND METHODS

Cell counts and biochemical analyses

Vials of the lyophilised platelet product were reconstituted with 8 ml sterile water for injection per the manufacturer's instructions and used immediately. All vials were from the same lot number (V-D-017–075). The platelets in each lot are derived from 18 different platelet apheresis donors. To evaluate the baseline properties, the reconstituted product was tested as follows. Particle counts were performed using a Z1 Dual Coulter counter fitted with a 50 µm aperture (Beckman Coulter, Brea, California, USA), the counter was set to count all particles between 2 and 8 µm diameter. These settings were designed to count single platelets, although small platelet aggregates with a diameter between the upper and lower bounds would have been counted as a single particle. Aliquots were removed and submitted for cell count and biochemical analysis by the institution clinical pathology laboratory. Blood cell counts were performed using an ADVIA 2120 (Siemens, Washington, DC, USA). Biochemical analyses were performed using a Cobas 501 (Roche Diagnostics, Indianapolis, Indiana, USA). Fibrinogen concentrations of the reconstituted product were measured by the Clauss method by use of a human thrombin reagent (STA-Fibrinogen, Diagnostica Stago, Parsippany, New Jersey, USA) and a standard curve derived from dilutions of a canine plasma standard. The fibrinogen concentration of the canine plasma standard was determined by a quantitative, gravimetric method.[27] Blood gas, acid-base, electrolyte and lactate concentrations were measured in the reconstituted product using a RapidPoint 400 benchtop point-of-care blood gas analyzer (Siemens, Malvern, Pennsylvania, USA) and a handheld lactate meter Lactate Pro (Arkray, Edina, Minnesota, USA), respectively.

Cytokine profile following platelet activation

To evaluate the effects of platelet activation on cytokine concentrations, aliquots of reconstituted product from three separate vials were activated for 10 minutes under non-stirred conditions using various platelet agonists. Specifically, the P2Y receptor pathways were stimulated with adenosine diphosphate (ADP) (final concentrations 20 µM, 100 µM, 1000 µM), the PAR receptor pathways were stimulated with gamma-thrombin (final concentrations 50 nM, 100 nM and 380 nM) and alpha-thrombin (final concentrations 0.2 U/ml, 1 U/ml, 5 U/ml) and the collagen-GPVI pathway was stimulated with convulxin (final concentrations 20 ng/ml, 50 ng/ml, 100 ng/ml). To overcome potential inhibition from inadequate extracellular calcium concentrations resulting from use of citrate-phosphate dextrose adenine solution for anticoagulation of the original platelet product,[28] calcium chloride was added to each aliquot of reconstituted lyophilised platelets before activation (final concentration 1 mM). Aliquots of the lyophilised platelet product were left unstimulated after reconstitution to act as untreated (basal) controls. To evaluate the effects of the agonist vehicles, aliquots were also combined with equal volume of phosphate buffered saline or with dimethyl sulfoxide (DMSO). Aliquots of the product were also treated with the calcium ionophores A23187, final concentration 50 µM and phorbol myristate acetate (PMA), final concentration 50 µM to non-specifically activate protein kinase C. All reactions were initiated within 40 minutes of product reconstitution. Following agonist stimulation, reactions were quenched by rapid cooling to 4°C immediately followed by centrifugation at 4°C (16 000 rcf, 5 minutes). The supernatants were then frozen

pending batch cytokine analyses. Pooled platelet-poor plasma collected from healthy normal dogs, anticoagulated using CPDA-1 (each containing plasma from three dogs) was used as control samples for the cytokine assays. These platelet-poor plasma samples were obtained from the institution blood bank.

Canine platelet-rich plasma preparation

To provide positive controls for the flow cytometry assays, canine platelet-rich plasma was prepared from freshly drawn blood samples collected in accordance with a protocol approved by the local institutional animal care and use committee (IACUC-2014–0052). Citrate (3.2 per cent) anticoagulated blood (2.7 ml) was obtained in a 1:9 ratio from the external jugular veins of healthy dogs and then diluted in a 1:1 ratio to a final volume of 1.2 ml with buffer saline glucose citrate (129 mmol/l NaCl, 13.6 mmol/l sodium citrate, 11.1 mmol/l glucose, 1.6 mmol/l KH_2PO_4, 8.6 mmol/l NaH_2PO_4, pH 7.31).[29] The cell suspension was gently mixed and then centrifuged at 1900×*g* (5300 rpm) for 30 seconds at room temperature (RT). The resultant dilute platelet-rich plasma supernatant was collected and rested for 10 minutes at RT before activation.

Cytokine measurements

Concentrations of proinflammatory and anti-inflammatory cytokines in the lyophilised platelet products were measured at baseline and following activation using two established immunoassay methods. The first method involved a commercial premixed canine-specific multiplex assay CCYTOMAG-90K (MilliporeSigma, Burlington, Massachusetts, USA) kit that uses bead-assisted immunocapture. This 13-plex assay was analysed by flow cytometry using a Bio-Plex System with Luminex xMap Technology (Bio-Rad, Hercules, California, USA) enabling simultaneous quantification of GM-CSF, IFN-ɣ, KC-like, chemokine (C-X-C) motif ligand 10 (CXCL10), IL-2, IL-6, IL-7, CXCL8, IL-10, IL-15, IL-18, CCL2 and tumour necrosis factor (TNF)-α. This assay has been validated for use on canine serum and plasma samples and has been widely used to measure canine blood cytokine concentrations by our group and others;[30 31] for review, see Richter and others.[32] Another immunoassay with overlapping spectra but with a distinct assay principle was used to augment cytokine detection. This second plate-based immunoassay, the Canine ProInflammatory Panel 3 Ultra-Sensitive Kit (Meso Scale Diagnostics (MSD), Rockville, Maryland, USA) measures four cytokines (IL-2, IL-6, CXCL8, TNF-α) using a proprietary electrochemiluminescent detection system, QuickPlex SQ 120 (MSD, Rockville, Maryland, USA) and has also been used by various groups to measure cytokine concentrations in canine serum and plasma samples.[30 33–35] For both assays, standard curves were generated in duplicate using manufacturer supplied reagents. All sample analyses were performed in duplicate and the mean values of duplicate readings used for statistical analyses. For the plate-based assay, where values were below the lower limit of detection, the manufacturer's stated minimum detectable concentrations (pg/ml) were imputed as follows: 1.3 (CXCL8), 7.6 (IL-2), 2.4 (IL-6), 0.2 (TNFα). For the bead-based assay, where values were recorded as below the measurable range of the assay the manufacturer's stated minimum detectable concentrations (pg/ml) were imputed as follows: 21.0 (CCL2), 21.7 (CXCL8), 3.2 (CXCL10), 9.2 (GM-CSF), 3.5 (IL-2), 3.7 (IL-6), 7.5 (IL-7), 8.5 (IL-10), 9.0 (IL-15), 5.8 (IL-18), 5.3 (KC-like), 6.1 (TNF-α). Values for IFN-ɣ are not reported, because it is reported that the assay does not reliably detect this cytokine in canine serum.[36]

Flow cytometry

Flow cytometry assays were performed using 10 µL aliquots of reconstituted product or fresh PRP (positive control), diluted in 1 ml buffer containing 2.0 mM calcium chloride (Annexin V binding buffer, R&D Systems, Minneapolis, Minnesota, USA) and 0.4 mM gly-pro-arg-pro-NH_2 acetate (Sigma Aldrich, St. Louis, Missouri, USA) final concentrations. Four separate aliquots were stimulated with bovine alpha-thrombin (Sigma) at 0.1 U/ml and 1.0 U/ml and convulxin (Neta Scientific, Hainesport, New Jersey, USA) at 100 ng/ml and 500 ng/ml final concentrations. The samples were incubated for 10 minutes in a 37°C water bath without stirring. Non-stimulated samples of reconstituted product or fresh PRP were used as negative controls, respectively. Platelets were then triple labelled with a 1:100 dilution of anti-CD61-APC (clone Y2/51, Dako-Agilent, Santa Clara, California, USA) to detect GpIIIa as a constitutive platelet membrane marker and anti-CD62P-PE (clone AC1.2, BD Biosciences, San Diego, California, USA) to detect P-selectin expression and FITC-lactadherin (Haematologic Technologies, Essex Junction, Vermont, USA) to detect phosphatidylserine (procoagulant phospholipid) externalisation. An isotype antibody conjugated with PE (BD, Biosciences) was used to define the boundary for positive labelling with CD62P-PE . Cell suspensions were incubated in the dark for 20 minutes at RT, quenched with 400 µL PBS and analysed within 30 minutes on a cytometer (FacsCalibur, BD Biosciences, San Jose, California, USA). Platelet populations were defined based on FCS, SSC and CD61 fluorescence, and 10 000 platelet-gate events were collected per experiment. Data were analysed using FlowJo V. 10.5 (BD Biosciences, San Jose, California, USA) to determine the percentage of CD61-positive platelets with positive results P-selectin and phosphatidylserine expression.

Light-transmission aggregometry

The ability of platelets in the lyophilised platelet product to aggregate in response to agonists was evaluated using light-transmission aggregometry. Aggregation studies were conducted using aliquots of reconstituted platelet product (245 µL at 5×10^8/ml) in a Born lumi-aggregometer (560-VS, Chrono-Log), stirred at 1000 rpm

Table 1 Descriptive statistics for complete blood count and chemistry parameters of the lyophilised platelet units

Parameter	Mean	SD	Parameter	Mean	SD
Haematocrit (%)	0.33	0.58	Neutrophils ($\times10^9$/l)	0.0	0.0
Haemoglobin (g/l)	0	0	Lymphocytes ($\times10^9$/l)	0.0	0.1
RBC ($\times10^{12}$/l)	0.07	0.06	Monocytes ($\times10^9$/l)	0.0	0.0
MCV (fL)	66.3	12.0	Eosinophils ($\times10^9$/l)	0.0	0.0
MCH (pg)	3.7	6.4	Basophils ($\times10^9$/l)	0.0	0.0
MCHC (g/dl)	5	9	Other ($\times10^9$/l)	0.0	0.0
RDW (%)	12.1	4.6	Platelets ($\times10^9$/l)	1648	124
Leukocytes ($\times10^9$/l)	0.1	0	MPV (fl)	14.6	0.8
Sodium (mmol/l)	72.7	1.5	Glucose (mmol/l)	2.8	0.1
Potassium (mmol/l)	6.2	0.2	ALT (U/l)	5.3	0.6
Chloride (mmol/l)	53.3	2.1	AST (U/l)	153.0	11.5
Bicarbonate (mmol/l)	1.0	1.0	ALP (U/l)	2.7	2.1
Anion gap (mmol/l)	25.0	2.6	GGT (U/l)	0.0	0.0
BUN (mmol/l)	0.5	0.6	Total bilirubin (µmol/l)	0.0	0.0
Creatinine (µmol/l)	17.7	0.0	Amylase (U/l)	33.7	1.2
Calcium (mmol/l)	0.3	0.0	Lipase (U/l)	5.7	0.6
Phosphate (mmol/l)	0.5	0.1	Cholesterol (mmol/l)	0.2	0.0
Magnesium (mmol/l)	0.6	0.0	CK (U/l)	2445.0	770.2
Total protein (g/l)	5.0	0.0	LDH (U/l)	1199.0	344.7
Albumin (g/l)	2.7	0.6	Iron (µmol/l)	2.6	0.3
Globulin (g/l)	2.3	0.6	Fibrinogen (g/l)	1.5	0.2

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBC, red blood cell.

(37°C). Traces were captured using a computer interface (Aggro/Link, Chrono-Log, Havertown, Pennsylvania, USA). The percentage maximum aggregation at 3 min was measured in response to a series of physiological agonists including ADP (100 µM), α-thrombin (5 U/ml, 1 U/ml), ɣ-thrombin (380 nM) and convulxin (100 ng/ml) and the pharmacological agents A23187 (50 µM) and PMA (50 µM). DMSO and saline were used as vehicle controls. Before each tracing the aggregometer was calibrated against a buffered saline blank, such that 100 per cent aggregation was considered to be equivalent to the light transmitted through this saline blank.

Statistical analysis

Descriptive statistics were calculated using commercial software (Prism 7e, GraphPad, La Jolla, California, USA). Cytokine concentrations were compared between agonists or agonist concentrations using one-way ANOVA for repeated measures. Cytokine concentrations in the lyophilised platelet product at baseline were compared with concentrations in control plasma using unpaired t-tests. Bonferroni corrections were applied posthoc to control for multiple comparisons. Corrected P values below 0.05 were considered significant.

RESULTS

Cell counts and biochemical analyses

The mean (±SD) particle count following reconstitution was $1.23\pm0.2\times10^9$/ml. The mean pH of the solution was 6.81±0.02. The mean lactate concentration was 3.4±2.7 mmol/l. Results of the complete blood counts and biochemical analyses are presented in table 1.

Platelet activation responses

Flow cytometry analyses indicated that platelets in the lyophilised platelet product expressed CD61 but were not able to externalise P-selectin (CD62-P) in response to either dose of thrombin or convulxin (figure 1E,F). The lyophilised platelets however had phosphatidylserine exposed on their outer membrane irrespective of activation status (figure 1D–F). The fresh platelet-rich plasma samples stimulated with thrombin and convulxin demonstrated a shift in forward scatter and side scatter (characteristic of activated platelets) and with a new population representing platelet fragments and microparticles evident (figure 1B,C). In contrast, the lyophilised platelets demonstrated the appearance of an activated platelet population (with respect to side scatter and forward scatter) and minimal to no apparent qualitative changes in forward scatter in the stimulated samples (figure 1E,F).

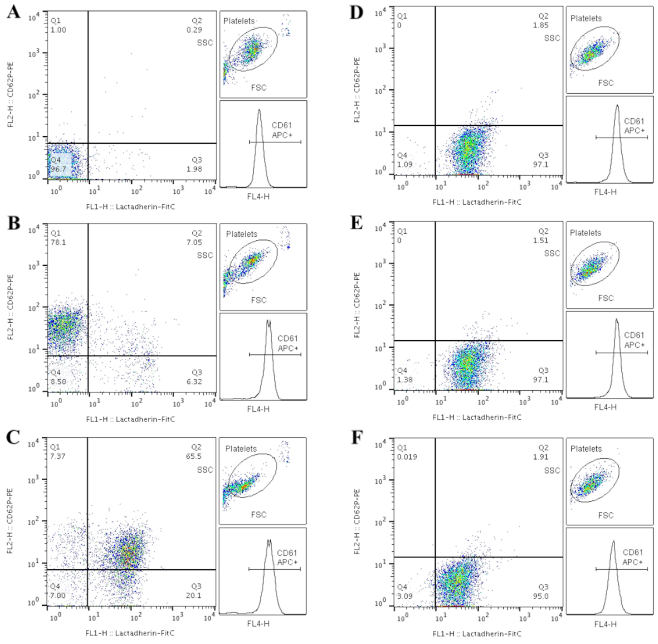

Figure 1 CD62-P (P-selectin, y axis) and phosphatidylserine expression (Lactadherin-binding, x axis) on the surface of platelet populations gated by means of side scatter and forward scatter (shown at top right, small ancestry panel) and by CD61-APC fluorescence (shown at bottom right small panel). (A–C) Depict density plots of triple-labelled fresh platelet-rich plasma and (D–F) depict triple-labelled lyophilised platelet samples. Non-stimulated samples are shown in the top row (A,D); thrombin stimulated (1.0 U/ml) samples are shown in the middle row (B,E); convulxin (500 ng/ml) stimulated samples are show on the bottom row (C,F). The platelet gate was defined from the side scatter and forward scatter properties of unstimulated fresh platelet-rich plasma (A). Representative plots of a single experiment are shown.

Platelet aggregometry demonstrated that the lyophilised platelet product aggregated in response to all concentrations tested of alpha thrombin and gamma thrombin (figure 2, table 2). In contrast, there was no aggregation in response to convulxin, ADP, A23187 or PMA stimulation.

Cytokine concentrations

The concentrations of cytokines in the lyophilised platelet product are summarised in tables 3 and 4; note that control samples represent pooled platelet-poor plasma each containing plasma from three dogs. Values for IL-2, IL-6, CXCL10 and TNFα were consistently at or below the lower limit of detection for the bead-based assay and hence are not displayed in the tables. Likewise, values for IFN-ɣ are not reported, because the assay does not reliably detect this cytokine in canine serum. In general, cytokine concentrations in the lyophilised platelet units measured by the bead-based assay were within published reference intervals for canine serum.[36] The exception was CXCL8 that was measured at high concentrations relative to published reference intervals. Although the values were not greater than controls, there was considerable variability in the control sample CXCL8 concentrations. The concentrations of KC-Like were significantly increased in the lyophilised platelet units compared with controls as measured by the bead-based assay (p=0.004). Values for all of the cytokines measured by the plate-based assay were above published values for healthy dogs.[33] The concentrations of CXCL8 in the lyophilised platelet units were significantly greater than in controls as measured by the plate-based immunoassay (p=0.029). Likewise, as measured by the plate-based immunoassay, the TNFα concentrations were significantly higher in the lyophilised platelet units compared with control plasma

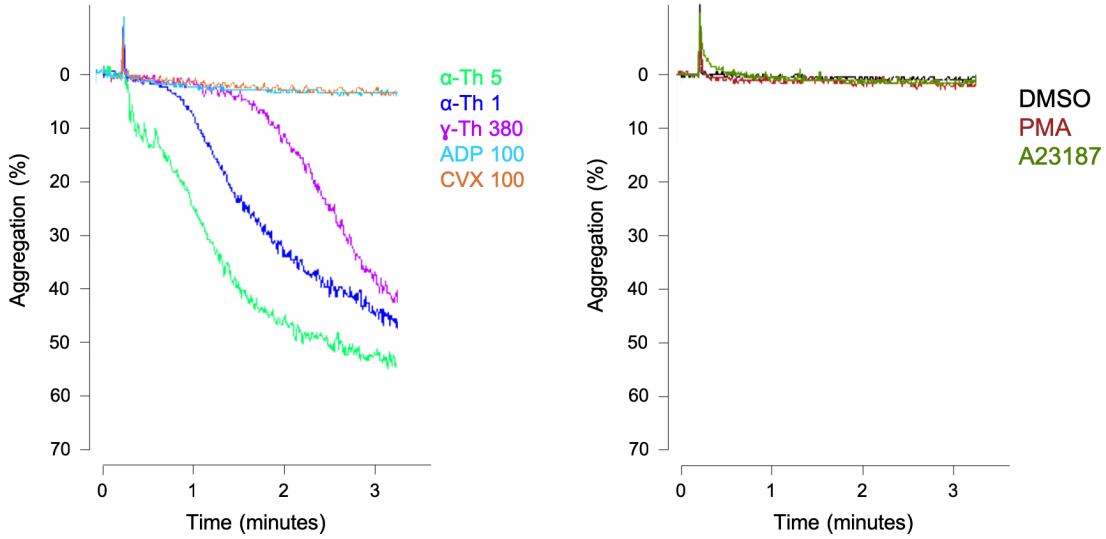

Figure 2 Representative light transmission aggregation traces from aliquots of lyophilised platelets (245 µL at 5×10^8/ml) stimulated in a Born aggregometer (560-VS, Chrono-Log), stirred at 1000 rpm (37°C). The percentage maximum aggregation at 3 minutes was measured in response to ADP (100 µM), α-thrombin (5 U/ml, 1 U/ml), ɣ-thrombin (380 nM) and convulxin (100 ng/ml) stimulation. Controls employed included A23187 (50 µM), PMA (50 µM) and DMSO as a vehicle control. DMSO, dimethyl sulfoxide; PMA, phorbol myristate acetate.

Table 2 Summary light transmission aggregometry data from analyses of the lyophilised platelet units

Agonist (final concentration)	Maximum aggregation (%)
α-thrombin (5 U/ml)	43.3±5.5
α-thrombin (1 U/ml)	40.3±8.1
γ-thrombin (380 nM)	27.0±20.0
Convulxin (100 ng/ml)	4.7±1.2
ADP (100 µM)	3.3±0.6
A23187 (50 µM)	2.7±2.5
PMA (50 µM)	1.7±1.5
DMSO (n=1)	0

0% aggregation represents no change in light transmission from baseline, while 100% aggregation was equivalent to light transmission through a buffered saline blank.
A23187, calimycin; ADP, adenosine diphosphate; DMSO, dimethyl sulfoxide; PMA, phorbol myristate acetate.

(p=0.008). Agonist stimulation did not significantly affect cytokine concentrations for any cytokine, for any agonist as measured with either assay.

DISCUSSION

This study aimed to determine the cytokine profile of a canine lyophilised platelet product and to characterise the changes in cytokine concentrations following activation by physiological agonists. The present study used two distinct assays for cytokine detection because these assays are reported to have varying sensitivity for individual analytes.[32] Comparisons with previously reported normal range for canine serum,[36] and normal canine values[33] suggest that several of the cytokines in the lyophilised product were mildly increased. Concentrations of IL-2, IL-6, CXCL8 and TNFα in the lyophilised platelets were all above the values previously reported for healthy dogs as measured by the plate-based assay, with increases typically twofold to threefold for cytokines except for CXCL8 which was approximately fivefold increased as measured by the plate-based assay.

Concentrations of CXCL8 and TNFα as measured by the plate-based assay, and KC-Like as measured by the bead-based assay were significantly greater in the basal, untreated lyophilised platelet product than in control plasma samples after correction for multiple comparisons. The increase in KC-Like while statistically significant is of questionable clinical relevance because the values remain within previously reported reference intervals for dogs. The concentration of TNFα is of unknown clinical significance. This proinflammatory cytokine is predominantly cell-associated[37] and hence mild increases in free TNFα might be more important than similar concentrations of other cytokines. Levels of TNF in the product were comparable to concentrations reported in dogs with sepsis,[38] and with IMHA,[39] and hence are noteworthy, but warrant confirmation and further study of any potential clinical impact.

Comparisons with stored red blood cell units might enable assessment of the potential clinical relevance of the cytokine concentrations measured in the lyophilised platelet units. Several groups have assessed the cytokine concentrations of stored canine red blood cells, with and without leukoreduction.[40–42] Compared with the study by Corsi and others,[40] the CXCL8 concentrations in the lyophilised platelets were similar to non-leukoreduced red blood cell units stored for ≥28 days, while the TNFα concentrations were 10-fold lower than in non-leukoreduced units and threefold lower than in leukoreduced units. Compared with the study by Yang and others,[42] the CXCL8 concentrations in the lyophilised platelets are lower than in red blood cell units stored for ≥14 days, while all TNFα concentrations were below the assay's detectable range. In all non-leukoreduced units in the Purcell study,[41] the CXCL8 concentrations were greater than in the lyophilised platelet units in the present study. Similarly, the TNFα concentrations were below the assay's detectable range in that study. Overall, the cytokine concentrations documented in the lyophilised platelets are comparable to those reported for other transfusion products and hence would not be expected to cause adverse effects following administration.

The increase in CXCL8 concentration is notable, because the values as measured by both assays were substantially greater than those reported for healthy dogs in recent studies.[33 36] It should be noted, however, that the concentrations of CXCL8 as measured by the bead-based assay were not significantly different from contemporaneous control plasma samples, which may have resulted from high variability in these control samples. As such, further investigation is needed to clarify the cause of this discrepancy and define any clinical implications of a true increase in CXCL8 concentration. The chemokine CXCL8 (also called interleukin-8) is a neutrophil chemoattractant and activator and also serves to recruit T-cells and basophils.[43] Concentrations of CXCL8 are increased in various inflammatory diseases in people including chronic obstructive pulmonary disease, asthma and cancer[44] and related diseases in veterinary species.[45] The pathophysiological role of CXCL8 in canine inflammatory processes is still under investigation, however.[38 46–48] This chemokine is produced by a wide variety of cell types including monocytes, T-cells, endothelial cell, platelets and neutrophils themselves.[49] The origin of the increased CXCL8 concentrations in the lyophilised platelet product are not clear, but release from the platelets themselves is a plausible source,[50] as human platelet alpha granules contain CXCL8.[49 50] Human platelet alpha granules also reportedly contain CCL2,[51] but proteomics studies suggest the concentrations of CXCL8 in platelets are higher than those of CCL2.[52] This difference in the granule content of these cytokines may explain why CXCL8 concentrations were found to be increased, while those of CCL2 were not. Our group recently demonstrated using a proteomics approach that, in contrast to human platelets, CXCL8 is highly abundant in canine

Table 3 Cytokine concentrations in the lyophilised platelet units as measured by the bead-based immunocapture assay/ Luminex

Cytokine	LLOD	RI[36]	Basal	α-Th 5 U/ml	α-Th 1 U/ml	α-Th 0.2 U/ml	ɣ-Th 380 nM	ɣ-Th 100 nM	ɣ-Th 50 nMnM
CCL2	21	0–317	64±43	92±85	77±49	74±76	71±70	99±68	90±61
CXCL8*	0.02	0–3.78	1135±801	1144±753	1077±815	560±204	775±303	2636±1481	1423±949
GM-CSF	9.2	0–332	10±1	37±22	28±13	27±15	31±15	26±14	25±13
IL-7	7.5	0–507	14±3	12±3	12±5	10±1	11±3	17±5	13±3
IL-10	8.5	0–2064	217±72	282±113	274±89	285±102	261±97	284±112	274±116
IL-15	9.0	0–578	94±50	90±41	118±62	90±44	124±57	147±71	109±50
IL-18	5.8	0–638	12±4	18±6	13±4	14±5	16±5	18±6	18±7
KC-Like	5.3	0–855	100±8	139±43	115±14	138±47	125±26	154±51	150±49

Cytokine	LLOD	RI[36]	Basal	CVX 100 ng/ml	CVX 50 ng/ml	CVX 20 ng/ml	ADP 1000 µM	ADP 100 µM	ADP 20 µM µM
CCL2	21	0–317	64±43	91±37	74±29	70±31	67±29	65±16	83±10
CXCL8*	0.02	0–3.78	1135±801	1277±733	630±212	789±423	602±212	1946±1471	1005±211
GM-CSF	9.2	0–332	10±1	36±15	31±15	27±14	19±10	25±12	17±8
IL-7	7.5	0–507	14±3	15±7	13±3	13±4	9±1	13±5	14±3
IL-10	8.5	0–2064	217±72	307±114	276±98	252±83	238±69	272±63	309±28
IL-15	9.0	0–578	94±50	168±92	118±54	91±46	82±37	131±35	139±21
IL-18	5.8	0–638	12±4	18±6	19±6	13±4	14±4	17±5	17±3
KC-Like	5.3	0–855	100±8	142±31	146±42	136±40	132±32	132±30	135±24

Cytokine	LLOD	RI[36]	Basal	A23187 50 µM	PMA 50 µM	PBS	DMSO	Control (n=2)
CCL2	21	0–317	64±43	57±9	80±34	67±23	63±26	287±86
CXCL8*	0.02	0–3.78	1135±801	556±51	599±226	397±104	446±134	3500±3500
GM-CSF	9.2	0–332	10±1	14±5	9±0	12±2	13±2	114±105
IL-7	7.5	0–507	14±3	10±1	11±3	11±3	17±6	42±35
IL-10	8.5	0–2064	217±72	264±29	225±80	203±55	224±59	27±18
IL-15	9.0	0–578	94±50	108±27	103±47	58±29	110±52	237±228
IL-18	5.8	0–638	12±4	14±1	16±5	13±4	11±3	52±43
KC-Like	5.3	0–855	100±8	103±6	98±11	101±5	103±10	5.3±0

All cytokine concentrations are in pg/ml, except for CXCL8 which is in ng/ml (denoted by *); this also means the LLOD value appears substantially smaller for CXCL8 than for other cytokines. Note that the basal (unstimulated) values have been reproduced adjacent to each set of stimulated values for ease of comparison.
A23187, calimycin; ADP, adenosine diphosphate; CCL, C-C motif chemokine ligand; CVX, convulxin; CXCL, chemokine (C-X-C) motif ligand; DMSO, dimethyl sulfoxide; GM-CSF, granulocyte-monocyte colony stimulating factor; IL, interleukin; KC-Like, keratinocyte chemoattractant-like; LLOD, lower limit of detection; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; RI, reference interval (from Johnson and others); Th, thrombin; TNF, tumour necrosis factor.

platelets and that canine platelets release CXCL8 during activation.[53] A strong correlation between serum CXCL8 concentration and platelet count was also described in a study of thrombocytopenic dogs, compatible with release from platelets as the source of this chemokine during the process of coagulation and serum formation. In contrast, CCL2 was not identified in our recent proteomics study of canine platelet releasate.

If platelet activation results in CXCL8 release, as is the case in people,[52] then it would be expected that stimulation of the reconstituted lyophilised platelet product could increase the measured CXCL8 concentrations. This was not the case with any of the agonists evaluated, and CXCL8 concentrations were fairly consistent across all of the samples and conditions. This was also the pattern for all of the other cytokines evaluated. This could be interpreted one of three ways: (a) platelets do not secrete cytokines on activation, (b) the platelets were not activated in our experiments or (c) the platelets were already activated and hence incremental cytokine release could not be detected. To investigate lyophilised platelet reactivity, we evaluated various aspects of platelet activation response including aggregation, P-selectin expression as a marker of degranulation and phosphatidylserine externalisation as an indicator of platelet procoagulant activity. The lyophilised platelet product was able to aggregate in

Table 4 Cytokine concentrations in the lyophilised platelet units as measured by the plate-based assay (ELISA)

Cytokine	LLOD	RI[33]	Basal	α-Th 5 U/ml	α-Th 1 U/ml	α-Th 0.2 U/ml	γ-Th 380 nM	γ-Th 100 nM	γ-Th 50 nM
IL-2	7.6	0–79	228±98	196±40	191±45	218±26	201±19	193±11	207±47
IL-6	2.4	0–65	117±77	82±17	115±35	109±49	98±32	130±37	84±26
CXCL8	1.3	0–4400	19 239±4902	22 518±5348	22 670±5242	22 179±4817	21 981±4541	22 182±4761	22 303±4893
TNFα	0.2	0–7	21±3	20±1	20±4	22±4	20±3	21±0	20±1

Cytokine	LLOD	RI[33]	Basal	CVX 100 ng/ml	CVX 50 ng/ml	CVX 20 ng/ml	ADP 1000 µM	ADP 100 µM	ADP 20 µM
IL-2	7.6	0–79	228±98	180±54	256±97	203±15	198±5	218±61	205±63
IL-6	2.4	0–65	117±77	100±27	109±19	97±29	84±18	96±6	93±32
CXCL8	1.3	0–4400	19 239±4902	22 704±5390	22 037±4994	22 087±4811	19 554±4961	19 322±4950	19 700±5405
TNFα	0.2	0–7	21±3	23±6	27±15	30±12	19±1	21±4	20±1

Cytokine	LLOD	RI[33]	Basal	A23187 50 µM	PMA 50 µM	PBS	DMSO	Control
IL-2	7.6	0–79	228±98	211±52	216±18	193±6	172±10	33±9
IL-6	2.4	0–65	117±77	112±49	101±17	89±18	115±11	30±13
CXCL8	1.3	0–4400	19 239±4902	19 472±5126	19 359±4857	19 244±4398	19 376±4490	7±7
TNFα	0.2	0–7	21±3	19±2	19±2	19±2	17±1	3±1

All cytokine concentrations are in pg/ml. Note that the basal (unstimulated) values have been reproduced adjacent to each set of stimulated values for ease of comparison.
A23187, calimycin; ADP, adenosine diphosphate; CVX, convulxin; CXCL, chemokine (C-X-C) motif ligand; DMSO, dimethyl sulfoxide; IL, interleukin; LLOD, lower limit of detection; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; RI, reference interval (from Kilpatrick and others); Th, thrombin; TNF, tumour necrosis factor.

response to high concentrations of thrombin. It should be noted that the aggregometry tracings did not display platelet shape change; thus, we cannot exclude agglutination, rather than true aggregation mediated by signalling through and activation of the fibrinogen receptor as the mechanism by which there was a change in light transmission in the aggregometer. We saw no evidence of platelet shape change or aggregation after treatment with any other agonist, further suggesting that platelet signalling and downstream response were impaired. The cause of the lack of responsiveness to ADP or convulxin is uncertain. Thrombin, ADP and convulxin all act on different receptors.[54] Thrombin is considered the most potent of these three agonists,[55] and canine platelets are generally less responsive to ADP than to thrombin.[56] It is also possible that the lyophilisation process affected the distinct receptors differently such that thrombin responsiveness through the protease activated receptors was preserved while reducing or disrupting expression of the P2Y and GPVI receptors.

Similarly, flow cytometry revealed minimal basal or stimulated P-selectin expression, (ie, a lack of platelet degranulation). The lyophilised platelets, however demonstrated expression of the high density, constitutive membrane protein GpIIIa (CD61) and a high percentage of the lyophilised platelets were positive for phosphatidylserine exposure in both unstimulated and stimulated reactions. Rapid platelet membrane phosphatidylserine externalisation is triggered by agonists that induce sustained high intracellular calcium levels; however, outer membrane phosphatidylserine exposure is also a feature of platelet senescence mediated by apoptotic stimuli.[57] It is possible that the process of lyophilisation induces changes in platelet calcium mobilisation or activates apoptotic pathways and that both phosphatidylserine externalisation and CXCL8 represent epiphenomenon of either process. On the basis of the forward and side-scatter profiles, the activated fresh platelets appeared to generate platelet fragments and microparticles in response to agonist stimulation, but similar small particles were not seen in analyses of the lyophilised platelets. This is less consistent with apoptosis. Additional analyses including caspase-3 activity would be required to determine what role apoptosis plays in the phosphatidylserine exposure identified in the unstimulated lyophilised platelets.

Overall, the platelet activation data suggest that the process of generating the lyophilised platelet product renders them procoagulant based on outer membrane phosphatidylserine exposure, but poorly responsive to most agonist stimuli. From the perspective of in vivo haemostasis, this characteristic might promote fibrin clot formation by providing a surface for enhanced assembly of procoagulant factor complexes and thrombin generation regardless of impaired degranulation response.

Other potential sources of the CXCL8, such as neutrophils or mononuclear cells are considered less likely. The apheresis process substantially reduces leukocytes in the final product, as confirmed by the complete blood counts we performed. While it is possible that there were increased concentrations of CXCL8 in the platelet donor

dogs, none of the other cytokines were increased above normal concentrations. The potential consequences of infusion of increased concentrations of CXCL8 into patients receiving the lyophilised platelet product are unclear. Cytokine and chemokine concentrations are dynamic and alterations are typically transient.[58 59] The ongoing clinical trial of this product will provide data to determine whether the lyophilised platelets generate NHFTR in recipients, potentially mediated by CXCL8.

The limitations of this study include our evaluation of only three lyophilised platelet product units. We cannot be certain that other units from distinct batches of preparation would generate identical results. Our study included only in vitro analyses of the products. Although the cytokine concentrations of the products themselves are generally low, it is not known what effect infusion of this allogeneic platelet product has on the cytokine concentrations of recipients who retain the capacity to mount an immunological response and generate cytokines de novo. This question is under investigation in the ongoing treatment trials. The present study used two multiplex assays to measure a wide array of cytokine concentrations. One key analyte that was not available in these panels and hence was measured in the present study is IL-1. As an important proinflammatory cytokine, measurements of IL-1 in the product might be of interest should any adverse inflammatory or febrile events be noted following administration of the lyophilised platelets.

In conclusion, the lyophilised canine platelet product has minimal contamination by other cell types (eg, leukocytes, erythrocytes) and was capable of aggregation or agglutination in response to thrombin stimulation and contains platelets with surface phosphatidylserine expression. These features support its potential use as a haemostatically active product. Most cytokine concentrations measured by the bead-based assay were within reference intervals published for canine serum. Cytokine concentrations as measured by the plate-based assay were higher than published values for healthy dogs. Concentrations of CXCL8, TNFα and KC-like were significantly increased compared with contemporaneous control samples. The highest cytokine concentrations identified in the lyophilised platelets were for CXCL8. The most likely origin of the high CXCL8 concentrations the platelets themselves potentially released during the process of lyophilisation. The potential consequences of infusion of these increased concentrations of CXCL8 into recipients are unknown and further study under experimental conditions and in clinical usage is warranted.

Contributors RG designed and conducted experiments, analysed data and drafted the manuscript. SC designed and conducted experiments, analysed data and edited the manuscript. MB assisted with experiment design and data interpretation and edited the manuscript.

Competing interests RG is the principal investigator on a multicentre clinical trial investigating use of StablePlate RX for use in dogs with thrombocytopenic bleeding that is sponsored by Bodevet LLC. BodeVet was not involved in the conduct, analysis or authorship of the studies reported here.

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