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Cryopreservation & Low Temperature Biology in Blood Transfusion

Cryopreservation and low temperature biology in blood transfusion

Proceedings of the Fourteenth International Symposium on Blood Transfusion, Groningen 1989, organised by the Red Cross Blood Bank Groningen-Drenthe

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FOREWORD

The theme of this 14th International Symposium on Blood Transfusion is closely related to the work and scientific contributions of the Dutch cryobiology pioneer Dr. Herman W. Krijnen of the Dutch Red Cross Central Laboratory.

Dr. Krijnen was known and respected in the national and international blood transfusion community as an extremely competent scientist and a beloved and admired colleague.

Dr. Krijnen was intentionally honoured with the invitation to open this symposium on cryopreservation and low temperature biology in blood transfusion and be the guest of honour at this event. Unfortunately, Dr. Krijnen suddenly died on the first of June 1989. In honour and memory of Dr. Krijnen this symposium will therefore be dedicated to him.

Since the 10th International Symposium on Blood Transfusion in 1985 highlighted the theme of "Future developments in blood banking", major changes have occurred in the blood banking world. Most of these changes were forced upon the Blood Banks by the fear of spreading AIDS through contaminated donations. This not only led to the widespread testing of blood, but also to a more appropriate counselling of the community and the blood donors in specific. Additionally, virus inactivation techniques were introduced for those components derived from multiple donations and intended for a regular transfusion in haemophilia patients and others.

In 1987 the State Secretary of Health in the Netherlands introduced a standard registration document on factor VIII preparations. Since January 1988 the production of all factor VIII preparations in our country, including cryoprecipitate for the treatment of haemophilia, have to observe the regulations of this document. Consequently, Good Manufacturing Practice was definitely introduced in blood banking. Advances in biotechnology have caused changes in interest and policy of Blood Banks, anticipating new approaches to blood transfusion.The concept of transfusion medicine has developed into an almost mature principle with noticeable acceptance and visible implementation.

Today, this symposium will focus on the principles and techniques of preservation of blood as a transplant in its cellular and plasma components

It will also lead the way into the rapidly extending field of organ preservation and banking.

When in two years time the bell will toll for Europe 1992, blood transfusion medicine needs to have reached the state of full maturity, and policies have to be developed and set for the future. At the turn of the 80-ties, at the start of the final decade of the 20th century, more than ever blood banking needs dynamic and creative leadership supported by wisdom and confidence. I have great confidence in the science to be presented and discussed in wisdom at this symposium, which will contribute to a tradition of leadership created by you all in contributing to this series of unique Red Cross Blood Bank events.

F. Brink
Chairman of the Board of the
Red Cross Blood Bank Groningen-Drenthe

I. PRINCIPLES AND FUNDAMENTALS

CONTENTS

METABOLISM AND PHYSIOLOGY OF CELLS AT LOW TEMPERATURES

W.J. Armitage

The strategies for preserving blood cells can be broadly devided into storage at hypothermic temperatures above 0°C and cryopreservation at –80°C or below. With tissues such as cornea there is a third option, namely that of normothermic storage by organ culture. The aim of all three approaches is to maintain viability, which may be defined as the ability of cells and tissues to perform their normal physiological functions when transfused or transplanted. Whereas organ culture aims to keep cells in as near normal a state as possible by supplying all the nutrients necessary to maintain metabolism, the use of cooling is an attempt to reduce or abolish cellular demand for energy.

Hypothermic storage

Effects of reduced temperature on cells

The effects of cooling on cellular metabolism are complex [1]. Cooling lowers the rate of chemical reactions and so has a general depressive effect on metabolism, as demonstrated by the fall in oxygen consumption that accompanies cooling [2]. Both the demand for energy, for activities such as active transport of ions and protein synthesis, and energy production, in the form of ATP synthesis, are reduced by cooling. The relation between absolute temperature, T, and the rate constant of a chemical reaction, k, is defined by the Arrhenius equation,

$$k = Ae^{-E/RT}$$

where A is a constant, E is the activation energy of the reaction and R is the gas constant [3]. Thus, for a simple reaction, an Arrhenius plot of $\log_e k$ as a function of $1/T$ yields a straight line with slope $-E/R$. In a sequence of reactions, however, each step may have a different activation energy making the overall effect of a change in temperature unpredictable. Discontinuities have frequently been reported in Arrhenius plots of complex biological reactions [e.g. 4], which emphasizes the fact that

many properties of a cell are altered by temperature, each of which may influence the overall "reaction rate" of the process being studied.

Inhibition of active and facilitated transport systems in membranes reduces the uptake of substrates by cells and upsets intracellular ionic composition resulting in the net gain of sodium and calcium and loss of potassium. The maintenance of intracellular ionic composition at normothermia is the result of a balance between passive fluxes of ions down their electrochemical gradients and the activity of pumps transporting ions against their electrochemical gradients. The activity of the Na-K pump in human erythrocytes at 5°C is only 0.25% of that at 37°C [5]. But passive fluxes tend to be less affected by temperature and may even display paradoxical behaviour such as that shown by the passive flux of potassium in human erythrocytes which falls to a minimum at 12°C and then increases at lower temperatures [6]. The net result is that the rate of ion pumping at hypothermia is unable to match the passive ion fluxes and, owing to the charge carried by intracellular proteins, there is a net gain of Na and Cl which osmotically draws in water and leads to cell swelling [7].

Hypothermic storage of blood cells

Erythrocytes can be stored at 4°C for several weeks in plasma containing an anticoagulant. They do not swell appreciably owing to the presence outside the cells of plasma proteins and citrate. But storage in an electrolyte solution without protein does lead to swelling, although this can be prevented by the addtion of mannitol [8]. The viability of erythrocytes, as determined by their survival in the circulation after transfusion, steadily declines during storage [9]. Both metabolic changes and alterations in structure and function of the plasma membrane play a role in this loss of viability [10,11]. During storage of erythrocytes, the ATP and 2,3-diphosphoglycerate (2,3-DPG) levels fall [12,13], and the loss of plasma membrane lipids is associated with reduced cell deformability [14]. The addition of adenine to the anticoagulant reduces the loss of ATP [15]. The loss of 2,3-DPG during storage increases the affinity of haemoglobin for oxygen and compromises the ability of stored erythrocytes to deliver oxygen to the tissues. Although 2,3-DPG levels are restored to a limited extend following transfusion [16], a wide range of additives has been investigated in an attempt to moderate the loss of 2,3-DPG during storage [17,18].

When platelets are stored at 4°C, they change shape from discs to spheres [19]. The disc form is a metabolically dependent state and is maintained by a circumferential bundle of microtubules that depolymerize at low temperature [20]. Reassembly of microtubules is severely compromised by only 24 hours of storage at 4°C [21]. Plasma membrane lipids are also lost during 4°C storage [22]. Storage at 4°C for only a few hours compromises platelet viability as shown by a reduction in survival following transfusion [23]. Storage at 22°C, on the other hand, also

results in morphological and metabolic changes and reduces the *in vitro* aggregation response to ADP, but subsequent *in vivo* survival following transfusion is better preserved than after storage at 4°C [24]. Even so, the duration of storage of viable platelets is stille only a few days.

Hypothermic storage of tissues

The success of a corneal graft is critically dependent on the viability of the monolayer of endothelial cells that covers the posterior surface of the cornea. This layer of cells controls corneal hydration both by acting as a passive permeability barrier to reduce the influx of solutes and water into the corneal stroma from the aqueous humour and by actively pumping HCO_3^- ions from the stroma thereby inducing an osmotic efflux of water [25,26]. Failure of endothelial function results in corneal oedema and loss of transparency. When whole eyes are placed at 4°C, the endothelial ion pump is inhibited and the cornea gradually thickens and becomes cloudy. Returning the eye to physiological temperature restores normal metabolic activity and activity of the endothelial HCO_3^- pump, and the cornea thins [27]. With increasing storage time at 4°C, however, endothelial function becomes compromised owed to insufficient supply of substrates for the albeit reduced metabolism and build up of waste products, principally lactate, in the aqueous humour. Removal of the cornea from the eye and storage in tissue culture medium containing 5% dextran partially overcomes these problems and increases the permissible period of hypothermic storage from 2 to 4 days [28,29].

Normothermic storage

Normothermic storage of tissues

One way to avoid the detrimental effects of hypothermia that limit the duration of hypothermic storage is to maintain the cells or tissue at normothermia. Cell culture techniques are well established but for technical and logistical reasons have found no place in blood banking. In the 1970s, however, organ culture techniques developed for skin were applied to the cornea [30]. Corneas maintained in tissue culture medium at 37°C were shown to be metabolically active and retained normal endothelial ultrastructure for at least 30 days [31]. Subsequent clinical results confirmed the efficacy of this technique [32].

The increase in storage time provided by this technique confers a number of significant advantages. Routine corneal grafts can be performed electively rather than as emergency procedures; a stock of corneas is always available for genuine emergencies; there is more time for tissue typing and matching where this is appropriate (immunological rejection is the main cause of corneal graft failure); the tissue is screened for microbiological contamination; and endothelial integrity is assessed [33].

Cryopreservation

The aim of cryopreservation is to cool living cells and tissues to sufficiently low temperatures to completely suppress cellular metabolism and so maintain them in a biologically stable state. Below about –130°C, no chemical reactions can take place in biological systems: cells stored at higher temperatures (e.g. –70°C) tend to be less stable, although useful long-term storage can be obtained depending on the type of cell and the conditions under which the cells are frozen. The survival of cryopreserved cells depends on their ability to cope with a range of physical, physicochemical, physiological and biochemical stresses encountered during freezing and thawing. Uncontrolled freezing is usually lethal to cells, but many cell types do survive freezing and thawing when a cryoprotectant is present in the bathing medium and when the rates of cooling and warming are controlled [34]. Different cell types vary markedly in their response to freezing and some, for example human granulocytes, have not yet been successfully cryopreserved. In contrast to isolated cells, there has been a marked lack of success in the cryopreservation of organized tissues and organs [35].

Effects of cooling and warming rates

When cell survival is plotted against cooling rate, an inverted U-shaped curve is often obtained showing that cells have an optimum cooling rate at which survival is maximal [36]. When an aqueous solution freezes, water is withdrawn from the solution to form ice thus concentrating the solutes in the liquid phase. The concentration of solutes is fixed by temperature, and as temperature falls there is a substantial rise in solute concentration: the higher the cooling rate, the more rapid is the rise in solute concentration. Mazur [37] showed that the fall in survival at cooling rates higher than the optimum was related to the inability of cells to lose water quickly enough to maintain osmotic equilibrium. Under these conditions, the cells supercool and eventually freeze internally, which is usually lethal [38]. When cells are cooled slowly, they can maintain osmotic equilibirum with their surroundings by losing water (i.e. they behave as osmometers) [39].

Thus cells cooled rapidly contain ice but are not appreciably shrunken, whereas cells cooled slowly are shrunken but do not contain ice. The mechanism of damage to slowly cooled cells has been ascribed to prolonged exposure at relatively high subzero temperatures to the adverse changes occurring in the extracellular medium, especially the increased concentrations of electrolytes [40].

Survival of frozen cells is also dependent on the warming rate. When cells are cooled at rates just higher than the optimum, they are likely to contain small amounts of intracellular ice: the survival of these cells is higher when they are warmed rapidly rather than slowly. Furthermore,

when cells are cooled at suboptimal rates, they survive better when warmed slowly rather than rapidly [41].

Cryoprotectants

Cryoprotectants are compounds that possess the remarkable property of reducing damage to cells during freezing. Although glycerol [42] and dimethyl sulphoxide (Me_2SO) [43] are perhaps the most commonly used additives for cell cryopreservation, there is a large range of chemically very diverse compounds that are able to protect cells against freezing injury [44,45]. Cryoprotectants are broadly divided on the basis of whether they permeate cells; for example, cells are usually permeable to glycerol and Me_2SO but impermeable to dextran and sucrose. As the concentration of a cryoprotectant such as glycerol is increased, cell survival rises and the optimum cooling rate becomes lower [46]. Thus cryoprotectants are effective against slow cooling injury but are ineffective against damage caused by intracellular ice formation.

But cryoprotectants can also be detrimental to cells as a result of chemical toxicity and osmotic stress. Protocols for the addition and removal of cryoprotectants can play a decisive role in determining the survival of cyropreserved cells. The osmotic stress arises because cells tend to be more permeable to water than to the cryoprotectant. When the extracellular concentration of permeating cryoprotectant is increased abruptly, osmotic equilibrium across the plasma membrane is initially restored by an efflux of water from the cell. Cryoprotectant and water then move into the cell which returns towards its normal volume. Abrupt dilution of the external concentration of cryoprotectant, on the other hand, initially causes a rapid influx of water followed by a slower return to normal volume as cryoprotectant and water leave the cell [47]. Thus rapid addition of permeating cryoprotectant causes transient cell shrinkage, while rapid dilution causes transient cell swelling. The size of these fluctuation in cell volume depends on the concentration gradient of cryoprotectant across the plasma membrane and the membrane permeability characteristics.

The extent to which a cell can tolerate changes in cell volume can be evaluated by exposing cells to a range of hypo- and hyperosmotic concentrations of a non-permeating solute [48]. Provided that the permeability characteristics of the cell have been determined, stepwise addition and dilution protocols can be calculated to maintain the cells within those tolerated volume limits [49]. Platelets are very sensitive to osmotic stress [50] and they are damaged by rapid addition and dilution of glycerol. At room temperature, platelets are approximately 4000-fold more permeable to water than to glycerol [51,52]; thus the osmotic effects of changes in extracellular glycerol concentration would be substantial. Platelet tolerance of 1 mol/l glycerol is substantially improved when the rates of addition and removal are such that platelet volume