Abstract

Human medicine studies have so far demonstrated that erythrocytes may be preserved and stored at low temperatures for decades retaining their metabolic and biochemical properties. However, detailed studies regarding this problem are not yet available in veterinary medicine. Therefore, the objective of the current study was to investigate time-dependent effects of long-term frozen storage of canine red blood cells.

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 by a cryogenic micro-centrifuge and packed RBC suspensions were obtained. The samples were prewashed three times in 0.9% NaCl solution and were allocated into three groups to be evaluated at three different time points (day 0 and month 4 and 6). The samples to be frozen were subjected to glycerolization and then stored at -80°C for 4 and 6-month periods. At the end of this period the packed RBC samples were thawed, centrifuged and then washed in a consecutive series of dextrose solutions. 2,3-Diphosphoglycerate (2,3-DPG), Adenosin triphosphate (ATP), supernatant hemoglobin (SupHb), sodium (Na+) and potassium (K+) levels, residual glycerol concentrations and hemograms were evaluated and compared. Sterility tests were performed on all samples for bacterial contamination. A statistically significant decrease was noted in potassium levels, which was the natural outcome of deglycerolization process. No significant change was observed in terms of other parameters due based on different time points. In conclusion, long-term frozen storage had no negative effect on the quality parameters of canine erythrocytes.

Key words: canine, cryopreservation, erythrocyte, glycerol, storage

Introduction

Novel diagnostic and therapeutic approaches have appeared in veterinary medicine with advance of technology. Likewise, transfusion medicine has gained importance in response to increased demands (Kim et al. 2004, Lucas et al. 2004). Developments in blood storage techniques are important scientific achievement for the maintenance of Blood Banks in terms of the availability of blood products for therapeutic pur-

Each component of blood has different and unique function in the organism. Selection of the proper component varies depending on the type of the disease and the requirements of the patient ( Battaglia et al. 2000, Sen and Khetarpal 2013). Blood samples collected from the donor animals should either be engaged for an instant use or be separated into the components and stored in most efficient way (Lucas et al. 2004).

With the advances in veterinary transfusion medicine, researchers have discovered the contraindications of the usage of Whole Blood and thus verged to use the blood components, instead, for the treatment of miscellaneous diseases in order to minimize the risks (Kim et al. 2004). The next step was to improve blood storage techniques in an attempt to compensate growing demands for blood transfusion, and therefore, new studies were designed investigating long-term cryopreservation of RBCs (Kim et al. 2004, Kim et al. 2007, Pallota et al. 2012). The aim was to prolong the shelf life of blood products (Pallota et al. 2012) and prevent economic losses.

Studies on cryopreservation of RBCs in human medicine prompted similar studies in veterinary transfusion medicine (Kim et al. 2007), however, research with respect to the determination of alterations or lesions associated with long-term preservation and frozen storage of canine RBCs are far from providing sufficient data. Further studies are still required to investigate the diversity of currently available cryopreservative agents, to improve washing procedures and to determine the storage-induced changes in blood products. Therefore, in the present study, we aimed to determine the time-dependent effects of long-term frozen storage of canine RBCs.

Materials and Method

Twelve healthy canine donors, meeting the criteria for blood transfusion, were used in the study. Blood samples of 450 ± 45 ml were obtained from each dog, centrifuged for 5 min at 4200 x g and 22°C and then packed RBCs were obtained. Samples were prewashed three times in 0.9% NaCl solution, each time with the addition of 1.5 fold of the initial samples (AABB Technical Manual 2005d p. 808) by a closed system device (Terumo TSCD-II). Then the samples were divided into three groups to be evaluated on day 0, and at month 4 and month 6. Samples to be frozen were subjected to three-stage glycerolization (Glycer-olyte 57, Fenwall) and stored at -80°C for 4 and 6-month periods (AABB Technical Manual 2005d p. 809). At the end of this period RBC units were thawed at 36-38°C in a water bath with manual stirrer and then washed via a series of centrifugation steps in 12%, 1.6% and 0.9% NaCl + 0.2 dextrose solutions for the deglycerolization process (Wagner et al. 2000). Quality parameters were assessed for the samples collected on day 0 and for the samples thawed and deglycerolized.

ATP content was measured by 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 with commercial kits (Roche Diagnostics) (Lecak et al. 2004).

Extracellular Na⁺ and K⁺ levels were determined by flame photometry (JENWAY) (Valeri et al. 2000).

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

Sup. Hb level was estimated by spectrophotometric method.

Residual glycerol concentration in final supernatant samples was estimated by refractometry (Atago surNh). For this purpose, Wong (2009)’s method has been modified and used. Because the supernatant Hb concentration is also effective on the refractive index (RI), the mathematical relationship between Hb and glycerol concentration has been defined by using dilution series and calibration curves. Therefore, dilution series of Hb and glycerol solutions have been prepared using the final rinsing buffer. RI measurements of the Hb and glycerol dilution series have been performed by using refractometer and the values plotted against the Hb and glycerol concentrations (Fig. 1A,B). Finally, residual glycerol formula has been constructed by combining the equations obtained from the calibration curves (Equation 1).

Equation 1

Equation from Fig. 1A: \( y = 27.586 \times x + 16 \) which means; \( RI = 27.586 \times \text{Hb} + 16 \)

Equation from Fig. 1B: \( y = 0.8 \times x + 16 \) which means; \( RI = 0.8 \times \text{Glycerol} + 16 \)

\( RI = a \times \text{Glycerol} + b \times \text{Hb} + c \)

\( RI = (0.8 \times \text{Glycerol}) + (27.586 \times \text{Hb}) + 16 \)

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

\( a / b : \text{Slope of calibration curves of Glycerol/Hb dilutions} \)

Sterility tests were carried out by using blood culture bottles (Oxoid) against bacterial contamination.
Results

The cryopreservation procedure was found to be effective in terms of ATP, DPG, Na⁺, RDW, MCV, and Sup. Hb levels. A statistically significant decrease was found 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. Mean K⁺ values decreased both in comparison with the day 0 and between the two different month intervals and this decrease was found to be statistically significant. An increase was monitored in Hct values only at month 6 although the changes in RBC, Hb, MCH and MCHC levels were of no statistical significance. Residual glycerol values did not differ between 4 and 6-month time points. (Table 1). No bacterial contamination was evidenced on the basis of sterility tests.

Discussion

The decreases in ATP and 2, 3 DPG concentrations after 4 months, which were found to be statistically significant (p <0.001), continued also in the storage period (between 4th and 6th month), however were not statistically significant. It is well known that while glucose concentration decreases due to ultra-low temperature during storage, pH value decreases as well as a result of an increase in lactic and pyruvic acid levels.

ATP and 2, 3 DPG concentrations are reduced accordingly (Hogman and Meryman 1999, Hess and Greenwalt 2002, Hess 2010).

The changes monitored due to the duration of the storage period following the freezing process were found not to be statistically significant (Table 1). The studies conducted on human (Valeri et al. 2000, Lecak et al. 2004) and canine RBCs (Contreras et al. 1979, Kim et al. 2004) revealed similar results (Sen and Khetarpal 2013).

On the basis of these findings it was suggested that canine RBCs were exposed to oxidative stress (Liu et al. 2002, Kim et al. 2004) during freezing process due to the glycerol solution used and thus a concomitant loss of ATP was observed. Furthermore, canine RBCs were found to be more fragile than the RBCs of other mammalian species (Matsuzava and Ikarashy 1979).

During storage, degradation of 2, 3 DPG, which is closely associated with that of ATP, was already anticipated as a result of the cessation of glycolysis and a drop in the pH value (Hess and Greenwalt 2002, Kurup et al. 2003, Holme 2005, Scott et al. 2005, Kor et al. 2006). This decrease in 2, 3 DPG level (Table 1) was compatible with the findings of the Valeri et al. (2000) and Lecak et al. (2004), in which human RBCs were stored by freezing and this effect was found to be statistically significant in this study.

In the study, intracellular K⁺ level decreased significantly in all groups due to different time periods. The findings were compatible with those of the
Table 1. Quality parameters of fresh and frozen dog erythrocytes.

<table>
<thead>
<tr>
<th>Storage period</th>
<th>ATP (nmol gHb)</th>
<th>2,3 DPG (g/l)</th>
<th>Na⁺ (mmol/l)</th>
<th>K⁺ (mmol/l)</th>
<th>Supernatant hemoglobin (g/dl)</th>
<th>Residual glycerol (g/l)</th>
<th>RBC (×10⁶ μL)</th>
<th>Hb (g/dl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>MCV (fl)</th>
<th>RDWc (%)</th>
<th>Het (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=11)</td>
<td>(n=12)</td>
<td>(n=12)</td>
<td>(n=12)</td>
<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
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<td>(n=8)</td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>0th day</td>
<td>23.48 ± 2.13</td>
<td>1.37 ± 0.08</td>
<td>15.69 ± 0.15</td>
<td>1.50 ± 0.09</td>
<td>0.67 ± 0.02</td>
<td>5.81 ± 0.2</td>
<td>13.02 ± 0.5</td>
<td>0.5 ± 0</td>
<td>22.45 ± 0.3</td>
<td>33.02 ± 0.4</td>
<td>68.38 ± 1.2</td>
<td>1.2 ± 0.17</td>
<td>39.60 ± 1.04</td>
</tr>
<tr>
<td>4th month</td>
<td>6.95 ± 1.1</td>
<td>0.80 ± 0.1</td>
<td>25.74 ± 0.85</td>
<td>0.93 ± 0.08</td>
<td>0.37 ± 0.04</td>
<td>34.84 ± 5</td>
<td>5.38 ± 0.4</td>
<td>13.15 ± 0.09</td>
<td>24.96 ± 2.2</td>
<td>29.35 ± 1.5</td>
<td>97 ± 4.6</td>
<td>19.21 ± 1.17</td>
<td>48.08 ± 4.54</td>
</tr>
<tr>
<td>6th month</td>
<td>3.53 ± 0.32</td>
<td>0.77 ± 0.08</td>
<td>27.96 ± 1.4</td>
<td>0.56 ± 0.05</td>
<td>0.50 ± 0.03</td>
<td>32.16 ± 3.30</td>
<td>6.47 ± 0.6</td>
<td>17.91 ± 2.1</td>
<td>27.66 ± 1.6</td>
<td>28.09 ± 1.3</td>
<td>101.5 ± 2.3</td>
<td>20.23 ± 1.03</td>
<td>65.15 ± 5.7</td>
</tr>
</tbody>
</table>

Significance

(P-value) <0.001 <0.001 <0.001 <0.001 <0.001 0.46 0.13 0.026 0.057 <0.001 <0.001 <0.001

a-b – There is a significant differences between the means indicated with different letters on the same column (p<0.05).

^ – Repeated measures analysis of variance for each group as a result significance.
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In the present study, freezing process revealed statistically significant changes in the quality parameters of canine RBCs. However, long-term storage of packed RBCs had no significant impact on these parameters, except for potassium levels and hematocrit values. Since alterations in potassium levels were considered to be a natural course of the deglycerolization process, it can be concluded that freezing and long-term frozen storage of canine RBCs has no negative effect on the quality of the product.
Acknowledgments

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References


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