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Freezing and storage of leukodepleted erythrocyte suspensions

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ABSTRACT: Studies on the frozen storage of human blood products have benefited veterinary transfusion medicine in recent years, but the long-term cryopreservation of canine red blood cells (RBCs) has not yet been thoroughly investigated. Further, no studies are available with respect to the frozen storage of leukocyte-depleted canine red blood cells (LD-RBCs). The objective of the current study was to investigate time-dependent effects of long-term frozen storage on leukocyte-depleted canine RBCs. Twelve healthy adult dogs meeting the criteria for blood transfusion were used in the study. Whole blood samples (450 ± 45 ml) collected from each dog were centrifuged for 5 min at 22 °C and 4200 × g in a cryogenic microcentrifuge and concentrated RBC (pRBC) suspensions were obtained. Leukocyte depletion was achieved by filtration (2.6 $\log_{10}$). Then, the filtrated samples were prewashed three times in 0.9% NaCl solution and were allocated into three subgroups to be evaluated at three different time points (Day 0, Month 4 and Month 6). The samples for cryopreservation were subjected to glycerolisation and then stored at –80 °C for 4- and 6-month periods. At the end of this period pRBC units were thawed by manual agitation in a water bath maintained at 36–38 °C, centrifuged and then washed in a consecutive series of 12%, 1.6% and 0.9% of NaCl + 0.2 dextrose solutions. 2,3-Diphosphoglycerate (2,3-DPG), adenosine triphosphate (ATP), supernatant haemoglobin (SupHb), sodium (Na^+) and potassium (K^+) levels, residual glycerol concentrations and haemograms of thawed and deglycerolised pRBC samples were evaluated together with those of Day 0. Sterility tests were performed on all samples for bacterial contamination. No statistically significant differences were noted except for Hct and SupHb levels. No bacterial contamination was noted in any of the samples on the basis of sterility tests. It was found that the described glycerolisation procedure could be a method of choice in the cryopreservation of leukocyte-depleted pRBCs (LD-pRBCs) since no negative effect was observed on the quality of the products and long-term frozen storage did not cause RBC destruction.

Keywords: canine; cryopreservation; leukocyte; quality; red blood cell

Innovations in transfusion medicine have underlined the rising significance of this procedure in veterinary medicine (Kim et al. 2004; Lucas et al. 2004). In parallel, more efficient storage techniques have been sought and new studies were carried out on the long-term frozen storage of red blood cells (RBCs) (Kim et al. 2004; Kim et al. 2007; Pallotta et al. 2012; Sen and Khetarpal 2013). Storage lesions during RBC preservation are manifested by increased lactic acid, extracellular K^+ accumulation and a subsequent reduction in pH, adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) levels. Additives applied prior to storage minimise these lesions (Scott et al. 2005) and thus offer a prolonged life-span for erythrocytes even after storage and during transfusion (Battaglia 2000; Scott et al. 2005; Holovati et al. 2009). Cryoprotectants protect against cold shock damage due to freezing, intracellular crystallisation and de-crystallisation as well as membrane destabilisation during the thawing process. Prior to freezing intracellular balance is sustained by the help of cryoprotectants (Palasz and Mapletopt 1996; Kim et al. 2004; Sputtek et al. 2007; Chaudhari 2009).

Moreover, it was also suggested that alterations in stored erythrocytes result not only from intracellular factors and storage conditions but also from contaminating leukocytes and their side products (Andreu 1991; Holovati et al. 2008). Therefore, the

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necessity of a closed system processing unit ensuring stringent control against bacterial contamination was particularly underlined (Battaglia 2000; Lucas et al. 2004). It was described that leukocytes and platelets were the blood components, which were responsible for immunosuppression and for certain transfusion reactions even in donor/recipient-compatible blood types (Kristensen and Feldman 1995; Vamvakas and Blajchman 2001; Lucas et al. 2004). For this purpose leukocyte-depleted (LD) concentrated RBC (pRBC) suspensions were obtained by the filtration of white blood cells (WBCs) (Hohenhaus 2005). The WBC count could be decreased down to 1×10^6 by the application of different filters (Kilic et al. 2004). By the advent of the 1990s most countries had created legislation to enforce the use of WBC-filtrated blood products for samples intended for transfusion. Buffy coat-derived leukocyte-deprived (BC-LD) blood products were used to investigate the adverse effects of blood transfusions and comparative studies were carried out in non-transfused versus BC-LD RBC-transfused patients (van de Watering et al. 1998; Dzik et al. 2000). In the last decade in North America, Europe, Asia and Australia (Vamvakas 2007), WBC reduction by filtration has been established as technique prior to the usage of blood cellular components (Dzik et al. 2000). LD-blood components have long been in use in transfusion medicine in order to eliminate or at least reduce the risks and side effects of alloimmunisation (Kirkley 1999; Bontadini et al. 2002; King et al. 2004).

The effects of storage on LD canine pRBC products have also been investigated. However, there is only a limited number of studies with respect to presumptive alterations caused by frozen storage of canine erythrocytes via glycerolisation (Contreras et al. 1979; Kim et al. 2004; Kim et al. 2007) Furthermore, there is no data available in terms of long-term storage of LD canine pRBC products.

Leukocytes and their side products in pRBC adversely affect the quality of the blood components. A remarkable decrease in transfusion-induced side effects in humans after pRBC transfusions is undoubtedly associated with the increasing use of leukodepleted blood products. Furthermore, leukodepleted blood products were shown to have longer shelf-life in blood banks (AuBuchon et al. 1997; Chin-Yee et al. 1997; Walter et al. 2000; Hess and Greenwalt 2002). The current study was designed to determine the effects of leukodepletion on the viability and function of canine pRBCs after long-term frozen storage. We hypothesised that leukodepletion will be beneficial for the freezing and storage of erythrocyte suspensions and therefore will cause quality control parameters to be improved.

MATERIAL AND METHODS

The study material initially consisted of fifteen 1- to 9-year-old, healthy male easy-going peaceful dogs which were seronegative for blood parasites (Battaglia 2000) such as *Dirofilaria*, *Ehrlichia*, *Anaplasma*, *Borrelia*, and *Leishmania*. The dogs were of two different breeds (Kangal and German Shepherd) and weighed ≥ 30 kg. Out of the initial 15, 12 dogs meeting the criteria for blood transfusion (Kerl and Hohenhaus 1993; Barr 2000) in terms of haematocrit value were selected for the study. Standard amounts of 450 ml of whole blood were collected from the dogs according to the established standards of several different blood banks (Kristensen and Feldman 1995; Battaglia 2000; Hohenhaus 2005).

Whole blood samples of 450 ± 45 ml were collected from each dog, centrifuged for 5 min at 4200 × g and 22 °C and then pRBCs were obtained. Leukocyte depletion was achieved by using leukocyte filters (Imugard III RC-4P). LD-pRBC samples were subjected to a leukocyte counting protocol using a haemocytometer method in order to determine the filtration efficiency. Filtration efficiency for each sample was estimated in percentage value in a nageotte (Superior Marienfeld) counting chamber. WBC counts of the samples before and after filtration process were assessed on thoma and nageotte counting chambers, respectively (Superior Marienfeld) using haemocytometric methods (Bontadini et al. 2002). Filtration efficiency percentage value was estimated by calculating the correlation between WBC counts before and after filtration and this value was defined on a $\log_{10}$ level (Brownlee et al. 2000). Then, the filtrated samples were prewashed three times in 0.9 % NaCl solution, each time with the addition of 1.5 fold of the initial samples (Brecher 2005) by a closed system device (Terumo TSCD-II). Finally, the samples were divided into three groups to be evaluated on Day 0, and at 4- and 6-month periods. Samples to be frozen and stored were subjected to glycerolisation and then stored at –80 °C for 4- and 6-month periods. At the end of this period LD-RBC units were thawed at 36–38 °C in a water bath with a manual

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stirrer and washed in a series of centrifugation steps in 12%, 1.6% and 0.9% NaCl + 0.2 dextrose solutions, respectively (Wagner et al. 2000). Quality parameters were assessed for the samples collected on Day 0 and for those, which were thawed and deglycerolised (Valeri et al. 2000; Kim et al. 2004).

ATP content was measured using a luminometric assay (Berthold Luminometer) with a commercially available kit (PROMEGA G7570 Cell Titer Glo Cell Viability Assay).

2,3-DPG concentration was assessed by spectrophotometry (Lecak et al. 2004) using commercial kits (Roche Diagnostics). Extracellular Na^+ and K^+ levels were determined via flame photometry (JENWAY) (Valeri et al. 2000).

Haematological parameters (RBC, Hb, Hct, RDW, MCV, MCHC, MCH) were assessed using an automatic blood cell counter (Abacus Junior Vet).

Supernatant haemoglobin (SupHb) levels were determined using the spectrophometric method.

Residual glycerol concentration was measured using refractometry (Atago surNα).

A modified method for measuring residual glycerol concentration was adopted from that of Wong et al. (2009), used in that study to calculate refractive index and to estimate the residual glycerol concentration on human RBCs. Glycerol solutions were prepared at different concentrations (0, 5, 10, 15, 20 g/l). The final rinsing buffers, 0.9% NaCl + 0.2 dextrose and 1.6% NaCl (1 : 1, depending on the RBC amount), were used as the main buffer solutions. The glycerol solutions at different concentrations were diluted in main buffers. Concentrated canine RBCs were haemolysed by centrifugation and the haemoglobin (Hb) levels of the product were measured. The suspension was then diluted 100, 200, 300, 400, 500 fold in buffer solutions. A total of 25 standard tubes were prepared with five from each dilution and finally, the refractive index was estimated for each sample.

The impact of Hb and glycerol concentration on refractive index was mathematically calculated and then an equation was established for the correlation between refractive index and residual glycerol concentration.

$$\text{Glycerol} = (\text{RI} - 27.586 \times \text{Hb} - 16)/0.8$$

Sterility tests were carried out using blood culture bottles (Oxoid).

For statistical analyses of the data obtained in the study, the Shapiro-Wilk test was used to verify in-group distribution of the parameters. Groups showing standard distributions were evaluated using the parametric ANOVA test, whereas groups with non-normal distribution of variables were evaluated using the non-parametric Mann-Whitney *U*-test. The significance of the measurement period was determined using Duncan and Games Howell tests.

RESULTS

The approximate intensity of filtration level of WBCs was 99.76% (2.6 $\log_{10}$) in canine LD-pRBC samples. The freezing process was found to be efficient in terms of ATP, 2,3-DPG, Na^+, K^+, RDW, MCV and SupHb values. A statistically significant decrease was noted in terms of the mean values with respect to these parameters at 4- and 6-month periods compared to those on Day 0. No statistically significant difference was found between these two months. An increase was detected in Hct values only at Month 6, despite the fact that the changes in RBC, Hb, MCH and MCHC levels were of no statistical significance. Residual glycerol values were observed to be slightly increased in comparison with the normal range. No bacterial contamination was noted in any of the samples on the basis of sterility tests.

Taking into account the effects of leukocytes and leukocyte-derived products on the cellular membrane of erythrocytes, it was determined that the frozen storage of leukocyte-filtrated concentrated canine erythrocytes through glycerolisation did not alter the quality parameters. Therefore, it was concluded that this freezing procedure could be used as a reliable method for the long-term storage of blood products.

DISCUSSION

Statistically significant decreases were found for ATP and 2,3-DPG levels after freezing and storage. The changes detected to be due to the effects of the duration of the storage period following freezing revealed similar results to those carried out on human blood products (Contreras et al. 1979; Valeri et al. 2000; Kim et al. 2004; Lecak et al. 2004; Sen and Khetarpal 2013). The reduction in 2,3-DPG concentrations could be associated with the decrease in pH values as a result of hydrogen ion accumulation due

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Table 1. RBC, Hb, MCH, MCHC, MCV, RDWc, Hct, ATP, 2,3-DPG, Na^+, K^+, SupHb and residual glycerol values of fresh and frozen canine leukodepleted packed red blood cells (means ± SD)

Storage Period	RBC ($\times 10^6$ µl) (n = 8)	Hb (g/dl) (n = 8)	MCH (pg) (n = 8)	MCHC (g/dl) (n = 8)	MCV (fl) (n = 8)	RDWc (%) (n = 8)	Hct (%) (n = 8)	ATP (nmol/gHb) (n = 10)	2,3-DPG (g/l) (n = 8)	Na^+ (mmol/l) (n = 9)	K^+ (mmol/l) (n = 10)	SupHb (g/dl) (n = 12)	Residual glycerol (g/l) (n = 12)
Day 0	5.93 ± 0.24	13.06 ± 0.47	22.13^a ± 0.27	31.94 ± 0.42	69^a ± 1.22	14.78^a ± 0.17	40.72^a ± 1.04	22.32^a ± 1.38	1.32^a ± 0.08	15.96^a ± 0.17	1.17^a ± 0.1	0.68^a ± 0.017	
Month 4	4.83 ± 0.38	12.88 ± 0.88	27.44^{ab} ± 2.15	27.16 ± 1.75	88.13^b ± 4.55	20.61^b ± 1.17	43.18^a ± 4.54	11.14^b ± 1.31	0.59^b ± 0.1	25.2^b ± 0.19	0.82^b ± 0.09	0.43^a ± 0.06	36.4 ± 10.91
Month 6	6.16 ± 0.63	15.7 ± 2.14	29.68^b ± 1.61	29.94 ± 1.58	94.88^b ± 2.32	22.39^b ± 1.03	58.15^b ± 5.7	4.57^b ± 0.97	0.53^b ± 0.08	30.64^b ± 1.61	0.5^b ± 0.06	0.49^b ± 0.032	32.93 ± 2.45
Significance^A (P-value)	0.176	0.399	0.012	0.089	0.002	< 0.001	0.012	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.46

^{a,b}significant differences between the mean values are indicated with different letters in the same column ($P < 0.05$)

^{A}repeated-measures analysis of variance for each group indicates significance

to the conversion of glucose into lactate (Hess and Greenwalt 2002; Holme 2005). Levels of 2,3-DPG are determined by the balance between destruction and synthesis. This balance depends on pH, and the degradation process starts when pH values drops below seven (Kurup et al. 2003). In this study, decreases in 2,3-DPG and ATP concentrations could be considered as a natural outcome due to the cessation of glycolysis and thus the fall of pH values during storage.

Since all metabolic and biochemical activities are suppressed within the cell during the freezing process (Holovati et al. 2008), a decrease in ATP concentration is not to be expected. However, thawing haemolysis (Kim et al. 2004) may cause a decrease in ATP levels, which is closely associated with that of 2,3-DPG (Hess and Greenwalt 2002; Holme 2005). It is well known that glycerolisation results in osmotic stress (Liu et al. 2002; Kim et al. 2004) for canine RBCs and concomitantly leads to ATP reduction.

In the present study, intracellular K^+ levels decreased among all groups depending on the time interval, which is in accordance with studies on human RBC products (Valeri et al. 2000; Holovati et al. 2008) and ATP reduction might be associated with intracellular K^+ depletion and Na^+ elevation. ATP loss at low temperatures was proven to be a consequence of the paralysis of the cellular membrane-associated sodium-potassium pump (Hess 2010) and thus leading to a decrease in its activity (Hess 2006; Hess 2010; Zubair 2010). Furthermore, electrolyte changes during the washing steps in the deglycerolisation process are an anticipated outcome (Holovati et al. 2008).

The findings regarding the changes in SupHb levels between 4- and 6-month periods were similar to those of Lecak et al. (2004), in which human RBCs were used (Table 1). Thawing haemolysis and osmotic stress during deglycerolisation (Kim et al. 2004) showed that erythrocytes underwent haemolysis to a certain extent but the duration of the freezing period had no effect on this process. Residual glycerol concentrations were unchanged between these 2-month periods.

Previously, changes in RBC, Hb and RDW values of canine RBCs after their storage under hypothermic conditions were described which are in agreement with our findings (Wardrop et al. 1994b). Although samples of pRBCs were evenly distributed, resultant cellular sedimentation might have caused an increase in RBC, Hct and Hb values (Wardrop et al. 1994a; Mudge et al. 2004). Furthermore, the increase in Hct values was directly correlated with

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those of MCV. On the other hand, it was proposed that osmotic effects of additional solutions cause certain morphological changes in erythrocytes and thus increase cellular volume (Mudge et al. 2004).

Increases in RDW values, similarly as observed by Pallotta et al. (2012), might be associated with anisocytosis and cellular deformity due to low temperature (Sollberger et al. 2002).

MCH and MCHC mean values, in parallel to Hb concentration (Table 1) were compatible with those from studies carried out with human erythrocytes (Lecak et al. 2004; Holovati et al. 2008).

MCV mean values increased significantly at 4- and 6-month periods, in comparison with those on Day 0 (Table 1). In a previous study, a slight increase was noted in leukocyte-depleted human erythrocytes after the freezing process, but this was not statistically significant (Holovati et al. 2008). The increase in MCV values, which was directly correlated with that of Hct, could be explained by the osmotic effect of deglycerolisation solutions. Mudge et al. (2004) pointed out the osmotic effects of additional solutions and anticoagulants and indicated the possibility of a rise in MCV values.

Since pRBCs were processed in a closed system in the study, there was no evidence of bacterial contamination.

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