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Quality control in veterinary blood banks: evaluation of canine platelet concentrates stored for five days

Camila Serina Lasta^{1,2*} , Nicole Hlavac³, Natália Aydos Marcondes⁴, Magnus Larruscaim Dalmolin⁵, Sílvia Resende Terra², Luciana de Almeida Lacerda⁵, Gustavo Adolpho Moreira Faulhaber^{6,7} and Félix Hilário Díaz González²

Abstract

Background: Platelets undergo structural, biochemical and functional alterations when stored, and platelet storage lesions reduce platelet function and half-life after transfusion. The objective of this study was to evaluate stored canine platelet concentrates with platelet aggregation, flow cytometry and biochemistry assays. Twenty-two bags of canine platelet concentrates were obtained by the platelet-rich plasma method and were assessed on days 1, 3 and 5 after collection. Parameters such as platelet counts, residual leukocytes, platelet swirling, glucose, lactate, pH, CD62P expression (platelet activation), JC-1 (mitochondrial function) and annexin V (apoptosis and cell death) were assessed.

Results: Over the five days of storage there was a significant decrease in glucose, HCO₃, pCO₂, ATP, pH, swirling and mitochondrial function, associated with a significant increase in lactate levels and pO₂. At the end of storage pH was 5.9 ± 0.6 and lactate levels were 2.8 ± 1.2 mmol/L. Results of the quality parameters evaluated were similar to those reported in human platelets studies. The deleterious effects of storage were more pronounced in bags with higher platelet counts (> 7.49 × 10¹⁰/unit), suggesting that canine platelet concentrates should not contain an excessive number of platelets.

Conclusions: Quality parameters of canine platelets under standard storage conditions were similar to those observed in human platelets. Our results have potential to be used for the routine evaluation and quality control in veterinary blood banks.

Keywords: Dog, Platelet concentrate, Platelet-rich plasma, Platelet storage lesions

Background

Recent advances in intensive care and oncology have increased the demand for blood components in veterinary medicine. However, there is limited availability of platelet concentrate (PC) because it is difficult to obtain and has a very short storage period. Although some studies evaluating the quality of canine PC obtained by the platelet-rich plasma (PRP) method under routine blood bank processing and storage have been reported [1–4], the quality control in veterinary transfusion medicine is still based on human blood bank protocols.

During storage platelets undergo structural, biochemical and functional changes, also referred as platelet storage lesions (PSL), which are caused by a multifactorial process that includes energy consumption, pH decrease, platelet activation and apoptosis [5, 6]. PSL reduce platelet half-life and function after transfusion, accordingly, one of the objectives of veterinary transfusion medicine is to understand and minimize them [7–10]. Consequently, understanding the mechanisms of canine PSL may serve as a basis for future research aimed at developing strategies to increase platelet viability. The objective of our study was to evaluate the main platelet quality parameters in stored canine PC under standard storage conditions.

Results

Data on the different parameters analyzed on days 1, 3, and 5 of storage are showed in Table 1 and Fig. 1. Mean

* Correspondence: camilaslata@gmail.com

¹Departamento de Saúde, Faculdade de Medicina Veterinária, Centro Universitário Ritter dos Reis – UniRitter – Campus FAPA, Av. Manoel Elias, 2001, 91240-261, Porto Alegre, Brazil

²Departamento de Patologia Clínica Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Full list of author information is available at the end of the article



Table 1 In vitro metabolism of canine platelets stored for 5 days ($n = 22$)

Parameters	Day 1	Day 3	Day 5
Platelet count ($\times 10^{10}$ /unit)	7.48 \pm 2.39	7.69 \pm 2.39	7.42 \pm 2.79
MPV (fL)	11.5 \pm 1.2	12.5 \pm 4.5	13.2 \pm 6.6*
PDW (fL)	13.0 \pm 7.9	14.0 \pm 5.5	15.1 \pm 2.0*
pH (22 °C)	7.0 \pm 0.4	6.4 \pm 0.5*	5.9 \pm 0.7**
pO ₂ (mmHg)	107.6 \pm 32.2	115.9 \pm 31.0	140.1 \pm 21.8*
pCO ₂ (mmHg)	43.7 \pm 3.1	25.5 \pm 2.5**	9.5 \pm 2.5**
Glucose (mmol/L)	25.3 \pm 2.3	18.8 \pm 4.4**	13.7 \pm 5.6**
HCO ₃ (mmol/L)	11.9 \pm 1.7	3.5 \pm 2.8**	0.6 \pm 1.2**
Lactate (mmol/L)	0.7 \pm 0.3	1.8 \pm 0.8*	2.8 \pm 1.2**
ATP (μ mol/ 10^{11} platelets)	1.7 \pm 0.6	1.2 \pm 0.3*	1.0 \pm 0.3**
CD61 (%)	91.5 \pm 4.8	93.4 \pm 4.3	91.8 \pm 5.7
CD62P (%)	2.7 \pm 1.5	2.6 \pm 3.6	2.6 \pm 2.2
LDH (U/L)	237.9 \pm 159.1	404.7 \pm 98.5	630.0 \pm 162.1
Annexin V (%)	2.4 \pm 2.5	1.6 \pm 1.1	3.5 \pm 4.2
Caspase (%)	10.2 \pm 11.4	6.7 \pm 5.9	12.0 \pm 13.9
$\Delta\psi_m$ JC-1 (%)	91.8 \pm 3.9	84.2 \pm 20.5	55.4 \pm 37.3**

Data are shown as mean \pm SD.

$\Delta\psi_m$ mitochondrial membrane potential, ATP adenosine triphosphate, HCO₃ bicarbonate, LDH lactate dehydrogenase, MPV mean platelet volume, pCO₂ partial carbon dioxide pressure, PDW platelet distribution width, pO₂ partial oxygen pressure.

ANOVA with Duncan's test.

* significantly different values between day 3 or 5 of storage compared to day 1 ($p < 0.05$). ** $p < 0.001$

platelet count was $7.48 \pm 2.39 \times 10^{10}$ /unit on day 1, and it remained stable over time. All PC showed adequate platelet counts, pH, swirling and leukoreduction (residual leukocytes $< 2.0 \times 10^8$ /unit) (data not shown) at the first day of assessment, in compliance with the Brazilian regulations for human PC [11]. Swirling decreased in all PC units over time ($p < 0.001$). None of the canine PC units presented aerobic or anaerobic microbiological contamination.

ATP levels and pH decreased over the storage period. Higher pO₂ levels were detected on day 5 compared with days 1 and 3, whereas a significant reduction of pCO₂ levels was detected as storage time increased. Glucose concentration gradually decreased whereas lactate levels increased. There was no significant difference in platelet aggregation when ADP and collagen were used (data not show). However, platelet aggregation increased between days 1 and 5 when arachidonic acid was used ($p = 0.0082$). Percentage of platelets positive for CD61 and CD62P remained stable during storage.

Mitochondrial membrane potential ($\Delta\psi_m$ – JC-1 aggregates) values remained stable between days 1 and 3 of storage. A reduction of $\Delta\psi_m$ was identified on day 5 when compared with days 1 ($p = 0.0016$) and 3 ($p = 0.0373$) (Fig. 2). No differences in the percentage of cells

with phosphatidylserine exposure or with expression of active caspase were observed during the evaluated period. LDH showed a trend to increase ($p = 0.051$).

There was a negative correlation between glucose and lactate ($\rho = -0.927$, $p < 0.001$), pH and lactate ($\rho = -0.871$, $p < 0.001$), and lactate and $\Delta\psi_m$ ($\rho = -0.846$, $p < 0.05$). There was a positive correlation between glucose and pH ($\rho = 0.936$, $p < 0.001$), glucose and swirling ($\rho = 0.828$, $p < 0.005$), glucose and $\Delta\psi_m$ ($\rho = 0.911$, $p < 0.001$), and pH and swirling ($\rho = 0.803$, $p < 0.05$).

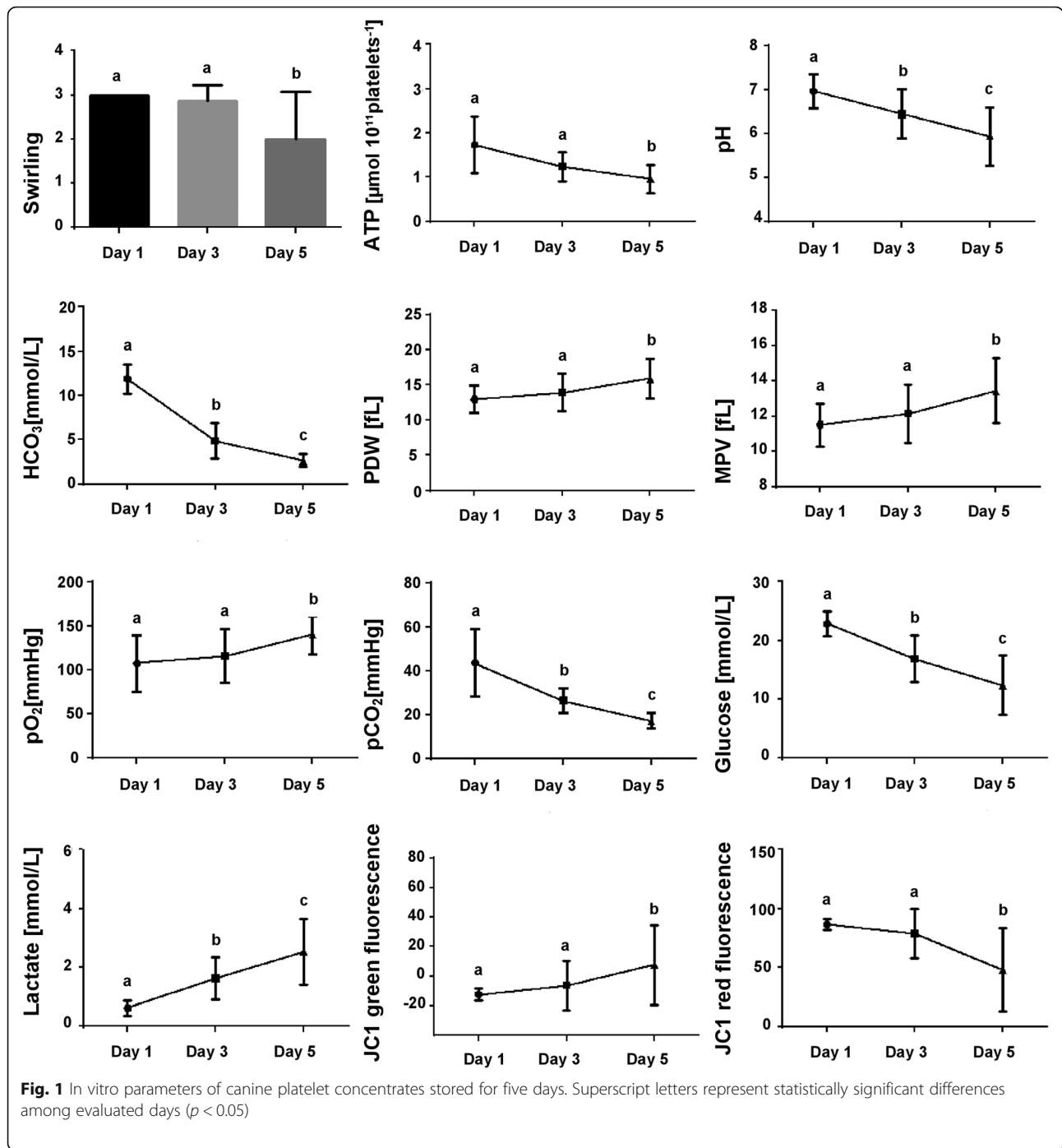
PC were divided into two groups: one with lower than average platelet count ($\leq 7.49 \times 10^{10}$ /unit, $n = 14$) and one with a higher than average platelet count ($> 7.49 \times 10^{10}$ /unit, $n = 8$). PC with lower platelet counts presented better quality parameters than those with higher counts on the last day of storage (Table 2).

Discussion

In our study, we evaluated stored canine PC on days 1, 3 and 5 of storage with platelet aggregation test, biochemical assays and flow cytometry analysis. Our results were consistent with previous reports from human [12, 13] and canine PC studies [1, 3, 4, 14]. Canine PC showed a decrease in pH, glucose, bicarbonate, swirling and pCO₂ values, and an increase in lactate and pO₂ values. Hence, our values for those parameters may be used as references for quality control of canine PC in veterinary blood banks (Table 3).

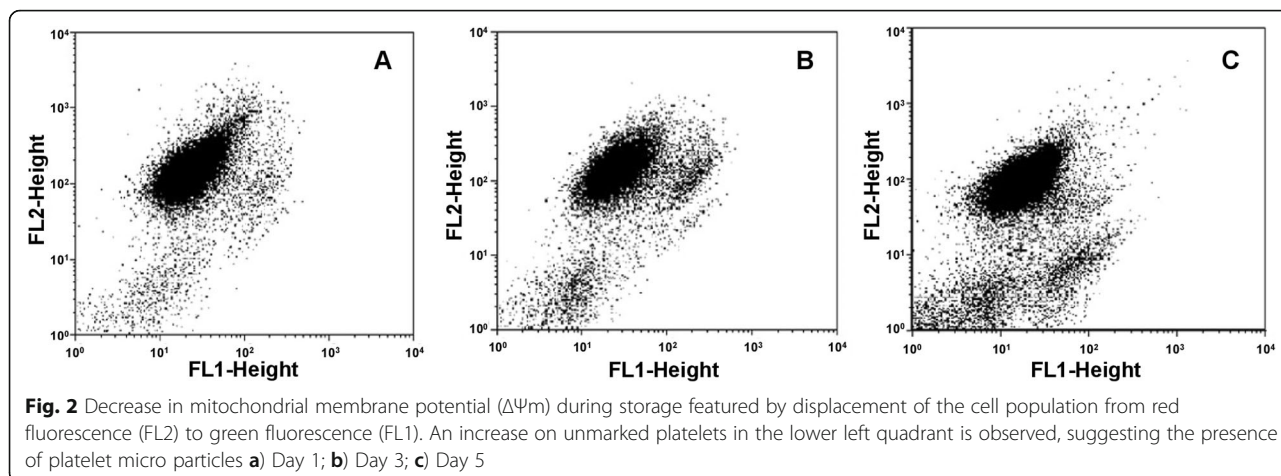
When PC with lower and higher than average platelet counts were compared, those with higher counts presented significantly lower pH, glucose, ATP and $\Delta\psi_m$ values, and higher lactate concentrations. All PC bags with lower than average platelet counts presented good quality until day 5 of storage, in agreement with recommendations of the Brazilian Health Surveillance Agency (ANVISA) [11]. Similar findings were reported in human PC, with the report of significant differences in glucose, lactate, pH, pCO₂, bicarbonate and ATP values in PC with high platelet counts on day 5 of storage [15]. Another study also reported a faster decrease of swirling, pH and glucose values in PC with high platelet counts when compared with those with lower platelet counts [16]. Our data on PDW and MPV indicate that platelets in the PC with lower than average counts may present less swelling and lower fragmentation rates. When PC contains a high number of platelets per volume, oxygen is consumed faster, and the metabolic pathway used by platelets becomes anaerobic with consequent lactate production and decrease in pH [15, 16]. Still, a higher plasma volume per bag increases the transfusion volume and the risk of transfusion reactions [7, 9].

The correlations among the studied parameters determined on day 5 are consistent with the alterations suffered by platelets during storage. A significant correlation between pH decrease and increase in MPV and PDW



indexes was observed. MPV increase was also correlated with swirling loss, indicating that platelets shape changed from discoid to spherical. The higher lactate concentration on day 5 of storage was correlated with the increase in MPV and strongly correlated with the decreases in swirling and pH. These correlations were expected due to the cause-effect relationship between medium acidity and morphological changes in platelets [17].

The high pO₂ values by the end of storage period indicates that glycolytic anaerobic metabolism superseded aerobic metabolism despite the presence of oxygen (Warburg effect) [15], as confirmed by lactate accumulation and pH reduction. When platelets divert their metabolism to anaerobic glycolysis, there is a decrease in pH and swirling loss. The positive correlation between swirling loss and $\Delta\Psi\text{m}$ decrease may explain the lower oxygen utilization, because the tricarboxylic acid cycle



occurs in the mitochondria. Along with pH decrease, bicarbonate values gradually and significantly decreased, because it is mobilized to buffer the accumulated H^+ ions [5]. As glucose is consumed, lactate levels increase, exceeding the medium buffering capacity and causing a decrease in pH followed by swirling loss [15].

Platelet aggregation capacity increased in response to arachidonic acid, whereas no differences were observed in response to ADP or collagen. However, light transmission aggregometry studies in the clinical practice require fresh platelets, with less than 4 h after blood sampling [18]. This is a limitation of the aggregation tests performed and could explain our results.

The percentage of CD61 expression remained stable during storage, indicating that there was no glycoprotein internalization by endocytosis [19]. P-selectin expression on canine platelets surface did not change over time, in contrary to previous studies with human platelets. However, PC acquisition method may influence platelet activation, since human studies have shown that buffy-coat or apheresis methodologies may increase platelet activation [13, 20] and our samples were obtained with PRP method.

Some authors suggest that $\Delta\Psi_m$ loss is the most sensitive marker of platelet quality during storage, because it signals the apoptosis process, even before caspase pathway activation or phosphatidylserine exposure [20–23]. We did not observe an increase in phosphatidylserine exposure, probably due to the short evaluation time [24]. Nonetheless, a reduction in $\Delta\Psi_m$ values was observed from day 3 of storage. Correlations were observed among $\Delta\Psi_m$ and classical PC quality markers, such as glucose, lactate and pH, as well as a high correlation between swirling loss and $\Delta\Psi_m$ reduction. These results suggest that swirling evaluation may be routinely used as a predictor of PC quality in veterinary blood banks, as it is an efficient and low-cost technique, requiring only a trained evaluator.

In the clinical setting, high platelet concentrations are desirable to achieve higher transfusion efficiency. Nevertheless, considering that PC with higher platelet counts experience more severe PSL during storage, it is suggested that canine PC stored in TOTM-plasticized bags with counts greater than 7.49×10^{10} per unit should be used in less than five

Table 2 Comparison of quality parameters in canine platelet concentrates with high and low platelet counts on day 5 of storage

Parameter	Platelet count ($\leq 7.49 \times 10^{10}$ /unit) (n = 14)	Platelet count ($> 7.49 \times 10^{10}$ /unit) (n = 8)	P
PDW (fL)	14.5 ± 4.3	18.4 ± 5.1	0.017
MPV (fL)	12.9 ± 2.6	15.0 ± 4.2	0.043
Glucose (mmol/L)	17.0 ± 4.6	7.9 ± 3.3	< 0.0001
Lactate (mmol/L)	2.1 ± 0.6	4.1 ± 0.9	< 0.0001
ATP ($\mu\text{mol}/10^{11}$ platelets)	1.1 ± 0.3	0.8 ± 0.1	< 0.0001
pH (22 °C)	6.3 ± 0.2	5.3 ± 0.4	< 0.0001
pO ₂ (mmHg)	126.6 ± 23.7	152.9 ± 32.6	< 0.0001
pCO ₂ (mmHg)	13.3 ± 3.7	9.1 ± 5.4	0.043
$\Delta\Psi_m$ JC-1 (%)	66.5 ± 10.1	15.3 ± 8.7	0.003
LDH (mg/dL)	442.7 ± 76.6	957.7 ± 100.0	0.022

$\Delta\Psi_m$ mitochondrial membrane potential, ATP adenosine triphosphate, LDH lactate dehydrogenase, MPV mean platelet volume, pCO₂ partial carbon dioxide pressure, PDW platelet distribution width, pO₂ partial oxygen pressure.

Table 3 Suggested reference values for canine platelet concentrates obtained through platelet-rich plasma method and stored for 5 days in TOTM-plasticized bags

Parameter	Expected value
Volume	50–70 mL
Platelet count ^a	$\leq 7.49 \times 10^{10}$ /unit
Residual leukocytes ($\times 10^8$ /unit)	< 2.0
pH	6.59–7.35
Swirling	$\geq 2+$
Microbiological analysis	Negative

^a Platelet concentrate bags stored in TOTM-plasticized bags with platelet counts $> 7.49 \times 10^{10}$ /unit should be used in less than five days

days, as exceeding this period may negatively affect PC quality and possibly transfusion efficacy.

Our study has some limitations. Samples were not evaluated daily and caspase should have been assessed for longer than 5 days of storage.

Conclusion

In conclusion, quality parameters of canine platelets under standard storage conditions were similar to those observed in human platelets. Moreover, as the results of the present study are specific of canine PC, they could be used for quality control in veterinary blood banks.

Methods

Animal selection

Twenty-two dogs aged between 2 and 7-years old, weighing among 30 kg and 60 kg, with no history of receiving transfusion and not taking any medication were selected. The animals were privately owned. Dogs were individually submitted to physical examination, complete blood counts (CBC), blood chemistry profile, and infectious disease screening (Snap 4Dx Test, Idexx Laboratories Inc., Westbrook, USA; Vetcheck, Tecsa Lab, São Paulo, BRA). Included breeds were Golden Retriever ($n = 12$), German Shepherd ($n = 4$), Giant Schnauzer ($n = 4$) and English Mastiff ($n = 2$). All included dogs had updated vaccines and deworming and were considered healthy.

Blood collection

After clinical and laboratory approval, a total of 450 mL of whole blood (WB) were collected in bags using a triple-bag closed system (CPD/SAGM, JP Indústria Farmacêutica, São Paulo, BRA). Primary collection bags had sodium citrate as anticoagulant; and citrate, phosphate and dextrose (CPD) solution as an additive solution for red blood cells. Dogs were kept under gentle physical restraint and with no anesthesia for blood collection. After trichotomy and 70% alcohol aseptis, WB was collected by puncturing the jugular

vein [25], using equipment with automatic control of homogenization, flow and donation volume (Biomixer 323, Ljungberg & Kögel AB, Helsingborg, SWE). After blood withdrawn, all included dogs received water and a tasty meal, and remained under observation for at least 30 min; subsequently they were released to their owners.

Platelet concentrates preparation using the platelet-rich plasma method

WB bags were allowed to stand at 22 °C for one hour after collection. The PRP method was applied to obtain the PC as follows: each WB bag was subjected to light centrifugation (1600 X g) for 6 min at 22 °C (Sorvall Legend RT + Centrifuge, Thermo Scientific, Waltham, USA). PRP was removed using a manual plasma extractor (ACS201, Terumo Medical of Brazil, São Paulo, BRA) and then subjected to a second centrifugation (3300 X g) for 8 min at 22 °C. Fluid weight and density (1.026 g/mL) were used to calculate the final volume of the bag [26]. Excessive plasma was removed with the aid of a plasma extractor until 50–70 mL of residual plasma remained at the bottom of the bag with the sedimented platelets [25]. PC were stored in polyvinyl chloride (PVC) bags plasticized with tri-2-ethyl-trimellitate (TOTM, JP Indústria Farmacêutica), which are specific for platelet storage and do not have anticoagulants nor additive solutions.

Storage and sampling of canine platelet concentrates units

PC bags were left undisturbed for one hour after preparation, and then were stored at 20–24 °C in a platelet environmental chamber (model CDCI 03, Indrel, São Paulo, BRA) under constant stirring (AP48L linear plate agitator, Presvac, BA, ARG). Aliquots were removed for analyses using a sampling site coupler previously sterilized with 70% alcohol (Fenwal, Lake Zurich, USA) on days 1, 3, and 5 [4]. All PC were kept out of store for sampling for a maximum of 60 s. On day 5 of storage, all 22 PC were submitted to aerobic and anaerobic microbiological culture in brain heart infusion (BHI) incubated at 37 °C for 10 days.

Qualitative variables

Immediately before aliquot sampling, platelet swirling was assessed. This technique consists in the visual evaluation of the PC against a light source to observe platelet movement. Swirling is classified according to a 0–3 scale, where 0 indicates no platelet swirling and 3 corresponds to platelets with very nice cloudy movements [27, 28]. Swirling was assessed in all PC bags by the same evaluator. Platelet count, PDW and MPV were determined using an automated hematology counter calibrated for canines (PocH-100iV Diff, Sysmex, Lincolnshire, USA). Residual leukocytes were counted in a Nageotte chamber (LO-

Laboroptik GmbH, Bad-Homburg, DEU) on day 1 of storage (24 h after collection), as previously described [15].

Biochemical analyses

Lactate and LDH concentrations were determined by dry chemistry (Vitros 250 Chemistry System, Johnson & Johnson, São Paulo, BRA). Glucose, HCO_3^- , pO_2 and pCO_2 values were determined using a portable blood gas analyzer (CG8, i-Stat, Abbott Point of Care, Mississauga, CAN), according to the manufacturer's instructions. On days 1, 3, and 5, ATP was extracted [29] and samples were frozen at -80°C until further analyses. Platelet ATP levels were determined by bioluminescence (Lite Luminescence ATP Detection Assay System, PerkinElmer, Waltham, USA), and samples were read in a multi-mode microplate reader (Spectramax M5, Molecular Devices Inc., San Jose, USA) in the same assay. The pH was measured using a calibrated pH meter (HI 99171, Hanna Instruments Inc., Woonsocket, USA) under controlled temperature (22°C) and according to the manufacturer's instructions. All samples were tested in duplicate.

Flow cytometry analyses

Platelet activation was determined according to CD62P (P-selectin) expression. About 500,000 platelets were incubated with titrated amounts of anti-human CD61 FITC (clone VI-PL2, BD Biosciences, San Jose, USA) and anti-human CD62P PE (clone AC1.2, BD Biosciences, San Jose, USA) monoclonal antibodies for 20 min at room temperature. Samples were resuspended in phosphate buffered saline (PBS) prior to acquisition. Percentages of platelets positive for CD61 and CD62P were recorded.

Assessment of $\Delta\psi_m$ was performed as described elsewhere [30]. Briefly, platelets were incubated with MitoScreen JC-1 (BD Biosciences, San Jose, USA). Cells were pelleted by centrifugation and resuspended in MitoScreen buffer for acquisition. Relative degrees of mitochondrial polarization were quantified by measuring the red-shifted JC-1 aggregates, which are favored under conditions of high membrane potential, and green-shifted monomers, which tend to predominate under conditions of low membrane potential [31].

Phosphatidylserine exposure was assessed with Annexin V. About 500,000 platelets were incubated with ApoFlowEx[®] FITC Kit (Exbio, Praha, CZE) for 15 min at room temperature, according to the manufacturer's instructions. Samples were washed once and resuspended in PBS before acquisition. The percentage of platelets binding to Annexin V was recorded.

CaspACE[™] FITC-VAD-FMK (Promega, Madison, USA) was used to determine caspases involvement in apoptosis induction. About 5,000,000 platelets were incubated with 1 mM of CaspACE[™] FITC-VAD-FMK for 20 min at 37°C . Samples were washed once and

resuspended in 1 mL of PBS before acquisition. Percentage of platelets presenting active caspase was recorded.

All flow cytometry experiments were performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, USA) using CellQuest[™] Pro software, after daily quality control procedures. At least 30,000 events were acquired for each analysis. All flow cytometry data were analyzed using FCS Express 5 software (De Novo, Software, Ontario, CAN).

Platelet aggregation test

Platelet aggregation test was performed with light transmission aggregometry (Agreg Myr4 aggregometer, Qualitem, Cesário Lange, BRA) using ADP ($5\ \mu\text{M}$), collagen ($5\ \mu\text{g/mL}$), and arachidonic acid ($1\ \mu\text{M}$) as inducers.

Statistics

Data analysis was carried out with ANOVA for repeated measurements, and means were compared with Duncan's test. Correlations among the evaluated parameters were tested with Pearson's correlation test. Results were considered significant at $p < 0.05$. All data were analyzed with GraphPad Prism 6.0 Software (GraphPad Software, La Jolla, USA).

Ethical aspects

This study was approved by the Ethics Committee on the Use of Animals of the Universidade Federal do Rio Grande do Sul (CEUA/UFRGS) under protocol #26102. The owners of all evaluated dogs provided written informed consent before inclusion in the study.

Abbreviations

ANVISA: Brazilian Health Surveillance Agency; CBC: Complete blood counts; PC: Platelet concentrate; PRP: Platelet-rich plasma; PSL: Platelet storage lesions; PVC: Polyvinyl chloride; WB: Whole blood; $\Delta\psi_m$: Mitochondrial membrane potential

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Authors' contributions

CSL, NRCH designed the project, standardized the tests, collected the blood bags, performed the experiments, collected the data, participated in data interpretation and wrote the manuscript. NAM, SRT, GAMF standardized and performed the flow cytometry experiments, were involved in drafting and critically revising the manuscript. MLD, LAL made substantial contributions to the design of the project and standardization of the tests, collected the blood bags, carried out the data collection and were responsible data analysis. FHDG made substantial contributions to the design of the project, performed data interpretation and reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Written informed consent was obtained from the owners of all evaluated dogs before inclusion in the study. All animals were included in the study in accordance with bioethics concepts applied to animal research. Animals were not euthanized, at the completion of their participation in the study the dogs were released to their owners. This study was approved by the Ethics Committee on the Use of Animals of the Universidade Federal do Rio Grande do Sul (CEUA/UFRGS) under protocol #26102 and was conducted in accordance with current laws in Brazil.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Departamento de Saúde, Faculdade de Medicina Veterinária, Centro Universitário Ritter dos Reis – UniRitter – Campus FAPA, Av. Manoel Elias, 2001, 91240-261, Porto Alegre, Brazil. ²Departamento de Patologia Clínica Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. ³Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia, Salvador, Brazil. ⁴Laboratório Zanol, Porto Alegre, Brazil. ⁵Blut's Centro de Diagnósticos Veterinários, Porto Alegre, Brazil. ⁶Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. ⁷Programa de Pós-Graduação em Medicina, Ciências Médicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

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