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The cryopreservation of composite tissues

Principles and recent advancement on cryopreservation of different type of tissues

Joseph Bakhach

U.m.I Urgence Main Liban; Bellevue Medical Center; Mansourieh, Beirut Lebanon

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Cryopreservation of human cells and tissue has generated great interest in the scientific community since 1949, when the cryoprotective activity of glycerol was discovered. Nowadays, it is possible to reach the optimal conditions for the cryopreservation of a homogeneous cell population or a one cell-layer tissue with the preservation of a high pourcentage of the initial cells. Success is attained when there is a high recovery rate of cell structures and tissue components after thawing. It is more delicate to obtain cryopreservation of composite tissues and much more a whole organ. The present work deals with fundamental principles of the cryobiology of biological structures, with special attention to the transfer of liquids between intra and extracellular compartments and the initiation of the formation and aggregation of ice during freezing. The consequences of various physical and chemical reactions on biological tissue are described for different cryoprotective agents. Finally, we report a review of results on cyropreservation of various tissues, on the one hand, and various organs, on the other. We also report immunomodulation of antigenic responses to cryopreserved cells and organs.

Introduction

Recent world wide advances in the field of composite organ allotransplantation and reconstruction by allotranfert of partial or total amputations of upper limbs and, more recently of the face, have allowed reconstructive surgery to make immense strides and a noted entry into the third millennium.[1] These innovative techniques are alluring, but are currently limited to reconstruction of total or partial amputation of an organ. They allow simultaneous restoration of anatomy, esthetics and function to an organ when no other classical operative procedure can do so. We studied the possibility of extending conditions of allotransplantation to the reconstruction of anatomical and functional units, like articular complexes, tendons and neural structures. Such structural reconstructions, which are in great demand and very important in daily clinical practice and can only be envisaged in planned operations. Cryopreservation of sampled organs is an indispensable step conditioning the feasibility of these new procedures. It would allow time to select the recipient from an anatomical, immunological and psychological standpoint and to prepare the allotransplantation procedure.

Correspondence to: Joseph Bakhach; Email: joebakhach@free.fr
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More than ever, cryopreservation of biological tissues and composite organs is stimulating the scientific community with the aim of conserving organs from a living donor pending a recipient. The object is to block, or at least slow down, cellular functions while preserving their physiochemical structures. The dream of creating an organ bank to make available a series of organs for tailored reconstructions seems to be at hand.

Our work is divided into two chapters:

• In the first, we describe the general fundamental bases of cryobiology, particularly, written for clinicians to get a general approach of the basic elements of cryopreservation processes.

• And in the second, we review current knowledge of the cryopreservation of various types of biological tissues, which can allow us when considering cryopreservation of a composite organ to select the adapted cryoprotective agents for each component tissue.

Fundamentals of Cryopreservation

By definition, cryopreservation is the maintenance of biological tissues in a living state of suspended animation at cryogenic temperatures. At a temperature of -196°C, that of liquid nitrogen, all chemical reactions, biological processes and physical intra and extracellular activities are suspended. Theoretically, a cryopreserved organ can be kept indefinitely. The principle of cryopreservation must be differentiated from mere freezing of a tissue, like bone, which is done without addition of cryoprotective agents and at a temperature no lower than -80°C.

For a group of uniform cells, cryopreservation allows suspension of all chemical processes by preserving the cell's three-dimensional architecture indefinitely. The aim is to adapt this technique to multi-tissue composite organs, in which each tissue has its own reaction and resistance to freezing. The goal is unifrom cryopreservation of all tissue components of a composite organ, from the outside to the inside. With our current state of experimental knowledge, cryopreservation is routinely used for uniform tissues (skin, cartilage), for tissues of which a single cellular fraction suffices to assure functioning of the initial cell population (sperm), and for tissues that can repopulate

from precursor cells (bone marrow). However, cryopreservation of composite organs composed of structures and tissues of varying nature (vessels, bone, cartilage, tendons, ligaments, nerves, etc.,) has yet to be validated. The main problem is the preservation of the different cellular elements of the organ using adapted cryopreservatives, while respecting the three-dimensional architecture of the tissue matrix,[2] both during freezing and thawing of the organ. It is also fundamental that the cryopreserving agents used be adapted and specific to the tissue in question and that the phases of administration to and extraction from the organ be limited in time so as to reduce risks of direct cellular toxicity.[3]

At temperatures between 0°C and -25°C, the enzymatic activity of cells is only slowed but remains active, while below -40°C physiochemical exchanges are frozen. For longer preservation of a cell in the presence of cryoprotectors, temperatures must be below -130°C.

One can easily imagine the damage to cells when temperatures fall from +37°C to -196°C. This is mainly due to about a 95% loss of intracellular water, a considerable increase of electrolyte concentrations in both intra and extracellular media[4,5] and possible ice crystal formation in the intracellular spaces that deform and compress cells and even destroy intracellular structures.

Our current state of knowledge about the physiochemical processes occurring during cryopreservation and the existence of experimental models of the cell's reaction to cryopreservation allowed the development of precise protocols adapted to a large number of cell types.[6]

Physiochemical Aspects of the Ice Crystal Formation

Under isotonic conditions, water in biological tissues becomes thermodynamically unstable at temperatures below 0°C and tends to crystallize. During cryopreservation, the transition of water to a solid state is a central concern that takes into account the proportion of liquid in biological tissues, which may reach 70% of the volume. To master the process of crystallization, it is very important to know the kinetics of ice formation in both the intra and extracellular media.

All liquid solutions tend towards a solid state when submitted to temperatures below their equilibrium point of fusion. This process of solidification corresponds to the phenomenon of crystallization. During this process, the portion of the solution still in a liquid state is excluded from the crystalline matrix and becomes more and more concentrated. This increase in concentration lowers the point of fusion of the remaining solution. This process continues until a thermodynamic equilibrium is established between the crystalline and the solution states. The process of crystallization then stops.

Therefore, the quantity of ice crystals that forms at a given temperature depends on the initial composition of the solution. This can be predicted by mathematical formulas. Consequently, the chemical composition of the initial liquid solution and the threshold of the thermodynamic equilibrium between the crystalline and liquid phases are the basic mechanisms regulating the crystalline portion, on the one hand, and unfrozen water, on the other.

Under certain conditions, ice formation depends on kinetic phenomena more than on thermodynamic constraints. Effectively, ice formation in a "super cold" state, a thermodynamically stable situation, can initiate an instantaneous, massive process of aggregation of water molecules, from which ice crystals propagate throughout the solution; this is the process of "super fusion".[7]

These two processes of ice formation, whether they depend on kinetic or thermodynamic parameters, are markedly slowed by an increase in the solution's viscosity. As one approaches the threshold of crystallization, a sufficiently high viscosity inhibits molecular diffusion and blocks transition from a liquid to a solid crystalline state. The solution will tend toward an amorphous, non-crystalline, meta-stable state. By cooling rapidly, the solution's fusion threshold is reached before crystallization can start. In this case, even if formed, the amount of crystals remains very limited and is inferior to 10^{-6}; this is so-called "vitrification".[8]

Theoretically, crystallization during cryopreservation of biological tissues can be controlled by increasing the viscosity of the liquid phase and its polymer concentration, and by using relatively rapid cooling speeds.

Biophysical Effects of Ice Formation

Crystallization, especially extracellular, plays a determinant role during cryopreservation of tissues. Indeed, ice formation in the intercellular space modifies the chemical environment, generating mechanical stress on cell walls by deforming them, which can initiate intracellular crystallization.

Cellular dehydration. The first consequence of ice crystal formation in the extracellular space is a change in chemical composition with an increase in ion concentration of the extracellular liquid. A concentration gradient therefore forms between the intra and the extracellular spaces, causing solutes to enter the cell and water to leave it. This cellular dehydration is more pronounced at low temperatures.[9,10] The cell membrane permeability coefficient is modified and it behaves like a semi-permeable membrane. Consequently, cells react to cold by a loss of cytoplasmic water.[11]

There are two types of cellular lesions due to dehydration:

• an increase in the concentration of salts in the extracellular medium, which modifies and denatures proteins and lipoprotein complexes that comprise the major part of cell membranes;

• crystallization of buffering salts leads to important variations of pH with consequent irreversible denaturation of certain proteins.

The process of intracellular dehydration has been formulated by Mazur. He demonstrated that the transfer of liquid is not a simple phenomenon of transmembrane diffusion, but depends wholly on modifications of the cell membrane.[12,13]

Mechanical stress. Extracellular ice formation generates mechanical stress that deforms the cell. The crystalline matrix sequesters cells into channels that contain residual liquid. The volume occupied by the liquid is a function of cell's volume.[14] As the temperature falls, the diameter of the channels shrinks, progressively deforming the cells more and more.[15]

Intracellular crystallization. In addition to mechanical and chemical stresses, extracellular ice formation appears to be

directly implicated in setting off intracellular crystallization.[13,16] The mechanisms of these interactions have not been elucidated, despite the elaboration of numerous hypotheses.[12,17-20]

A mathematical equation models the aggregation of intracellular ice nuclei into macroscopic crystals.[21] The speed of aggregation is proportional to dehydration of the intracellular compartment and to the drop in temperature in the cytoplasm.

When cells are cooled slowly, the flow of water into the extracellular medium is increased, which avoids intracellular supercooling and limits ice aggregation and intracellular crystallization. Cellular dehydration dominates the process of intracellular crystallization. In contrast, with rapid cooling, diffusion of water is relatively low and intracellular crystallization predominates. Thanks to mathematical models that take into account the gradient of water diffusion and parameters of intracellular aggregation and crystallization, one can predict what portion of cells will be subjected to crystallization and the fraction of intracellular liquid involved.[21]

Thermal shock. A brisk change in temperature during cooling can by itself cause cellular lesions, even in the absence of ice crystal formation; this is "thermal shock". Shock occurs essentially between +37°C and +15°C, but also between 0°C and -80°C. Lesions of the cell membrane occur by differential contraction of its various components. Lesion are linked to changes in the anion composition of the extracellular medium, especially of acetate, chloride, nitrate, iodine and sulfate anions. Thermal shock is clearly reduced by adding cryoprotective agents and specific phospholipids (phosphatydl serine) combined with controlled, relatively slow cooling.

Biological effects of ice formation. Chemical, mechanical and thermal stresses perturb the biological functions of tissues. Although the suspension of metabolic reactions at a cryogenic temperature (-196°C), which theoretically preserves tissues indefinitely, the phases of freezing and thawing can be nefarious. While the mechanics of cell and tissue lesions are not clearly understood, the level of cell survival at a given freezing speed is identical whatever the cell type. This level is low using either very slow or very rapid freezing speeds and is optimal at intermediate rates.

Stemming from these observations, Mazur et al.[22] proposed a hypothesis to explain the link between freezing speed and its effect on cells:

• At low speeds, lesions appear to be partially due to exposure to highly concentrated cell solutes, aggravated by cell dehydration, the so called "solution effect"[23] and by mechanical interactions between cells and extracellular ice crystals;

• At high speeds, there are evident correlations between cell lesions and the formation of intracellular ice crystals.[4] Cell lesions of mechanical origin[17] appear either at the level of the plasma membrane,[23] or at the level of the membranes of intracellular organelles.[24] Intracellular crystals may be small and relatively unstable. During slow thawing, they may aggregate to form larger crystals that are harmful; this is the "nucleation" phenomenon.

The optimal cooling speed should be adapted to allow sufficient dehydration of the cell and avoid premature intracellular crystallization.

Other damaging effects of a non mechanical nature[20] are induction of the formation of gas bubbles in the intracellular space[25,26] and osmotic effects linked to fusion of ice crystals during thawing.[27] However, such intracellular ice crystals may remain inoffensive if their extension is controlled.[17,28-30]

Lovelock[31] showed that a hypertonic solution can cause denaturation of lipoproteins resulting in red blood cell hemolysis. Other authors focused on the phenomena of cell contraction and retraction in response to hypertonicity of the extracellular medium. There seems to be a minimum vital threshold of the cell's volume[32] below which retraction is irreversible. Destruction of cell membrane lipids rigidifies the membrane and prevents it from returning to its initial volume.[33]

In addition to the "solution effect", extracellular ice generates lesions in places where it contacts the plasma membrane;[27] this is the "stress" phenomenon.[34,35] These lesions are due to dendritic expansion of ice crystals.[36]

Further experimentation is necessary to better understand the damaging mechanisms occurring during cryopreservation, especially as concerns slow freezing and thawing speeds.

Cryoprotective substances. Cryobiology was born in 1949 with the discovery by Polge of the cryoprotective properties of glycerol used to preserve sperm.[37] Since then, other substances with similar properties have been discovered without fully understanding their mechanisms of action in protecting the cell during cryopreservation.

They can be classified into two categories:

• Diffusible or intracellularly active cryoprotectors: They have molecular weights less than 400 and cross the cell membrane. The most commonly used are dimethyl sulfoxide (DMSO), glycerol and 1,2-propanediol.

• Non diffusible or extracellular cryoprotectors:, like polyvinylpyroldone, hydroxyethyl starch and certain sugars. These do not cross the cell membrane due to their high molecular weights.

Their mechanisms of action are the subject of several hypotheses. Schematically during cooling, cryoprotective substances form hydrogen bonds with water allowing maintenance of a liquid state at temperatures lower than the temperature at which it freezes. This unfrozen solute constitutes a solvent for electrolytes. Simultaneously, these cryoprotectors block crystallization by inactivation of condensation nuclei; this is the "chemical poisoning" phenomenon.

Certain cryoprotectors act to reduce the solution effect that precedes an important increase of the intracellular concentration of electrolytes. Cryoprotectors also protect cellular proteins from denaturation,[38,39] especially those of the plasma membrane by DMSO, through electrostatic interactions.[40] It is accepted that the presence of high concentrations of cryoprotectors, both in the extra and intracellular media, increases viscosity and considerably reduces nucleation and aggregation of crystals.[41] However, high cryoprotector concentrations themselves may induce cellular lesions via direct toxic effects.[42] This risk may nevertheless be limited by reducing the time of tissue exposure to cryoprotectors at ambient temperatures.

Paradoxically, the cryoprotective solution can generate cytoplasmic retention of water and consequently increase intracellular ice formation.[21,43] Moreover, the application and removal of cryoprotective solutions before and after cryopreservation can cause cellular lesions due to osmotic phenomena. Indeed, the immediate cellular response to contact with a cryoprotective substance is dehydration, with a loss of water due to exosmosis. To avoid rapid transmembrane transfer of water, cryoprotectors are applied and removed progressively by stepwise dilution of the concentration in the extracellular medium. On the other hand, the choice to use slow transfer of cryoprotectors must be weighed against the increased duration of cellular contact with the cryoprotector. A mathematical model of the transfer of water as a function of the speed of cryoprotector transfer has been formulated so as to optimize the application and removal protocols of cryoprotective solutions.[3]

Review of Experimental Work on Cryopreservation of Various Tissues

Different tissues and cells react differently to the freezing process. Ideal cooling parameters depend sometimes on contradictory factors and much remains unknown. Experimentation often allows determining the best cooling conditions (speed, levels, cryoprotectors). The aim is to avoid thermal shock, increase of saline concentrations to destructive levels, and alteration of the colloidal state of the cellular structure.

The optimal speed of cooling is that which maximizes cell survival. The cooling speed must avoid formation of intracellular ice, on the one hand, and important cellular dehydration, on the other, both of which can distort the cell irreversibly. Maximal survival is in a zone of speed called the "transition zone", in which the combined effects of both mechanisms are attenuated.

Experimental work specific to each tissue, which we review below, has defined optimal conditions of cryopreservation for each tissue.

Cryopreservation of Cutaneous Tissue

Most experimental studies concern dermal-epidermal skin segments exposed to cryoprotective substances. These grafts are of either bovine[44,45] or human origin;[46-48] Two publications concern samples of isolated keratinocytes[44] and microsurgical skin strips with their nutritive vascular pedicle, through which cryopreservatives were administered.[49]

Dermal-epidermal grafts are easy to study, but are limited, particularly as concerns inhomogeneous diffusion of the cryoprotector throughout the thickness of the graft. The tissue concentration of the cryoprotector within the graft, as measured by ^{1}H nuclear magnetic spectroscopy, does not exceed 44% to 69% of the concentration in the exterior medium.[45] Keratinocytes in the basal layer were the last to be impregnated.

Tissue and cell viability have been measured by:

- the rate of oxygen consumption.[44,45]
- activity of tetrazolium reductase.[44,46,47]
- Nucleation and ice crystal aggregation in cellular and interstitial compartments using optical and electron microscopy.[44]
- The take of grafted cutaneous segments and revascularization of microsurgical allotransplants.[48,49]

Results

1,2 propanediol. 1,2 propanediol,[46] a diffusible intracellular cryoprotector, has the main advantage of low toxicity for cutaneous tissues, even at high concentrations. The best levels of survival were obtained with concentrations of 10% to 20% and slow cooling speeds (-1°C/min).

2 M glycerol. This intracellular cryoprotector in contact with dermal-epidermal grafts for 10 minutes at 4°C or 3 minutes at 22°C, allowed optimal survival (100%) when using rapid cooling (-5,100°C/min) and reheating (5,400°C/min). Effectively, optical microscope examination of samples showed a clear reduction of crystallization in the derma and epidermis, expect for crystals with a diameter no greater than 0.5 µm. Moreover, the dermal conjunctiva and structure of collagen fibers were completely conserved. This protocol offers condition of optimal protection of dermal-epidermal structures and can be applied to other fine tissue and membrane structures.[45]

Trehalose. The non-diffusible extracellular type of trehalose was tested in association with dimethyl sulfoxide (DMSO) and compared under the same conditions to cryopreservation protocols using glycerol alone, DMSO alone, or a combination of both. The results demonstrated the advantage of associating an extracellular cryoprotector in the sugar family with classic diffusible cryoprotectors.

An experimental study published by a Japanese group in 1993 reported a cryopreservation protocol for microsurgically revascularized cutaneous strips from rats.[49] These strips were stored at -196°C after perfusion and immersion with glycerin. Upon thawing, glycerin was completely removed and the strips were allografted and revascularized by microsurgery.

Finally, it should be noted that skin sampled for cryopreservation is systematically treated with a mixture of antibiotics to avoid development of latent microbes. The antibiotics used most often have a wide spectrum and associate an aminoside (gentamycin) and rifampicin. Experiments with cutaneous segments incubated with amphotericin B show a significant reduction of tissue and cell survival. This anti-fungal should not be used in anti-infection protocols.[47] *(Note: anti-fungals are strong DNA synthesis inhibiters).*

Cryopreservation of Blood Vessels

Blood vessels have been widely studied with the purpose of defining the best parameters of conservation and improving levels of endothelial cell survival.

Initially, cryopreservation studies of rabbit carotid arterial segments used DMSO, applied and removed in a stepwise manner to avoid osmotic changes, in association with controlled cooling, storage at -180°C, and rapid thawing. Fracture of the circumference of the arterial walls occurred systematically.[50,51] These

fractures occurred during the thawing phase between -150°C and -100°C. They were linked to thermal stress occurring in the cryopreserving solution upon "supercooling" around -123°C, the transition threshold temperature. Fractures could be avoided by controlled thawing of arterial segments at a speed of 50°C/min between -180°C and -100°C. Thereafter, thawing could be done rapidly in a water bath at 37°C. More recently, others have confirmed the advantage of slow thawing speeds that protect arterial segments from parietal fractures.[52,53]

Most experiments on the cryopreservation of vessels evaluated two parameters: survival rate of endothelial cells and recovery of the contraction-relaxation functions of parietal smooth muscle fibers.

The best endothelial cell survival rates were obtained with arterial segments cryopreserved in DMSO using a cooling speed of 1°C/min, storage at -145°C and slow thawing.[54,55] Survival was around 89% and the cell injuries (oedema, ergastoplasmic reticulum deformation, mitochondrial oedema, nuclear chromatin condensation) were completely reversible. Studies of cryopreservation of endothelial cells in suspension tend towards slow thawing speeds.

In order to avoid direct toxicity, it is fundamental to reduce times of exposure of smooth muscle fibers to cryopreservative substances.[57] All studies agree that recuperation of contraction of smooth muscle fibers upon stimulation with noradrenalin, 5-hyrosytryptamine, norepinephrine and thromboxane is no greater than 30–40%, while the relaxation response to acetylcholine, papaverin and bimakalin is conserved.[55,58,59]

Protocols for cyropreservation of vascular segments, as for all others tissues, use antibiotics. Inclusion of imipenem and fluctosine[60] during incubation at 37°C or cryopreservation is without risk for endothelial cell survival.

Finally, cryopreserved arterial segments have been used as shunts on femoral arteries of rabbits. Permeability reached 66%.[61] More recently, a clinical study reported the use of cryopreserved tibial artery allografts for the reconstruction of arterial axes in the legs of 35 patients. The results are of interest and showed 75% permeability of allografts at 12 months and 59% at 18 months, with a 80% rescue rate of legs at 12 months and 73% at 18 months.[62]

Bone Tissue Cryopreservation

In tissue banks, it is customary to freeze long bone segments for the reconstruction of large bone substance loss and femoral heads for natural filling material, in particular around whole hip prostheses.[63] Stored at -80°C, bone behaves like a simple graft after implantation with revascularization by the surrounding tissues. Spongy tissue is restored and revascularized in about three weeks, whereas the process may require several years for the cortex. The mechanical properties of cryopreserved bone are comparable to those of fresh bone, except for increased fragility of the diaphysis. Lastly, the main advantage is a lack of antigenicity of the protein-mineral complex. An antigenic activity could arise from leukocytes still present in the bone marrow of the donor tissue.

The aim of bone cryopreservation is to respect the mineral and phosphocalcium framework of the bone. Once grafted, the framework is progressively restored with osteoblasts from the recipient's bone. Some experimental studies also show that it is possible to preserve osteoblasts in cryopreserved bone.[64] Osteoblasts can be cryopreservation in segments of iliac crests at -80°C in DMSO for 48 hours, followed by progressive thawing in a water bath at 37°C. The major advantage of this resides in the acceleration of osteoblast integration and consolidation of the bone graft, while reducing cortical fragility.

Cryopreservation of Periosteum

In 1993, a Canadian group studied cryopreservation of perostial tissue. Thanks to its osteogenic capacity, this tissue can be used to reconstruct areas in damaged articulations and devascularized bone.[65]

In rabbits, periostial grafts, cryopreserved in DMSO at -192°C, retain their ability to synthesize proteins when the cooling speed is -1°C/min. It was identical to that of fresh periostial grafts, even if the grafted tissue had been frozen for 4 months.[65]

Cryopreservation of Cartilage

Today, there are no curative treatment or satisfactory reconstruction procedures for lesions of cartilage. Attempts to isolate, culture and graft chondrocytes have yet to be successful. Numerous experimental studies have tried to develop cryopreservation of cartilage, either by studying whole cartilage segments like the meniscus, or by exploring the activity of isolated, cryopreserved chondrocytes.

Studies on the meniscus did not show a significant difference between cryopreservation and simple freezing. Effectively, comparison of cryopreserved and frozen menisci showed:[66]

- Preservation of the macroscopic appearance of menisci and maintenance of a normal healing process in areas of capsular contact, with no sign of rejection in either group;
- Similar increased water content with a progressive decrease of glycoaminoglycans in both groups.

The only difference was a more marked decrease of chondrocytes during the first week of cryopreservation of menisci.[67] Cellular repopulation was restored after three months of cryopreservation and approached a normal level after one year in both groups. Despite protection of chondrocytes by cryopreservation of menisci in 10% DMSO, cartilage cells underwent damage, especially at the level of intermediate cartilage. Chondrocyte damage tended not to heal and evolved towards definitive necrosis.[69]

Cyropreservation studies done on chondrocytes reveal the fragility of chondrocytes to the freezing procedure.[70] Indeed, even if the rate of chondrocyte survival is greater in the presence of a cryoprotector (10% DMSO), it remains insufficient. Freezing has a certain deleterious effect on the chondrocyte.

Some authors tried to study the viability of chondrocytes in cartilage grafts "hibernated" at low temperature (4°C), then cultured in Dulbecco's modified Eagle's medium.[71] Potential residual protein synthesis by chondrocytes, as measured by ^{35}S-methionine

uptake, showed a decreased uptake of radioactivity of only 0.8% after 24 hours and of 6.4% after 48 hours. Contrary to freezing and cryopreservation, hibernation at low temperature preserved an adequate chondrocyte population, provided storage was no longer than 48 hours.

Cryopreservation of Nervous Tissue

Cryopreservation of peripheral nerve allografts was studied by a group in Toulouse, France. As a model, they used a series of 4 cm long nerve samples taken from rat paws.[72] These specimens were cryopreserved in a mixture of several cryoprotectors (2,3-butanediol, 1,2 propanediol, polyethylene glycol and UW solution). Viability of Schwann cells was studied in function of a series of parameters:

- The concentration of cryoprotective agents (diluted 100, 50 or 30% in 4% albumin);
- Duration of exposure to cryoprotective agents (10, 15 or 30 minutes);
- Cooling speeds (3, 12 or 231°C:min);
- Extraction of cryoprotectors after reheating, either by rapid extraction, or progressively with a replacement solution.

The highest rate of Schwann cell survival was obtained for preparations exposed for 10 minutes to a 50% solution of cryoprotectors and progressive extraction, whatever the cooling speed used. The six nerve specimens in this group were allografted and compared to control fresh autografts. All allografts were rehabilitated with axonal growths of central origin.

Cryopreservation of Corneas

The cornea has greatly benefited from cryopreservation studies using numerous cryoprotective substances, in particular those with an intracellular action, like DMSO and glycerol.

DMSO. The use of DMSO as cryoprotector was disappointing. Indeed, attempts to xenograft human corneas previously cryopreserved in 2.5 M DMSO at -196°C to cats[73] failed, with as much as a 48% loss of the epithelial reserve, while control corneas lost only 8%. Moreover, these xenografted corneas thickened with apoptotic keratinocytes.

In 1999, Bourne et al.[74] demonstrated that the use of DMSO led to a 10% loss of the epithelial population after 24 hours of culture and a 27% loss after 36 hours. While preliminary results after 24 hours approximate those obtained with cryopreserved bone marrow cells used in clinical practice, the results obtained after 36 hours deserve further investigation, since cell loss appears to be very significant.

Polyvinylpyrrolidone (PVP). Experimental results obtained with PVP are more encouraging.[75] Effectively, rabbit corneas cryopreserved in a hypercalcium solution containing 3 mol/l DMSO and 40% PVP and cooled at speeds of less than 1°C/min show an 81% survival of the cell population. Hypocalcaemia limits ionic imbalances and epithelial edema during hypothermic phases. The association of DMSO and PVP was not toxic at 0°C. Although the cell population was preserved, electron microscopy showed the occurrence of detachment and loss of epithelial cells adhering to the basal membrane.

1,2 diol Propane (PROH). Rabbit corneas cryopreserved in 1.4 mol/l v/v PROH, cooled at 0.2°C/min to -80°C, and rapidly reheated at 20°C/min conserved the morphology and architecture of their epithelial cells.[76] These results confirm those of earlier experiments.[77]

Chondroitine sulfate. Chondroitine sulfate was used experimentally to evaluate survival of pig corneal epithelial cells.[78] Various parameters were studied:

- The concentration of chondroitine sulfate and of fetal calf serum in the cryoprotective solution,
- The cooling speed,
- The incubation period of corneas in the cryoprotective solution before beginning cooling.

After reheating cryopreserved corneas and culturing at 31°C, their density was evaluated by trypan blue and alizarin S red staining. The results showed that corneas cryopreserved in 2% chondroitine sulfate and 20% fetal calf serum, cooled at 1°C/min had a greater epithelial density (2,430°C/mm^2) than control corneas (3,395°C/mm^2). This technique proved superior to conventional preservation techniques.

Dextran. Results showed that corneas suffered minimal cell loss when cryopreserved in a milieu containing 10% dextran without fetal calf serum, with cooling at 1°C/min and reheating to 37°C. Dextran thus appears to be a cryoprotector adapted to the epithelial layer of corneas.[79]

Cryopreservation of whole organs. Cryopreservation of whole organs has been attempted with kidneys,[80,81] liver,[81,82] the heart,[83] trachea,[84-86] lung,[87] parathyroid glands,[88] embryos and ovarian tissue.[89,90] However, for the time being results and advances remain experimental.

Immunomodulation Due to Cryopreservation

Cryopreservation seems to modify the antigenicity of allografted tissues. Herein, we review publications that support this hypothesis.

Two reports analyzed the immunogenic behavior of whole organs after cryopreservation. The first concerns cardiac valves of "RT1n on RT1 Lewis" Brown Norway rats.[91] Indeed, cryopreservation protocols using cooling speeds greater than 10°C/min led to a significant decrease in immunogenicity compared to fresh valves. Immunomodulation was measured by cross-matching tests between cryopreserved cardiac valves from donor rats (RT1n) and recipient rat (RT1) lymphocyte responders. At this cooling speed, cardiac valve viability, evaluated by uptake of H^3-proline, was unaltered, whereas it was at lower cooling speeds.

The second experimental work concerned skin graft immunogenicity.[92] The stimulatory activity of C3H (H-2^k) skin-derived epidermal cells was measured after being mixed with responder lymphocytes from either BALB/c (H-2^d) or CBA (H-2^k) mice. Three different types of cryopreservation protocols were used:

- Cooling speed of 1, 30, 64 and 100°C/min in 15% v/v DMSO or
- Cooling speed of 30°C/min in 5% v/v DMSO, or

• Cooling speed of 30°C/min in 20% v/v glycerol.

The first protocol seemed to be the most efficient both for allograft viability and immunomodulation.[92] Effectively, the stimulatory activity of cryopreserved epidermal cells was no greater than 5%, compared to fresh epidermal cells. The number of Langerhans cells in cell suspensions apparently did not correlate with the stimulatory capacity of fresh or cryopreserved epidermal cells. This phenomenon is apparently due to a functional alteration of the immunogenic stimulatory capacity of the epidermal cells.

Other experimental studies concern the behavior of cells implicated in the immunogenic process. It seems that cryopreservation of bone marrow macrophage and mononuclear cells does not affect their morphology or their biological properties.[93] Their adherence is stable and they retain their ability to produce superoxide (O_2) anions, tumor necrosis factor (TNF) and interleukin-1 (IL-1).

Circulating T lymphocytes were tested before and after cryopreservation at -196°C for periods of 3, 14, 21, 35 and 50 days.[94] Whatever the duration of cryopreservation, lymphocyte recovery was close to 90%. Usually, resting human lymphocytes do not secrete cytokines. Stimulation, in particular with PHA, is therefore necessary. Their ability to produce cytokines (IL-2, IL-6, TNFα, interferon-γ, GMCSF) is not affected by cryopreservation. Production of IL-2 by cryopreserved T lymphocytes is greater after PHA stimulation than that by fresh lymphocytes.

The capacity to produce IL-6 was used to study peripheral mononuclear cells from healthy donors.[95] It appeared that cryopreserved mononuclear cells produced more IL-6 than did their fresh homologues.

Conclusion

Crypreservation trials with different types of tissue allow defining the ideal cryopreservation protocol for each of them. The synthesis of these protocols allows selecting parameters adapted to the cryopreservation of composite organs in order prepare an ideal combination of cryoprotectors adapted to each organ and its component tissues and cells.

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