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CRYOPRESERVATION OF SHEEP RED BLOOD CELLS

1. EXPERIMENTS WITH POLYVINYLPYRROLIDONE AND OTHER PROTECTIVE AGENTS

By

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MYHRVOLD, VESLEMØY: *Cryopreservation of sheep red blood cells. 1. Experiments with polyvinylpyrrolidone and other protective agents.* Acta vet. scand. 1979, *20*, 525—530. — The protection of sheep erythrocytes at freezing temperatures was investigated using glycerol, dimethylsulfoxide (DMSO), glucose and four different types of polyvinylpyrrolidone (PVP) as cryoprotective agents. Depending on type (molecular weight) and concentration good protection was obtained with PVP, whereas glycerol, DMSO and glucose were unsatisfactory. Recovery of cells after thawing was most successful when the cells had been frozen at a concentration of $1—2 \times 10^9$ cells/ml. No cells tolerated freezing at —20°C. Best results were obtained when the cells were frozen directly in liquid nitrogen (—196°C).

sheep red cells; preservation; low temperature.

Human erythrocytes can be successfully stored in liquid nitrogen in the presence of cryoprotective agents such as glycerol, dimethylsulfoxide, polyvinylpyrrolidone and hydroxyethyl starch. Except for early studies on the ability of sheep erythrocytes to survive freezing (*Woodcock et al.* 1941) and the cryopreservation of sheep erythrocyte intermediates for complement titrations (*Spurlock et al.* 1974) few data are available concerning storage of sheep blood at low temperature.

Sheep erythrocytes are a useful reagent in titrations of several serum constituents, and a practical procedure for long time storage would greatly facilitate the laboratory use of sheep

blood. The present paper is concerned with storage of sheep erythrocytes in the presence of polyvinylpyrrolidone (PVP) and other protective agents.

MATERIALS AND METHODS

Sheep red blood cells were collected aseptically into anticoagulant solutions and stored for one week at 4°C before freezing. The following anticoagulants were used: Acid-citrate-dextrose (ACD) containing (g/l): Glucose 14.7, trisodium citrate (dihydrate) 13.2, citric acid 4.8. One volume of blood was drawn into four volumes of ACD solution. Citrate-phosphate-dextrose (CPD) containing (g/l): Trisodium citrate (dihydrate) 29.2, sodium dihydrogen phosphate (monohydrate) 0.16, glucose 2.0. Fourteen ml of CPD was mixed with 100 ml blood. Alsevers solution (g/l): Glucose 20.5, trisodium citrate (dihydrate) 8.0, sodium chloride 4.2. Equal volumes of blood and Alsevers solution were used.

For the experiments using ACD solution one volume of cryoprotective agent was added to one volume of the stored blood, the mixture distributed (1.5 ml) to 2 ml plastic serum test tubes No. 1076 (Nunc) and plunged direct into liquid nitrogen in an LR-35 liquid nitrogen container (Linde Company). Some experiments were also done by freezing at —20° and —90°C. Dimethylsulfoxide (DMSO Sigma) was used in a concentration of 20 % in 0.15 M-NaCl (i.e. final conc. 10 %). Several PVP polymers (Fluka AG) were used. These are characterized by their relative viscosities (K) and the average molecular weights. The PVP polymers K 15 (MW 10 000), K 25 (MW 24 000), K 30 (MW 40 000) and K 60 (MW 160 000) were used in concentrations of 15, 20 and 30 % in 0.15 M-NaCl (i.e. final concentrations 7.5, 10 and 15 %, respectively). Other cryoprotectants were also tested as indicated in Table 1.

Thawing of cells kept at —20° and —90°C was done in a 43°C water bath the tubes being shaken by hand. To avoid breakage, tubes kept at —196°C were kept for 15 s at room temperature before thawing at 43°C.

After thawing the tubes were centrifuged at 4°C for 10 min at 700 × g, the supernatant was removed and the cells were washed three to five times with one volume of 1.4 × isotonic GVBSM^{++}-sucrose of ionic strength 0.065 (veronal-buffered sa-

line containing 9.7 % sucrose, 0.1 % gelatin, 0.001 M-Mg^{++} and 0.00015 M-Ca^{++}).

The loss of cells by the procedure was calculated by the release of hemoglobin read at OD 541 nm. Hemoglobin was determined in the supernatant after thawing (loss by thawing) and in the combined washings (loss by washing). Cells recovered after washing were estimated after hemolysis with distilled water and centrifugation at 700 × g to remove stroma.

To study the effect of red cell concentration on cell recovery the cells in ACD buffer were washed two to five times with GVBSM^{++}-sucrose and diluted in the same buffer before adding cryoprotectant.

Table 1. Cryopreservation of sheep red blood cells at —196°.

Cryoprotectant	Conc. final	Loss thawing %	Loss washing %	Recovery %
Glycerol	10	32.4	67.6	0
Glycerol	20	22.3	77.7	0
		25.7	74,3	0
Glycerol sorbitol		46.1	53.9	0
		49.9	50.1	0
DMSO	10	10.3	89.7	0
Glucose	5.4	64.4	4.4	31.2
Glucose	8.1	63.1	6.2	30.7
PVP K*15 MW** 10 000	7.5	25.7	10.5	63.8
	10	14.9	12.7	72.4
	15	7.7	25.6	66.7
PVP K*25 MW** 24 000	7.5	20.0	17.1	62.9
	10	10.6	26.1	63.3
	15	3.8	54.7	41.5
PVP K*30 MW** 40 000	7.5	13.0	30.7	56.3
	10	7.9	31.3	61.8
	15	2.8	57.6	39.6
PVP K*60 MW** 160 000	7.5	50.9	29.6	19.5
	10	38.8	32.5	28.7
	15	30.8	45.3	23.9

Final conc. of sheep red cells by freezing: 4×10^9 cells/ml.
* K = Relative viscosities.
** Average molecular weights.

The results obtained by use of ACD buffer were compared with cells collected in CPD buffer and in Alsevers solution. For these experiments the cells were washed several times with GVBSM^{++}-sucrose, until OD 541 nm in the washings was less than 0.030, and then diluted to 4×10^9 cells/ml in the GVBSM^{++}-sucrose before adding cryoprotectant.

RESULTS AND DISCUSSION

The experiments showed (Table 1) that only PVP was a satisfactory cryoprotective agent. With some of the other cryoprotectants tested varying amounts of red cells survived thawing, but hemolyzed during the washing procedure. The molecular weight of PVP was of importance for the cryoprotective effect, and best results were obtained with PVP K 15 (average molecular weight 10 000).

Best cell recovery was seen when PVP K 15 was used in a final concentration of 10 %. With this PVP type the recovery was best when the cells were frozen in a concentration of $1—2 \times 10^9$ cells/ml (Table 2).

Table 2. Cryoprotection of sheep red blood cells with 10 % PVP K 15 at —196°. Effect of cell concentration on cell recovery.

Red cells $\times 10^9$/ml	Loss thawing %	Loss washing %	Recovery %
8	62.2	11.3	26.5
4	14.9	12.2	72.1
3	14.6	9.4	76.0
2	7.6	9.3	83.1
1	7.4	8.9	83.7
0.5	10.0	12.0	78.0

In the above-mentioned experiments the red cells had been stored in ACD buffer for seven days before freezing. Table 3 shows that the storage time before freezing could be extended to 48 days without significant decrease in cell recovery after freezing, and that storing in ACD, CPD or Alsevers solution before freezing gave identical results.

Table 3. Cryoprotection of sheep red blood cells with 10 % PVP K 15 at —196°. Effect of storage time at 4°C before freezing.

Buffer	Storage time days	Loss thawing %	Loss washing %	Recovery %
Alsevers	4	10.3	5.8	83.9
	21	12.0	5.4	82.6
	28	9.2	6.1	84.7
ACD*	4	7.7	9.2	83.1
	21	7.6	9.4	83.0
	42	10.2	7.8	82.0
	48	7.1	8.9	84.0
CPD**	4	8.2	6.4	85.4
	21	9.5	6.6	83.9
	42	7.2	8.8	84.0

* Acid-citrate-dextrose.
** Citrate-phosphate-dextrose.

Freezing at —20°C was not tolerated by any cells, and best cell recovery was seen when the cells were frozen at —196°C (Table 4).

Table 4. Cryoprotection of sheep red blood cells with 10 % PVP K 15. Effect of the temperature of freezing.

Temperature	Loss thawing %	Loss washing %	Recovery %
—20°	71.1	28.9	0
—90°	23.9	6.5	69.6
—196°	6.2	8.6	85.2

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SAMMENDRAG

Beskyttelse av røde saueblodlegemer ved frysing. 1. Virkning av polyvinylpyrrolidone og andre stoffer.

Bevaring av røde saueblodlegemer etter nedfrysing med glycerol, dimetylsulfoxide (DMSO), glucose og polyvinylpyrrolidone (PVP) som kryoprotektive midler ble undersøkt. Avhengig av type (molekylvekt) og konsentrasjon ble god cellegjenvinning oppnådd etter nedfrysing med PVP, mens glycerol, DMSO og glucose var uegnete. Flest celler ble bevart når cellene ble frosset i en konsentrasjon av 1—2 $\times$ 10^9 celler/ml. Ingen celler overlevet nedfrysing ved —20°C. Best resultat ble oppnådd ved nedfrysing direkte i flytende nitrogen (—196°C).

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