Original Contribution

NEW CRYOPROTECTIVE MEDIA FOR CRYOPRESERVATION OF MAMMAL ERYTHROCYTES

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ABSTRACT

PURPOSE: To compare the effectiveness of a newly designed multi-component cryoprotective medium (15% PEO-1500, 10% DMSO, 5% 1,2-propanediol, 5% sucrose) with three known single- and two-component ones containing DMSO (20 %), PEO-1500 (30 %), and glycerol-mannitol (30 % and 4 %, respectively) in the cryopreservation of human, equine, bovine and rabbit erythrocytes. **METHODS:** Cryopreservation was achieved plunging the cryotubes with erythrocyte suspensions into liquid nitrogen (-196°C) at an average cooling rate of 200 deg/min. During the three cryopreservation stages; equilibration, freeze-thaw and final removal of cryoprotectants, the survival of erythrocytes was estimated by the level of hemolysis, measured spectrophotometrically. **RESULTS:** With the erythrocytes of all investigated mammals the designed medium produced a higher level of survival at all cryopreservation stages. **CONCLUSION:** The tested multi-component cryopreservation medium, combining both penetrating and non-penetrating cryoprotectants, diminished their concentration and accompanying toxic effects and achieved a higher survival of human and mammal erythrocytes at all stages of their cryopreservation.

Key words: human, bovine, equine and rabbit red blood cells, penetrating and non-penetrating cryoprotectants, survival, hemolysis

INTRODUCTION

Recent demand in the resources of animal blood components is stipulated by the needs of veterinary medicine (1, 2). Long-term storage of biological objects is carried out in low-temperature banks using various cryopreservation techniques. The conventional methods for cryopreservation of erythrocytes (red blood cells) and other components of human blood have been extensively elaborated. Concerning the cryopreservation of erythrocytes of domestic mammals however, the development of new techniques is still required. For example, the standard cryoprotectants for human erythrocytes, based on glycerol or 1,2-propanediol (1,2-PD) (3,4), are found to be ineffective for erythrocytes of many animals (5, 6). Compared to glycerol and

1,2-PD, dimethyl sulfoxide (DMSO) provides better results in the cryopreservation of equine, bovine, feline and canine erythrocytes, however, the level of hemolysis after all stages of cryopreservation is still quite high (5, 6). This could be due to the accompanying toxic effects of DMSO (7). This necessitates the improvement of existing methods of cryopreservation of mammal red blood cells.

With this in mind, we have developed a multi-component cryopreservation medium containing 15% PEO-1500, 10% DMSO, 5% 1,2-PD, 5% sucrose (8). The aim of the presented study was, compared to several broadly used single- and two-component cryopreservation media, to test the effectiveness of above mentioned multi-component medium in the cryopreservation of human, bovine, equine and rabbit erythrocytes.

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MATERIALS AND METHODS

All studies were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine and have been performed in accordance to the ethical standards of 'Declaration of Helsinki, 1964', as well as 'European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes' (Strasburg, 1986).

The erythrocyte suspensions were prepared with erythrocytes isolated from human, bovine, equine and rabbit blood. Human blood (male, A (II) +) was obtained from healthy adult volunteers with their informed consent at the Kharkiv Regional Center of Blood Service. Animal's blood was obtained from healthy mature males at the experimental farm of the Kharkiv State Zoo-Veterinary Academy using a preservative "Glucicir" solution in a ratio of 1:1 (v/v). Erythrocytes were isolated from the plasma and white blood cells by centrifugation at 1500 x g for 5 min. The next two washings were carried out in an excess volume of phosphate- buffered saline (PBS, 0.01 M Na-phosphate in 150 mM NaCl), pH 7.4.

Cryopreservation media were prepared to dissolve the indicated ingredients in the above mentioned PBS. The effectiveness of tested multi-component solution was compared with that of the following media which have been broadly used for cryopreservation of human and animal erythrocytes: A variant was 30% glycerol and 4% mannitol, B variant was 20% DMSO, and C variant was 30% PEO-1500.

Each cryopreservation medium was added to a paste of washed erythrocytes at a ratio of 1:1 (v/v), stirred in placed in cryotubes. The obtained erythrocyte suspensions were equilibrated incubating cryotubes at 21 °C for 15 minutes. The suspensions were frozen plunging the cryotubes into liquid nitrogen (-196°C) at an average cooling rate of about 200 °C/min and kept frozen for 24 hours. Thawing of frozen suspensions was carried out in a water bath at 40-42 °C until the appearance of liquid phase. After thawing, the erythrocytes were thrice washed with the cryoprotectants gradually reducing tonicity. The first washing solution was 0.6 M NaCl, 10 mM Na-phosphate buffer (pH 7.4), while the second and third ones were 0.150 M NaCl, 10 mM Na-phosphate buffer (pH 7.4) (9).

The level of erythrocytes preservation (survival) was evaluated at three points (stages); after the equilibration of erythrocytes with cryoprotective medium, immediately after thawing and after final washing. Survival was estimated by the percentage

of cells that did not hemolize. The hemoglobin content of the supernatant was determined by the optical density at 540 nm by a Pye Unicam SP 8000 spectrophotometer (UK).

RESULTS AND DISCUSSION

The survival of humanity, as well as mammal, erythrocytes under the cryoprotection of the four cryoprotectant media is shown in **Figures 1-4**. From the first to the third stage of cryopreservation it gradually decreased with different rates depending on the erythrocyte species and the composition of the cryoprotective medium. At the first stage of cryopreservation the survival was almost complete indicating no species differences for all media. This indicates rapid and perfect equilibration of tested erythrocytes towards various media within the chosen time for equilibration (15 min, 21 °C). The cryoconservation of human red blood cells is usually conducted in an A medium of 30% glycerol and 4% mannitol. In our experiment, the application of this cryopreservation medium also yielded good survival of human erythrocytes at the final stage (**Figure 1**). However, the cryopreservation of tested mammal erythrocytes by this medium was too low (**Figures 2-4**) putting forward the need to use another cryoprotective media. In this respect, we measured the cryoprotection of human and mammal erythrocytes by three known media compared to that of our multi-component cryoprotective medium (15% PEO-1500, 10% DMSO, 5% 1.2-PD, 5% sucrose).

The glycerol containing medium ensured almost 100 % survival of equine erythrocytes at the stage of equilibration due to the high permeability of these cells to glycerol (12). Nevertheless, survival at the final stage was very low (**Figure 2**). This low cryopreservation efficiency of the glycerol-containing medium could result from the high concentration of glycerol and its low-temperature toxicity to these cells.

Compared to glycerol much better but still insufficient results with equine erythrocytes were obtained under the cryoprotection of DMSO-containing medium, confirming the data of others (10). The protection of equine erythrocytes by PEO-1500 yielded a relatively good survival level at the freezing-thawing stage, while at the final stage the survival was poor. This high-molecular non-penetrating cryoprotectant causes dehydration of cells at the equilibration stage, thereby diminishing the intracellular crystallization at subzero temperatures. On the other hand, due to its high extracellular concentration this cryoprotectant immobilizes the molecules of a huge amount of extracellular water, resulting in an inhibition of extracellular crystallization and a decrease of

membrane damage by ice crystals (11). Despite these beneficial impacts of PEO-1500 against freezing, its final removal from the erythrocyte suspension resulted in almost full hemolysis (Figure 2). By contrast, compared to other cryopreservation media the use of our multi-component cryopreservation medium achieved a significantly high survival level of equine erythrocytes at all stages of cryopreservation (Figure 2).

The cryopreservation of bovine erythrocytes under the protection of all media yielded almost the same survival data as these obtained with the equine erythrocytes (compare Figure 3 and Figure 2). With these erythrocytes the best survival (96.8 % at the second and 76.6 % at the final stage) was again obtained using the multi-component cryopreservation medium (Figure 3).

Figure 1. Survival of human erythrocytes at different stages of cryopreservation. * - statistically significant difference relative to 30% glycerol ($p < 0.05$), $n = 5$; # - statistically significant difference relative to 20% DMSO ($p < 0.05$), $n = 5$; ^ - statistically significant difference relative to 30% PEO-1500 ($p < 0.05$), $n = 5$.

Figure 2. Survival of equine erythrocytes at different stages of cryopreservation. * - statistically significant difference relative to 30% glycerol ($p < 0.05$), $n = 5$; # - statistically significant difference relative to 20% DMSO ($p < 0.05$), $n = 5$; ^ - statistically significant difference relative to 30% PEO-1500 ($p < 0.05$), $n = 5$.

Figure 3. Survival of bovine erythrocytes at different stages of cryopreservation. * - statistically significant difference relative to 30% glycerol ($p < 0.05$), $n = 5$; # - statistically significant difference relative to 20% DMSO ($p < 0.05$), $n = 5$; ^ - statistically significant difference relative to 30% PEO-1500 ($p < 0.05$), $n = 5$.

The effectiveness of the glycerol-containing medium on rabbit erythrocyte cryopreservation was close to that of DMSO-containing medium (**Figure 4**). This may be due to the high permeability of rabbit erythrocytes for glycerol (12). Unfortunately, rabbit erythrocytes rapidly coagulated on contact with the single-component medium of PEO-1500 (data not shown). This impeded the collection of correct data about the cryopreservation of rabbit erythrocytes by this

medium (**Figure 4**). This could be due to the very high concentration of PEO-1500 in the former medium as the presence of PEO-1500 at reduced concentration in our multi-component medium did not cause coagulation of rabbit erythrocytes. **Figure 4** demonstrates successful preservation and high survival of rabbit erythrocytes under the cryoprotection of the latter medium.

Figure 4. Survival of rabbit erythrocytes at different stages of cryopreservation. * - statistically significant difference relative to 30% glycerol ($p < 0.05$), $n = 5$; # - statistically significant difference relative to 20% DMSO ($p < 0.05$), $n = 5$; ^ - statistically significant difference relative to 30% PEO-1500 ($p < 0.05$), $n = 5$.

Thus, in contrast to the single- and two-component media, the use of a multi-component medium combining cryoprotectants with both exo- and endocellular action can

achieve sufficiently high cryopreservation of mammal erythrocytes. This could be explained by diminishing the concentrations and, thereby, the adverse effects of all components as well as by the complex synergistic actions of these components towards the better cryopreservation of mammal erythrocytes.

CONCLUSIONS

The presented results demonstrate that, compared to the single- and two-component media, the use of a combined cryoprotective medium containing exo- and endocellular cryoprotectants can significantly improve the cryopreservation of human, bovine, horse and rabbit erythrocytes.

ABBREVIATION

1.2-PD – 1.2-propanediol, DMSO – dimethyl sulfoxide, PEO-1500 – polyethylene oxide of molecular weight 1500, PBS – phosphate-buffered saline.

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