Cryopreservation of Erythrocytes, Thrombocytes, and Lymphocytes

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Key Words

Cryopreservation · Freezing · Erythrocytes · Thrombocytes · Lymphocytes · Glycerol · Dimethyl sulphoxide · Hydroxyethyl starch

Summary

The cryopreservation of blood cells can be regarded as a classical field of development and application of low temperature biology. Cryopreservation methods have been developed for erythrocytes, which are commonly frozen with glycerol as the cryoprotective additive although hydroxyethyl starch (HES) shows considerable promise. Cryopreserved erythrocytes for transfusion are of advantage in the case of patients with rare blood groups, adverse antibody problems, autologous use and civil as well as military disasters. Additionally they can be used for blood typing, antibody screening and compatibility testing. Cryopreservation methods for thrombocytes, lymphocytes and hematopoietic stem cells usually involve dimethyl sulfoxide (DMSO) as the cryoprotective additive. Low temperature preservation of thrombocytes offers the possibility of making HPA- and/or HLA-typed platelet concentrates available in blood banks at any time. The use of cryopreserved lymphocytes is well established and a routine procedure for clinical laboratory testing. Recently there is a growing clinical interest in cryopreserved lymphocytes in addition to hematopoietic progenitor cells for the supplemental treatment of patients after blood stem cell transplantation. Despite occasional reports, it is our opinion that no clinically suitable method for the preservation of human granulocytes has been developed so far.

Schlüsselwörter

Kryokonservierung · Einfrieren · Erythrozyten · Thrombozyten · Lymphozyten · Glycerin · Dimethylsulfoxid · Hydroxyethylstärke

Zusammenfassung

Die Kryokonservierung von Blutzellen stellt eine klassische Entwicklung und Anwendung der Tieftemperaturbiologie dar. Die für Erythrozyten entwickelten Methoden bedienen sich im Allgemeinen des Kryoprotektivs Glycerin, obwohl auch die Verwendung von Hydroxyethylstärke (HES) vielversprechend ist. Kryokonservierte Erythrozyten zur Transfusion sind von Vorteil bei Patienten mit seltenen Blutgruppenmerkmalen, bei Problemen mit bestimmten Antikörpern, zur Bevorratung mit Eigenblut und im Falle ziviler oder militärischer Katastrophen. Darüber hinaus können sie aber auch bei der Blutgruppenbestimmung, der Antikörpersuche und der Verträglichkeitstestung eingesetzt werden. Kryokonservierungsverfahren für Thrombozyten, Lymphozyten und hämatopoetische Blutstammzellen verwenden im allgemeinen Dimethylsulfoxid (DMSO) als Kryoprotektiv. Die Tieftemperaturkonservierung von Thrombozyten erlaubt die Bevorratung von HPA- und/oder HLA-typisierten Plättchenkonzentraten. Der Gebrauch von tiefgefrorenen Lymphozyten ist etabliert für die Durchführung bestimmter Tests in klinischen Laboratorien. In den letzten Jahren hat das Interesse an kryokonservierten Lymphozyten zur ergänzenden Therapie nach der Blutstammzell-Transplantation deutlich zugenommen. Trotz bisweilen in der Literatur auftauchender Berichte wurde aber nach unserer Einschätzung noch kein zufriedenstellendes Verfahren zur Kryokonservierung von humanen Granulozyten gefunden.

Introduction

Frozen erythrocytes, thrombocytes, lymphocytes and hematopoietic progenitor cells (from peripheral blood as well as from bone marrow) are being used for various diagnostic and therapeutic purposes [reviews in e.g. 1–3]. A variety of cell-specific cryopreservation protocols have evolved so far. The methods differ with regard to i) cell concentrations, ii) protective solutions used (cryoprotectants and their concentrations), iii) temperature-time histories (TTH) during cooling and re-warming, and iv) storage temperature. Additionally, some of the cryoprotectants are not well tolerated clinically in the concentrations required for cryopreservation (e.g. dimethyl sulfoxide (DMSO) for platelets) or lead to an osmotically induced lysis of the cryoprotectant-loaded cells when transfused into an isotonic individual organism (e.g. glycerol for erythrocytes). In these cases, a washing procedure is required after thawing to remove the cryoprotective additive prior to the application/transfusion.

Erythrocytes

Clinically Applied Methods Using Glycerol

In principle, three different methods have been established for routine clinical use of frozen red cells (RBC) [4]; these are:

- i) The Huggins [5] technique utilizes ~40% [wt/v] glycerol as the cryoprotective additive. Prior to transfusion the glycerol is removed by reversible agglomeration of the RBC using nonionic sugar solutions. Initially used by many hospitals, this procedure is rarely used now because of unavailability of required equipment and disposable supplies.
- ii) The ‘high glycerol slow cooling technique’ according to Meryman and Hornblower [6] utilizes ~40% [wt/v] glycerol and washing with concentrated salt solutions prior to transfusion. This is the dominant method in use in the USA [for details see 7, 8].
- iii) The ‘low glycerol-rapid cooling technique’ according to Rowe [9, 10] and Krijnen et al. [11] utilizes ~17–19% [wt/v] glycerol and rapid cooling in liquid nitrogen (LN₂). Storage of frozen material must be in LN₂ (–196 °C) or its vapor (≤ -165 °C). This is the dominant method for cryopreservation of RBC in Europe [detailed description in 8].

Methods Using Macromolecular Additives

The utilization of macromolecular cryoprotectants is an alternative approach to using glycerol for cryopreservation of erythrocytes. Watersoluble, cryoprotective macromolecules such as albumin, dextrans, modified gelatin, polyvinylpyrrolidone (PVP), polyethylene oxide (PEO), polyethylene glycol (PEG) and hydroxyethyl starches (HES) exhibit the principal advantage of not entering into the cells. This property significantly

Fig. 1. Patented freezing container [19]. This container generates automatically the cooling rate (200–250 °C/min) required for the HES procedure as described in [8] when immersed in LN₂.

facilitates their removal after thawing. In the case of emergencies, this step could be omitted if the additive, e.g. albumin, dextrans, modified gelatin or HES, is biodegradable and tolerated by the human organism.

Cryopreservation with Hydroxyethyl Starch

Early studies on macromolecular cryoprotectants go back to Rinfret and coworkers [12] who utilized PVP successfully for freezing erythrocytes. In 1967 Knorpp and co-workers [13] described the successful cryopreservation of human RBC using HES and liquid nitrogen (LN₂). Comparing the efficacy of HES to that of PVP, they preferred the colloid HES to PVP as the latter is retained to a considerable extent in the recipient (as are PEO and PEG). Moreover, in the case of hypovolemia, albumin, dextrans, modified gelatins and HES are often transfused to serve as blood volume substitutes.

After several in vitro investigations and optimizations of the original HES procedure [14, 15] and after in vivo experiments in dogs, Sputtek and coworkers [16] conducted a successful in vivo study including seven healthy volunteers. They subsequently performed a systematic clinical trial in patients [17]. A detailed description of the method for the clinical application can be found in [8], and a review of the development of the procedure in [18]. *Nota bene:* The patented freezing container (fig. 1 [19]) which must be used has a wall thickness of 2 mm. The exterior is pasted with a microporous textile tape to improve the heat transfer during the cooling process in boiling LN₂. Cooling rates of approximately 70 °C/min that result from the use of uncoated containers are too low compared with those required (i.e. 200–250 °C/min in the temperature range between 0 °C and –30 °C). Additionally, the closed container produces a well defined flat geometry of the bags and a homogenous sample thickness (approximately 5–6 mm). LN₂ is not allowed to come into contact with the samples during

Fig. 2. Shaking water bath, modified with a pouch for rapid thawing as described in [8].

the initial cooling process. Please note that you will not be able to reproduce our results when not using the patented freezing container. Rapid thawing is needed as well: This can be achieved by using a shaking water bath with a pouch (fig. 2 [8]). The reason is that the composition of the cell suspension (e.g. hematocrit, HES concentration, electrolyte content), sample thickness, geometry and cooling rate are interdependent: A change in one of these parameters will lead to a less favorable result.

Based on the work published by Robson [20], Thomas and co-workers [21, 22] have developed a modified procedure for the freezing of RBC using HES. The major differences compared to our procedure are: i) no pre-freeze washing, ii) different HES modification, iii) lower HES concentration, iv) higher electrolyte concentration, v) higher hematocrit, vi) larger freezing bag, vii) smaller sample thickness, viii) smaller volume, ix) higher viscosity, x) different freezing container, and xi) uncontrolled thawing. Unfortunately, because of high viscosity, they must dilute or wash out the HES prior to transfusion because of poor flow rate of the concentrated HES-erythrocyte mixture.

As we have pointed out, it is essential to remove white cells and platelets prior to freezing. Thomas et al. [22] have speculated that the contents of the white cells and platelets are highly thromboplastic. As these cells are destroyed on freezing, thereby liberating their contents into the HES/RBC mixture, failure to filter could cause a disseminated intravascular coagulopathy when the RBC are subsequently thawed and transfused without post-thaw washing.

Cryobiological researchers and clinical colleagues interested in our HES method for freezing erythrocytes have posed questions regarding the nature of HES and where it can be obtained. The following 2 paragraphs address some of these issues.

Structure of HES

HES is a modified natural polymer of branched amylopectin (one of the two components of starch, the other component is the linear amylose). Its physical and chemical characteristics are mainly defined by i) the degree of hydroxyethylation (DS), i.e. replacement of hydroxyl groups of the anhydroglucose units by hydroxyethyl groups) and ii) the molecular

weight distribution. Whereas native starch is hardly watersoluble, hydroxyethylation increases water solubility. DS is determined by measuring the number of substituted anhydroglucose units and dividing this number by the total number of anhydroglucose units in the molecule. The molar substitution (MS) is calculated by measuring the total number of hydroxyethyl groups present and dividing this by the total number of anhydroglucose units. DS and MS are not the same, but they are often incorrectly used interchangeably in the literature. As hydroxyethylation can occur at carbon positions 2, 3 or 6 of the anhydroglucose unit, depending of the manufacturing process, the substitution pattern can vary greatly. HES molecules show a great polydispersity (in contrast to other cryoprotectants such as glycerol and DMSO, which have molecular weights of 92.09 g/mol and 78.13 g/mol, respectively). The molecule sizes of HES usually follow a sort of bell-shaped distribution, ranging from some thousand g/mol to over a million g/mol. Consequently 'the molecular weight' can be regarded as an 'average' molecular weight only. There are two ways for the calculation: i) arithmetic mean, i.e. total weight of all molecules divided by the number of molecules (M_n) and weight averaged mean (M_w). For example, an HES specified as '450/0.7' should be an HES with an M_w of 450,000 g/mol and an MS of 0.7 (i.e. 70 hydroxyethyl groups per 100 anhydroglucose units of the polymer). A comprehensive overview on the structure of HES can be found in Banks et al. [23]. Of major importance is that HES, a plasma volume expander, is metabolized by the body and excreted unlike some other macromolecules (e.g. PEO, PVP).

HES Solutions

For historical and marketing reasons, predominantly slowly degradable, high M_w HES (450/0.7) is available in the USA. In Europe, a large variety of HES solutions is available, dominated by medium M_w , easily degradable HES (200/0.5) [24]. The optimized HES solution used in our clinical trials with patients (KryoHAES®, manufactured by Fresenius, Bad Homburg, Germany, according to our specifications) had an HES concentration of 23% (wt/wt) which equals 25% (wt/v). This is higher than the 3% (wt/v), 6% (wt/v) or 10% (wt/v) solutions available for clinical use. The sodium chloride concentration of 60 mmol/l was lower than the 'standard' 155 mmol/l. The average M_w and the DS were 200,000 g/mol and 0.5, respectively. Although it is commercially not available at present, it can be prepared from dry HES powder or commercially available HES solutions after dialysis and freeze drying [25, 26]. The dry substance can be dissolved in distilled water and the appropriate amount of sodium chloride has to be added. Suppliers of dry HES powder (with different amounts of low molecular weight impurities (= LMWI, see below) are e.g. Ajinomoto (Japan), Fresenius Kabi (Germany), and Serum Werk Bernburg (Germany). Suppliers of HES solutions for infusion in Germany are e.g. Baxter, B. Braun, Fresenius Kabi, Serag-Wiesner, and Serum Werk Bernburg. In principle any

dialysis tubes from any supplier can be used, as long as the cut-off is about 10,000–14,000 g/mol. HES concentrations should not be higher than 10% (wt/wt), as otherwise the tubes may crack during the procedure. *Nota bene*: If the decision is made to use a ready-made solution provided by one of the above mentioned companies, it has to be determined by trial and error if it works. If the solution contains too many LMWI (i.e. electrolytes, oligosaccharides) it may not work. A product named ‘Hydroxyethyl starch’, product code H 6382, from Sigma-Aldrich should never be used for another reason. Only an average of 10 per 100 anhydroglucose units are substituted with a hydroxyethyl group (DS = 0.1). As a consequence it is poorly soluble in water. A product which is called ‘Hetastarch’ [for nomenclature of HES in the USA see 24], product code H 2648, from the same company can be used. It comes as a 6% solution in 0.9% sodium chloride. However, this is very expensive (approximately EUR 15.– per gram HES) compared to the 3, 6 or 10% solutions used in the intensive care units in hospitals. HES has been used for many decades as a volume replacement after blood loss. The cost for HES in plasma substitutes is approximately EUR 0.33 per gram HES). The modifications familiar for this purpose in Europe are 450/0.7, 200/0.5, 130/0.4 and 70/0.5. The concentrations vary from 3 to 10% (wt/v) and the electrolyte content is adjusted to isotonicity (mostly by adding sodium chloride). The solutions available for volume replacement may contain various amounts of LMWI, these may differ from one HES modification to the other, and sometimes even from one lot to the next from the same manufacturer. If this is not taken into account, it will be difficult to reproduce the work.

Determination of RBC Recovery/Viability

The determination of cell recovery/viability after thawing in the presence of HES poses a problem. HES coats the surface of the erythrocytes and may provide a scaffolding for damaged membranes so that some cells appear intact, though they will rupture if diluted with isotonic saline. Viability after thawing, in terms of ‘saline stability’, can be determined as follows [14]: 250 µl of the RBC suspension is diluted 40-fold in a buffered isotonic saline solution. After 30 min the suspension is separated into a supernatant (destroyed RBC) and sediment (intact RBC) by centrifugation. Saline stability is then calculated using:

$$\text{Saline stability [\%]} = (1 - \text{Hb}_s / \text{Hb}_T) \times 100, \quad (1)$$

where Hb_T corresponds to the total hemoglobin and Hb_s to the hemoglobin in the supernatant. The determination of the 2 hemoglobin concentrations can be performed spectrophotometrically at 546 nm using Drabkin’s solution. A correction for the hematocrit is not required, as the volume fraction of erythrocytes after 40-fold dilution is less than 2%. Plasma stabilities (i.e. when plasma is used as diluent in the test instead of isotonic saline) may be slightly higher than saline stabilities

because plasma factors allow a few very slightly damaged cells to recover. In the presence of HES (and other macromolecular cryoprotectants!) the post-thaw recovery of intact cells, determined by simply spinning down the suspension without any dilution and measuring the percentage of ‘free’ hemoglobin, should not be used as a quality control procedure because the result is misleading, irrespective of the hematocrit being taken into consideration or not.

Thrombocytes

Since the first reported attempt in 1956 to stop thrombocytopenic bleeding by the infusion of previously frozen platelets by Klein [27], a broad variety of *in vitro* and *in vivo* studies on cryopreserved platelets have been published. Djerassi et al. [28] in 1966 were the first to report on the use of 5% DMSO and cooling at 1 °C/min for successful cryopreservation and transfusion of human platelets. As no removal of DMSO after thawing was performed, the recipients suffered from problems arising from infusion of this substance. The introduction of a post-thaw washing procedure by Lundberg et al. [29] helped to overcome the symptoms of nausea, vomiting, local vasospasm and a garlic-like odor and taste. The most widely used method for the cryopreservation of platelets is a ‘10% DMSO-slow cooling’ method tested clinically by Schiffer [3, 8, 30, 31] while another approach is a ‘low glycerol/glucose-intermediate cooling’ method described by Dayian and Rowe in 1976 [1, 32].

HES-cryopreserved platelets (frozen in the presence of 4% (wt/v) HES at 1 °C/min), turned out to be hemostatically effective when using a 4% HES method [33]. Optimum results can only be achieved when increasing the cooling rate from 1 °C/min as described by Choudhury [33] to 15 ± 5 °C/min and reducing the sodium chloride concentration in the cryoprotective solution from isotonic to 120 mmol/l [8, 34]. When comparing the optimized HES method to Schiffer’s technique [30, 31], both protocols turned out to be highly effective regarding the post-thaw numerical platelet recovery (approximately 90%). However, functional *in vitro* parameters showed that the DMSO-protected frozen platelets were inferior to fresh controls but superior compared to the HES-protected ones [35].

Lymphocytes

The use of cryopreserved mononuclear cells (which includes lymphocytes) is well established and a routine procedure for clinical laboratory testing. Cohen and Rowe [36] were the first to report on detection of leukoagglutinins with frozen lymphocytes for transfusion compatibility, a forerunner of tissue typing plates in common use for detection of HLA (human leukocyte antigen) antibodies. Today frozen lymphocytes are

used for various diagnostic purposes, e.g. HLA typing, detection of HLA antibodies in patients on waiting lists for organ/bone marrow transplantations and mixed lymphocyte reactions/cultures. They are also of interest with respect to look-back procedures in transfusion medicine or diagnosis in patients. The methods for freezing mononuclear cells reported in the literature vary from one author to another [1–3]. Recently, there is a growing clinical interest in cryopreserved lymphocytes for the supplemental treatment of patients after blood stem cell transplantation making use of the ‘graft-versus-leukemia’ effect in the case of relapse. Usually, those peripheral donor lymphocytes are frozen according to methods which are more or less modifications of a technique which was first described for bone marrow by Ashwood-Smith [37] in 1961 using 10% DMSO. During cooling, the heat is removed either by computer-controlled and LN₂-operated machines or in mechanical (–80 °C) refrigerators. Cryopreserved autologous and homologous blood stem cells – which are mononuclear cells as well – have become a ‘standard’ blood component for the treatment of several malignant diseases. Stiff and co-workers [38] have reported that the addition of 6% HES reduced the ‘original’ concentration of DMSO (10%) by one half. Optimum results for peripheral blood stem cells can be obtained when using cooling rates between 1 and 5 °C/min and at least 5% DMSO are present [39]. A detailed review of the various procedures for hematopoietic stem cells (in German) can be found elsewhere [40].

If mononuclear cells are to be frozen for laboratory purposes, cell concentrations may range from 0.5×10^6 to 50×10^6 /ml, the most frequently used medium is RPMI 1640 supplemented with human or fetal calf serum or plasma, and the cryoprotectant of choice is 5–10% DMSO. Cooling is performed in 1 or 2 ml vials at 1–2 °C/min down to a temperature of –30 °C or less by means of a programmable LN₂-operated freezer, whereas thawing is usually performed in a water bath of 37 °C. Numerical recoveries reported vary from 60 to 90%. We believe that cooling rates at temperatures below –40 °C are not as critical as in the upper temperature region (i.e. above –40 °C) and can be increased up to 10 °C/min to save time. Additionally, we do not think that a programmable LN₂-operated freezer is always required to generate the appropriate cooling rate: –80 °C refrigerators may be suitable as long as

provision is taken (e.g. by using card board insulations) that the cooling rate in the upper temperature region does not exceed 5 °C/min. It is best to document the cooling rate of 1–5 °C/min with a suitable temperature recorder. For long-term storage (i.e. months or years), however, we recommend liquid nitrogen LN₂ (–196 °C) or the vapor (≤ -165 °C). In any case temperatures well below –123 °C are needed, which is the glass transition temperature of maximally freeze concentrated aqueous DMSO solutions [41]. However, storage for a few days or weeks in –80 °C freezers may also be acceptable but prolonged storage (months or years) requires lower temperatures.

Granulocytes

There have been publications in the past which claim the successful cryopreservation of granulocytes [reviews in e.g. 42, 43]. Despite reports appearing now and then in the newer literature (mostly as abstracts), it is our opinion that no clinically suitable method for the preservation of granulocytes has been found. The huge variation of the in vitro results shows how cumbersome the viability assays are, and how unsuitable they will be to predict anything that is going to happen in vivo. Membrane integrity tests (i.e. staining tests, often referred to as ‘viability tests’) measure only a *conditio sine qua non* (i.e. an intact cell membrane). Mature granulocytes are end stage cells designed to break down and liberate their lysosomes and lysosomal enzymes causing cell destruction. However, what is the meaning of these results if tests measuring typical granulocytic functions (e.g. chemotaxis, bactericidal activity) fail to detect any significant activity? Takahashi and co-workers [44] have proposed some explanations why granulocytes are so unrewarding regarding their cryopreservation. Already at temperatures below –5 °C without the formation of ice, a significant loss of function can be observed. This could be prevented by the addition of DMSO, whereas glycerol failed to show this effect. Because of their limited osmotic tolerance (already a two-fold increase compared to isotonicity caused a significant loss of function), they are highly susceptible to the electrolyte enrichment taking place during ice formation. Granulocytes also showed a limited tolerance to hypotonic stress which may occur upon thawing.

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