A Comparative Study of the Effects of Glycerol and Hydroxyethyl Starch in Canine Red Blood Cell Cryopreservation

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ABSTRACT. Hydroxyethyl starch (HES) is a nonpenetrating extracellular cryoprotectant. In contrast to glycerol, it does not require labor-intensive removal from thawed red blood cells (RBCs) prior to transfusion. In this study, we compared glycerol and HES, and assessed HES as a substitute for glycerol in cryopreserved canine RBCs. The RBCs were preserved for 2 months in liquid nitrogen using a 20% (w/v) glycerol solution, and variable concentrations of HES solution. We evaluated the two cryoprotectants by the percentage of post-thaw hemolysis from the total free hemoglobin, saline stability, osmotic fragility, and by observing the erythrocyte morphology using a scanning electron microscope after thawing. The optimal concentration of HES was 12.5% (w/v) for the cryopreservation of canine RBCs. The thaw hemolysis, saline stability, and osmotic fragility index were 25.6 ± 4.7%, 87.8 ± 6.9%, and 0.445 ± 0.024% NaCl respectively. These parameters resemble the results of RBCs frozen in a 20% (w/v) glycerol solution, which are 24.7 ± 5.2%, 99.2 ± 0.1%, and 0.485 ± 0.023% NaCl respectively. From a morphological point of view, 12.5% (w/v) HES showed the best cryoprotection of RBCs compared to the other concentrations of HES. These results suggest that HES could be a possible substitute for glycerol for the cryopreservation of canine RBCs.

KEY WORDS: canine red blood cell, cryopreservation, glycerol, hydroxyethyl starch.

FULL PAPER

In the past few years, interest in veterinary transfusion medicine has grown rapidly. Many practitioners use whole blood collected using CPD (citrate-phosphate-dextrose solution) or CPDA-1 (citrate-phosphate-dextrose solution containing adenine) as the anticoagulant [8]. Some studies show that canine red blood cells (RBCs) stored in CPDA-1 drop below the Food and Drug Administration standard of 75% post-transfusion viability after only 20 days of storage at 4°C [15], even though human RBCs are routinely stored for up to 35 days. Whole blood used to be the mainstay of veterinary transfusion therapy, but the rare need for whole blood, the recognized need for specific therapy to decrease the risk of adverse reactions, the increased availability of storage facilities, and a need for more efficient use of available donor blood have made component therapy the choice in transfusion-dependent critically ill patients [8].

Over the past few decades, a considerable number of studies have been conducted on blood cryopreservation in human medicine. It has been recognized that RBC cryopreservation is the ideal method of long-term storage [23]. The usual procedures use glycerol as a cryoprotectant. Glycerol protects RBCs by limiting the rise in electrolyte concentration which occurs when water is converted to ice, and by inhibiting the rate of ice crystal growth [10]. This type of cryopreservation allows RBCs to be stored for a long time, but requires that the thawed RBCs be thoroughly washed to remove glycerol before transfusion. The addition and removal of glycerol can also cause potentially damaging transient osmotic stress. This can be circumvented by using extracellular additives which do not penetrate the RBC membrane. Knopp et al. [7] reported the successful cryopreservation of human RBCs with hydroxyethyl starch (HES), which is a substituted polysaccharide and a nonpenetrating cryoprotectant. HES is slowly metabolized in vivo to a utilisable carbohydrate by α-amylases. It is nontoxic, poorly antigenic, and often used as a plasma expander [19]. There are several papers on canine RBC cryopreservation using HES, but the experiments were conducted with short storage terms [5, 22] and small sample sizes [5]. We were unable to find any other papers on morphological observation of canine RBC cryopreservation.

This study evaluated the protective capacities of glycerol and variable concentrations of HES in canine RBCs by assessing the percentage of thaw hemolysis, saline stability, osmotic fragility, and morphological changes after freeze-thawing. Furthermore, we determined the best concentration of HES for canine RBC cryopreservation and confirmed HES as a substitute for glycerol.

MATERIALS AND METHODS

Cell preparation: Sixteen mature beagle dogs were used in this study. They were housed indoors and maintained according to Yamaguchi University Animal Care and Use Committee regulations. Dogs were 3 to 5 years of age and weighed 10 to 14 kg. Each dog was subjected to a physical examination and blood tests prior to blood collection.

Whole blood collected in CPDA-1 (Terumo Blood Bag CPDA, Terumo Co., Tokyo, Japan) was centrifuged for 5
min at 1,500×g and then washed three times in an isotonic saline solution (0.9% (w/v) NaCl). After the final washing, RBCs were packed by centrifugation to a hematocrit value of about 80%.

**Freezing, thawing and washing procedures for 20% (w/v) glycerol:** The 20% (w/v) glycerol cryopreservation procedure was similar to the freezing and deglycerolization procedures described by Contreras et al. [2], except that the equilibration time for deglycerolization was extended to 5 min. Thirty milliliters of glycerolized RBCs were transferred to a freezing bag (Cryogenic Storage Container, CharterMed Inc., Lakewood, NJ, U.S.A.). This bag was then placed in a closed aluminum container, submerged in liquid nitrogen, and stored for 2 months.

The frozen RBCs were thawed by manual agitation in a water bath maintained at 47°C. A 2 ml sample of the thawed cells was taken for thaw hemolysis, as described below. Glycerol was removed from the remainder of the cell suspension by serial dilutions with decreasingly concentrated saline solutions.

**Freezing, thawing and washing procedures for the HES groups:** Fifteen milliliters of washed RBCs were mixed in the same volume of HES (molecular weight about 200,000, Ajinomoto Co. Inc., Tokyo) solution manually. The HES solutions had concentrations of 5, 15, 25, 35, and 45% (w/v) with 60 mM NaCl. The mixture was transferred to a freezing bag (final HES concentrations: 2.5, 7.5, 12.5, 17.5, 22.5% (w/v)), then frozen, stored, and thawed as described above. A 2 ml sample of the thawed cells was taken for thaw hemolysis. The remainder of the cell suspension was washed with the same volume of isotonic saline solution.

**Thaw hemolysis:** Free hemoglobin (Hb) concentrations of washed RBCs were measured using a cell-free sample isolated by centrifugation for 35 min at 12,000×g. The cell free sample and total Hb contents were determined with a Hb measurement indicator for spectrophotometric analysis at 540 nm (Hemoglobin B-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Thaw hemolysis (%) = (Supernatant Hb content/Total Hb content) × 100 [17]

**Saline stability test:** Two hundred fifteen microliters of deglycerolized RBCs or washed RBCs taken from each HES group were diluted 50 fold in an isotonic saline solution and mixed gently. Ten milliliters of the red cell/saline mixture was transferred into a test tube and then centrifuged for 10 min at 1,500×g after waiting for 30 min. The total Hb was estimated by transferring 2 ml of the remaining mixture to another tube and making the total volume 10 ml with distilled water (DW). The optical density (OD) of the supernatant was read at 540 nm. The saline stability was calculated as follows:

Saline stability (%) = 100 – Supernatant OD × 100 × 0.2/Total Hb OD [23]

### RESULTS

**Thaw hemolysis and saline stability test:** The percentage of thaw hemolysis depended on the protective agent used and its concentration (Table 1). The use of 2.5% (w/v) HES resulted in greater than 80% lysis during the freeze/thaw cycle and nearly complete lysis following post-thaw processing.

Saline stability was over 80% except in 2.5% and 22.5% (w/v) HES.

**Osmotic fragility test:** Osmotic fragility was determined for deglycerolized RBCs, washed RBCs of HES groups, and non frozen fresh RBCs (control), as described by Jain [6]. The percentage of hemolysis was calculated by assuming the hemolysis in DW to be 100%. The concentration of saline necessary to induce 50% hemolysis was determined from these curves. This value, termed the fragility index of the cell suspension, was used to quantify the degree of fragility. Using this method, a larger fragility index corresponds to a more fragile cell.

**Scanning Electron Microscopy:** Thawed and washed RBCs were fixed with 1.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 hr, washed with the same buffer three times, and dehydrated with an ascending ethanol series (50 to 100%). The specimens were dried using a critical point dryer, coated with gold in an ion-coater apparatus, and observed under a scanning electron microscope (JSM 6100, Japan Electron Optics Laboratory Co. Ltd., Tokyo) at an accelerating voltage of 15 kV.

### Table 1. Thaw hemolysis and saline stability

<table>
<thead>
<tr>
<th>HES concentration</th>
<th>Thaw hemolysis (%)</th>
<th>Saline stability (%)</th>
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</thead>
<tbody>
<tr>
<td>20% Glycerol</td>
<td>24.7 ± 5.2</td>
<td>99.2 ± 0.1</td>
</tr>
<tr>
<td>2.5% HES</td>
<td>82.6 ± 6.6b</td>
<td>50.6 ± 13.Oa,c</td>
</tr>
<tr>
<td>7.5% HES</td>
<td>33.9 ± 6.6</td>
<td>85.3 ± 5.6</td>
</tr>
<tr>
<td>12.5% HES</td>
<td>25.6 ± 4.7</td>
<td>87.8 ± 6.9</td>
</tr>
<tr>
<td>17.5% HES</td>
<td>27.9 ± 4.6</td>
<td>80.3 ± 7.5</td>
</tr>
<tr>
<td>22.5% HES</td>
<td>31.7 ± 7.2</td>
<td>66.9 ± 7.5</td>
</tr>
</tbody>
</table>

mean ± SD, n=5

a) Denotes a significant difference (p<0.05) with respect to 20% (w/v) glycerol. b) Denotes a significant difference with respect to 12.5% (w/v) HES. c) Denotes a significant difference with respect to 7.5% and 17.5% (w/v) HES.
The fragility curve for 20% (w/v) glycerol shows a shift to the right compared to the control group (Fig. 1A). The 12.5% (w/v) HES group was more stable than the other concentrations of HES. The fragility indices (Fig. 1B) were 0.485% NaCl in the 20% (w/v) glycerol and 0.445% NaCl in the 12.5% (w/v) HES group. The fragility index of the control group was 0.427% NaCl.

Cell morphology: In 20% (w/v) glycerol (Fig. 2A), there were fewer echinocytic cells than the HES groups, but more cells with some hemispherical protrusion at the center. The use of 12.5% (w/v) HES limited the appearance of echinocytic, spherocoenocytic, and spherocytic shapes compared to the other HES groups (Fig. 2B, 2C, 2D). In 17.5% (Fig. 2D) and 22.5% (w/v) HES, there were some deformed cells with stick-like shape on their surfaces. The percentage of discocytes for 20% (w/v) glycerol and 12.5% (w/v) HES were 75 ± 5.01% and 65.6 ± 5.08%, respectively and the other HES groups showed under 45% discocytes (p<0.05).

DISCUSSION

In this study, we conducted an experiment on the cryopreservation of canine RBCs for 2 months with 20% (w/v) glycerol and variable HES concentrations. HES is a non-penetrating cryoprotectant, so it does not need to be removed after thawing [7]. However, Horn et al. [4] reported that a washing step before the transfusion of cryopreserved RBCs using HES decreased the amount of transfused free plasma Hb in human RBCs. Therefore we washed HES cryopreserved RBCs once with an isotonic saline solution after thawing.

We mimicked the 20% (w/v) glycerol freezing and deglycerolization procedures described by Contreras et al. [2], where they added and removed glycerol in a step-wise manner to reduce osmotic damage. The 30 min saline stability mirrors the in vivo 24 hr post-transfusion survival rate [24]. Our study had in vitro stabilities of approximately 99.2% (Table 1) as compared to 90% in their study [2]. One possible explanation for this difference is the longer equilibration time for deglycerolization in our study [9].

RBCs frozen in 12.5% (w/v) HES are better protected after thawing compared to other HES concentrations. The fragility indices of this concentration and 20% (w/v) glycerol were similar to that of fresh RBCs (Fig. 1). However, the glycerol percentage does not take into account the hemolysis and loss of cells which occur during the post-thaw washings to remove glycerol.

In 20% (w/v) glycerol, there were some cells with a hemispherical protrusion at the center (Fig. 2A). Liu et al. [9] reported osmotic stress on canine RBCs when using glycerol. This stress could be the cause of the morphological changes during glycerolization and deglycerolization.

The morphology of thawed RBCs shows that freezing with concentrations lower and higher than 12.5% (w/v) HES leads to more deformities (Fig. 2). In 17.5% and 22.5% (w/v) HES, there were some deformed cells with a stick shape on the surface. To the best of our knowledge, there are no other reports about these deformed cells or why high HES concentrations cause damage to RBCs. A possible reason for this is that HES concentrations over 12.5% (w/v) seemed to cause dehydration of RBCs during freezing and thawing due to high osmolarity. In contrast to this, HES concentra-
tions under 12.5% (w/v) proved insufficient for protecting RBCs from freezing stress.

When blood is being frozen, the extracellular fluid becomes hypertonic, causing water to flow out of the red cells, which leads to a reduction in cell volume [11]. It is suggested that the consequent damage is directly related to either intracellular dehydration or stress on the cell membrane [12]. HES is thought to achieve its cryoprotectant effect by delaying the outflow of water from the cell during freezing [24].

The 12.5% (w/v) HES group showed the same value of thaw hemolysis but more deformities than the 20% (w/v) glycerol group. Some studies showed that adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglyceric acid (DPG) affect the assembly of the cytoskeleton [18, 20], and that a change in ATP level affects cell shape [13]. In a report about the effect of glycerol and HES on ATP and 2,3-DPG in RBC cryopreservation, RBCs using HES had significantly reduced ATP concentrations compared to glycerol after thawing [5, 16]. This is one possible reason why 12.5% (w/v) HES showed the same value of thaw hemolysis but more deformities than glycerol. The results of this study show that RBC shapes after thawing depend on the cryoprotective agent and its concentration. However, even though abnormal RBC shapes were seen, one study reported that the morphological changes after thawing using HES recovered rapidly in fresh autologous plasma [21]. Further research is required to determine the effects that ATP and 2,3-DPG have on RBC membranes using rejuvenation [1, 3, 14, 24] and to determine the biochemical changes to RBC membranes.

Blood cryopreservation has several distinct advantages over traditional refrigerated storage. There are many reports on cryopreservation of human blood using glycerol. Unfortunately this approach has been labor- and cost-prohibitive for animal transfusions. HES is a simple and cost-effective substitute for glycerol.

In conclusion, 12.5% (w/v) HES was the optimal concentration for canine RBC cryopreservation. It also has a thaw hemolysis value and osmotic fragility index similar to 20% (w/v) glycerol. From a morphological point of view, 12.5% (w/v) HES offers superior protection for RBCs from frozen stress as compared to other HES concentrations. This data suggests that HES is a viable substitute for glycerol in canine RBC cryopreservation.

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