Comparison of the effects of glycerol, dimethyl sulfoxide, and hydroxyethyl starch solutions for cryopreservation of avian red blood cells

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OBJECTIVE
To compare effectiveness of glycerol, dimethyl sulfoxide (DMSO), and hydroxyethyl starch (HES) solutions for cryopreservation of avian RBCs.

SAMPLE
RBCs from 12 healthy Ameraucana hens (Gallus gallus domesticus).

PROCEDURES
RBCs were stored in 20% (wt/vol) glycerol, 10% (wt/vol) DMSO freezing medium, or various concentrations of HES solution (7.5%, 11.5%, and 20% [wt/vol]) and frozen for 2 months in liquid nitrogen. Cells were then thawed and evaluated by use of cell recovery and saline stability tests, cell staining (7-aminoactinomycin D and annexin V) and flow cytometry, and scanning electron microscopy.

RESULTS
Percentage of RBCs recovered was highest for 20% glycerol solution (mean ± SE, 99.71 ± 0.04%) and did not differ significantly from the value for 7.5% HES solution (99.57 ± 0.04%). Mean saline stability of RBCs was highest for 10% DMSO (96.11 ± 0.25%) and did not differ significantly from the value for 20% HES solution (95.74 ± 0.25%). Percentages of cells with 7-aminoactinomycin D staining but without annexin V staining (indicating necrosis or late apoptosis) were lowest for 10% DMSO freezing medium (3%) and 20% glycerol solution (1%) and highest for all HES concentrations (60% to 80%). Scanning electron microscopy revealed severe membrane changes in RBCs cryopreserved in 20% HES solution, compared with membrane appearance in freshly harvested RBCs and RBCs cryopreserved in 10% DMSO freezing medium.

CONCLUSIONS AND CLINICAL RELEVANCE
Cryopreservation of avian RBCs with HES solution, regardless of HES concentration, resulted in greater degrees of apoptosis and cell death than did cryopreservation with other media. Transfusion with RBCs cryopreserved in HES solution may result in posttransfusion hemolysis in birds. (Am J Vet Res 2015;76:487–493)

Abbreviations

7-AAD 7-aminoactinomycin D
DMSO Dimethyl sulfoxide
HES Hydroxyethyl starch
OD Optical density

dextrose solution with adenine). In all preparations, potassium concentrations increased substantially (> 30 mEq/L) after storage for 7 days, and stored blood was deemed unsafe for transfusion. To the authors’ knowledge, no other investigations of the effects of storage media on avian RBCs have been reported.

Outcomes of RBC transfusion have been investigated in pet bird species. In cockatiels (Nymphicus hollandicus), transfusions with RBCs harvested from the blood of autologous and homologous donors resulted in longer RBC survival times (10.5 to 16.8 days) than did transfusions with blood from heterologous donors (0.1 to 2.6 days). Results were different in conures of the genus Aratinga, in which RBC transfusions from autologous and homologous donors resulted in a half-life for RBCs of 9.9 and 8.5 days, respectively, and those from heterologous donors had a half-life of 4.5 days. Differences between RBC half-lives for transfusions with blood from autologous and homologous donors were not significant in that study. Consequently, homologous donors have been recommended...
as a source for blood transfusions in pet bird species,\textsuperscript{3} but heterologous donors may still be an option when homologous donors are not available.

Cryopreservation of RBCs allows for long-term (months to years) storage,\textsuperscript{2} providing a source of RBCs when other blood products are unavailable. Cryopreservation of cells typically involves use of glycerol or DMSO as a cryoprotectant; however, these media are toxic and must be removed from RBC samples prior to transfusion. The process for removal of cryoprotectants is time consuming, and addition and removal of glycerol or DMSO can cause osmotic stress to cells.\textsuperscript{4} Although DMSO has been used to successfully cryopreserve RBCs from hens for use as reagents in virus titration studies, glycerol is considered unfit for use in cryopreservation because the cell recovery rate after thawing and washing is poor.\textsuperscript{5} Effects of extracellular additives that do not penetrate RBCs have also been investigated. Hydroxyethyl starch solution has been used for cryopreservation of human and canine RBCs,\textsuperscript{6,7} with the advantage that HES is nontoxic and RBCs preserved with it can be administered to patients without the need for cell washing prior to transfusion.

A study\textsuperscript{8} revealed that human RBCs stored in HES solution may need to be washed prior to transfusion to reduce the amount of free hemoglobin in the transfused product. However, a later study\textsuperscript{9} revealed that autologous reinfusion of human RBCs that had been cryopreserved in HES solution led to no adverse reactions, regardless of whether cells were washed after thawing. In another study,\textsuperscript{10} some humans developed mild leukocytosis and a moderate increase in serum bilirubin concentration after transfusion with RBCs stored in HES solution, but these effects were no longer evident 20 hours after transfusion.

Elimination of HES from the bloodstream reportedly follows first-order kinetics in dogs and humans.\textsuperscript{11} Intravenous infusion of HES solution has been successfully used in the resuscitation of birds with shock caused by severe blood loss.\textsuperscript{12} Interestingly, oxyglobin was more effective for resuscitation than was HES or crystalloid solutions in that study.\textsuperscript{12} It follows that RBCs cryopreserved with HES solution may also be more effective than HES or crystalloid solution alone for resuscitation of birds with severe blood loss.

Concentration of an HES solution can affect degree of RBC recovery after cryopreservation. In dogs, a 12.5% (wt/vol) concentration was identified as yielding a greater proportion of viable RBCs after cryopreservation than did other concentrations of HES solution.\textsuperscript{13} In humans, transfusion with washed or unwashed RBCs cryopreserved in 11.5% HES solution reportedly yields results similar to those of transfusion with RBCs stored in solution containing phosphate, adenine, glucose, guanosine, saline solution, and mannitol.\textsuperscript{8} Another study\textsuperscript{13} revealed a cell recovery rate of 97% and saline stability > 80% when human RBCs were cryopreserved in 14% HES solution. An in vivo study\textsuperscript{14} in dogs revealed no difference in 24-hour and long-term survival rates between radiolabeled, autologous RBCs cryopreserved with HES solution and freshly donated autologous RBCs, with > 95% of HES-cryopreserved cells surviving 24 hours after transfusion.

Integrity of RBCs can be assessed on the basis of several criteria, including degree of stability in isotonic saline (0.9% NaCl) solution, plasma stability, percentage of cells recovered, mean corpuscular volume, extracellular electrolyte concentrations, hemoglobin content, intracellular and extracellular pH, intracellular lactate concentration, density gradients, osmotic fragility, and cell morphology as assessed via scanning electron microscopy.\textsuperscript{7,15} In general, percentages of RBCs recovered after freezing should be > 80% after any washing or cryoprotectant-removal process, and degree of RBC stability in saline solution during a 30-minute period should be > 85%.\textsuperscript{16}

The reagents 7-AAD and annexin V are used to identify cellular death and apoptosis, respectively,\textsuperscript{17,18} and are therefore also used to assess cell viability following cryopreservation.\textsuperscript{19} Markers of apoptosis are used to detect translocation of phosphatidylserine to the outer plasma membrane, which occurs in the early stages of apoptosis and necrosis. Apoptosis and erythrocyte senescence (eryptosis) result in exposure of phosphatidylserine in the outer membrane of cells.\textsuperscript{20} Although 7-AAD and annexin V have not specifically been used to measure viability of avian erythrocytes, they have been used together to characterize the presence and stage of apoptosis in suspensions of avian lymphocytes.\textsuperscript{21} The objective of the study reported here was to compare the effectiveness of glycerol, 10% DMSO, and HES solutions for the cryopreservation of avian RBCs. Our hypothesis was that effectiveness would differ significantly among media.

**Materials and Methods**

**Animals**

Twelve healthy 3-year-old Ameraucana hens (\textit{Gallus gallus domesticus}) were used for blood sample collection in the study. Mean ± SD body weight was 1.91 ± 0.18 kg (median, 1.90 kg; range, 1.64 to 2.27 kg). All chickens were from a farm colony at Cummings School of Veterinary Medicine at Tufts University. All were assessed as healthy on the basis of results of complete physical examinations and CBCs performed at the beginning of the study. Chickens were housed as a group in a large house (5 X 5 X 15 m) equipped with perches and nest boxes. All were fed a ration formulated for layer hens and had access to water ad libitum. Ground oyster-shell calcium was available at all times in a feeder. A cycle of 12 hours of light and 12 hours of darkness was maintained, and the hen house was mechanically ventilated to control and maintain air quality and ambient temperature to support optimal poultry health. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Cummings School of Veterinary Medicine.
Preparation of RBCs

Ten milliliters of blood (or no more than 1% of body weight) was collected from each chicken by use of a 12-mL syringe containing 1.0 mL of acid-citrate-dextrose solution as anticoagulant and a 22-gauge, 1-inch butterfly needle, mixed thoroughly, and transferred into 15-mL polypropylene collection tubes. Each blood sample was centrifuged for 5 minutes at 1,500 × g, then washed 3 times with isotonic saline solution. After the final wash, RBCs were concentrated by centrifugation to achieve an Hct of approximately 68%. Harvested cells were then dispensed into 1-mL aliquots such that RBCs from each bird were available for each of 5 cryopreservation conditions.

To assess the effects of 20% (wt/vol) glycerol solution on RBCs, a procedure similar to the freezing and glycerol removal process described elsewhere was used, except that 250 μL of 40% glycerol solution was added in a dropwise manner to 250 μL of RBCs during a 10-minute period; and samples were then transferred into 1.2-mL cryopreservation tubes. Tubes were held at –80°C for 2 hours, then submerged in liquid nitrogen and stored for 2 months. Tubes of frozen RBCs were thawed by manual agitation in a water bath maintained at 37°C. A 250-μL sample of thawed cells was removed for assessment of cell recovery. To assess the effects of 10% (wt/vol) DMSO freezing medium on RBCs, 250-μL RBC samples from each chicken were mixed with 750 μL of medium that contained 10% DMSOb, 60% fetal bovine serum, and 30% enriched modified Eagle medium. Freezing medium was added in a dropwise manner during a 10-minute period, and samples were transferred into 1.2-mL cryopreservation tubes. Tubes were held, frozen, and thawed as described for 20% glycerol solution. A 0.5-μL sample of the thawed cells was removed for later assessment of cell recovery.

To assess the effects of various concentrations of HES solution on RBCs, washed RBC samples from each chicken were manually mixed in tubes containing various volumes of sterile filtered 23% HES solution during a 10-minute period to achieve final HES concentrations (wt/vol) of 7.5% (163 μL of 23% HES solution mixed with 337 μL of RBCs), 11.5% (250 μL of 23% HES solution mixed with 250 μL of RBCs), and 20% (435 μL of 23% HES solution mixed with 65 μL of RBCs). The mixtures were transferred to cryopreservation tubes and then frozen, stored, and thawed as described for 20% glycerol solution. A 250-μL sample of the thawed cells from each HES preparation was removed for later assessment of cell recovery. The remaining cell suspensions were washed with the same volume of isotonic saline solution.

RBC recovery

Percentage of RBCs recovered after cryopreservation was measured by comparing the hemoglobin concentration in sample supernatants with that of whole blood. First, 250 μL of each thawed RBC sample was centrifuged individually at 12,000 × g for 35 minutes. The supernatant was transferred to another test tube, and 125 μL of supernatant was diluted with 4,875 μL of Drabkin reagent. After contents were mixed adequately, the OD of the mixture was measured at 540 nm with an absorbance reader; 25 μL of RBCs from the original thawed tube was mixed with 4,975 μL of Drabkin reagent, and the OD was measured again. The PCV was determined by use of the microhematocrit method. From these results, percentage of lysed cells was calculated as follows:

\[
\text{Percentage of lysed cells} = \left( \frac{\text{OD}_{\text{supernatant}} \times 2}{\text{OD}_{\text{total hemoglobin}}} \right) \times (100\% - \text{PCV}) \times 0.1
\]

where ODtotal hemoglobin represents the OD of RBCs in the Drabkin reagent.

The percentage of lysed cells was then subtracted from 100% to obtain the percentage of cells recovered.

Degree of stability in saline solution

A 250-μL portion of each thawed RBC sample was centrifuged for 5 minutes at 1,500 × g, then washed 3 times in isotonic saline solution. The washed RBCs were diluted in 12.5 mL of isotonic saline solution and mixed gently. Five milliliters of each mixture was transferred into 2 test tubes and set aside at room temperature (approx 20°C) for 30 minutes and 2 hours, respectively. For measurement of total hemoglobin concentration, another 2 mL of mixture was added to a tube containing 10 mL of distilled water and mixed gently. Tubes were set aside for 30 minutes, and tubes for total hemoglobin measurement were centrifuged at 1,902 × g for 10 minutes. The supernatant was removed and transferred into a flat-well ELISA plate. Optical density was measured in 30-minute and total hemoglobin supernatants by use of a plate reader at a wavelength of 540 nm. The tubes that had been set aside for 2 hours were centrifuged at 1,902 × g for 10 minutes, supernatant was removed and transferred to flat-well plates, and supernatant OD was once

<table>
<thead>
<tr>
<th>Solution*</th>
<th>Recovery (%)</th>
<th>Absolute difference in means</th>
<th>P value†</th>
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</thead>
<tbody>
<tr>
<td>20% Glycerol</td>
<td>99.71 ± 0.04</td>
<td>Referent</td>
<td>—</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>99.37 ± 0.04</td>
<td>0.34</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>7.5% HES</td>
<td>99.57 ± 0.04</td>
<td>0.14</td>
<td>0.20</td>
</tr>
<tr>
<td>11.5% HES</td>
<td>99.52 ± 0.04</td>
<td>0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>20% HES</td>
<td>99.43 ± 0.04</td>
<td>0.28</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
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*Concentration represents wt/vol. †Values of P < 0.05 were considered significant. — = Not applicable.

The RBCs had been harvested from blood samples obtained from each chicken such that RBC samples from each chicken were represented in each test condition. Mixed linear modeling was performed to account for the lack of independence associated with multiple tests performed on the same samples.
again measured at a wavelength 540 nm. Degree of saline stability was calculated as follows:

\[
\text{Percentage saline stability} = \left(100 - \frac{\text{OD}_{\text{supernatant}}}{\text{OD}_{\text{total hemoglobin}}} \times 0.2\right) \times 100\%
\]

**Apoptosis assay and flow cytometry**

Thawed, washed RBC samples for each test condition were stained to identify apoptosis. Briefly, each sample was centrifuged for 5 minutes at 1,500 X g, then washed twice with PBS solution. Washed RBCs were suspended in 1 mL of 1X binding buffer and distributed into tubes to provide sufficient 100µL aliquots for testing. Sets of aliquots representing each chicken were left unstained or stained with annexin V and 7-AAD, or both. For annexin-V staining, 5 µL of annexin V was added to appropriate tubes, which were then incubated at room temperature for 10 minutes in the dark. Afterward, tubes were centrifuged at 475 X g for 3 minutes, and contents were washed 2 times with PBS solution. Washed cells were resuspended with 100 µL of 1X binding buffer. Staining with 7-AAD was performed next by addition of 5 µL of 7-AAD to appropriate tubes. Samples were analyzed immediately thereafter by use of flow cytometry. Forward scatter to side scatter and forward scatter to FL1 and FL2 scatter were assessed. Percentage of cells with fluorescent staining for each preparation was calculated. Samples with negative results for 7-AAD and annexin V staining were classified as live cells. Samples with negative results for 7-AAD staining but positive results for annexin V staining were classified as being in early apoptosis. Samples with positive results for both stains were classified as being in mid apoptosis, and samples with positive results for 7-AAD staining and negative results for annexin V staining were classified as being necrotic or in late apoptosis.

**Scanning electron microscopy**

Freshly harvested RBCs, thawed and washed RBCs that had been stored in 10% DMSO freezing medium, and thawed and washed RBCs that had been stored in 20% HES solution and 20% HES solution were fixed by incubation with 2.0% glutaraldehyde in 0.1M PBS solution (pH 7.2) for 1 hour, washed 3 times with the same type of PBS solution, and dehydrated by exposure to increasing concentrations of ethanol (10% to 100%). Specimens were dried by use of a critical point dryer, coated with 4 nm of gold and palladium (80:20) in a high-resolution sputter coater, and evaluated with the aid of a scanning electron microscope at an accelerating voltage of 10 kV. Cell morphology was assessed by examination of scanning electron photomicrographs (1,000X to 10,000X magnification) of arbitrarily selected areas in each sample. Severity of cellular membrane changes, including deformed cells and hemispheric surface protrusions, was evaluated and compared among media by examination of the same photomicrographs.

**Statistical analysis**

Results are reported as mean ± SE. Generalized linear mixed models were created with the aid of statistics.
tical software to determine least squares means and differences in means for cell condition, percentage of cells recovered, and degree of saline stability among the 5 preservation media, with 20% glycerol solution as the referent treatment. This statistical approach accounted for the lack of independence among multiple RBC samples from the same chicken. Values of \( P < 0.05 \) were considered significant.

Results

Percentage of RBCs recovered varied with the cryoprotectant used but was > 99% for all tested media (Table 1). Saline stability of RBCs during a 30-minute period was > 94% for all media (Table 2). Significantly lower percentages of cells were recovered after cryopreservation in 10% DMSO freezing medium and 11.5% and 20% HES solutions, compared with percentages of cells recovered after storage in 20% glycerol solution. Significantly higher degrees of saline stability were identified for 10% DMSO freezing medium and 20% HES solution, compared with values for 20% glycerol solution.

Cryopreservation of RBCs in all concentrations of HES solution (7.5%, 11.5%, and 20% HES) resulted in a significantly higher mean percentage of late-apoptotic and necrotic cells (ie, with staining for 7-AAD but not for annexin V; 60% to 80% of cells), compared with cryopreservation in 20% glycerol solution (1%) or 10% DMSO freezing medium (3%; Figure 1). No significant difference in mean percentage of 7-AAD–positive cells was identified between 10% DMSO freezing medium and 20% glycerol solution. Cryopreservation in 20% HES solution resulted in a significantly (\( P < 0.001 \)) higher mean percentage of cells with staining for both 7-AAD and annexin V (indicating mid apoptosis) than did cryopreservation in 20% glycerol solution (24% vs 2%). No significant difference in mean percentage of cells with staining for 7-AAD and annexin V was evident between the other media (10% DMSO freezing medium and 7.5% and 11.5% HES solutions) and 20% glycerol solution. Significantly (\( P < 0.001 \)) fewer live cells (ie, negative for 7-AAD and annexin V staining) and early apoptotic cells (7-AAD-negative and annexin V-positive) cells were detected in RBC samples cryopreserved in various concentrations of HES solution, compared with results for samples cryopreserved in 20% glycerol solution. No other significant differences were detected.

Scanning electron microscopy revealed severe deformation and hemispheric protrusions in RBCs cryopreserved in 20% HES solution (Figure 2). Fresh RBCs and thawed and washed RBCs stored in 10% DMSO freezing medium were similar in morphological appearance, with minimal to no observable cell deformation.

Figure 2—Representative scanning electron micrographs of RBCs obtained from healthy 3-year-old Ameraucana hens. A—Cells freshly harvested from a whole blood sample. B—Cells that had been stored in 10% DMSO freezing medium, frozen in liquid nitrogen for 2 months, and thawed. Notice the morphological similarity to the cells in panel A. C—Cells that had been stored in 20% HES solution, frozen in liquid nitrogen for 2 months, and thawed. Notice the deformed cell shape with hemispheric protrusions. Sputter coated with gold and palladium; bar = 50 \( \mu \)m for panel A and 10 \( \mu \)m for panels B and C.
Discussion

Standards of the American Association of Blood Banks indicate that the percentage of RBCs recovered after cryopreservation and thawing should exceed 80% and saline stability during a 30-minute period should exceed 85% after any washing or cryoprotectant-removal process. Results obtained in the present study for avian RBCs stored in 20% glycerol solution, 10% DMSO freezing medium, or various concentrations of HES solution (7.5%, 11.5%, and 20%) and frozen for 2 months in liquid nitrogen exceeded these standards. For RBCs cryopreserved in 20% HES solution, percentages of cells recovered and values for saline stability were comparable with values for RBCs cryopreserved in 10% DMSO freezing medium.

On the other hand, RBCs stored in HES solution, regardless of concentration, had high degrees of 7-AAD and annexin V staining, suggesting that a high proportion (> 60%) of the HES-cryopreserved cells had died or were in late apoptosis. Because 7-AAD binds to double-stranded DNA, it is generally used to assess viability of nucleated cells, which is the reason the 7-AAD staining method was chosen to evaluate viability in nucleated avian RBCs. However, erythrocyte-derived microvesicles might have transferred phosphatidylserine to the surface of nucleated cells, falsely marking them as apoptotic. To determine the fate of HES-cryopreserved RBCs after infusion in birds, radiolabeling techniques would be needed to measure the percentage of radiolabeled RBCs recovered 24 hours after IV administration. Nevertheless, results of the present study suggested that avian RBCs stored in HES solution would have a short circulation period following transfusion.

The aforementioned findings were supported by results of scanning electron microscopy, which revealed considerable membrane changes in RBCs cryopreserved in 20% HES solution, compared with membrane appearance in fresh RBCs or RBCs cryopreserved in 10% DMSO freezing medium. These membrane changes, specifically the hemispheric protrusions, were similar to changes identified in canine RBCs cryopreserved in HES solution in another study. However, it should be considered that morphological changes in human RBCs cryopreserved in HES solution are rapidly restored to typical RBC morphology when placed in fresh autologous plasma after thawing.

Findings in another study suggested that glycerol solution is unfit for use in the cryopreservation of avian RBCs, with 77.4% and 85.6% hemolysis after cryopreservation of avian RBCs in 20% and 10% glycerol solutions, respectively. After RBCs cryopreserved in glycerol solution were washed, the final percentage of cells recovered was 0% in that study. Although additional investigation would be required to determine the reason for the low percentage of cells recovered, it is possible that the glycerol addition rate, freezing rate, or cryoprotectant-removal process was responsible for the low cell survival rate after cryopreservation.

In the present study, 10% DMSO freezing medium and 20% glycerol solution were effective media for cryopreservation of avian RBCs. Whereas results for 20% HES solution were similar to results for 10% DMSO freezing medium with respect to cell recovery and saline stability values, the low cell viability identified for HES solution indicated it was the less effective cryoprotectant. In vivo studies are needed to evaluate RBC viability after transfusion of HES-cryopreserved RBCs in birds.

Acknowledgments

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Footnotes

1. Fisherbrand Easy Reader polypropylene centrifuge tubes (flat bottom, sterile, in rack), 15 mL, Fisher Scientific, Pittsburgh, Pa.
2. Sigma Chemical Co, St Louis, Mo.
3. HyClone defined fetal bovine serum (SH30070.03), 500 mL, GE Healthcare Life Sciences, Logan, Utah.
4. MEM alpha Eagle medium, BioWhittaker Cell Culture Media, Lonza, Allendale, NJ.
5. KryoHAPS (molecular weight, 200,000 Da; degree of substitution, 0.5; concentration, 25% wt/wt), Fresenius, Bad Homburg, Germany.
6. ELix808 Absorbance Reader, BioTek Winooski, Vt.
7. BD Biosciences, San Jose, Calif.
8. Biotium Inc, Hayward, Calif.
9. 10X Annexin V binding buffer, BD Biosciences, San Jose, Calif.
10. FEI Quanta 200 FESEM MK II, FEI, Hillsboro, Ore.
12. The authors thank Airiel Davis for technical assistance.

References


