Original Study

In vitro hemolysis of stored units of canine packed red blood cells

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Abstract

Background – Hemolysis is an important quality parameter of packed red blood cells (pRBCs) that is used to assess the cellular integrity of stored blood units. According to human standards, hemolysis at the end of storage must not exceed 1%, as otherwise it may be responsible for decreased transfusion effectiveness and acute life-threatening reactions.

Objectives – This prospective study was designed to evaluate the hemolysis of canine pRBCs stored in an additive solution containing adenine, dextrose, mannitol, and sodium chloride, and to assess its associations with storage time, duration of the collection process, collection disturbances, and with the final volume and PCV of the pRBCs units.

Methods – One hundred eighty pRBCs units were collected from canine donors. Hemolysis of the pRBCs units was determined immediately after processing ($t = 0$). The units were then stored and retested ($t = 1$) either before administration (during weeks 2, 3, 4, 5, or 6 of storage) or at the end of the storage period (42 d) if not used.

Results – Mean hemolysis at $t = 0$ was 0.09% (SD 0.06) and increased during storage, at a more pronounced rate from the 5th (mean values of 0.52%, SD 0.29) to the 6th week (1.2%, SD 0.72). Almost 51% of the units with 36–42 days of shelf-life showed more than 1% hemolysis. Disturbances in the collection process, the volume of the whole blood units, and the volume of stored pRBCs units or their PCV were not related to pRBCs hemolysis.

Conclusions – According to human blood bank recommendations regarding acceptable hemolysis, canine pRBCs stored for more than 35 days should be tested to ensure <1% hemolysis prior to administration.

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Keywords: dogs, pRBC, quality control, storage

Abbreviations

HGB	hemoglobin
pRBCs	packed red blood cells
SAG-MAN	additive solution containing adenine, dextrose, mannitol, and sodium chloride
WB	whole blood

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Introduction

The use of blood components has become a common procedure in small animal medicine. To ensure a constant availability of blood products, blood banks have to process and supply an increasing number of units stored and store them for longer periods. Thus, a new research area related to storage lesions and the hemolysis of packed red blood cells (pRBCs) is emerging in veterinary transfusion medicine.

Hemolysis is a limiting factor for the shelf-life of stored RBCs, not only because it significantly decreases the ability of RBCs to transport and deliver oxygen to hypoxic tissues, but also because it may be responsible for transfusion reactions, commonly described as nonimmune-mediated hemolytic transfusion reactions. These are usually benign and require no treatment, although they contribute to a decreased transfusion effectiveness.[1] However, a recent study reported acute life-threatening transfusion reactions in 4 dogs, with 3 fatalities after administration of hemolyzed pRBCs units due to inappropriate storage.[2] Reported clinical signs were similar to an acute hemolytic reaction (eg, hemoglobinemia, hemoglobinuria, fever, prolonged

coagulation time, thrombocytopenia, petechiae and ecchymosis, melena, hematemesis, and pulmonary infiltrates associated with progressive dyspnea).[2]

When the extracellular hemoglobin (HGB) concentration exceeds normal plasma and cellular binding capacities, its plasma concentration increases, causing important vasoactive and oxidative effects that lead to acute kidney injury and vascular dysfunction.[3] A total of 3 g of free HGB (approximately 5 units of pRBCs with 1% hemolysis) can be infused into a healthy human adult without evidence of hemoglobinuria, which usually appears when HGB plasma levels reach 10 g/dL.[4] Renal injuries were described in a 1947 experimental study of healthy dogs after intravenous administration of 20 mL/kg of canine HGB in a 5% solution.[5] Although excessive hemoglobinemia (over 3.7 g/dL or an average of 2.2 g/dL during 24 h) is associated with renal injury in healthy dogs, it is probably an uncommon cause of renal failure by itself but instead likely enhances the risk in canine patients with concomitant shock, dehydration, renal ischemia, or infection.[6,7] Other detrimental effects of high plasma concentrations of free HGB were recently reported after administration of older RBCs. These transfusions were related to higher risks of shock and lung injury, along with a high mortality rate, in dogs with severe pneumonia.[8,9] Due to the reported harmful effects of free HGB, the Council of Europe and the American Association of Blood Banks stipulate that the maximum hemolysis percentage of human blood units at the end of storage must not exceed 0.8% and 1%, respectively.[10,11] There is currently insufficient information for a similar recommendation in dogs. The shelf-life of pRBCs with citrate-based anticoagulants and additive solutions was reported to vary between 33 and 42 days if stored at temperatures of $4 \pm 2°C$ ($39.2 \pm 35.6°F$).[12–19] The objectives of this study were to evaluate the effect of storage on canine pRBCs hemolysis and to determine the potential influence of the collection procedures and the pRBCs characteristics (total volume and PCV) on the hemolysis of stored pRBCs. Furthermore, the study aimed to determine if prestorage hemolysis allowed for predicting the magnitude of the hemolysis at the end of storage.

Material and Methods

The present prospective study was approved by the ICBAS-UP Ethics Committee (project number 008/2012). A total of 92 healthy mixed-breed dogs, weighing 35–60 kg, were chosen by the investigator from the list of dogs included, for the first time, in a blood donor program. All dogs had been vaccinated and properly dewormed. The donors also demonstrated a negative polymerase chain reaction analysis[a] for the following agents: *Anaplasma* spp., *Ehrlichia* spp., *Babesia canis*, *Leishmania*

infantum, and *Dirofilaria immitis*. CBCs and chemistry profiles were within normal reference ranges. Blood collections were held under the regular donation program from a blood bank. All venipunctures were performed by a specially trained veterinarian with 10 years of experience in blood banking.

Blood collection

Whole blood units were collected in commercial triple blood bags[b] without leukocyte depletion filters, consisting of a primary bag for the collection of $450 \pm 10\%$ mL of blood with 63 mL of CPD anticoagulant (tri-sodium citrate, sodium phosphate, and dextrose), an empty bag for plasma storage, and 1 bag with 100 mL of additive solution containing adenine, dextrose, mannitol, and sodium chloride (SAG-MAN). After a complete physical exam, the animals were placed in lateral recumbency and the puncture area over a jugular vein was clipped of hair and aseptically prepared (using chlorhexidine and alcohol). Jugular venipuncture allowed blood to flow by gravity into the collection bag, which was gently inverted to mix the blood with the anticoagulant at every 50 mL of collected volume. Approximately 11–13 mL/kg of blood was collected, and if an interruption in blood flow was noted, the needle was immediately repositioned. Once the total volume was obtained, the tubing was manually stripped to allow for anticoagulation of the tubing and sealed.[c] The volumes of blood units were calculated on the basis of their weight, assuming that 1 mL of whole blood (WB) weighs 1.053 g and that 1 mL of pRBCs weighs 1.085 g.[14] The duration of the collection process, from the venipuncture to the removal of the needle, was recorded. The need for needle repositioning, animal movement during the procedure, and irregular blood flows or the presence of macroscopic blood clots in the collection system were also recorded and collectively considered disturbances in the collection process (animal movement included all movements of the head, neck, thoracic limbs, or thorax that hampered the blood flow by changing the position of the jugular vein or the needle; irregular blood flow refers to a quick interruption of the blood flow, lasting 1–5 s). The collected WB units were immediately stored using refrigerated butanediol plates,[d] keeping a constant temperature of $22 \pm 2°C$ ($71.6 \pm 35.6°F$), and processed within 12 hours. Whole blood unit volumes, including the anticoagulant solution, were registered.

Processing and evaluation of the pRBCs units

After gentle mixing, the triple bag system was placed in the centrifuge cups,[e] avoiding plastic folds and ensuring a symmetrical distribution of weight with a tolerated difference of 0.2 g between opposite cups. The units were

centrifuged at 2,000 × *g* for 20 minutes, with 80 seconds of acceleration and 110 seconds of deceleration, at 6°C (42.8°F). Plasma was expressed into the transfer bag using a manual plasma extractor.[f] A volume of 100 mL of SAG-MAN was added to the primary pRBCs bag. To evaluate hemolysis in the lower volume units (half units), blood was extracted from 88 pRBCs units with an initial volume above 350 mL: 100 mL ± 10% was transferred into a secondary transfer bag, using a sterile connection.[g] These lower volume pRBCs units were evaluated as individual units. For quality analysis, after gentle mixing by inversion of the pRBCs unit, an 8 mL aliquot was aseptically separated into a sample bag,[h] using a sterile connection, and immediately analyzed ($t = 0$) for PCV, total HGB, and supernatant HGB. Packed cell volume was obtained using a microhematocrit centrifuge according to standard methodology.[20] Total HGB was measured using a specific analyzer,[i] according to the manufacturer's protocol. After centrifugation,[j] the supernatant HGB was determined by spectrophotometry using an analyzer for low values of HGB,[k] also according to the manufacturer's protocol. The percentage of hemolysis was obtained using the following formula:[4]

$$\%\,\text{hemolysis} = \text{supernatant HGB (g/dL)} \times (100 - \text{PCV})/$$
$$\text{total HGB (g/dL)}$$

Packed RBCs units were stored at 4°C (39.2°F) in a dedicated refrigerator[l] and retested for hemolysis either before their administration or at the end of the maximum storage period of 42 days, if not used ($t = 1$). Bacterial contamination was also investigated at $t = 1$ by the sterile addition of 5 mL of pRBCs to aerobic culture bottles with specific growth medium,[m] followed by incubation at 37°C (98.2°F) and continuous examination for 7 days using a specific analyzer.[n]

Statistical analysis

For comparison by storage times, the units were grouped according to $t = 1$: 7–14 days (week 2), 15–21 days (week 3), 22–28 days (week 4), 29–35 days (week 5), and 36–42 days (week 6) of storage. To evaluate the influence of different parameters on the level of hemolysis after different storage times, the pRBCs units were also grouped according to the duration of the blood collection (<300, 300–360, 361–420, and >420 s), the presence of collection disturbances, the volume of initial WB units (<460, 460–510, and 511–560 mL), and the volume (90–100, 200–250, and 251–350 mL) or PCV (55—60%, 61—65%, and 66–70%) of the pRBCs units. The results were analyzed with statistical software.[o] Normal distribution of the data was assessed with the Kolmogorov–Smirnov test. The Pearson correlation coefficient was used to assess a possible linear relationship between hemolysis percentage, PCV or total HGB, and the time of evaluation. Additionally, this test allowed for the evaluation of the relation between hemolysis measured before and after storage ($t = 0$ and $t = 1$). A one-way ANOVA was used to compare differences in the hemolysis percentage according to the following variables: PCV of the pRBCs, volume of the WB unit, volume of the pRBCs unit, and duration of the blood collection process. An independent-sample Student *t*-test was used to compare differences in hemolysis between normal and abnormal blood collections. Values were considered significant at $P \leq 0.05$.

Results

A total of 92 WB units were collected from an equal number of canine blood donors, with each unit from a different donor. After processing, 180 pRBCs units were tested ($t = 0$), of which 88 were low volume units (half units). All units were stored and retested once before their use or disposal. All pRBCs units tested negative for aerobic bacterial cultures.

The data for all variables were normally distributed. Hemolysis, PCV, and total HGB from pRBCs units are displayed in Table 1 and Figures 1 and 2, respectively. Statistically significant positive correlations between storage time and hemolysis ($r = 0.73$; $P < 0.001$) and PCV ($r = 0.55$; $P < 0.001$) were found. Hemolysis measured at $t = 0$ ranged from 0.00% to 0.32%. Its subsequent increase during storage was nonlinear, with a steeper increase from the 5th (hemolysis ranged from 0.12% to 1.34%; mean 0.52%, SD 0.29) to the 6th week (hemolysis ranged from 0.23% to 2.47%; mean 1.2%, SD 0.72). By week 5, only 3 units (6%) exhibited hemolysis above 1%, the maximum acceptable limit according to the American Association of Blood Banks,[11] while by the 6th week of storage, 41 units (51%) surpassed this value. Total HGB concentration values were constant during the storage period without any statistically significant differences.

Table 1: Hemolysis, packed cell volume (PCV), and total hemoglobin concentration (HGB) measured in canine packed red blood cells units stored at 4°C for varying periods of time before and after storage

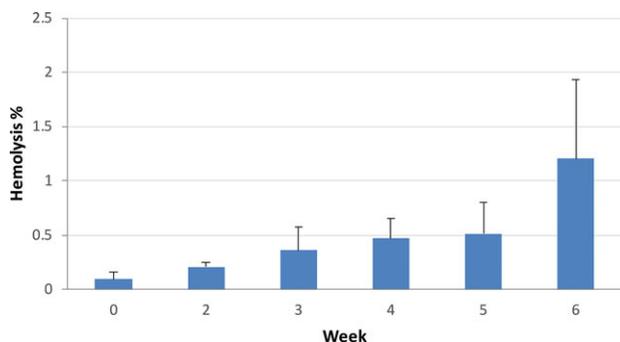|	*n*	Hemolysis (%) Mean	Hemolysis (%) SD	PCV (%) Mean	PCV (%) SD	Total HGB (g/dL) Mean	Total HGB (g/dL) SD
Before storage	180	0.09	0.06	63	4	21.37	1.58
Week 2	15	0.21	0.04	62	4	21.26	1.72
Week 3	19	0.36	0.22	63	5	21.05	1.63
Week 4	18	0.47	0.18	67	4	21.01	1.34
Week 5	48	0.52	0.29	68	4	21.75	1.37
Week 6	80	1.20	0.72	70	5	21.64	1.45

Figure 1: Hemolysis (%) during storage of canine pRBCs units stored at 4°C. There is a positive significant correlation between storage times and hemolysis ($r = 0.73$; $P < 0.001$). Statistically significant differences were also found between hemolysis before storage (week 0) and hemolysis at week 3, 4, 5, and 6 ($P < 0.001$), between hemolysis during week 6 and every other measurement ($P < 0.001$), and between hemolysis registered at week 2 and week 5 ($P < 0.05$).
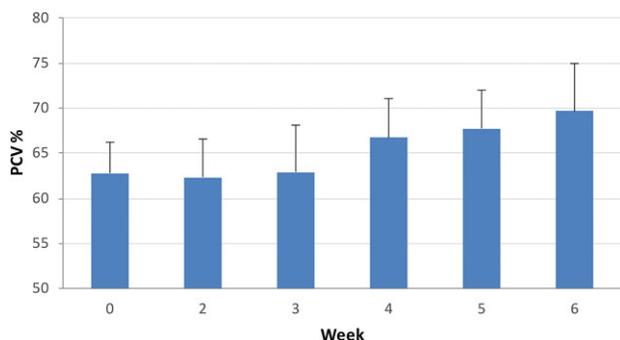
Figure 2: Packed cell volume during storage of canine pRBCs units stored at 4°C. There is a positive significant correlation between storage times and PCV ($r = 0.55$; $P < 0.001$). Statistically significant differences were also found between hemolysis before storage (week 0) and hemolysis at week 4, 5, and 6 ($P < 0.001$), between hemolysis during week 2 and hemolysis at week 4 ($P < 0.05$), 5, and 6 ($P < 0.001$), and between hemolysis during week 3 and hemolysis at week 4 ($P < 0.05$), 5, and 6 ($P < 0.001$).

The hemolysis percentage of pRBCs units grouped according to the duration of the blood collection, the evidence of disturbances during collection (abnormal collections), the volume of WB units, and the volume of pRBCs units are displayed in Table 2. There were no significant differences in the hemolysis of the different groups within each category.

No correlation was found between pre- and poststorage hemolysis ($r = 0.09$; $P > 0.05$).

Table 2: Hemolysis (%) of canine packed red blood cells (pRBCs) units measured immediately after processing (poststorage) and after up to 6 weeks of storage (week 6). Data were grouped according to the duration of the blood collection, evidence of disturbances during collection (abnormal collections), volume of whole blood units, and volume of pRBCs units

	Before storage ($n = 180$)		Week 6 ($n = 80$)	
Duration of the collection (s)	Mean	SD	Mean	SD
<300	0.07	0.04	1.45	1.19
300–360	0.09	0.08	1.15	0.60
361–420	0.10	0.11	1.07	0.72
>420	0.08	0.03	1.04	0.47
Evidence of disturbances during collection				
Normal collection	0.09	0.09	0.08	0.05
Abnormal collection	1.20	0.73	1.09	0.52
Volume of WB (mL)				
<460	0.09	0.06	1.47	0.95
460–510	0.09	0.05	1.39	0.66
511–560	0.09	0.10	1.30	0.72
Volume of pRBCs (mL)				
90–100	0.1	0.10	1.12	0.62
200–250	0.09	0.08	1.26	0.76
251–350	0.09	0.08	.35	0.65

$P > 0.05$

Discussion

The present study was intended to evaluate the hemolysis of canine pRBCs during storage and to address the influence of the collection process, pRBCs volume, and units PCV in hemolysis.

Corroborating previous veterinary and human studies, hemolysis increased during storage.[13,21–26] The cumulative effect of proinflammatory substances, mostly released from leukocytes and platelets, along with the ATP depletion that reduces the integrity of RBCs membranes, may explain this finding.[27–29] A higher increase in hemolysis was found during the 6th week of storage. If the human guidelines had been applied to dogs, only 3 units (6%) would have been discarded by week 5, but 41 units (51%) would have been discarded by week 6.[11] Despite the reported shelf-life of 42 days considered by some authors,[14,18,19] these findings highlight the importance of a regular quality control program to evaluate hemolysis in stored units older than 35–42 days. In the present study, the prestorage pRBCs hemolysis was not correlated with the hemolysis found after weeks 5 and 6 of storage. Disturbances in the collection process, the volume of the WB units, and the volume of the stored pRBCs units or their PCV, were statistically unrelated to the end-of-storage hemolysis, in opposition to the direct relation found in humans between PCV and end-of-storage hemolysis. To the authors' knowledge, there are no previous veterinary investigations addressing these

factors in canine pRBCs units, or the relationship between pre- and poststorage hemolysis.

The anticoagulants and additive solutions available for humans also seem to ensure the viability of canine RBCs during storage.[15,16] Optimal additive solutions provide cells with nutrients, such as dextrose, adenine, mannitol, and sodium chloride.[15,30] These nutrients help the RBCs maintain their function and increase their viability by preventing lysis during storage.[15,30] After plasma separation, the additive solution is added to the pRBCs units, extending their lifespan to 42 days, in both canine[14,18,19] and human[12] units, and facilitating their administration by reducing their viscosity.[16] The present study evaluated hemolysis in canine pRBCs prepared with the SAG-MAN additive solution (also known as AS-5). Previous veterinary studies evaluated pRBCs units with the additive solution AS-1[p] and reported hemolysis of $0.32 \pm 0.12\%$ in units stored for 35 days ($n = 6$),[24] $0.59 \pm 0.11\%$ at day 37 ($n = 4$)[13] and $0.71 \pm 0.57\%$ at day 42 ($n = 5$).[25] These values are lower than the ones obtained in the present study, possibly due to the small number of units evaluated in former studies and to differences in the mannitol concentration of the additive solutions.[13,24,25] Furthermore, it may also be related to different methods of determining the supernatant plasma HGB concentrations.[26,31] Additionally, the study by Brownlee et al. used prestorage leukoreduction filters,[24] which could extend the viability of RBCs, as the presence of leukocytes in the pRBCs units accelerates hemolysis during storage.[23,32]

As previously reported in human studies, the pRBCs units' PCV increased during storage. This could be related to an increase in RBCs volume with time, due to degenerative changes that permit the ingress of water into the cells.[33–35] The cell morphology, which changes progressively during storage, from biconcave disks to speculated or sphere-shaped cells (echinocytes or spherocytes, respectively), may also be related to higher PCV.[35] Mannitol stabilizes RBCs membranes by an unknown mechanism that delays swelling and hemolysis.[36] However, it seems insufficient to completely halt this degenerative mechanism and the consequent PCV increase. In our study, HGB concentrations were very stable throughout storage, meaning that it may be a more accurate measurement of the oxygen carrying capacity of the pRBCs units than PCV, as it does not depend on erythrocyte morphology. Thus, as the HGB measurement becomes more accessible to veterinary practitioners, it could replace the PCV measurements.

The visual inspection of the pRBCs supernatant color to evaluate hemolysis could be inaccurate.[37] In fact, even when the supernatant is red-tinged, this does not indicate that the 1% level of hemolysis has been surpassed, and thus the unit can still be effectively transfused with no clinical consequences.[4] Thus, in addition to its value in identifying and rejecting unsuitable units, the hemolysis quantification also prevents the rejection of useful units that seem visually inappropriate for transfusion.[4,23,37]

The results of the present study highlight the need to implement a strict quality control program in veterinary blood banks to be able to detect hemolyzed pRBCs units that may be harmful to canine patients. Thus, despite the generally described shelf-life of 42 days for canine pRBCs units, one should be aware that each pRBCs unit stored for more than 5 weeks should be evaluated for hemolysis as an indicator of its viability. However, our results do not support the practice of disposing of units stored for more than 4 or 5 weeks, as almost half of them had acceptable hemolysis values during the 6th week of storage.

The small number of units evaluated could hamper the significance of some results, which is a limitation of this study. Consequently, further larger studies are warranted, to identify other factors, besides storage period, that influence hemolysis during storage.

In conclusion, the results of the present work support the principle that pRBCs stored for 36–42 days should be tested for hemolysis to ensure that the level has not exceeded the recommended standards for transfusion to human patients. Other than storage time, no other blood collection or unit characteristics were related to hemolysis of the pRBCs, and the prestorage hemolysis was insufficient to predict its value at the end of the storage period.

Footnotes

[a] Idexx Laboratories, Barcelona, Spain.
[b] Terumo blood bag – triple, Terumo Medical Corporation, Leuven, Belgium.
[c] Composeal, Fresenius Kabi, Hesse, Germany.
[d] Compocool, Fresenius Kabi.
[e] Megafuse 40R, Thermo Scientific, Waltham, MA.
[f] Terumo component extractor, Terumo Medical Corporation, Somerset, NJ.
[g] CompoDock, Fresenius Kabi.
[h] Macopharma, Mouvazux, France.
[i] Hb 201 System, HemoCue Inc., Brea, CA.
[j] Centrifuge IEC Centra CL3R, Thermo Scientific.
[k] Plasma Low Hb, HemoCue Inc.
[l] Medika 250, Fiocchetti, Luzzara, Italy.
[m] Bact/Alert PF, Biomerieux, Marcy l'Etoile France.
[n] Bact/Alert 3D, Biomerieux.
[o] SPSS, version 22.0.0, IBM, Chicago, IL.
[p] Adsol, Fenwal Laboratories, Deerfield, IL.

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