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Effect of cooling rate and its interaction with pre-freeze and post-thaw tissue culture on the in vitro and in vivo function of cryopreserved pancreatic islets

J. Foreman, H. Moriya*, M. J. Taylor

MRC Medical Cryobiology Group, University Department of Surgery, Douglas House, Trumpington Road, Cambridge CB2 2AH, UK

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Abstract. Rapid cooling destroys passenger lymphoid cells during cryopreservation. We now describe improved in vivo survival of rat islets after rapid cooling by adding pre-freeze tissue culture. Islets were equilibrated with 2M dimethylsulphoxide, cooled at 0.3°, 20°, or 70°C/min, and stored at −196°C. The culture was kept at 37°C for 2 or 72 h before and/or after preservation. When cooled at 0.3°C/min, keeping the culture for 72 h gave the highest proportion of dye-excluding cells, but more than 50% were viable under all culture conditions. Islets cooled at 20° or 70°C/min (rapidly) required 72 h of culture for a survival rate of more than 50%. When islets were cultured for 72 h before cryopreservation, their in vitro insulin secretory ability was similar to that of slowly cooled islets and they were able to sustain normoglycaemia in diabetic animals, although more islets were needed. Extended tissue culture before freezing improves the survival of rapidly cooled islets and is therefore important for combined immunomodulation and cryopreservation.

Key words: Pancreatic islets, cryopreservation – Cryopreservation, pancreatic islets

Introduction

It is well recognised that long-term banking of isolated pancreatic islets provides several advantages for islet transplantation, and this has led to the development of a variety of cryopreservation procedures [19, 29]. As with other multicellular tissues, slow cooling (< 1°C/min) has generally been favoured for effective preservation of islets from a number of species, yet few studies have undertaken to evaluate the effect of a wide range of cryobiological variables in an attempt to derive a truly optimized procedure [4, 19, 29, 31]. An earlier study from this laboratory sought to resolve the interaction between variables by examining, in tissue from a single species, the effect of a wide range of cryobiological conditions, particularly cooling rate, on the insulin secretory function of frozen islets [31].

It was shown that, providing full equilibration with 2 mol/l Me_2SO was achieved and warming was rapid, the cooling rate was not a major determinant of survival. The observations from this single study therefore corroborated the findings of a number of unrelated studies in which fast cooling was shown to be compatible with the functional survival of cryopreserved islets [4]. The suggestions from a number of investigations that islets can tolerate cooling at rates as high as 60°–70°C/min raised the possibility of using such a technique to preserve the islets whilst selectively depleting any contaminating lymphoid cells; the possibility of modulating the immunogenicity of islets by such a process, and the important clinical implications of achieving this, have been explained in several previous communications [30, 32, 35–37]. These studies showed that lymphocytes, macrophages and, most importantly, dendritic cells retain a high level of survival when cooled slowly (< 1°C/min) by the cryopreservation protocol used for freezing islets. Moreover, it was established that fast cooling (> 50°C/min) was necessary to facilitate a high degree of destruction of these immunostimulatory cells. Since techniques for altering the immunogenicity of isolated islets are now regarded as essential for preventing rejection of transplanted islets without reliance upon continuous immunosuppression, the precise functional capability and immunogenicity of rapidly cooled islets must be determined. This paper describes a detailed evaluation of the functional status of rapidly cooled islets as a prelude to an investigation of the possible effects of preservation on the immunogenicity of isolated islets.

Tissue culture of islets in vitro can influence both the immunogenicity of the tissue and the outcome of

* *Present address:* First Department of Surgery, St. Marianna University, 2-16-1 Sugao, Miyanmae-ku, Kawasaki, Kanagawa, Japan

Correspondence to: M. J. Taylor, Department of Neurosurgery Research, Medical College of Pennsylvania (Allegheny Campus), 9th Floor, South Tower, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15212, USA

cryopreservation [24, 25, 32], and we have recently reported that extended tissue culture before and after cryopreservation was found to be essential for the retention of insulin secretory function of rapidly cooled islets [8]. This corroborated a number of earlier reports that a period of tissue culture prior to freezing at 5 °C/min afforded improved survival of cryopreserved islets [14, 24, 26]. The scope of the present study is broader, using a spectrum of viability assays, and it was designed to examine the interaction of pre-freeze and post-thaw culture with the rate of cooling upon both the in vitro and in vivo status of frozen/thawed islets.

General viability of islets was assessed by measuring membrane integrity using fluorescent dyes, and in vitro function was assessed by measuring insulin secretion during both a static batch incubation assay and during perifusion. Transplantation studies were also included to assess the in vivo survival of rapidly cooled islets since this has not previously been reported and has important implications for attempts to reduce tissue immunogenicity during cryopreservation.

Materials and methods

Isolation and in vitro culture

Wistar albino Glaxo (WAG) rats, of either sex, weighing between 150 and 200 g were used in this study. Anaesthesia was induced with diethyl ether and maintained by sodium pentabarbitone (40 mg/kg body weight IP). The technique for the isolation of islets was based on the method described by Sutton et al. [28], which itself was based on a technique developed by Gray and his colleagues for the isolation of human islets of Langerhans [9]. The pancreas was exposed through a transverse abdominal incision and the distal end of the bile duct ligated as close to the duodenum as possible. The bile duct was cannulated at the proximal end using a 23-gauge butterfly needle that had been slightly blunted to avoid tearing the bile duct. It was necessary to tie the cannula in place to prevent leakages. The donor was sacrificed by exsanguination and 3–4 ml of a solution consisting of 3 mg/ml collagenase (Sigma Type 1A) in Dulbecco's phosphate buffered saline (PBS) at 0 °C was slowly injected into the pancreas via the bile duct cannula. The pancreas was then excised and held on ice in a pre-cooled beaker prior to incubation at 37 °C in PBS. Tissue digestion was arrested after approximately 20 min by replacing the digestion solution with 20 ml of cold PBS containing 10% fetal calf serum (PBS/FCS). The pancreases were then disrupted by gentle aspiration from a 10-ml syringe through a 14-gauge stainless steel needle and washed several times with PBS/FCS. Free islets were then separated from undigested tissue and exocrine cells by centrifugation at 1000 *g* for 15 min in a solution of Ficoll/Hypaque (SG 1.078) [39]. The harvested islets were washed twice in PBS/FCS and finally in RPMI 1640 medium containing 11 mmol/l glucose and 10% FCS (RPMI/FCS) before being transferred to culture dishes (30 mm; Sterilin, UK) containing RPMI/FCS. The yield per pancreas ranged from 800 to 1200 islets of size varying between 75 and 350 µm. The islets were incubated at 37 °C in an atmosphere of 95% air/5% CO_2 and 95% humidity, either for a minimum period of 2 h prior to freezing or were cultured for an extended period of 72 h, in which case the culture medium was changed after 48 h.

Preservation procedures

Exposure to cryoprotectant. In preparation for freezing, the islets were fully equilibrated with 2M dimethyl sulphoxide (Me_2SO) using a similar technique to that described previously [31, 34]. Batches of 400–500 islets of random size, in 250 µl RPMI 1640/10% FCS, were added to 5-ml polypropylene freezing vials (Sterilin, UK) and fully equilibrated with 1M Me_2SO during 30 min of exposure at room temperature, followed by 10 min of exposure to 2M Me_2SO at 0 °C in a final volume of 1.0 ml.

Cooling procedures. The ampoules were transferred to a cryostat bath thermostated at −10 °C and freezing was initiated by nucleating the samples after a further 10 min. After allowing release of the latent heat of crystallisation (5 min), samples were cooled at one of three controlled rates (0.3°, 20° or 70 °C/min) to −65 °C and then plunged directly into liquid nitrogen for storage. The lowest rate, 0.3 °C/min, was achieved in a small volume, programme-controlled cooling machine [11] that gave a linear rate of cooling to −65 °C. A rate of 20 °C/min was achieved by suspending a 5-ml polypropylene freezing vial containing islets in a 20-ml polystyrene universal container (Sterilin, UK) which, in turn, was placed inside a 50-ml polypropylene centrifuge tube and the whole immersed in liquid nitrogen until −65 °C was reached. A rate of 70 °C/min was achieved by supporting a 5-ml polypropylene freezing vial in a larger polypropylene vial (63 × 26 mm) containing 15 ml methanol. Temperature was measured with a copper/constantan thermocouple inside a 'dummy' sample consisting of the freezing mixture alone in similar tubes.

Thawing and removal of cryoprotectant. Samples of islets stored in the gas phase of a liquid nitrogen container were thawed rapidly, with gentle agitation, in a 37 °C water bath until the last crystals of ice had just melted. The cryoprotectant was removed gradually by stepwise dilution with RPMI 1640/10% FCS over a period of 15 min. During the dilution period, the addition medium was maintained at 0 °C in a container of ice while the islet suspension was placed on the bench at room temperature, thus allowing for a gradual warming from 0 °C to room temperature. Following the final dilution step, the islets were washed twice and centrifuged gently at 50 *g* for 5 min, followed either by incubation at 37 °C for 2 h or extended culture for 72 h prior to assessment of viability.

Islet recovery and membrane integrity

The extent of recovery of intact islets following the different preservation conditions was assessed in a separate group of parallel experiments in which batches of 100 randomly selected islets were processed. In this way a quantitative estimate of the recovery of the numbers of islets following each treatment was obtained.

For every batch of islets processed for the in vitro insulin secretion assays described below, the membrane integrity of the cells within a sample of the recovered islets was also assessed by supravital staining, using a combination of the fluorescent probes acridine orange (AO) and propidium iodide (PI). As discussed previously [35] and elaborated upon by Bank [5, 6], the assay differentiates between viable and non-viable cells by the simultaneous use of inclusion and exclusion dyes. Living cells are permeable only to AO, which binds to nucleic acids, causing the DNA to fluoresce green. PI, on the other hand, is excluded from living cells but can freely permeate through the membranes of dead or dying cells, excluding AO competitively, and produces a red fluorescence.

A group of ten randomly selected islets were placed, in a single drop, in a 30-mm tissue culture dish and mixed with an equal volume of a double strength AO/PI solution containing 20 µmol/l AO and 30 µmol/l PI prepared in PBS. The suspension was incubated for 10 min at room temperature before transferring the islets with a drop of fresh PBS, without stains, to the well of a 3″ × 1″ glass microscope slide having a central depression. The islets were examined using a mercury vapour light source fitted to a microscope (Reichert-Jung) equipped for epi-illumination. Two combinations of FITC filters were used for fluorescence microscopy: a module with a 490-nm excitation filter and 520-nm barrier filter (American Optical No. 2077) was optimum for the green fluorescence of AO and a module with a 546-nm excitation filter and 590-nm barrier filter

Table 1. Recovery of islet numbers[a] and gross structural appearance following culture and cryopreservation. C, Compact and rounded; L, discrete islets having a loose structure; F, fragmentation; D, cellular debris

Controls				
Freshly isolated	100			
2 h	100			
72 h	107 ± 2			
Culture time (hours)		Cooling rate (°C/min)		
Pre-freeze	Post-thaw	0.3	20	70
2	2	125 ± 7 (L, F)	103 ± 3 (F, D)	111 ± 25 (F)
2	72	109 ± 2 (C)	15 ± 3 (D)	11 ± 1 (F, D)
72	2	117 ± 9 (C, some L)	121 ± 32 (F, D)	120 ± 30 (L, F)
72	72	92 ± 3 (C)	80 ± 5 (D, some C)	82 ± 3 (C, some D)

[a] Indices represent the average (± range) number of discrete islet structures counted in duplicate samples after each treatment. Aliquots of 100 islets were dispensed at the beginning of each procedure

(American Optical No. 2074) was optimum for the red fluorescence from PI. Islets generally required exposure to UV illumination for a minimum of 5 min to permit development of full fluorescence before a semi-quantitative method was employed to assess the relative proportions of viable and non-viable cells in each islet. To maximise the objectivity of this test, the assessment was carried out without prior knowledge by the person performing the assay as to which experimental treatment each sample had experienced. Islets were assigned a membrane integrity index (grade 1–7) on the basis of the proportional estimate of viable (green fluorescence) and non-viable (red fluorescence) cells. This method of assessment is an extension of the three-grade procedure that has been shown by Bank to correlate well with the insulin content of isolated islets [6].

In vitro insulin secretion tests

The functional survival of islets was determined by their ability to secrete insulin, both statically and dynamically in vitro, in response to a graded challenge with D-glucose in a modified Krebs-Henseleit solution pre-gassed with 95% O_2/5% CO_2.

Static insulin release assay. Static insulin release was measured during batch incubations in a shaking water bath at 37 °C in groups of ten randomly selected islets during four consecutive periods (30 min, 30 min, 60 min and 60 min). The islets were placed in 2-ml capped incubation tubes (Eppendorf, Hamburg, Germany) and bathed in 1 ml of a modified Krebs-Henseleit solution containing 119.1 mM NaCl, 4.75 mM KCl, 1.26 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 15.00 mM $NaHCO_3$, 10.00 mM HEPES, 1 mg/ml bovine serum albumin (Sigma, RIA grade) and, in all but the third incubation period, containing a non-stimulatory level of glucose (2.8 mM). During the third period, the islets were challenged with a similar medium in which the glucose concentration had been increased to 16.7 mM and included 5 mM theophylline as a supplementary secretagogue to conform with previous studies [2, 8]. At the end of each incubation period, the medium was removed and stored at −20 °C for subsequent analysis of insulin content. Mean insulin output was calculated from five replicate samples of ten islets from each of four replicate experiments.

An additional group of experiments was included to evaluate the effect of extended tissue culture on the concentration of insulin measured during the initial phase of the static insulin release assay. We have previously reported that preserved and cultured islets often exhibit a high indiscriminate release of insulin during the initial non-stimulatory phase of the static release test [8]. One possible explanation not previously tested in our system is the contamination of the initial assay samples with insulin carried over with small amounts of culture medium when islets were transferred to the assay tubes. This was investigated in two sets of experiments by introducing either an extra period of incubation in basal medium or by washing the islets with low-glucose medium prior to the static release test. Samples of culture medium were also collected after 2, 24, 48 and 72 h for subsequent analysis of insulin content.

Dynamics of insulin release during perifusion. The time-course of glucose-induced insulin secretion was measured during in vitro perifusion using the method described previously [33, 34]. This technique involved pumping the medium at a rate of 1 ml/min over batches of 65 islets retained on a filter supported in a perifusion chamber. Effluent samples were collected at either 1- or 5-min intervals during three discrete periods. During the first and third periods, the chamber was immersed in modified Krebs-Henseleit medium containing a non-stimulatory concentration of glucose (2.8 mM); during the middle period the islets were perifused with a stimulatory medium containing 16.7 mM glucose + 5 mM theophylline.

A comparison of the total amount of insulin secreted during the different phases of the assay was made by measuring the areas under the perifusion curves, which were digitised using a Graf Bar GP7 ultrasonic digitizer (SAC, Southport Conn., USA).

The insulin content of assay samples and culture medium was determined by radioimmunoassay using the double antibody method of Hales and Randle [10] and a rat insulin standard (Novo Alle, Denmark).

Transplantation

Syngeneic transplants of islets were performed using male WAG recipients that were previously rendered diabetic by a single intravenous injection of streptozotocin (55 mg/kg). Under ether anaesthesia a midline laparotomy was performed and the portal vein was cannulated with a 25-gauge butterfly needle primed with the islet suspension. A purse string suture was used to close the hole in the portal vein after the islets in 1 ml of medium had been injected slowly and the needle withdrawn. The surgical wound was closed with a continuous suture (3-0 cat gut). Animals were fed ad libitum and allowed free access to water.

Intravenous glucose tolerance tests (IVGTT) were performed 1 month after transplantation. Glucose solution (1.0 g/kg) was administered via the penile vein of rats anaesthetised with Nembutal (40 mg/kg) and blood samples were collected from the tail vein at the following time intervals: −10, −5, 2, 5, 10, 20, 30, 40, 50, 60, 90 and 120 min. The rate of decay of serum glucose (K-value) was calculated using the method of Moorhouse [17]. Serum glucose concentrations were measured using the GOD-Perid (Boehringer) method after blood had been deproteinized with uranyl acetate.

Results

Recovery of islet numbers

The numbers of islets recovered after each experimental procedure and the general gross structural appearance of each population is given in Table 1. With the exception of the groups of islets cooled at 20° or 70 °C/min after only 2 h pre-freeze culture and cultured for 72 h after thawing, there was no significant reduction in the number of discrete islets recovered from any of the procedures. In some cases more than 100 islets were counted following culture

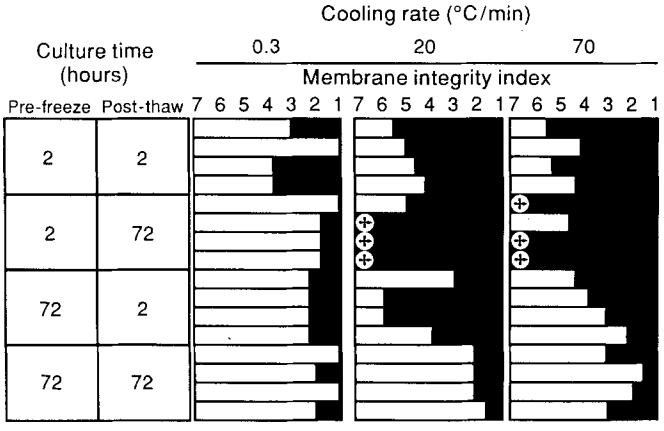

Fig. 1. Graphical representation of the membrane integrity assessment by the AO/PI fluorescence assay of cryopreserved islets showing the interaction between pre-freeze and post-thaw culture with the effect of cooling rate. Each *bar* indicates the average membrane integrity index assigned to individual groups of ten islets sampled from each batch used in the static insulin release test. Each *panel* shows the relative proportions of viable (green fluorescences; □) and non-viable (red fluorescence; ■) cells based upon a semi-quantitative method of grading. Islets were assigned a viability grade on a scale of 1–7. A score of 1 was assigned to islets that showed completely green fluorescence but no red/orange fluorescence, whilst islets that were completely red with no green fluorescence were graded 7. An index of 4 represented islets that contained equal proportions of green and red fluorescing cells; intermediate scores represented proportionately greater or lesser numbers of viable cells. The availability of insufficient numbers of islets in some groups to conduct the assay is indicated (+)

and preservation; this was assumed to be due to some fragmentation of the original islets. It was also observed that all groups of cryopreserved islets appeared 'loose' with 'ragged outlines' when observed immediately after thawing and dilution of the cryoprotectants. However, following 72 h post-thaw culture there was a marked change to more compact, rounded structures having a 'smoother profile'. Again the exceptions were islets cryopreserved using fast cooling (20° or 70°C/min) after only the minimum period of pre-freeze culture; under these circumstances extended post-thaw culture resulted in apparent disintegration of the recovered islets.

Membrane integrity test

Samples of cryopreserved islets from each batch used in the insulin release tests were assayed by the AO/PI test as described above. Figure 1 shows the semi-quantitative assessment of islet viability, and each panel indicates the average viability grade assigned to a minimum of ten islets from each thawed and cultured batch.

Measurements were made on four separate groups of islets for each experimental group except for the two groups with a minimal 2 h pre-freeze incubation period and an extended 72 h post-thaw culture period following either 20° or 70°C/min preservation protocols. In neither of these groups were there consistently sufficient intact islets remaining at the end of the experiment to conduct the assay. Islets cooled slowly at 0.3°C/min had more than 50% viable cells, irrespective of the combination of culture conditions. However, culturing for 72 h before and/or after freezing yielded the highest survival with islets containing few or no cells permeable to PI.

Islets cooled more rapidly at either 20° or 70°C/min showed less than 50% survival when a minimal pre-freeze culture period was employed. As was mentioned above, when the same islets were cultured for 72 h after freezing and thawing, there were insufficient islets in some experiments to allow for assessment.

In contrast, islets cultured for 72 h prior to rapid cooling (20° and 70°C/min) contained a much higher propor-

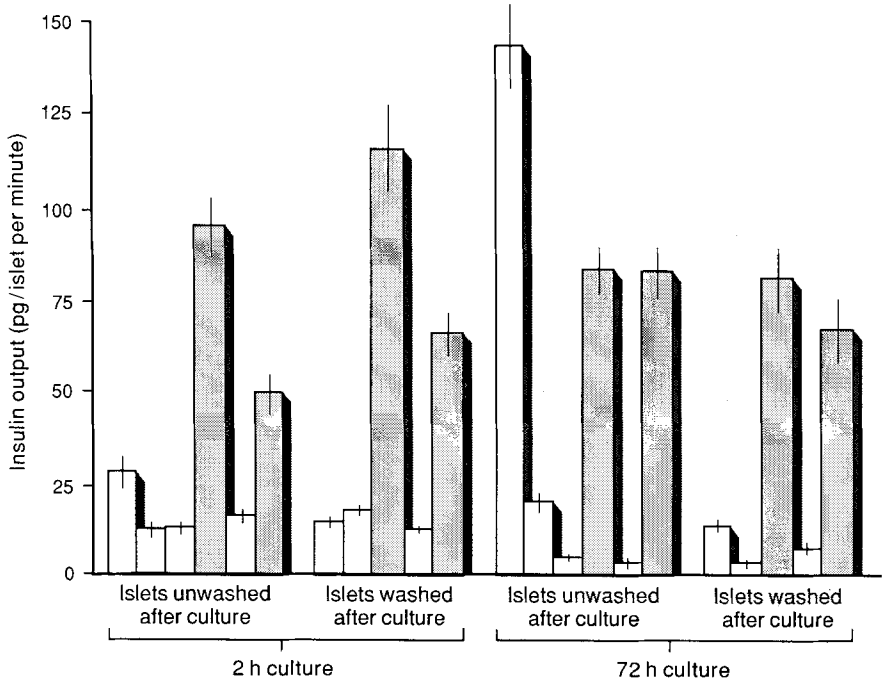

Fig. 2. The insulin secretory ability of unfrozen control groups of islets cultured in RPMI 1640 medium for either 2 h or 72 h and tested in the static insulin release assay with or without prior washing as described in the text. Insulin output from replicate batches of islets was measured during consecutive periods of incubation in either non-stimulatory (□) or stimulatory (■) medium ($n = 20 \pm 1$ SEM)

Table 2. Insulin content of culture medium during tissue culture of isolated islets

Culture time (hours)	Insulin content[a] (ng/ml)
2	316 ± 26
24	1838 ± 219
48[b]	2824 ± 212
72	1241 ± 297

[a] Mean (± SEM) insulin content of RPMI 1640 (containing 11.1 mM glucose) from duplicate islet cultures
[b] After 48 h of tissue culture, the medium was routinely changed for fresh medium

Table 3. Interaction of cooling rate and the time in tissue culture before and after cryopreservation on the insulin secretory capacity of frozen islets

Culture time (hours)	Static (S) or dynamic (D)		Stimulation factor[a]		
Pre-freeze	Post-thaw		Cooling Rate (°C/min)		
			0.3	20	70
2	2	S	2.8 ± 0.2	1.2 ± 0.1	1.4 ± 0.2
		D	2.9 ± 0.8	0.4 ± 0	0.8 ± 0.2
2	72	S	5.3 ± 0.8	3.0 ± 0.2[b]	1.3 ± 0.2[c]
		D	–	–	–
72	2	S	4.2 ± 0.4	3.2 ± 0.4	2.7 ± 0.3
		D	4.5 ± 0.2	1.0 ± 0.4	1.9 ± 0.2
72	72	S	6.1 ± 0.5	3.8 ± 0.5	5.4 ± 1.0
		D	8.0 ± 0.6	2.1 ± 0.3	4.2 ± 1.7

[a] Ratio of insulin secreted during stimulation to basal insulin release. Mean (± SEM) $n = 20$. Stimulation indices for non-frozen controls were as follows:
Cultured for 2 h: static = 7.2 ± 0.7; dynamic = 5.1 ± 0.6
Cultured for 72 h: static = 10.7 ± 1.3; dynamic = 4.1 ± 1.9
[b] $n = 5$. Sufficient numbers of recovered islets were not available in all replicate experiments for assaying in these groups
[c] $n = 10$

tion of viable cells; as shown in Fig. 1, the proportion of cells able to exclude PI was generally greater than 50%. Islets cultured for 72 h, both before and after freezing at these same rates, demonstrated very high recovery indices with very few cells showing permeability to PI.

In vitro insulin release

Measurement of basal insulin release following extended culture

Levels of insulin secreted by non-frozen control islets during the stimulation and non-stimulatory phases of a static release assay are shown in Fig. 2. The insulin output from islets cultured for only 2 h after isolation increased five to sixfold over basal levels during the first period of stimulation. Figure 2 shows that these groups of islets were able to return to basal levels of secretion and retain their ability for subsequent stimulation by further incubation in high-glucose medium. It should be noted that basal insulin release was similar during all non-stimulatory phases except during the first phase for batches of islets unwashed after culture when significantly higher concentrations of insulin were measured. This effect was significantly more pronounced for batches of islets cultured for 72 h and unwashed prior to the static release assay. The rate of secretion during the first non-stimulatory phase was greater than 140 pg/islet per minute and this declined rapidly during the subsequent non-stimulatory phases. Islets washed with low-glucose medium after the extended period of culture and before the static release test showed normal low levels (less than 20 pg/islets per minute) of basal insulin release during the non-stimulatory phases. The abnormally elevated concentration of insulin measured during the first non-stimulation phase of unwashed islets was almost certainly due to contaminating insulin carried over from the culture medium. Table 2 shows the very high concentration of insulin that accumulates in the medium of islets cultured in RPMI 1640 containing a stimulatory concentration of glucose (11.1 mM).

Static insulin secretion during batch incubations

The effect of cooling rate and its interaction with the length of time in tissue culture before and after freezing is illustrated by the insulin secretory indices given in Table 3. Insulin secretion is expressed as stimulation factors that reflect islet function by providing an index of the ratio of insulin secreted during stimulation to the non-specific basal insulin release and, therefore, indicates the ability of batches of islets to respond to the secretagogue.

All groups showed a discriminating response to the glucose challenge, but the stimulation index was low for islets cooled at either 20° or 70°C/min when assayed after the short period of post-thaw culture. By comparing the indices for groups of islets frozen and thawed in protocols involving either the minimum (2 h pre-freeze and 2 h post-thaw) or maximum (72 h pre-freeze and 72 h post-thaw) culture conditions, it is clear that for each cooling rate islet function was significantly improved by incorporating the extended periods of tissue culture. However, the specific effects of extending either the pre-freeze or post-thaw culture periods is seen to depend upon the cooling rate employed. A marked improvement ($P < 0.01$) in the stimulation factor was observed at each of the cooling rates when the period of pre-freeze culture was extended to 72 h. Under these circumstances when the protocol included 72 h pre-freeze culture, extending the post-thaw culture period to 72 h led to higher stimulation factors at each cooling rate. On the other hand, for islets cooled at the highest rates, post-thaw culture had no beneficial effect upon subsequent function when islets were cultured for only the minimum period prior to freezing. For islets cooled at 70°C/min, extending the pre-freeze culture to 72 h, with or without an extended post-thaw culture, resulted in a markedly higher stimulation index.

Dynamics of insulin secretion during perifusion

The dynamic pattern of insulin secretion from cultured control islets and for batches of islets frozen at 0.3°, 20° or 70°C/min are shown in Fig. 3. Each of these groups, which

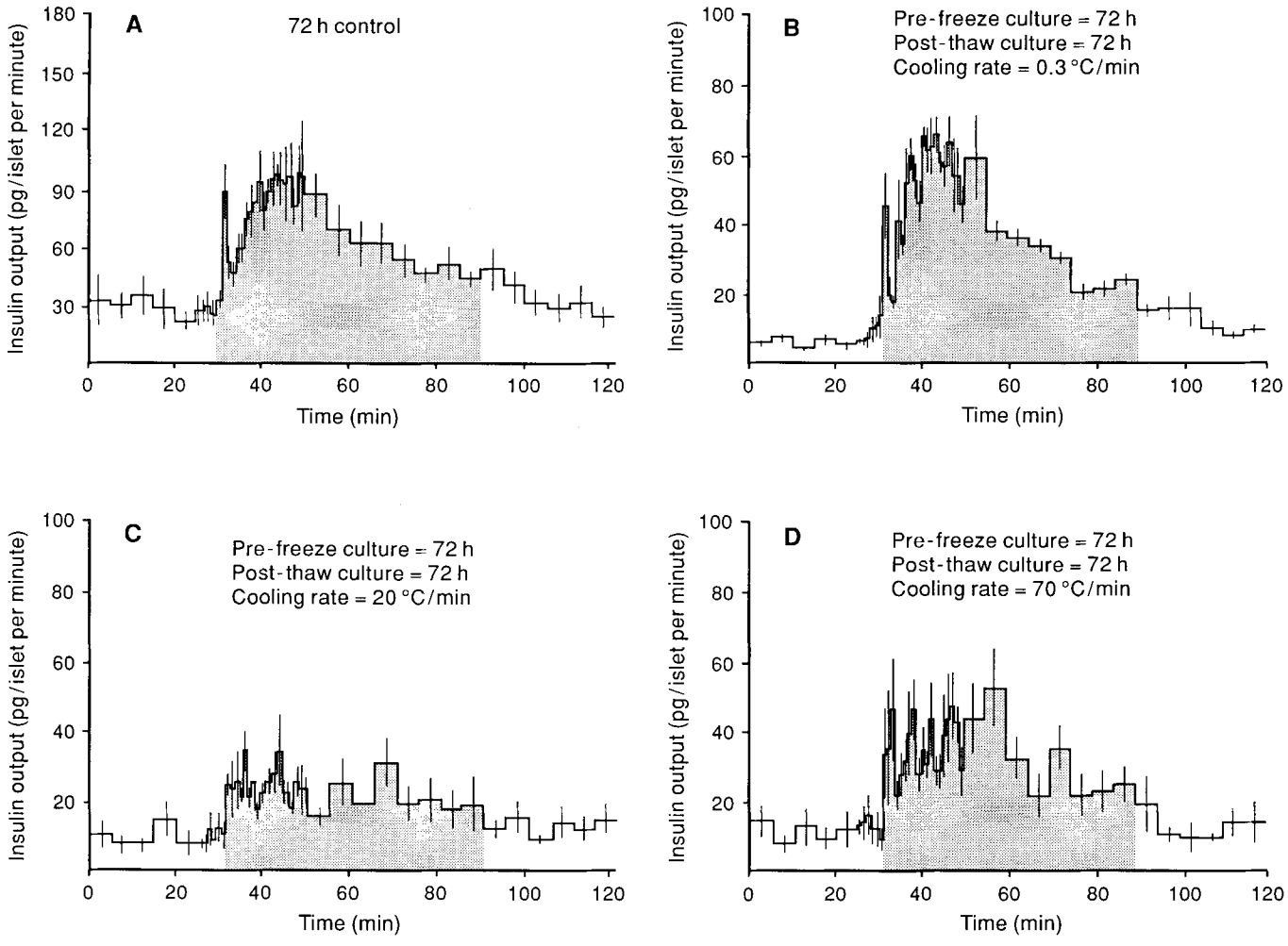

Fig. 3 A–D. The dynamic patterns of insulin secretion from groups of: **A** control and cryopreserved islets cooled at **B** 0.3 °C/min, **C** 20 °C/min, or **D** 70 °C/min. During perifusion islets were exposed either to non-stimulatory medium (□) or stimulatory medium (▨) as described. Graphs represent the averaged responses from duplicate perifusions, each of which included duplicate assay samples measured in a radioimmunoassay that included quadruplicate standards

were also subjected to the maximum culture conditions (72 h pre-freeze and 72 h post-thaw), exhibited a typical biphasic response to the glucose challenge. It is noticeable, however, that in all of these groups insulin output was not maintained throughout the stimulatory period. This contrasts with the sustained response for non-cultured, freshly isolated islets as we have demonstrated previously [8, 34] and discuss later in this paper.

Indices reflecting the comparative ability of groups of islets to maintain insulin secretion during stimulation were calculated from the areas under the perifusion curves and are given in Table 4. This analysis shows clearly that non-cultured islets, with or without freezing, were able to maintain insulin secretion, as reflected by an index of approximately 1 or less. Ratios for groups of islets subjected to extended culture before being perifused were significantly greater than unity.

The secretory function of perifused islets was also calculated as a stimulation factor in a similar way to that described above for data from the static release assay. In this case areas under the curves during the first 30 min of stimulation were expressed as a ratio of the areas calculated from the preceding 30 min of basal insulin release. Table 3 shows that for cryopreserved islets these stimulation factors very closely parallel those generated from the static assay, thus corroborating the significant benefits of extended tissue culture. For example, at each cooling rate the stimulation index increased significantly ($P < 0.01$) between the minimally and maximally cultured batches of cryopreserved islets. Again, this assay verified that pre-freeze tissue culture was essential for the maintenance of function for islets cooled at 70 °C/min; in this case the stimulation index of the recovered population was not significantly different from that of the non-frozen cultured control islets.

It was noted that the mean stimulation indices calculated using data from the static insulin release test were invariably higher than the comparable indices calculated from the perifusion curves. The largest difference between these respective stimulation indices was observed for groups of non-frozen islets cultured for 72 h before the insulin assay. An explanation for this difference is not readily apparent, but a contributory factor would have been the very low levels of basal insulin release measured

Table 4. Maintenance of stimulated insulin secretion during perifusion

Culture time (hours)		Stimulated insulin-secretion ratio[a]		
		Non-frozen controls		
2		0.93 ± 0.05		
72		1.53 ± 0.12		
		Cryopreserved Cooling rate (°C/min)		
Pre-freeze	Post-thaw	0.3	20	70
2	2	0.86 ± 0.04	1.15 ± 0.07	1.10 ± 0.23
2	72	–	–	–
72	2	0.90 ± 0.09	0.78 ± 0.03	1.01 ± 0.2
72	72	2.07 ± 0.37	1.11 ± 0.11	1.62 ± 0.09

[a] The ratio of the amount of insulin secreted during the first half of the stimulatory phase to that secreted during the second half was calculated from digitised areas under the respective portions of the perifusion curves. An index ≤ 1 indicates sustained insulin secretion; indices > 1 indicate declining secretion

Table 5. Glucose clearance indices measured in animals receiving islet transplants

Group	Post-transplant decay constant[a]	Correlation coefficient	IVGTT K value[b]
Controls	0.038	0.83	1.6–2.5
Cryopreserved (5000 islets)	0.022	0.82	0.6
Cryopreserved (8000 islets)	0.044	0.90	1.5–1.7

[a] Linear regression coefficients were calculated from semi-logarithmic plots of serum glucose against time to yield the rate of decline of serum glucose during 14 days following transplantation

[b] K-values calculated by the method of Moorhouse et al. [24]: K < 1.0 = severe diabetes 1.0 < K < 1.5 = mild diabetes and K > 1.5 = normal

during the non-stimulatory phase in the static assay using islets cultured for 72 h (Fig. 2); the quantities were markedly lower than for non-cultured islets. This would be reflected in a much higher stimulation index, even though the absolute levels of insulin secreted during the stimulatory phase were comparable.

Transplantation of rapidly cooled islets

The in vivo function of batches of islets cryopreserved using fast cooling after the 72-h culture was assessed by isograft transplantation in diabetic rats. Changes in serum glucose and body weight following transplantation are shown in Fig. 4 for groups of animals that received either cryopreserved islets or fresh, non-frozen islets. Figure 4 A shows that 2000 untreated islets were sufficient to restore normoglycaemia in a group of control isografts. By comparison the serum glucose in animals that received 5000 cryopreserved islets declined during the immediate post-operative period, but the animals remain diabetic (Fig. 4 B). A total of 8000–9000 cryopreserved islets were required to achieve a sustained normoglycaemic state. Figure 4 B also shows that these animals gained weight at a rate similar to those in the control group. The relative ability of the transplanted islets to function in vivo was also compared using two indices of glucose clearance, as shown in Table 5.

These data showed that during 14 days following transplantation the rate of decline of serum glucose in animals that received 5000 rapidly cooled cryopreserved islets was inferior to controls but was equivalent to the control group when the yield from cryopreserving approximately 8000 islets was transplanted. Animals in the latter group also had normal K-values in the glucose tolerance test that was carried out 1 month after transplantation.

Discussion

We have already explained that successful cryopreservation of isolated pancreatic islets has been achieved using a wide range of cooling rates and that methods employing fast cooling are of particular interest since these are more

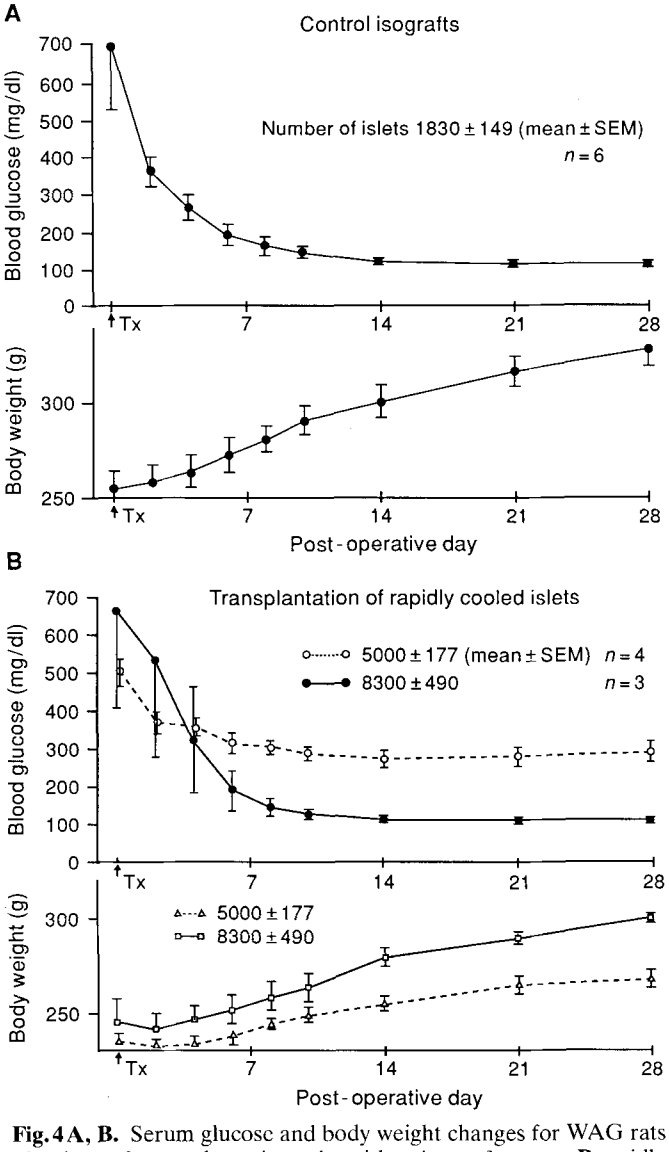

Fig. 4 A, B. Serum glucose and body weight changes for WAG rats after isograft transplantation using either **A** non-frozen or **B** rapidly cooled cryopreserved islets

likely to facilitate a reduction of tissue immunogenicity on the basis of selective depletion of passenger leucocytes than procedures relying upon slow cooling [30, 32, 35–37]. Furthermore, the importance of tissue culture for both the immunomodulation of the stored tissue and the outcome of cryopreservation is also well documented [24, 25, 32]. Following several independent reports showing a beneficial effect of tissue culture upon the functional integrity of cryopreserved islets [14, 16, 21, 26], Sandler and Andersson carried out the first systematic examination of the influence of pre-freeze and/or post-thaw culture on the outcome of islet cryopreservation [24]. Their study clearly demonstrated improved in vitro function after cryopreservation as determined by proinsulin biosynthesis and insulin secretion in islets cultured for a minimum of 2 days prior to freezing. They also showed that pre-freeze culture had a more significant influence on the viability of preserved islets than a period of post-thaw culture.

Previous studies from this laboratory have sought to resolve the interaction between several cryobiological variables [31], and in view of the apparent importance of tissue culture, we have reported preliminary findings that extended tissue culture is essential for the retention of in vitro insulin secretory function of rapidly cooled islets [8]. The present study analyses in more detail the interaction of pre-freeze and post-thaw culture with the effect of cooling rate as a prelusory study for subsequent investigations of the possible immunomodulatory effects inherent in the preservation procedures. It is apparent on the basis of these in vitro assessments that there are clear benefits from incorporating periods of tissue culture in the islet cryopreservation procedure. However, the extent to which the outcome of cryopreservation is improved by tissue culture is seen to depend upon both the cooling rate used and whether the tissue was cultured before or after freezing. When low rates of cooling were employed, islets retained their insulin secretory ability, even when culture times were minimal, but highest survival was obtained when islets were cultured for 72 h prior to freezing, with and without post-thaw culture. High survival of rapidly cooled islets, however, was dependent upon the tissue being cultured after isolation and prior to freezing; under these circumstances both membrane integrity and in vitro insulin secretory ability was similar to that of slowly cooled islets and non-frozen control islets cultured for 72 h. Moreover, the in vivo function of rapidly cooled cryopreserved islets has been demonstrated for the first time in this study.

The mechanism by which islets held in tissue culture following isolation are better able to withstand fast cooling remains uncertain, but it seems likely, as suggested by Sandler and Andersson [24], that culture helps to stabilise cell membranes previously altered or sensitised to freezing injury by the action of digestive enzymes during the process of isolating the islets. The AO/PI test of freshly isolated islets examined prior to being placed in tissue culture always showed that the component cells of the islets were intact on the basis of this assay following the isolation procedure. Only surface adherent cells, presumed to be residual exocrine cells, fluoresced red as a result of permeation of PI. This assay, which indicates when the integrity of cell membranes is severely compromised, allowing the passage of PI, is presumably not sufficiently sensitive to reveal modest membrane alterations that might be sufficient to sensitise cells to cryoinjury. On the other hand, sensitisation of the cells by some mechanism other than via an effect on the plasma membrane cannot be ruled out. Whilst the basis upon which the tissue culture effect is mediated will require further specific investigation for precise elucidation, the phenomenon of a marked improvement in the viability of islets cultured after isolation and prior to freezing is nevertheless clearly established. Furthermore, retrospective examination of the literature reporting survival of isolated islets following rapid cooling reveals that, although the effect of tissue culture was not examined specifically in these studies, the tissue was, indeed, cultured for between 1 and 3 days prior to freezing [2, 7, 12, 13, 15, 27, 31]. In many of these reports the significance of the pre-freeze culture was neither recognised nor specified as important for good recovery of rapidly cooled islets, but the subsequent specific studies of Sandler et al. [24, 27] and our own, reported here and elsewhere [8], have confirmed the importance of pre-freeze tissue culture for high survival of rapidly cooled islets. In contrast, Warnock and Rajotte have reported that tissue culture prior to cryopreservation does not provide any additional benefit for the survival of isolated rat islets following freezing using a slow cooling regimen [40]. Our results in this study are not at odds with this finding since we have shown that tissue culture was less crucial for slowly cooled islets than for rapidly cooled tissue. Nevertheless, in our study, markedly improved survival of slowly cooled islets was obtained when tissue was cultured for 72 h prior to freezing, and the highest survival was obtained when maximal culture before and after freezing was employed.

Rajotte et al. have also reported attempts to corroborate the findings of several published studies showing survival of cryopreserved islets by techniques involving fast cooling. However, they were unable to demonstrate normal patterns of insulin secretion during in vitro perifusion of islets after fast cooling [23]. Their observations support rather than contradict our own findings since it appears that they included only a period of post-thaw tissue culture and omitted the pre-freeze culture period which, as we have shown, is essential for the survival of islets cooled at a high rate.

The present experiments also confirm that islets cooled at an intermediate rate of 20 °C/min also benefit from being pre-cultured prior to freezing; this corroborates the findings of Sandler et al. [26], who have previously studied the survival of cryopreserved islets using a similar cooling rate (25 °C/min). We note, however, in the present study that islets processed using the maximum culture conditions and cooled at 20 °C/min had an inferior stimulation factor measured in both the static and dynamic assays compared with either the more slowly (0.3 °C/min; $P < 0.02$) or rapidly (70 °C/min; $P < 0.05$) cooled islets; this is consistent with the observations of Taylor and Benton [31].

In order to provide a detailed and balanced assessment of the functional status of cryopreserved islets, especially after fast cooling, which we know to provide the best op-

portunity to facilitate immunomodulation by freezing, a range of viability tests have been applied.

Vital staining has long provided a quick and convenient method of assaying the viability of preserved cells and tissues [18], and in this study we have shown that the fluorescent dyes AO and PI provide a simple, rapid and reliable assay for isolated islets that complements the in vitro measures of insulin secretion. The semi-quantitative method of evaluating islet cell integrity devised for these experiments is an extension of the qualitative procedure published by Bank in which a three-grade assessment using the AO/PI assay was shown to agree well with estimates of the insulin content of the same islets [5, 6].

In the static insulin release assay, high levels of indiscriminate insulin release during the first of the non-stimulatory phases of the batch-type incubation is a common phenomenon noted by several investigators (personal communications) and reported by some [8, 13, 34]. We have shown previously [8] that the apparent release of very high concentrations of insulin during the initial non-stimulatory phase of the static batch assays was predominant in those batches of both control and experimental islets held in tissue culture for 72 h prior to the assay. It is now established in the present study that, in our system, this was largely due to contamination of the initial samples in the assay by insulin carried over from the culture medium. This likelihood was verified by the determination that the culture of islets in RPMI 1640 containing 11.1 mmol/l glucose led to a marked accumulation of insulin in the medium. The sustained challenge of islets during culture may also be the most significant factor leading to the observed decline in insulin secretion during the later stages of the stimulatory period in the perifusion assay. The supposition is that the insulin reserves in islets cultured for 72 h in a moderately stimulatory medium become depleted such that they are unable to maintain a higher level of secretion when challenged with a stronger stimulus during perifusion. It is presumed that net insulin synthesis during 72 h culture in 11.1 mmol/l glucose is insufficient to maintain insulin reserves. It was established in the 1970s by Andersson et al. that insulin biosynthesis is markedly affected by the conditions of tissue culture [1, 3], and we have demonstrated here (Tables 3, 4) and reported earlier [8] that non-cultured, non-frozen islets maintain a high level of insulin secretion throughout the stimulatory period of the perifusion assay.

For the in vivo assessment of islet function, islets were not cultured after thawing but were incubated for the minimum period of 2 h only prior to transplantation as it was considered that the in vivo environment would provide the ultimate conditions for tissue recovery. This may not be strictly true if the diabetic state of the recipient, which would impose an immediate demand upon the transplanted tissue, hindered recovery. Nevertheless, functional survival of rapidly cooled islets was clearly demonstrated, provided sufficient numbers of cryopreserved islets were transplanted. In these experiments the quoted number of islets transplanted are estimates before the cryopreservation procedure, and since islets were not recounted prior to transplantation the loss during cryopreservation procedures was not estimated. Nevertheless, it was necessary to freeze approximately four times as many islets using this protocol to achieve the same clinical result as that in animals given approximately 2000 freshly isolated islets. This clearly indicates that this cryopreservation procedure, with cooling at 70 °C/min, is suboptimal for islet survival. Slow cooling (0.3 °–1 °C/min) has been preferred in most attempts to cryopreserve isolated islets because slow cooling is generally mandatory for adequate preservation of large cells and multicellular tissues [19, 29]. However, even using slow cooling, Rajotte et al. [20, 22] found that under some circumstances it was necessary to increase the number of cryopreserved islets by as much as 70 % in order to ameliorate diabetes in rat isografts. Clearly, evidence from this and other sources suggests that there remains a need for the development of improved and optimised procedures of cryopreservation in order to maximise the yield of viable islets.

If cryopreservation is also to play any part in the immunomodulation of donor pancreatic islets, as we have proposed on the basis of the presumed mechanism of selective inactivation or destruction of immunocompetent antigen-presenting cells, then it will be necessary to use fast cooling (> 50 °C/min), as we have already demonstrated [30, 32, 35–37]. This study shows that although cryopreservation using fast cooling might be suboptimal in terms of the yield of functional islets, a significant proportion of islets survive and are able to re-establish and sustain normoglycaemia when transplanted. It now needs to be determined in further studies to what extent islets processed and preserved in this way are able to resist acute rejection in the allograft situation. Preliminary studies have shown that modest prolongation of islet allograft survival in non-immunosuppressed rats is facilitated by preservation procedures involving rapid cooling and culture prior to cryopreservation, but not by conventional methods of cryopreservation involving slow cooling in the absence of prior tissue culture [38].

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