Normalization of Hyperglycemia in Diabetic Rats by Intraportal Transplantation of Cryopreserved Islets From Four Donors

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Techniques for freezing rat islets have been examined by the intensive use of the supravital stains fluorescein diacetate and ethidium bromide. By the use of a simple scoring system, the effect of the cooling rate, treatment with dimethyl sulfoxide (DMSO), rate of thawing, and postthaw culture were examined. These studies showed the most effective method to be a 24-h culture of islets, followed by partial incubation with 20% DMSO at 0°C, followed by seeding at −8°C in an alcohol bath. The islets were then cooled at a rate of −0.25°C/min to −40°C followed by quenching in liquid nitrogen at −196°C. Rapid thawing at 37°C was then followed by a 24-h culture. Islets from four Lewis rat donors were cryopreserved, counted, and transplanted intraportally into streptozocin-induced diabetic Lewis rats. Corresponding control transplants were performed with islets from four donors only cultured for 48 h. The results showed that reversal of hyperglycemia in severely diabetic rats was obtained at 5, 5, 6, 6, 6, or 8 days with cryopreserved islets from four donors, compared to reversal of diabetes at 1, 4, 5, 6, 7, and 12 days with islets from four donors subjected to culture alone. The new cryopreservation technique has several small modifications over previously described methods and results in a significant improvement in islet survival. *Diabetes* 38 (Suppl. 1):185–88, 1989

The development of successful techniques for isolation and transplantation of pancreatic islets of Langerhans has led to a search for techniques that would allow long-term storage of islet tissue, which would have a number of advantages for clinical application (1). Cryopreservation remains the most practical method for long-term storage, but despite many studies, an ideal method for freezing islets has not emerged, although a wide range of techniques have been described (2). In this study, we used a new approach to examine the interaction of some of the key cryobiological variables to achieve a modified procedure that would provide optimum conditions for islet cryopreservation.

Previous studies to assess the viability of isolated islets have used in vitro assays such as insulin secretion in static or perifusion systems (3,4), morphological assays (5), measurement of oxygen uptake (6), or radiolabeled amino acid incorporation (7). All of these techniques are time-consuming and expensive, and considerable time elapses before results are obtained. An alternative approach is the use of supravital stains such as fluorescein diacetate (FDA) and ethidium bromide (EB), which have been shown to distinguish living from dead islets and to allow discrimination of degrees of viability (8). We used this approach to investigate the key variables for cryopreservation. The variables investigated were the freezing rate, the concentration and duration of treatment of islets with dimethyl sulfoxide (DMSO; 10%, 20%, 2 M, 3 M; partial or full equilibration), the thawing rate (slow/fast), and the effect of 24 h of postthaw culture. We then took the optimal cryopreservation method, as shown by the staining technique, and tested the efficacy in an isogeneic rat transplantation model.

MATERIALS AND METHODS

Animals. Inbred female Lewis rats (RT1^l) weighing 190–250 g from our own colony were used as islet donors and recipients. During the experimental period, rats were allowed free access to tap water and laboratory chow.

Tissue culture medium. RPMI-1640 medium containing 11 mM glucose (Gibco Europe, Paisley, UK), 10% fetal calf serum (FCS; vol/vol), 45 μg/ml penicillin, 45 μg/ml streptomycin, and 90 μg/ml kanamycin was used throughout all the procedures and the subsequent culture of isolated islets. Glutamine at a concentration of 20 mM was added on the day of use. Working dilutions of cryoprotectant were obtained by diluting stock DMSO in isotonic medium containing RPMI-1640.

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Isolation and culture of islets. Islets of Langerhans were isolated by an intraductal collagenase digestion/Ficoll separation technique as previously described (9). Two hundred isolated islets were placed in a sterile (50- by 13-mm) plastic petri dish (Sterilin, Feltham, UK) containing 5 ml RPMI-1640 medium. The islets were cultured for a maximum period of 18–20 h at 37°C in 95% humidified air/5% CO_2.

Assessment of viability by supravital staining. Isolated islets were stained with stock solutions of FDA (5 mg/ml in acetone) and EB (200 μg/ml in HBSS + HEPES) as previously described (8). For each set of islets to be stained, a fresh mixture of FDA/EB was prepared each time by addition of 10 μl FDA stock solution to 1 ml EB stock solution. Islets to be stained were held in a drop of culture medium in a plastic petri dish cover to which was added an approximately equal amount of FDA/EB mixture. After 2 min, the islets were washed twice to remove excess stain and examined immediately under a Leitz fluorescence microscope.

The viability of islets was determined by three criteria: *1*) observation of unstained islets by direct stereoscopic microscopy and assessment of structural integrity, fragility, and peripheral damage; *2*) observation of stained islets under a fluorescence microscope and assessment of fluorescence in inner, middle, and outer layers; and *3*) classification of FDA/EB-stained islets into class A for perfect islets, class B for islets showing mild to minimal cell death, or class C for dead or very damaged islets. For the second criterion, FDA/EB-stained islets were placed in a flat petri dish and viewed under a fluorescence microscope with ×4 objective and with the focus racked to show the entire islet. Viable cells were stained green, and the nuclei of dead cells were stained red. A previously devised scoring system was used (8). The islet was viewed as having three concentric layers: inner, middle, and outer. Each layer was scored on a scale of 0–5, depending on the amount of fluorescence observed; thus, a fully viable islet was scored (+5, +5, +5) 15 and a dead islet (+0, +0, +0) 0.

Experimental protocol. The conditions of cryopreservation examined by supravital staining are listed in Table 1. Ten medium-sized islets (150–250 μm) per cryotube (×5) were used for each set of conditions in the experiment. For any given set of experimental conditions, 50 islets were frozen, thawed, and assessed for viability by FDA/EB staining. Partial equilibration of islets was performed with either 10 or 20% DMSO, and the procedure used was essentially that described by Taylor and Benton (10). For full equilibration of the islets, the methods described by Rajotte et al. (11) were used. Nucleation before commencement in both of these methods was performed in a seeding alcohol bath at −8°C with a pair of tongs precooled in liquid nitrogen by gripping the cryotube tightly at the liquid/air interface for ~3 s. Flash-cooling was by direct plunging in liquid nitrogen. Five minutes were allowed for release of latent heat of fusion for all cooling rates <1000°C/min. The cryotubes were transferred to the cooling chamber of a minifreezer previously programmed at −8°C (type 202/200R; Planer Products, Sudbury on Thames, UK). Controlled cooling was commenced at one of the freezing rates listed in Table 1. After completion, the tubes were removed and quenched in liquid nitrogen and held there at −196°C.

Frozen cryotubes containing islets were thawed either by standing the cryotubes on a bench at room temperature (slow warming rate) or by agitating the cryotubes in a water bath at 37°C (fast warming rate). All samples were transferred to an ice bath at 0°C, just before lysis of the last ice crystals. The contents of each thawed cryotube (10 islets/cryotube) at 0°C were then poured into a small sterile culture dish (50 by 13 mm) containing 10 ml RPMI-1640 at 0°C. After counting the recovery yield of each cryotube, the islets were pooled together (contents of 5 tubes) and transferred gently into another similar culture dish containing 5 ml RPMI-1640 for a 24-h postthaw culture in 95% humidified air/5% CO_2 (12). Islet viability was assessed with the criteria described above immediately after thawing and after the 24-h culture.

Intraportal islet transplantation. Islets obtained from four donors were subjected to either 48 h of culture alone or 24 h of culture followed by cryopreservation by the optimal method determined with FDA/EB, with a further 24-h postthaw culture period. Before transplantation, the islets were counted and pooled. Lewis rats were made diabetic by injection of streptozocin (65 mg/kg body wt), and diabetes was confirmed by a serum glucose level >22 mM on two consecutive occasions 2 wk after injection. Islets were transplanted into the portal vein as described previously (13), and serum glucose was measured daily until graft function was confirmed by serum glucose levels <12 mM and at regular intervals up to 150 days thereafter.

RESULTS

Supravital staining. Slow freezing at −0.25°C/min to −40°C yielded islets that were superior at all concentrations

TABLE 1
Conditions examined by fluorescein diacetate and ethidium bromide supravital stains

Cryopreservation variable	Condition
Cooling rate	
Fast	Flash-cooling vitrification, then direct to −196°C
	Incubation at −8°C, then direct to −196°C
	−50°C/min to −140°C, then direct to −196°C
	−60°C/min to −70°C, then direct to −196°C
Slow	−5°C/min to −140°C, then direct to −196°C
	−0.3°C/min to −70°C, then direct to −196°C
	−0.25°C/min to −40°C, then direct to −196°C
Two step (fast)	−2°C/min to −20°C, then −5°C to −140°C, then direct to −196°C
Two step (slow)	−0.5°C/min to −20°C, then −5°C to −140°C, then direct to −196°C
Thawing rate	Slow at room temperature (10°C/min)
	Fast at 37°C (200°C/min)
Dimethyl sulfoxide	10% for 6 min
	20% for 6 min
	2 M + 3 M for 45 min
Culture period	No postthaw culture period
	24-h postthaw culture period

Each cooling rate was examined for every combination of thawing rate, dimethyl sulfoxide incubation, and postthaw culture period listed.

TABLE 2
Clinical response in severely diabetic rats transplanted with a known number of cultured or cryopreserved islets from 4 donors

Pair	Islets transplanted (*n*)	Prediabetic serum glucose (mM)	Pretransplant serum glucose in diabetic rats (mM)	Serum glucose 1 day posttransplant (mM)	Normoglycemia posttransplant*		Follow-up serum glucose on posttransplant days (mM)					
					Days (*n*)	Serum glucose (mM)	15	33	48	68	114	148
1												
Cultured control	1872	8.7	37.9	24.1	12	11.1	9.9	8.9	9.7	10.6	9.4	9.3
Cryopreserved	1963	8.8	37.8	21.9	5	11.1	10.1	9.5	9.2	10.9	9.6	10.1
2												
Cultured control	1440	10.9	36.2	32.0	7	11.0	10.6	9.3	9.4	10.9	9.9	9.2
Cryopreserved	1890	8.8	38.5	31.0	6	11.2	11.1	9.8	12.6	10.2	16.1	11.1
3												
Cultured control	1464	9.5	36.5	9.7	1	9.7	10.1	7.9	8.7	10.5	9.2	9.8
Cryopreserved	1631	9.6	41.6	15.8	8	9.2	10.2	9.8	10.8	11.1	11.1	10.2
4												
Cultured control	1687	10.0	39.9	10.2	4	9.8	9.8	9.2	11.1	11.1	9.8	10.1
Cryopreserved	1897	8.7	38.9	10.7	6	10.1	11.3	11.1	10.2	11.0	10.2	10.8
5												
Cultured control	1396	10.7	33.2	18.8	6	10.9	10.7	9.2	9.8			
Cryopreserved	1512	10.6	34.8	34.8	6	9.6	9.4	9.5	9.9			
6												
Cultured control	1738	8.8	32.8	10.1	5	8.7	9.9	9.7	10.3			
Cryopreserved	1639	10.8	32.3	10.3	5	9.7	11.2	10.1	10.9			

*Recipients were considered normoglycemic when levels of serum glucose <12 mM were attained. No significant difference between recipients of cultured or cryopreserved islets on the day normoglycemia was achieved in the 6 pairs (Wilcoxon's signed-rank test).

of DMSO used. Fast thawing rates always gave better viability than slow thawing. Only one combination (islets treated with 20% DMSO for 6 min followed by cryopreservation at the slow rate, thawing at a fast rate, and a 24-h postthaw culture period) resulted in islets that were physically well preserved and robust when observed by direct microscopy and were scored as class A islets throughout, with an average score of 14.8 per islet. This combination was chosen for transplantation studies and for the purpose of description is termed the *optimal method*.

Intraportal transplantation of cultured and cryopreserved islets. Transplantation of islets from four donors cryopreserved by the optimal method resulted in reversal of hyperglycemia in severely diabetic rats in a manner similar to that obtained by islets subjected to culture alone from four donors (Table 2). There was no significant difference between recipients of cultured or cryopreserved islets on the day at which normoglycemia was achieved in the six pairs of recipients (Wilcoxon's signed-rank test).

DISCUSSION

One of the obstacles preventing successful clinical pancreatic islet transplantation is the difficulty in isolating a sufficient number of islets from a pancreas. In addition to other benefits (1,14), cryopreservation offers the possibility for long-term storage of a sufficient amount of islet tissue pooled together from multiple donors for donor-recipient matching and transplantation. A clear understanding of the cryobiology of islet tissue has failed to emerge over the years, partly as a result of the different freezing protocols used, the varied sources of islet tissue, and differing methods of islet preparation—issues recently reviewed by Bank (2). However, in these investigations we standardized the methods of isolation and culture of islet tissue, preparation, of treatment, and of removal of DMSO from islet tissue and then investigated the cryobiological variables that are known to influence islet viability after thawing over a range of freezing rates.

The use of supravital stains FDA and EB has allowed a rapid assessment of the effects of key variables that influence survival of islet cell tissue after freezing and thawing in a range of freezing rates. In addition, it has allowed the identification of a pattern in which the influence of one variable has resulted in better viability of islet tissue no matter what other variables were in use. For instance, thawing islets at a fast rate (37°C) always resulted in an improved viability over slow thawing, whatever other conditions were used.

The function of islets cryopreserved by the optimal method (as defined by FDA/EB) was shown to be comparable with that from unfrozen islet tissue, as demonstrated by reversal of severe hyperglycemia in diabetic rats after transplantation. These findings compare well with those in previous reports (15,16). The procedures for cryopreservation in the optimal method described here are similar in part to procedures employed and described by other researchers. The concentration of DMSO used (20% vol/vol, which is approximately equivalent to a 2-M concentrate) was similar to that used by Rajotte et al. (11); the procedure for partial equilibration of islets with DMSO was similar to that used by Bank et al. (16) and Taylor and Benton (10), the freezing rate being identical to that of Rajotte et al. (11,15); and the thawing and removal of DMSO procedures were similar to those described by Sandler and Andersson (12). From our preliminary data (not shown), treatment of thawed islets with a hyperosmolar sucrose solution resulted in increased fragility and central necrosis of the thawed islets, and the procedure was therefore discontinued.

Although an ideal method for cryopreserving islets has not evolved, the results presented in this article are encouraging.

It is possible that further changes to the optimal method may be investigated with the supravital stains to refine the technique. Whether the technique described here can be applied to other species, including humans, remains to be seen. However, the use of the FDA/EB stain approach will certainly allow rapid reassessment of the method if adjustments are required.

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