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CRYOPRESERVATION OF HEN RED BLOOD CELLS

By

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MYHRVOLD, VESLEMØY: *Cryopreservation of hen red blood cells.* Acta vet. scand. 1980, *21*, 498—503. — The cryoprotection of hen erythrocytes, used as reagent in virus titration, was investigated. The cryoprotective agents tested were neutralized polyvinylpyrrolidone (PVP), dimethylsulfoxide (DMSO) and glycerol. Good results were obtained with PVP, especially with PVP K15 (average molecular weight 10 000), and with DMSO, especially when used in a final concentration of 10 %, whereas glycerol was unfit for use in the concentrations tested. The red cell concentration, the suspending buffer before freezing and the washing procedure after thawing were of importance. The cells could be frozen and stored for at least three months without any significant effect on the virus titer.

hen red cells; freezing at —196 °C; storing at —90 °C; virus titrations.

Few data are available concerning freezing and storage of hen red blood cells at low temperature. A method of freezing erythrocytes from various species is, however, described by *Wesslén* (1978). His procedure was successful with respect to African green monkey, sheep, guinea pig, horse and ox erythrocytes, but red blood cells from pigeon, newborn chickens and rooster, all carrying nuclei, underwent hemolysis on thawing.

Previous experiments (*Myhrvold* 1979a,b,c, 1980) showed that polyvinylpyrrolidone (PVP) and some starch products were satisfactory cryoprotective agents for unsensitized and sensitized sheep erythrocytes. No protection was found when the intracellular agents glycerol and dimethylsulfoxide (DMSO) were used as cryoprotectants.

The present paper describes similar experiments with hen erythrocytes. Hen erythrocytes is a useful reagent for virus ti-

trations. The normal functional life of the cells is only about one week when stored at 4°C, and a practical procedure for long time storage would greatly facilitate the laboratory use of hen blood. This paper is concerned with freezing of hen erythrocytes in the presence of PVP and other protective agents. Fresh hen erythrocytes and hen erythrocytes recovered after freezing at —196°C and storage at —90°C were then used for viral titrations.

MATERIALS AND METHODS

Freezing procedure

One volume hen red cells (Plymouth Rock) was drawn aseptically from the wing vein of a hen into four volumes of acid-citrate-dextrose (ACD) containing (g/l): glucose 14.7 g, trisodium citrate (dihydrate) 13.2 g, citric acid 4.8 g. The cells were washed two to five times either with 0.15 M-NaCl or with GVBSM^{++}-sucrose prepared as follows: 486.1 g sucrose, 5.095 g Na-5,5 diethyl barbiturate and 18 g NaCl were dissolved in about 1.5 l of distilled water. pH was adjusted to 7.35 ± 0.05 with 1 M-HCl, the acid being added slowly with vigorous mixing of the solution. Five g gelatin dissolved in distilled water and 2.5 ml of a solution containing 1.0 M-$MgCl_2$ and 0.15 M-$CaCl_2$ were added. The volume was brought to 2 l with distilled water. This stock solution was kept at —20°C and diluted two and a half times with distilled water before use. The cells were then suspended in 0.15 M-NaCl or GVBSM^{++}-sucrose to concentrations of 20, 10 and 5 %, respectively. One volume of the cryoprotective agent (in 0.15 M-NaCl) was added to one volume of the suspended cells. Dimethylsulfoxide (DMSO Sigma) and glycerol (Merck) were used in concentrations of 20 and 40 % in 0.15 M-NaCl (i.e. final concentrations 10 and 20 %). 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) and K 30 (MW 40 000) were adjusted to pH 7.3 with 4 M-NaOH and used in concentrations of 15, 20 and 30 % in 0.15 M-NaCl (i.e. final concentrations 7.5, 10 and 15 %, respectively). The mixture was distributed (1.75 ml) to 2 ml plastic serum test tubes No. 1076 (Nunc) and plunged directly into liquid nitrogen in an LR-35 liquid nitrogen container (Linde Company). For thawing the tubes were kept for 15 s at room temperature to avoid breakage, and then in a 43°C water bath for about 2½ min the tubes being shaken by hand. With 0.15 M-NaCl as suspending solution the thawed cells were washed with this solution only. With GVBSM^{++}-sucrose as suspending buffer the thawed cells were washed with this buffer and most frequently also with 0.15 M-NaCl. This was done because the diluent usually used in viral titrations was 0.15 M-NaCl. All washings were performed until the supernatants were colorless. Loss of cells was calculated by the release of

hemoglobin read at OD 541 nm. Hemoglobin was determined in the supernatants after thawing (loss by thawing) and in the combined washings (loss by washing). Cells recovered after washing were estimated after hemolysis with 0.1 % Na_2CO_3 and centrifugation at 700 × g. For virus titrations the red cells were diluted to 0.5 % in 0.15 M-NaCl or in GVBSM^{++}-sucrose. Virus titration in GVBSM^{++}-sucrose was done because a great lot of the cells survived washing with this buffer but hemolyzed during washing with NaCl. Therefore these cells were washed with GVBSM^{++}-sucrose only.

Hemagglutination assay

Many viruses can agglutinate red blood cells. This property allows a simple rapid method for viral titrations. The hemagglutination assay used in these experiments was carried out by the endpoint procedure. Serial twofold dilutions of the virus sample (in 0.15 M-NaCl or GVBSM^{++}-sucrose) were each mixed with a standard suspension of red cells. These suspensions were left undisturbed for 4 h at 4°C, and the last dilution showing complete hemagglutination was taken as the endpoint. The titer estimated by this method has an inherent imprecision at least as large as the twofold dilution step used.

RESULTS AND DISCUSSION

It was shown (Table 1) that the red cell concentration by freezing had some consequence for cell recovery, and in further experiments the cells were frozen in a final concentration of 5 %. The experiments also showed that the buffer used for suspending the cells before freezing as well as the washing procedure for the thawed cells were of importance for cell recovery. With GVBSM^{++}-sucrose as the suspending buffer and PVP as the

Table 1. Cryoprotection of hen erythrocytes. Cryoprotective agent: 10 % (final conc.) PVP (MW 10 000).

Suspending solution before freezing	Red cell conc. %	Loss by thawing %	Loss by washing in susp. sol. %	Loss by washing in NaCl %	Recovery %
0.15 M-NaCl	10	26.9	20.2		53
	5	27.8	12.1		60.1
	2.5	22.8	18		58.2
GVBSM^{++}-sucrose	10	18.9	8.1	26.7	46.3
	5	12.2	2.4	23.1	62.4
	2.5	19.4	3.2	23	54.3

cryoprotectant much of the cells survived thawing and washing with GVBSM^{++}-sucrose, but hemolyzed during the washing with NaCl (Tables 1 and 2). This was not seen with 10 % DMSO as cryoprotectant, and DMSO in a final concentration of 10 % was a satisfactory cryoprotective agent independent of the washing procedure providing GVBSM^{++}-sucrose was the suspending solution (Tables 2 and 3). Table 2 also shows that glycerol was unfit for use in final concentrations of 10 and 20 % and that the concentration of PVP was of little importance for the cryoprotective effect. The type (molecular weight) of PVP was, however, of some importance for the protection, with best results with PVP K 15 (average MW 10 000) as cryoprotectant. Table 3 shows that the cells could be stored at —90°C for periods of 1 h to at least three months (the longest time studied) without further loss in cell recovery.

Table 3 also shows that the cells could be frozen at —196°C and stored at —90°C for at least three months without any significant effect on the virus titer. This was true independent of suspending solution, washing procedure and the cryoprotectant used.

Table 2. Cryoprotection of hen erythrocytes. Suspending solution: GVBSM^{++}-sucrose. Red blood cell concentration 5 % (final conc.).

Cryo-protectant	Final conc.	Loss by thawing	Loss by washing in		Recovery after washing in susp. sol. only	Recovery after washing in susp. sol. and NaCl
			sucrose	NaCl		
	%	%	%	%	%	%
PVP K 15	7.5	14.7	1.3	23.9	84.0	60.1
	10.0	11.9	2.2	18.4	85.9	67.5
	15.0	10.5	3.2	20.1	86.3	66.2
PVP K 25	7.5	28.1	1.7	27.4	70.2	42.8
	10.0	21.9	3.6	25.0	74.5	49.5
	15.0	17.0	5.5	29.3	77.5	48.2
PVP K 30	7.5	21.4	2.3	27.4	76.3	48.9
	10.0	18.9	2.8	27.4	78.3	50.9
	15.0	18.5	11.1	22.0	70.4	48.4
DMSO	10.0	13.4	1.8	3.6	84.8	81.2
	20.0	15.6	12.0	14.7	72.4	57.6
Glycerol	10.0	85.6	12.0	2.4	0	0
	20.0	77.4	20.2	2.4	0	0

Table 3. Cryoprotection of hen erythrocytes. Red blood cell concentration 5 % (final conc.). Virus titration with frozen and fresh (control) cells.

Susp. sol. before freezing	Cryo-protectant final conc. 10 %	Time in storage (—90°)	Recovery after washing in susp. sol. only %	Recovery after washing in susp. sol. and NaCl %	Virus titer
GVBSM^{++}-sucrose	PVP K 15	1 hour	85.3		1600
		13 days	85.8		800 (1600)
		27 days	89.9		1600 (800)
		3 months	86.2		1600 (800)
GVBSM^{++}-sucrose	DMSO	1 hour	86.1		1600
		13 days	84.9		800 (1600)
		28 days	86.9		1600
		3 months	85.0		1600
GVBSM^{++}-sucrose	DMSO	1 hour		79.5	1600
		13 days		76.6	1600
		28 days		80.9	3200
		3 months		80.5	1600 (3200)
NaCl	PVP K 15	1 hour	66.6		6400
		13 days	71.2		6400
		27 days	63.0		3200
		3 months	69.2		3200
NaCl	DMSO	1 hour	20.9		
Control (fresh cells suspended in GVBSM^{++}-sucrose)					1600
Control (fresh cells suspended in 0.15 M-NaCl)					3200

Concerning virus titration the pattern of aggregated and non-aggregated cells was very significant and the virus titer easy to determine when the titration was carried out in 0.15 M-NaCl. In GVBSM^{++}-sucrose the aggregated cells formed a thicker film which at the edge had a serrated appearance, and the titer was a little more difficult to estimate with certainty. With PVP as the cryoprotectant the advantages using this buffer were still greater than the disadvantages and should be recommended. Because few cells hemolyzed during washing with 0.15 M-NaCl with 10 % DMSO as cryoprotectant (3.6 %, Table 2), these cells can be used in virus titrations using 0.15 M-NaCl as diluent. PVP and DMSO were both easy to prepare and the recovery before washing with NaCl was about the same.

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SAMMENDRAG

Beskyttelse af røde høneblodlegemer ved frysing.

Bevaring av røde høneblodlegemer mot skade etter nedfrysing til —196°C ble undersøkt. Slike celler brukes som reagens for titrering av virus. Som kryoprotektive midler ble brukt nøytralisert polyvinylpyrrolidone (PVP), dimetylsulfoxide (DMSO) og glycerol. God cellegjenvinning ble oppnådd med PVP, især med PVP K 15 (molekylvekt 10 000), og med DMSO når denne ble brukt i endelig konsentrasjon 10 %, mens glycerol var uegnet. Cellekonsentrasjonen, suspensjonsmiddelet før frysing og vaskeprosedyren etter tining var av betydning. Virustitreringer med frosne celler ga samme resultat som når ufrosne celler ble brukt som reagens.

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