Cryopreservation: In Vitro Results in Rat Pancreatic Islets

J.M. González-Clemente, C. Goyti, J. Mendola, J.I. Conget, R. Casamitjana, R. Gomis, and E. Vilardell

Cryopreservation is an effective method of islet storage and may facilitate clinical trials of islet transplantation. It was the aim of the present study to evaluate the in vitro viability of cryopreserved rat islets, including the response to nonglucose secretagogues and glucose oxidation. After pancreatic digestion via intraductal injection of collagenase, 75- to 200-μm Wistar rat islets were handpicked and cultured in RPMI 1640 (glucose 11.1 mmol/L) and randomized into two groups: control (cultured 20 to 24 hours at 37°C) and cryopreserved (after 20 to 24 hours of culture at 37°C, islets were cryopreserved according to Rajotte's protocol: freezing velocity, −0.25°C/min; thawing velocity, 200°C/min). In the two groups, we evaluated recovery, insulin content per islet, staining viability (ethidium bromide/orange acridine; semiquantitative scoring, measuring the viable area of the islet from 0 = less viable to 3 = more viable), insulin secretion after glucose and nonglucose secretagogues, and oxidation of D-[U-^{14}C]glucose. The results for the control group were always higher for the following: recovery (95.4% ± 1.2% *v* 83.0% ± 2.1%, *P* = .00), insulin content (2,203.9 ± 335.2 *v* 1,443.3 ± 171.8 μU/islet, *P* = .03), insulin secretion after 5.5 mmol/L glucose (61.3 ± 8.0 *v* 28.3 ± 3.4 μU/islet/90 min, *P* = .00), 16.7 mmol/L glucose (151.4 ± 16.1 *v* 98.7 ± 14.1 μU/islet/90 min, *P* = .03), 10 mmol/L L-leucine + 10 mmol/L L-glutamine (125.6 ± 27.9 *v* 56.8 ± 6.4 μU/islet/90 min, *P* = .05), and 10 mmol/L L-arginine (202.5 ± 27.5 *v* 128.8 ± 14.2 μU/islet/90 min, *P* = .01), and glucose oxidation at 5.5 mmol/L (12.5 ± 1.1 *v* 7.9 ± 0.6 pmol/islet/120 min, *P* = .00) and at 16.7 mmol/L (26.1 ± 2.6 *v* 14.3 ± 1.6 pmol/islet/120 min, *P* = .00). No significant differences in staining viability were found between groups (2.35 and 2.48, respectively, *P* = .55). However, cryopreserved and control islets showed a significant increase in insulin secretion and glucose oxidation after increasing the glucose concentration from 5.5 to 16.7 mmol/L. We conclude that when glucose is increased, cryopreserved islets keep the capacity to increase insulin secretion, but cryopreservation produces a significant decrease in several islet viability characteristics. This decrease may be due to a decline of β-cell number per islet and/or a decrease in the content of insulin per β cell.

DEVELOPMENT OF successful techniques for the isolation and transplantation of pancreatic islets has led to a search for procedures that allow long-term storage of islets, which would have a number of advantages for clinical applications.[1] Cryopreservation remains the most practical method for long-term mass storage of islets because it offers many advantages: storage in a tissue bank to provide sufficient islets to transplant to a single recipient; modulation of tissue immunogenicity; purification; and shipment from center to center. A wide range of cryopreservation techniques have been described, and the technique used by Rajotte et al,[2] supported by other studies, is the best method to cryopreserve pancreatic islets.[1] The cryopreservation procedure modifies the in vitro function of rat islets,[2] but insulin release after nonglucose secretagogues and D-[U-^{14}C]glucose oxidation still need to be studied. The present study evaluates recovery, insulin content, insulin release after glucose and nonglucose secretagogues, and [U-^{14}C]glucose oxidation in cryopreserved rat islets. The resulting data will enable us to assess the minimum number of cryopreserved islets needed to reverse insulin-dependent diabetes in rat models of the disease.

From the Endocrinology and Nutrition Unit and Hormonal Laboratory, Hospital Clínic, School of Medicine, University of Barcelona, Barcelona, Spain; and the Medicine Department, School of Medicine, Orient Division, University of Santiago de Chile, Santiago, Chile.

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Address reprint requests to R. Gomis, MD, Endocrinology and Nutrition Unit, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain.

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MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200 to 300 g were used as islet donors. The animals were fed standard rat chow ad libitum and had free access to tap water.

Tissue Culture Medium

The culture medium was RPMI 1640 (Glucose 11.1 mmol/L; Flow Laboratories, Ayrshire, UK) supplemented with 2.0 mmol/L L-glutamine (Flow Laboratories), 10% (vol/vol) heat-inactivated (56°C) donor calf serum (Flow Laboratories), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (Fungizone, 1 μg/mL). Working dilutions of cryoprotectant were obtained by diluting stock dimethylsulfoxide (DMSO) in isotonic medium containing CMRL-1066 (Flow Laboratories) supplemented with 2.0 mmol/L L-glutamine, 10% (vol/vol) heat-inactivated (56°C) donor calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (1 μg/mL), and D-glucose to a final concentration of 5.5 mmol/L.

Isolation and Culture of Islets

Following ductal injection of collagenase (1 mg/mL, collagenase type V; Sigma, St Louis, MO), the pancreases were removed and digested at 37°C for 15 to 20 minutes.[3] The disrupted pancreases were washed several times with Hanks balanced salt solution. The free islets were identified with a stereomicroscope, and those with individual sizes of 75 to 200 μm and morphologically intact were handpicked free of exocrine contaminants. Batches of 100 to 300 islets were maintained in sterile (100 × 100 mm) plastic petri dishes (Soria-Greiner, Valdemoro, Spain) containing 10 mL RPMI 1640 medium. The islets were immediately cultured after isolation for a maximum period of 20 to 24 hours at 37°C in a humidified atmosphere of air:CO_2 (95%:5%).

Islet Groups

After the culture period (20 to 24 hours), batches of islets were randomly allocated into two groups: (1) control group, islets that were not cryopreserved. Viability studies were performed at this time; and (2) cryopreserved group. These islets were immediately cryopreserved.

Viability studies were performed after 20 to 24 hours of postthawing culture.

Freezing Protocol

For cryopreservation, we adapted Rajotte's method, adding DMSO stepwise to a final concentration of 2 mol/L.[2,4] Following the culture period, groups of 150 to 300 islets were transferred to 1.8-mL cryotubes (Nunc, Roskilde, Denmark) and centrifuged to form a pellet. This pellet was resuspended in 0.2 mL CMRL-1066 medium supplemented as already described. To this suspension, 0.1 mL 2-mol/L DMSO (0.67 mol/L) was added for 5 minutes at 25°C, followed by addition of 0.1 mL 2-mol/L DMSO (1.0 mol/L) for 25 minutes at 25°C. After the initial 30-minute equilibration period, 0.4 mL 3-mol/L DMSO (2 mol/L) was added for 15 minutes at 0°C. The cryotubes were transferred to a −7.5°C seeding bath and gently agitated before nucleation so that all the islets were suspended in this medium. After 5 minutes in the seeding bath, nucleation was achieved by grasping the cryotubes with forceps cooled to −196°C. Afterward, a period of 15 minutes was allowed for release of the latent heat of fusion. Later, a BV-10 freezer system (Cryoson; Carburos Metalicos, Barcelona, Spain) was programed to achieve a −0.25°C/min freezing velocity to −40°C. The rate of freezing was monitored by placing a thermocouple in a dummy sample. Finally, flash-cooling was performed by directly plunging cryotubes into liquid nitrogen.

Thawing Protocol

The samples were rapidly thawed (warming rate, 200°C/min) by agitating the tubes in a 37°C water bath. All samples were transferred to an ice bath at 0°C when the last ice had just melted. Extracellular DMSO was removed by centrifugation, and intracellular DMSO was extracted by adding to the cryotube 1 mL CMRL-1066 with 0.75 mol/L sucrose at 0°C. The sucrose was diluted at 25°C with RPMI 1640 media in four steps at 5-minute intervals. Finally, the islets were gently centrifuged and resuspended in the isotonic RPMI 1640 culture medium supplemented as described earlier. Islets were cultured for 20 to 24 hours at 37°C in a humidified atmosphere of air:CO_2 (95%:5%) before assessment of recovery and viability.

Viability Studies

Recovery. The number of islets was assessed by counting the islets in the culture medium under a stereomicroscope side-illuminated with a white acaloric light against a black background (SV8; Zeiss, Wetzlar, Germany), and after the culture period (control group) or after the culture–cryopreservation–culture protocol (cryopreserved group).

Insulin content. Groups of 10 islets, either cultured or cryopreserved, were disintegrated by an ultrasonic procedure (Sonifer 250; Branson, London, UK) at 4°C in 0.5 mL acid-alcohol solution (75% vol/vol ethanol, 23.5% bidistilled water, and 1.5% 10N HCl). The homogenates were kept at −20°C until insulin content was assayed by radioimmunoassay (CIS; Biointernational, Gif-Sur-Yvette, France). The method allows detection of 2.5 μU/mL with a coefficient of variation within and between assays of 6% and 8%. Insulin content was expressed in microunits of insulin per islet. Results are from at least five different experiments.

Insulin secretion. Insulin release was measured statically during batch incubations in groups of six randomly selected control or cryopreserved islets placed in small incubation vials. These batches were incubated in a shaking water bath for 90 minutes at 37°C in 1.0 mL bicarbonate-buffered medium containing bovine serum albumin (5.0 mg/mL; Fraction V, Sigma, St Louis, MO) and either 5.5 mmol/L D-glucose, 16.7 mmol/L D-glucose, D-glucose (16.7 mmol/L) plus forskolin (0.005 mmol/L; Sigma), L-leucine (10 mmol/L) plus L-glutamine (10 mmol/L) or L-arginine (10 mmol/L). During the first 10 minutes of incubation, the vials were gassed with O_2:CO_2 (95%:5%). At the end of the incubation period, supernatants were separated into aliquots and stored at −20°C until assayed for insulin by a radioimmunoassay method (CIS; Biointernational). Insulin release is expressed as microunits of insulin per islet per 90 minutes. Secretion results are from at least five independent experiments.

Assessment of viability by supravital staining. Immediately after insulin secretion studies, isolated islets were stained with a stock solution in phosphate-buffered saline of acridine orange (3 μg/mL) and ethidium bromide (10 μg/mL). Groups of 10 to 30 islets in 100 μL bicarbonate-buffered medium (see insulin secretion studies) were stained with 100 μL acridine orange/ethidium bromide mixture. In every viability staining study, a minimum of 30 islets were examined under a fluorescence microscope (Laborlux D; Leitz, Wetzlar, Germany). Viability was scored by evaluating the islets viewed under a 125× 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. Islets that have 75% to 100% green area were scored as 3, between 50% and 75% as 2, between 25% and 50% as 1, and between 0% and 25% as 0 (Fig 1). For every viability experiment, the mean value of the islets evaluated was scored.

Glucose oxidation. Groups of 18 to 20 islets were incubated in duplicate or triplicate in glass vials containing 1.0 mL bicarbonate-buffered medium containing bovine serum albumin (5.0 mg/mL, Fraction V; Sigma) with D-[U-^{14}C]glucose (0.2 mCi/mL, CFB.96, batch 198; Amersham, Amersham, UK) and nonradioactive glucose to yield a final concentration of 5.5 mmol/L or 16.7 mmol/L. The vials were inserted into glass scintillation flasks with 250 μL Hyamine hydroxide (Carlo-Erba, Milano, Italy), gassed with CO_2:O_2 (5%:95%), and tightly sealed. The flasks were shaken for 120 minutes in a 37°C water bath. Oxidation was stopped by injection of 20 μL of a solution with rotenone 0.01 mmol/L (Sigma), antimicine A 0.01 mmol/L (Sigma), and KCN 0.0625 mmol/L (Merck, Darmstadt, Germany) in citrate buffer 400 mmol/L (pH 4.0). $^{14}CO_2$ formed by glucose islet metabolism was released from the incubation medium and subsequently trapped in the Hyamine for 60 minutes at room temperature. Ten milliliters of Optiphase "Hi Safe" II (LKB Scintillation Products, Leicester, UK) was added to each flask, and radioactivity was measured by counting liquid scintillation. Oxidation of D-[U-^{14}C]glucose was expressed as picomoles per islet per 120 minutes.

Statistical Analysis

Quantitative results are expressed as the mean ± SEM, and P less than .05 is considered statistically significant. Study groups were compared using the unpaired or paired Student t test or the Mann-Whitney test.

RESULTS

Recovery

Five thousand four hundred seventy-eight islets in 36 batches were analyzed in the control group. The overall recovery in this group was 95.94% ± 1.19% (Table 1). The cryopreserved group was initially composed of 5,466 islets (40 batches), but only 3,430 islets in 23 batches were finally evaluated (947—six batches—were excluded because of a bacterial contamination episode, and 1,089—seven batches—because of a flaw in the freezing protocol). Following the culture–cryopreservation–culture process, 81.8% ± 3.0% of these islets were retrieved (Table 1).

Insulin Content

The mean insulin content of cryopreserved islets was 34.5% lower than in the control group, and this difference reached statistical significance (Table 1).

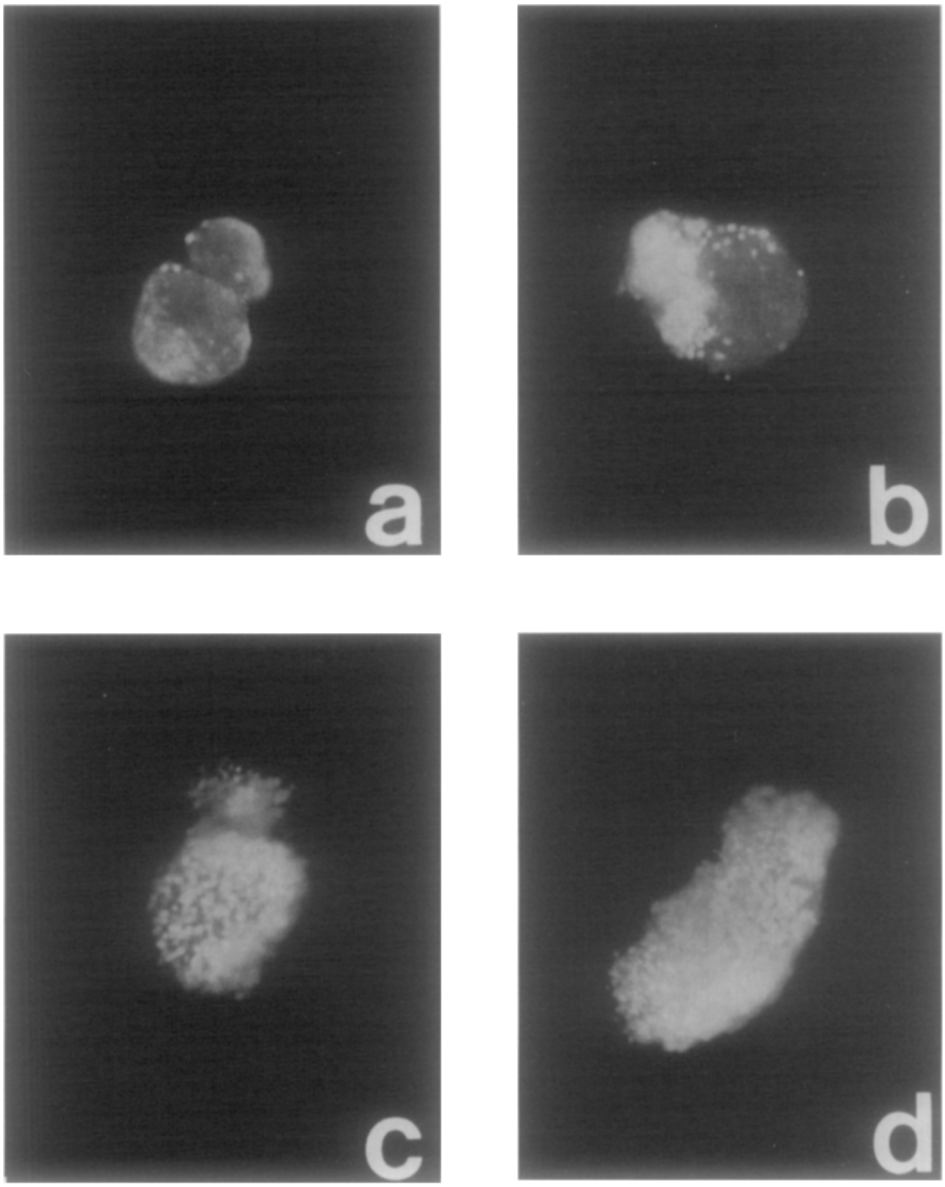

Fig 1. Fluorescence microscopy of cryopreserved islets according to the following scoring system (original magnification ×500): (a) islet scored as 3 (100% to 75% of its area is green); (b) islet scored as 2 (75% to 50% of the area is green); (c) islet scored as 1 (50% to 25% of the area is green); (d) islet scored as 0 (<25% of the area is green). In all cases, islets were stained with acridine orange/ethidium bromide dye.

Table 1. In Vitro Viability Results for Control and Cryopreserved Islets

Parameter	Cryopreserved		Control		*P*
	Mean ± SEM	No.	Mean ± SEM	No.	
Recovery (%)	95.9 ± 1.2	36	81.8 ± 3.0	23	.00
Insulin content (μU/islet)	2,203.9 ± 295.1	18	1,443.3 ± 171.8	21	.03
Insulin release (μU/islet/90 min)					
5.5 mmol/L glucose	61.3 ± 8.0	27	28.3 ± 3.4	17	.00
16.7 mmol/L glucose	151.4 ± 16.1	26	98.7 ± 14.1	22	.03
16.7 mmol/L glucose + 0.005 mmol/L forskolin	263.8 ± 37.0	12	178.7 ± 25.3	17	.08
10 mmol/L L-leucine + 10 mmol/L L-glutamine	125.6 ± 27.9	12	56.8 ± 6.4	14	.05
10 mmol/L L-arginine	202.5 ± 27.5	19	128.8 ± 14.2	33	.01
Viability by supravital staining	2.4 ± 0.2	8	2.5 ± 0.3	7	.12
Glucose oxidation (pmol/islet/120 min)					
5.5 mmol/L glucose	12.5 ± 1.1	25	7.9 ± 0.6	26	.00
16.7 mmol/L glucose	26.1 ± 2.6	24	14.3 ± 1.6	22	.00

NOTE. No. refers to the number of different experiments in each condition.

Insulin Secretion

Both cultured and cryopreserved islets demonstrated a discriminatory response to the increase in glucose concentration (both groups, $P = .00$). In the two groups after 0.005 mmol/L forskolin and 16.7 mmol/L glucose, a significant increase of insulin secretion (both groups, $P = .01$) was found.

In comparison to the control group, cryopreserved islets had a 53.8% mean decrease in insulin response after 5.5 mmol/L glucose, 34.8% after 16.7 mmol/L glucose, 54.8% after 10 mmol/L leucine + 10 mmol/L glutamine, 36.4% after 10 mmol/L arginine, and 32.8% after 16.7 mmol/L glucose + 0.005 mmol/L forskolin. These differences achieved a significant level in all conditions, with the exception of the last (Table 1).

Supravital Staining

A total of 495 islets (corresponding to eight experiments) were analyzed in the control group and 427 islets in the cryopreserved group (seven experiments). No significant differences were found between the two groups (Table 1).

Glucose Oxidation

Both study groups showed a significant increase in glucose oxidation when glucose was increased from 5.5 to 16.7 mmol/L (control group, $P = .00$; cryopreserved group, $P = .00$). However, cryopreserved islets oxidized 36.8% less glucose at 5.5 mmol/L and 45.2% less at 16.7 mmol/L than the control group; these differences are significant (Table 1).

DISCUSSION

Studies of the in vitro characteristics of cryopreserved islets, such as recovery, insulin content, insulin secretion, or glucose oxidation, are of interest to assess the theoretical minimum islet mass necessary to reverse insulin-dependent diabetes mellitus. Our results show that these characteristics are diminished in cryopreserved islets because of deterioration during the freezing-thawing procedure. These findings have to be taken into account in transplantation of cryopreserved islets.

In cryopreserved islet recovery, results are strongly affected by the time of evaluation. When counting just after thawing, loss is approximately 4% (data not shown) and may be related to the islets that remain in the containers used.[5] After thawing, if islets are cultured for 24 hours, the loss increases considerably up to 20% with 80% of total recovery, similar to previous studies in rat and mouse islets.[6-11] This loss of islet detection may correspond to islets that fragment. These islets may not be recognized as such because of their size or unusual shape, or they might have disintegrated completely due to loss of viability.[5] In any counting system, the number of islets (after cryopreservation or culture) may be falsely incremented because bigger islets may fragment into minor particles that may be counted as new islets. To overcome this problem, only islets between 75 and 200 μm in diameter were considered, as has been previously described.[6] In the case of a large volume of endocrine tissue, it is more useful to count the number of islet equivalents.[12] DNA content per islet can also be quantified,[10,13] but this technique is only related to the total number of cells in the islet and does not take into account the viability or cell type (endocrine or nonendocrine).

The insulin content per islet in the cryopreserved group showed a mean decrease of 34%, as previously described in rodent islets[10,13-16] and this may be due to a decrease of β-cell number per islet[10,15,16] and/or to a decline in the content of insulin per β cell.[10,13] The decrease in the number of β cells per islet may be a consequence of their destruction during the freezing-thawing procedure and is approximately 30% to 40%.[17] The decline in the content of insulin per β cell may be due to a deterioration of biosynthesis, secretion, and/or degradation of insulin.[13] The most prominent alteration may be the capacity of insulin storage and not the biosynthesis capacity.[10] However, studies in cryopreserved human islets, in which a decrease in second-phase insulin secretion after a glucose stimulus has been observed, suggest that the capacity to produce new insulin may be affected.[12]

A number of previous studies of insulin secretion in cryopreserved islets have been performed using the perifusion system,[18] but evaluation of nonglucose secretagogues is scarce. We used static incubations with different nonglucose secretagogues. This system is reliable in physiology studies of pancreatic islets[19] and is easier, quicker, and less "islet-consuming" than the perifusion system.[14] As segretagogues, we tested glucose (5.5 and 16.7 mmol/L), L-leucine 10 mmol/L + L-glutamine 10 mmol/L, forskolin 0.005 mmol/L + glucose 16.7 mmol/L, and L-arginine 10 mmol/L. Glucose, L-leucine, and L-glutamine stimulate insulin secretion through its metabolism: glucose enters glycolysis, and L-leucine and L-glutamine enter directly at Krebs cycle. As a nonnutrient secretagogue, we tested forskolin, which stimulates insulin secretion through activation of adenylate cyclase and cyclic adenosine monophosphate (cAMP) production.[20] This effect is different from the action of methylxanthines that increase cAMP levels after phosphodiesterase inhibition.[21] It has been suggested recently that 80% of the action of cAMP on insulin secretion is caused by its direct action on the secretory machinery of the β cell.[22] Therefore, the response after forskolin may be taken as a parameter of the secretory function. L-Arginine was also evaluated. Its mechanism of action is unknown. Because it is a cationic amino acid, it may increase insulin secretion after accumulating in the β cell and cause a membrane depolarization.[23,24] Other mechanisms have been proposed, like the activation of adenylate cyclase, synthesis of polyamines that are substrates of the transglutaminase, activation of protein kinase, or generation of nitric oxide.[24,25] Our results show that cryopreserved islets have a significantly lower insulin secretion response after 5.5 and 16.7 mmol/L glucose and that these decreases are in the same range as the decline in insulin content per islet. The same applies to the response to L-leucine 10 mmol/L + L-glutamine 10 mmol/L and L-arginine 10 mmol/L.

Several patterns of insulin secretion corresponding to cellular lesion have been described in cryopreserved islets.[18] However, comparison of the results for insulin secretion to previous studies is difficult because differences in the cryopreservation technique or in the studies of insulin secretion are common. For example, culture after cryopreservation may improve the response of insulin secretion.[11,18,26] But Flesch et al,[9] without a

culture period after thawing, described an increase in insulin secretion after 2.8 mmol/L glucose, although this increment could be due to a nonspecific leakage of insulin, reflecting some degree of cellular damage. Furthermore, Ishihara et al[27] describe, in rat islets frozen to −1°C/min and cultivated 1 day before cryopreservation and without culture after cryopreservation, a normal response to 3.3 mmol/L glucose but a diminished response to 16.7 mmol/L. The type of culture medium is also important. In dog islets, culture with CMRL 1060 after thawing instead of RPMI 1640 better preserves the kinetics of insulin secretion.[26] In general, in perifusion studies of rodents or human islets frozen slowly (<−1°C/min), insulin secretion at low glucose can be incremented or diminished, while it is decreased at high glucose.[12,28,29] These findings have been attributed, at least in part, to the high concentrations of DMSO (2 and 3 mol/L) used during the cryopreservation technique, since DMSO may modify proinsulin synthesis at these concentrations.[30]

The decrease in second-phase insulin secretion in cryopreserved islets may be related to a secretory machinery deficiency.[12,18] The decrease in insulin secretion after glucose + forskolin was in the same range as the decrease after testing the other secretagogues. This result does not support a significant deleterious effect of cryopreservation on the secretory machinery. Results with theophylline have been controversial (decreased in mouse islets cultivated 1 week before and 1 week after cryopreservation[14] and increased in cryopreserved human islets[31]).

Assessment of membrane integrity with supravital dyes has been used as a rapid method for leukocyte viability testing. This technique can be of value in cryopreservation, because damage to the plasma membrane is a prominent component during freezing injury.[32] In previous studies, propidium iodine/acridine orange or fluorescein diacetate/ethidium bromide have been used to test this viability.[33-35] Nevertheless, we use acridine orange/ethidium bromide after testing their utility in the isolation of human islets[36,37] and we developed a semiquantitative scoring system to better define islet viability. No significant differences were found between cryopreserved and control islets. However, this technique only takes into account the membrane integrity. Studies on correlations between these results and insulin secretion capacity still have to be made.

D-[U-^{14}C]glucose oxidation after increasing the glucose concentration in cryopreserved islets has never been tested before. Glucose oxidation in pancreatic islets depends mainly on β-cell glucose oxidation, because the other endocrine cell types only participate in total glucose oxidation in a minor way.[38] Therefore, the measure of glucose oxidation is a good parameter of β-cell function. The glucose oxidation rate at 5.5 mmol/L and at 16.7 mmol/L was decreased by the same amount as the decrease in insulin content and insulin secretion. These findings also match glucose oxidation results in mouse islets at 16.7 mmol/L glucose.[14] This suggests that these results may be related to a decrease in the number of β cells per islet and/or insulin content per β cell. However, direct or indirect freezing injuries on the mitochondria during the freeze-thawing procedure cannot be excluded.[39] Another aspect is that cryopreserved islets showed a significant increment in insulin secretion upon increasing the glucose concentration from 5.5 to 16.7 mmol/L, and the same was observed when D-[U-^{14}C]glucose oxidation was evaluated, indicating that the capacity to recognize increments in glucose concentration is preserved.

Finally, assuming a mean recovery of 80% and a decrease of insulin content, insulin secretion, and glucose oxidation capacities of about 40%, we suggest that to achieve in transplantation experiments the same metabolic effect as in 100 overnight-culture islets, at least 300 islets should be cryopreserved. This observation may also be important for considering the transplantation of freeze-thawed human islets in type I insulin-dependent diabetic patients.

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