20 Cryopreservation of human pulmonary tissues

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Pharmacological studies normally require freshly obtained tissues. These are usually taken from various animal species, although results obtained from human tissues are naturally the most predictable for human pharmacology. However, the main problems with use of human tissues are the irregularity of its supply and the quantity of material that can be utilized at one time, as the tissues change their *in vitro* characteristics progressively and deteriorate rapidly after removal from the body [1, 2]. Furthermore, while the usefulness of human tissues may be limited by factors such as age of the patient, concomitant disease, anaesthesia and medication, certain pharmacological questions can be only addressed when rare tissues, e.g., bronchi from asthmatic patients are available. Cryopreservation allows storage of living cells for virtually indefinite time and has become an important tool for the storage of human vascular tissues in pharmacological research [3, 4]. Recently, evidence has been presented that human airway smooth muscle preparations may also be stored by this technique with a wide variety of functional activities being well preserved [5–8].

Mechanisms of freezing injury

Freezing of living mammalian cells without cryoprotective additives generally induces severe cell damage and only few if any cells survive [9–11]. During cooling to subzero temperatures water tends to flow out of the cell to freeze externally and the cells shrink during this process (Fig. 20.1). If cooled too fast, cells are injured by the formation of intracellular ice crystals; if cooled too slowly, the cells will be injured by the "solution-effect", i.e., by changes in the composition of extra- and intracellular solutions since the concentration of extracellular salts in the residual unfrozen medium increases as ice is formed [9].

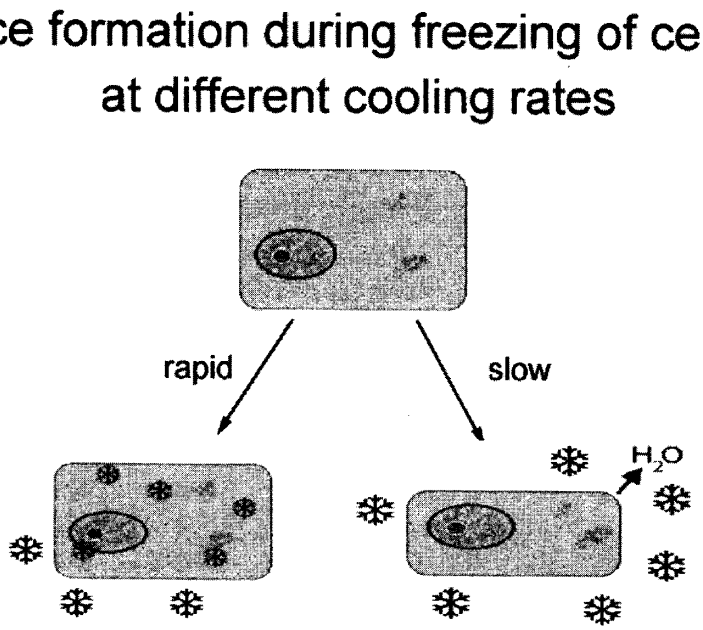

Figure 20.1. Ice formation during freezing of cells at different cooling rates
Ice nucleation is initiated around −5 °C outside the cell. If cooling is performed rapidly, small ice crystals form within the cell. During thawing these small ice crystals will fuse to form larger crystals and damage the cell membrane. If cooling is performed slowly, ice crystals are formed outside the cell and the environment of the cell becomes hyperosmotic. Consequently, the cell loses water and shrinks. During thawing these cells will be rehydrated.

Cryoprotective agents and cryomedia

To reduce the freezing injury during cryopreservation tissues must be suspended in a cryomedium consisting of a vehicle solution containing the cryoprotecting agents. Optimal protection against cryoinjury will be obtained by the combined action of a permeating and a nonpermeating cryoprotectant. The most commonly used permeating cryoprotectants are dimethyl sulfoxide (DMSO) and glycerol. It is assumed that these agents protect the cell from cryoinjury by entering the cell, thereby replacing some water [10]. Nonpermeating cryoprotectants such as sucrose, trehalose and hydroxyethyl starch, are suggested to stabilize the cell volume by retaining more liquid water at low temperatures, thereby reducing the external electrolyte concentration [9]. Both types of cryoprotectants act directly at the level of the cell membrane, yet their protective action may be synergistic

[12]. Most commonly used vehicles for the cryomedia are Dulbecco's modified Eagle's medium [13–15], fetal calf serum [7, 8, 12, 16] and Krebs-Henseleit solution [5, 6, 17, 18]

Freezing procedure and storage temperature

The optimal cooling rate differs from cell to cell and is dependent on both the water permeability of the cell membrane and the surface area to volume ratio of the cell. The presence of many different cell types within a tissue or organ implies, therefore, that no single freezing/thawing procedure can satisfy them all [10]. Moreover, in many peripheral arteries cells are quite densely packed together which may also reduce cell survival. Veins and pulmonary vessels are considerably less compact and show often better post-thaw recovery than peripheral arteries. To minimize the effects of cryoinjury, tissues must equilibrate for 10–30 min with the cryomedium before being slowly frozen. Prolonged prefreezing exposure to a DMSO-containing medium may attenuate the post-thaw functional activity [17, 18]. For mammalian cells the optimal cooling rate may range from 0.3 to 10 °C per minute, but once a sample is cooled to about –70°C, it can be transferred directly into liquid nitrogen (–196°C) and stored there for virtually infinite time until use [9, 10]. Although storage at higher temperatures (–70 to –85 °C) of both vascular [19] and airway tissues [14] has been shown to allow short-term storage for 3–4 weeks, this temperature range does not provide truly long-term survival of mammalian cells [15, 20].

Thawing procedure

During thawing ice is converted into free water, cells are exposed to an extracellular hypotonic solution and the dehydrated cells must rehydrate in order to remain in osmotic equilibrium. Serum or other high molecular weight polymers in the medium are suggested to reduce the damaging effect of this dilution shock [9]. Indeed, experimental evidence suggests that for many cell types, e.g., for endothelial cells in dog coronary arteries, it seems to be important to have at least 20% serum in the cryomedium [21]. However, considerable species and/or organ differences appear to exist. Thus, in human mesenteric and coronary arteries

endothelium-dependent responses to bradykinin and substance P are not modified by serum supplementation of the cryomedium [22]. The same applies for human bronchi, the post-thaw functional recovery of which is unchanged by the addition of serum to the cryomedium [5].

With isolated cells in general a rapid rate of warming, which limits the growth of ice crystals in the frozen samples, is applied. Thawing of isolated tissues at a high warming rate, however, may lead to mechanical stress inducing fractures which occur at temperatures between –100 °C and –150 °C [23]. This can be prevented by a slow rewarming procedure, i.e., by allowing the sample to reach slowly a temperature of about –70 °C prior to thawing in a 40 °C waterbath [23–25].

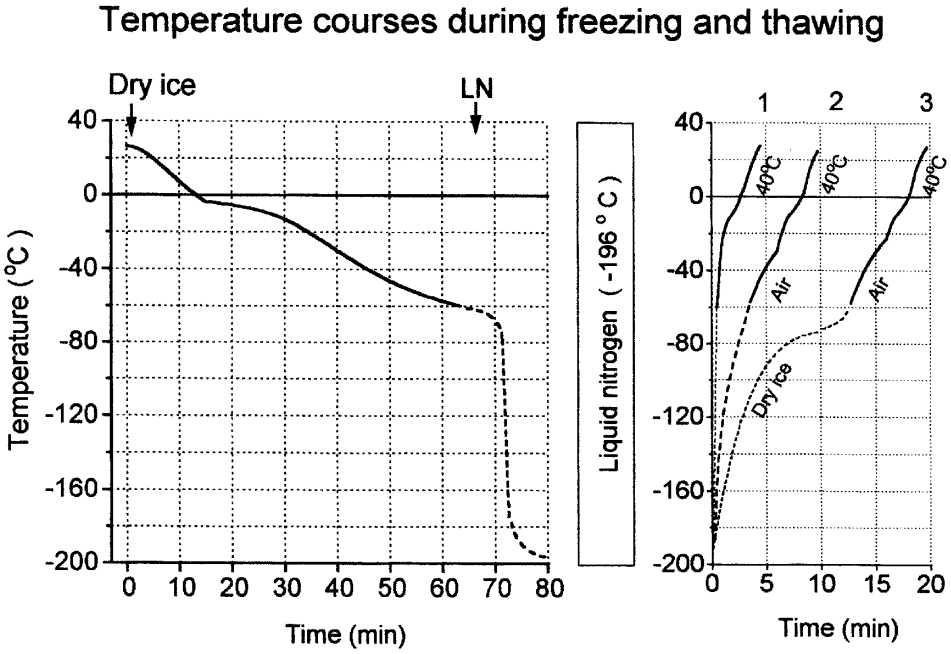

Figure 20.2. Temperature changes recorded during freezing and thawing within small segments of human internal mammary artery suspended in 2 ml plastic cryotubes containing Krebs-Henseleit solution with 1.8 M DMSO and 0.1 M sucrose

Left: The samples were placed in a polystyrol box and slowly frozen to –70 °C (dry ice) before being transferred into liquid nitrogen (LN). Right: Different thawing procedures: 1) the samples were thawed rapidly by placing the cryotubes during 4 min into a 40 °C waterbath, or 2) samples were first exposed for 6 min to room temperature before being thawed within 4 min in a 40 °C waterbath, or 3) samples were placed during 10 min on dry ice and then exposed for 6 min to room temperature before being thawed within 4 min in a 40 °C waterbath. The dotted lines indicate extrapolated time-courses.

Temperature courses during freezing and different thawing procedures are illustrated in Figure 20.2. After thawing of isolated mammalian cells often a stepwise dilution protocol is used, in order to avoid osmotic shock which may kill cells when returned to isotonic solution [10]. In isolated venous tissues such as canine saphenous and rat portal veins, which had been frozen in fetal calf serum containing 1.8 M DMSO, this procedure did not improve the post-thaw contractile function (E. Müller-Schweinitzer, unpublished observations). However, recent experiments on arterial tissues have shown that adding and removing cryoprotectants in a manner that avoids severe osmotic consequences, may improve the post-thaw function of both smooth muscle and endothelial cells in rabbit carotid arteries [25].

Methods, material and equipment

Material and equipment for cryopreservation of isolated tissues are summarized in Table 20.1.

Cryopreservation

Freezing procedure

1) Specimens of human lung, (obtained from surgery or multiple organ donors), are immersed in buffered physiologic salt solution, e.g., Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, pH 7.4), to be transported to the laboratory as soon as possible (within 24 h) after retrieval.
2) Small bronchi and/or pulmonary blood vessels (inner diameter $\approx 1-4$ mm) are excised and cleaned of surrounding tissue.
3) Tissues are cut into segments or rings suitable for an *in vitro* experiment and placed in 2 ml Liquid Nitrogen Storage Ampoules (Nunc Products) containing about 1.5 ml cryomedium.
4) After an equilibration time of 10–30 min at room temperature the samples are frozen slowly to −70 °C at a mean cooling rate of

Table 20.1. Material and equipment for cryopreservation

Tissues, media and equipment	Facts	Comments
Human tissues	Lung samples containing bronchi and pulmonary arteries (2–4 mm ∅) obtained from surgery for cancer or from multiple organ donors	Material should be processed as soon as possible (<24 h) after retrieval
Cryomedium	Krebs-Henseleit solution (with 1.2 mM $CaCl_2$, pH 7.4) containing 1.8 M DMSO + 0.1 M sucrose	Higher concentrations of $CaCl_2$ will precipitate
Cryotubes	2 ml plastic cryotubes with screw-top closure (internal threads).	To minimize explosion potential during thawing.
	Single-use volumes of 1–1.5 ml cryomedium may be prepared and stored at –20 °C to be readily available if required	This volume is sufficient to store material for up to six rings for *in vitro* studies
Polystyrol box (outer size about 5×7×15 cm)	Wall thickness about 1–2 cm required for slow freezing of the samples to –70 °C in a freezer or in dry ice	Cryotubes must be densely packed, empty space filled with paper tissue
–70 °C Freezer	For slow freezing of samples in a polystyrol box	If a programmable freezing apparatus is not available
Cryocanister	Container filled with liquid nitrogen to store the samples at –196 °C	The level of liquid nitrogen must be continuously monitored and refilled regularly
Liquid nitrogen		One cryocanister for 600 samples requires about 750 l liquid nitrogen per year
Dry ice (–70 °C)	For transportation and slow re-warmingof the samples before use	

1 °C/min. The ideal cell cooling rate can be provided by a programmable-rate freezing apparatus. If this is not available, slow freezing can be achieved by packing the ampoules into a polystyrol box (5×7×15 cm) which then is placed in a freezer maintained at –70 °C.

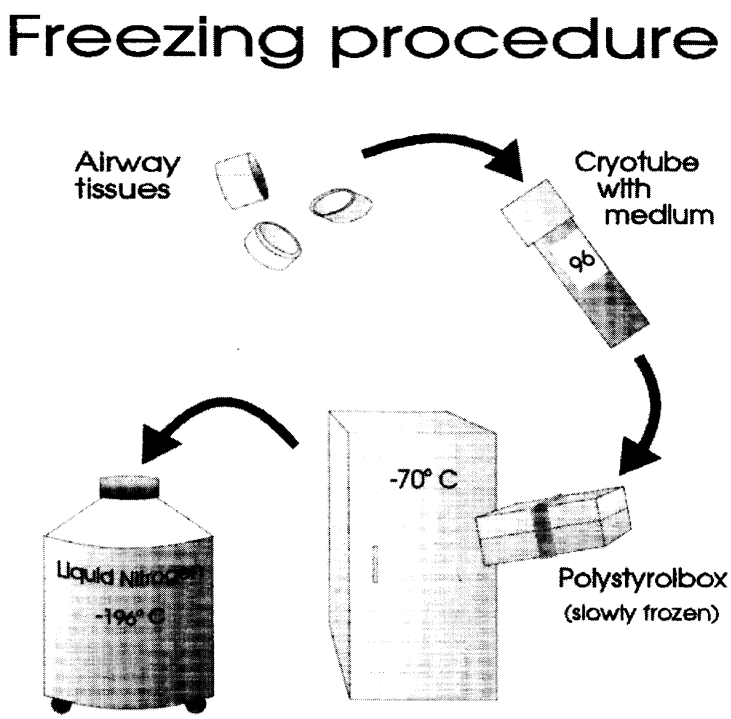

Figure 20.3. Illustration of the freezing procedure for isolated tissues to be used for* in vitro *experiments

5) About 1.5–15 h later the ampoules are transferred into liquid nitrogen (–196 °C) where they can be stored until used for *in vitro* experiments.

Temperature and sample handling during storage

The level of liquid nitrogen in the cryocanister must be continuously monitored and refilled if required. Make a note on a calendar whenever liquid nitrogen was added to the unit. Each time when a frozen sample is exposed to a warmer environment, even briefly, it experiences a change in temperature (Fig. 20.2) which may have detrimental effects on the viability of the frozen sample. Box stacking systems necessitate exposure of boxes at the top to warmer temperatures when retrieving samples from

lower boxes. If possible, use a cryocanister with aluminum canes to store the samples. The cryotubes are pressed onto the cane which is then placed into an aluminum or cardboard cryosleeve to eliminate the potential for the tubes to fall from the cane. This system maximizes the available space and needs considerably less liquid nitrogen than a box stacking system. When retrieving a frozen sample the cane should be lifted only to a level that exposes the requested sample without removing the remaining ampoules from the cryosleeve. Work as quickly as possible!

Thawing procedure

1) The selected sample (2 ml cryotube containing the frozen tissue) is removed from the liquid nitrogen and placed for 10 min on dry ice to warm up slowly to –70 °C.
2) Thereafter the sample is exposed for 6 min to room temperature.
3) Rapid thawing is achieved within 4 min by placing the ampoule into a 40 °C waterbath. Some gentle agitation of the tube during this period may accelerate the thawing process.
4) The whole content of the cryotube is then added to about 20 ml Krebs-Henseleit solution at room temperature before the tissue samples are transferred into fresh Krebs-Henseleit solution to be prepared for the *in vitro* experiment.

Inventory control

Numerous information may be important for the future and should be kept on appropriate records. Each vial must be labeled, e.g., by consecutive numbering, to allow identification of type and origin of the content, preservation methodology used, and location of the stored material in the cryocanister. Ideally three records are kept:

1) One record ("*donor record*") lists informations about each patient or organ donor such as age, sex, disease, drug therapy and time between retrieval and cryopreservation of the tissues.

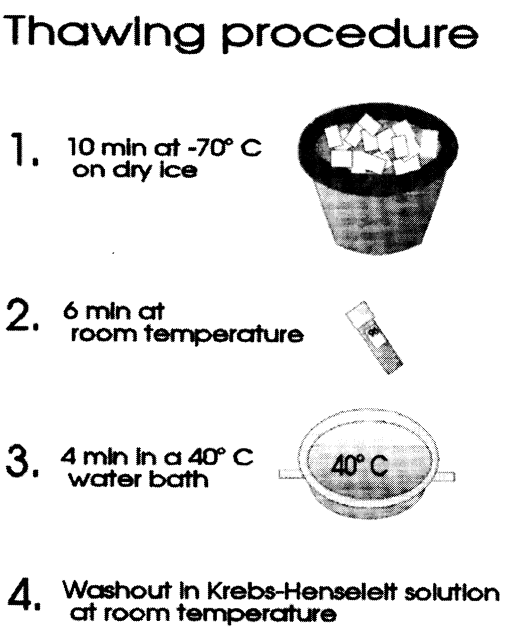

Figure 20.4. Illustration of the thawing procedure for the frozen samples to be used for* in vitro *experiments

2) A second record ("*sample record*") should contain the identification numbers of the samples, patient's identification number, source, type and amount of the tissue, date of cryopreservation, applied freezing procedure and date of use.
3) A third record ("*location record*") shows the location of each frozen sample in the cryocanister allowing rapid retrieval of a cryopreserved tissue with minimal risk of warming other samples.

Post-thaw functional recovery

Following suspension of *human bronchi* in the organ bath the tissues normally develop spontaneous tone. After cryopreservation this spontaneously developed tone amounts to about 50% of that observed in unfrozen bronchi [26]. However, the same is true for unfrozen human bronchi after storage overnight at 4 °C [27]. In some studies, after maximal re-

laxation of cryopreserved human bronchi by the combined action of papaverine with isoprenaline or bimakalim, the remaining passive resting tension in frozen/thawed bronchi was about 70% higher than in unfrozen controls suggesting that the cryopreservation process attenuated the bronchial elasticity [5]. However, other investigators found no evidence for any cold-induced reduction in the elasticity of human bronchi [7].

Functional responses of cryopreserved human bronchi to a large variety of contractile and relaxant agonists and antagonists have been tested and compared to those of unfrozen control tissues. Though eventually maximal responses to some agonists of cryopreserved bronchi tended to be slightly diminished, these differences rarely reached statistical significance. The same was true for the sensitivity as assessed by the pD_2 values (negative logarithm of the molar concentration of an agonist producing 50% of maximal response) of these agents [5–8]. It has been shown, furthermore, that accumulation of [3H]-inositol phosphate during incubation with acetylcholine was similar in both unfrozen and frozen/thawed human bronchi indicating well preserved phosphoinositide turnover in cryopreserved human bronchi [7]. In addition to a well maintained preservation of post-thaw smooth muscle function, the integrity of human bronchial epithelium has been shown to be largely maintained after cryopreservation as well. Evidence for this has been presented by both light microscopy [7] and by demonstration of the ability to produce epithelium-derived inhibitory factor(s) (EpDIF) of cryopreserved human bronchi [6]. However, in spite of a good preservation of post-thaw contractile and relaxant responses to various agonists, the contraction following application of antigen to bronchi from sensitized donors was not preserved when tested after cryopreservation, indicating that cryopreserved airway tissues would be unsuitable for studies in which the effect of sensitization status is pertinent [8].

As has been documented for various human blood vessels [1, 3, 4, 18, 19, 22], well preserved functional activity has also been shown for cryopreserved *human pulmonary arteries* [16]. These comparative studies were performed with a cryomedium consisting of fetal calf serum containing 1.8 M DMSO without addition of sucrose. Moreover, in that study no attention was paid to the pre-freezing equilibration time. Despite some reduction, by about 25%, of the contractile responses to various

agonists of the cryopreserved pulmonary arteries, there was a significant correlation of the apparent pD_2 values. The same was true for different relaxant agonists tested on arterial rings which had been precontracted by a submaximal concentration of the stable thromboxane analogue U46619. Although in these comparative experiments the preservation of endothelial function was not specifically investigated, a relaxant response to low concentrations of histamine in some of the frozen/thawed arteries suggested that the endothelial function may also be preserved after cryostorage of human pulmonary arteries [16].

Conclusion

Despite the relevance of human isolated tissue for human pharmacology, its use is still very much the exception rather than the rule. The major reason for this is that the supply of fresh human material is both irregular and unpredictable, and once removed from the patient, the tissue has a very short life span. Storage of isolated blood vessels in physiologic salt solution at 4 °C induces rapid and progressive changes of physiologic and functional properties within a few days. The method of cryopreservation and storage at –196 °C offers the prospect of virtually indefinite storage of both human airway and vascular tissues and may be used in pulmonary research and drug development. It may also be used to investigate pathophysiological mechanisms in diseased airway tissues such as bronchi from asthmatics and from patients who died during an asthmatic attack [26]. The method ensures the availability of human vascular and airway smooth muscle preparations with well maintained functional activity of enzymes, contractility and epithelial function.

In summary, despite certain problems such as maintenance of responses to antigen and some reduction in contractile force, affinities of most agonists and antagonists, phosphoinositide turnover and epithelial function have been shown to be well preserved in human bronchi after cryopreservation. Hence, this technique offers clear potential for ensuring the supply of both human airway and vascular material for pharmacological studies.

Safety recommendations

- Use buffered salt solution or culture medium (never 0.9% NaCl solution) for storage and transportation of tissues before cryopreservation.
- Avoid accidental rewarming during handling of frozen samples, e.g., when transferring ampoules from −70 °C into liquid nitrogen or when retrieving a selected sample for an experiment.
- Use plastic ampoules with internal thread screw-top closures to minimize explosion potential during thawing.
- Be cautious when handling human material which might contain infectious viral agents.
- Be cautious when handling DMSO which is quickly absorbed through the skin and might facilitate transport of potentially harmful substances into the body.
- Wear isolating gloves and a fullface shield whenever working with liquid nitrogen.

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