

## Effect of thawing rate and post-thaw culture on the cryopreserved fetal rat islets: Functional and morphological correlation

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### Abstract

The ability of the fetal pancreatic islet cells to multiply rendered them a potential tissue for transplantation studies to cure diabetes. A bank of fetal islets could be created with proper storage in liquid nitrogen. The aim of this study is to evaluate the effect of thawing rate and post-thaw culture on the structural and functional integrity of isolated cryopreserved islets of rat fetuses. Fetal rat islets were isolated by the collagenase digestion, cultured for three days, and then cryopreserved using dimethylsulphoxide as cryoprotectant and the step-rate cooling to  $-40\text{ }^{\circ}\text{C}$  before immersing them in liquid nitrogen. The islets were thawed by the slow or fast warming rates using hyperosmolar sucrose solution and then cultured for 1 or 2 days. Insulin and C-peptide contents of the slow thawed islets were higher than those of the control. In the fast thawed islets the contents were similar to those of the control. Insulin and C-peptide release in response to glucose for the slow thawed islets were lower than those of the control and in the fast thawed islets they were similar to that of the control. Histological examination showed irregular periphery and fragmented central part of the large slowly thawed islets, which showed also variable immunohistochemical reaction to anti-insulin serum, ranging from strongly positive reaction to markedly weak reaction. Fast thawed islets showed mostly regular periphery and their reaction to the anti-insulin serum was slightly weaker than that of the control islets. It was concluded that fast thawing and post-thaw culture is much better than slow thawing, as indicated by nearly normal insulin and C-peptide content and release and intact structural integrity.

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### Introduction

Successful clinical trials of pancreatic islet transplantation could be achieved if ways are found to effectively store the islets. The most appropriate method for long term storage of islets is to preserve them in a deeply frozen state (cryopreservation) at  $-196\text{ }^{\circ}\text{C}$ . At this low temperature, storage of islets would have minimal impact on viability (Ashwood-Smith, 1980). This approach would allow establishing an islet bank, thus providing a sufficient amount of islet tissue which could

be pooled together from multiple donors to treat individual patients. The use of fetal islets in the transplantation procedures offers several advantages to the diabetic recipient; including the potential for further growth and differentiation of B-cells either in culture before transplantation or in the recipient after transplantation (Morris, 1988). They also offer the advantage of relatively low immunogenicity, susceptibility to immunomodulation, and the availability of a source of fetal islets from aborted fetuses.

It was assumed that freezing the pancreatic islets requires proper cooling and thawing rates to avoid damage of B-cells by intracellular ice crystal formation (Rajotte and Mazur, 1981; Taylor et al., 1983; Taylor and Benton, 1987; Vasir et al., 1989; Hullett et al., 1989). Functional studies have shown that fast thawing of fetal islets (Mazur et al., 1976; Kemp et al., 1978;

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Hegre et al., 1984; Simonsen et al., 1987; Otonkoski et al., 1992; Beattie et al., 1993; Liu et al., 1993; Miyamoto et al., 1994) is superior to slow thawing (Rich et al., 1993). However, correlation of the functional studies with the structural integrity of the cryopreserved-thawed fetal islets was not documented. Similarly, the effect of post-thaw culture on the cryopreserved fetal islets was not properly documented.

The present work was therefore designed to study the effect of the thawing rate (fast or slow) and post-thaw culture (24 or 48 h) on the viability of the isolated cryopreserved fetal rat islets. Viability of the islets was assessed by measuring their content and release of insulin and C-peptide. Moreover, the effect of cryopreservation on the structural integrity of the B-cells of the fetal islets was assessed.

## Materials and methods

### Isolation and culture of the pancreatic islets

Full-term pregnant Lewis rats (21 days) were obtained from King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Pancreatic islets were isolated from fetal rats by the collagenase digestion and culture technique of Hellerstrom et al. (1979). The pancreata were removed, chopped into 1 mm<sup>3</sup> pieces and digested with collagenase (Sigma type V) at a concentration of 1.5 mg/ml for 10 min.

The isolated islets were cultured in 94 mm sterile polystyrene culture dishes, in groups of approximately 100 islets per dish. The culture medium consisted of RPMI-1640 (Sigma), pH 7.4, osmolality between 310 and 340 mOsmol/kg containing 11.1 mM glucose, 20 mM *N*-2-hydroxyethyl piperazine–*N*-2-ethane sulfonic acid buffer (HEPES buffer), penicillin 100 U/ml, streptomycin 0.1 mg/ml, gentamycin 10 µg/ml and nystatin 100 IU/ml. The medium was supplemented with 3% L-glutamine, and 5% heat inactivated, fetal rat serum. The cultured islets were maintained at 37 °C in a humidified atmosphere of 5% (v/v) carbon dioxide in air. The islets were cultured for 3 days before cryopreservation and the culture medium was changed daily.

### Cryopreservation

The procedure used is the method developed by Rajotte et al. (1984) and modified by Taylor and Benton (1987). About 100 islets were transferred to each sterile 1.8 ml cryotube, allowed to sediment, and the supernatant was removed leaving 0.2 ml of the medium in the tube. At room temperature, 0.1 ml

of 2M dimethylsulphoxide (DMSO) in culture medium was added and the tubes were allowed to stand for 5 min. A second 0.1 ml of 2 M DMSO was added, and equilibration was allowed for 25 min. Then 0.4 ml of 3 M DMSO was added and mixed to produce a final concentration of 2 M DMSO and the cryotubes were placed on ice for 20 min.

The cryotubes were then placed in a programmable step-rate freezer (Mini-Cool 40 P.C. Embryo Freezer) at a chamber temperature of –8 °C. The tubes were kept for 15 min to equilibrate, and then ice nucleation was induced, using a pair of tongs precooled in liquid nitrogen, by gripping the cryotube lightly at the liquid/air interface for approximately 3 s. The tubes were then returned to the chamber and held at –8 °C for an additional 10–15 min, to permit the release of the latent heat of fusion. The chamber temperature was then decreased at a rate of 0.25–0.5 °C/min until the chamber temperature reached –40 °C. At this point, the vials were transferred to liquid nitrogen and stored at –196 °C.

### Thawing

The islets were thawed either by standing the cryotubes on the bench at room temperature for approximately 10 min (slow warming rate, 10 °C/min) or by agitating the cryotubes in a water bath at 37 °C (fast warming rate, 200 °C/min). All samples were transferred to an ice bath at 0 °C, just before disappearance of the last ice crystal. The tubes were then centrifuged at 400 ×g for 2 min at 4 °C. The supernatant was decanted, and 2.5 ml of 0.75 M sucrose in M199 was added to each tube to draw DMSO from the intracellular compartment (4 °C). Thirty minutes later, the sucrose was diluted stepwise by addition of 2.5, 2.5, 5 and 10 ml fresh M199 at 5 min intervals. The islets were resuspended in culture medium, transferred to plastic dishes and cultured for 24 or 48 h.

### Functional evaluation

Aliquots from the islet preparations (control islets and cryopreserved/thawed islets) were functionally evaluated. Insulin and C-peptide were extracted from the isolated islets by acidified ethanol, and their concentration measured by ELISA counter using Insulin and C-Peptide ELISA kits (Dako, CA, USA). The results were expressed as units of insulin or C-peptide per gram of protein. The protein content of islet preparations was determined by the procedure of Lowry et al. (1951).

Table 1  
Insulin content (µIU/µg protein) of islets after slow and fast thawing

	D-1	D-2	D-3/thawed	D-4	D-5
Control	0.0583±0.0031 (30)	0.0425±0.0029 (30)	0.0335±0.0027 (10)	0.0311±0.0015 (10)	0.0258±0.0014 (10)
Slow-thaw			0.0569±0.0054 (10)	0.0686±0.0040 (10)	0.0616±0.0007 (10)
( <i>t</i> -test)			( <i>p</i> <0.05)	( <i>p</i> <0.05)	( <i>p</i> <0.05)
Fast-thaw			0.1094±0.0018 (10)	0.0376±0.0014 (10)	0.0225±0.0008 (10)
( <i>t</i> -test)			( <i>p</i> <0.05)	NS	NS

Note: NS = non significant.

Data are presented as mean±SEM (number of specimens).

Table 2  
C-peptide content ( $\mu\text{g}/\mu\text{g}$  protein) of islets after slow and fast thawing

	D-1	D-2	D-3/thawed	D-4	D-5
Control	0.0122 $\pm$ 0.0005 (30)	0.0106 $\pm$ 0.0005 (30)	0.0087 $\pm$ 0.0003 (10)	0.0079 $\pm$ 0.0003 (10)	0.0076 $\pm$ 0.0004 (10)
Slow-thaw ( <i>t</i> -test)			0.0119 $\pm$ 0.0004 (10) NS	0.0130 $\pm$ 0.0005 (10) NS	0.0265 $\pm$ 0.0003 (10) ( <i>p</i> <0.05)
Fast-thaw ( <i>t</i> -test)			0.0113 $\pm$ 0.0002 (10) NS	0.0110 $\pm$ 0.0005 (10) NS	0.0085 $\pm$ 0.0003 (10) NS

Note: NS = non significant.

Data are presented as mean $\pm$ SEM (number of specimens).

Insulin and C-peptide release from cryopreserved/ thawed islets was measured in response to glucose challenge. The isolated islets were incubated in a 0.5 ml syringe barrel containing medium M199 with 1% (w/v) bovine serum albumin (fraction v). The incubation chambers were gassed with O<sub>2</sub>/CO<sub>2</sub> (19/1) and incubated at 37 °C in a shaking water bath at 50–60 oscillations/min. An equilibration period of 30 min was allowed and then the islets were challenged for 30 min each with medium M199 supplemented with 15 and then 30 mmol/l glucose, separated by a 60 min resting phase with medium M199 only. After a further resting phase of 60 min, a final challenge with 15 mmol/l glucose together with 15 mmol/l theophylline were used to elicit a maximal response from the islets.

#### Histological and immunohistochemical examination

Islet specimens were taken from the different samples of the cryopreservation/thawing protocol, fixed in buffered neutral formalin and embedded in paraffin with 57 °C melting point. Five micrometer serial sections were cut from each specimen and stained with hematoxylin and eosin. Paraffin sections were also prepared from islets cultured without cryopreservation/thawing, and used for comparison.

To localize the insulin producing B-cells, the indirect immunohistochemical technique was used (Sternberger, 1979), as described by Dayal and Duhamel (1989). The primary antibody, guinea pig anti-swine insulin serum (Sigma, St. Louis, MO, USA), was diluted in phosphate-buffered saline (PBS) with 1% normal rabbit serum. Sections were incubated in the primary antibody (dilution 1:500) for overnight in a humidity chamber, at 4 °C. The secondary antibody used was rabbit anti-guinea pig immunoglobulin conjugated with peroxidase (dilution 1:200) (Sigma, St. Louis, MO, USA). The chromogen substrate used was 3, 3-Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and the sections were counterstained with Harris' hematoxylin to facilitate the nuclear identification. The specificity control for the immunohistochemical staining method was monitored by incubating sections with a non-immune serum, omission of the secondary antiserum, and absorbing antiserum with 10 mg insulin/ml for 24 h prior to staining.

#### Morphometric analysis

Paraffin sections of pancreatic islets were systematically analyzed at a magnification of 450 $\times$ . The profile diameters ( $d$ ) of the islets were calculated from the equation  $d = 2\sqrt{ab}$ ,

where  $a$  and  $b$  are the major and major at right angle semi-axis, respectively (Williams, 1977).

The results were presented as the mean $\pm$ standard error of mean (SEM). Student's *t*-test for non-paired observations was used for statistical evaluation of the data.

## Results

#### Functional evaluation

Insulin content of islets 1 and 2 days after slow thawing was significantly higher ( $P<0.05$ ) than that of the control islets (Table 1). The fast thawed islets showed insulin content higher than that of the slow thawed islets, immediately after thawing. However, the insulin contents of the fast thawed islets were not significantly different from those of the control islets 1 and 2 days after thawing (Table 1).

The C-peptide content of slow thawed islets was only changed at two days post-thaw giving results significantly higher ( $P<0.05$ ) than those of the control (Table 2). The fast thawed islets showed C-peptide contents similar to those of the controls (Table 2).

Insulin release from slow-thawed islets, in response to glucose challenge, was significantly lower ( $P<0.05$ ) than that of the control islets, 1 and 2 days post-thaw (Figs. 1 and 2). The fast-thawed islets, 1 and 2 days post-thaw, showed responses that were significantly higher ( $P<0.05$ ) than that of the control

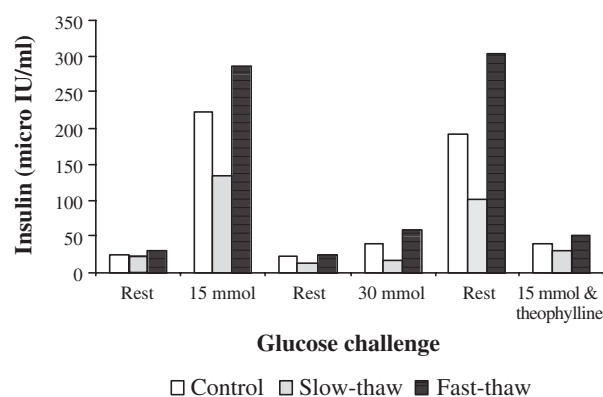


Fig. 1. Insulin release from control, slow and fast thawed islets, 1 day post-thawing. An equilibration period of 30 min was allowed in medium M199 and then the islets were challenged for 30 min each with 15 and then 30 mmol/l glucose, separated by a 60 min resting phase with medium M199 only. After a further resting phase of 60 min, a final challenge with 15 mmol/l glucose together with 15 mmol/l theophylline were used to elicit a maximal response from the islets.

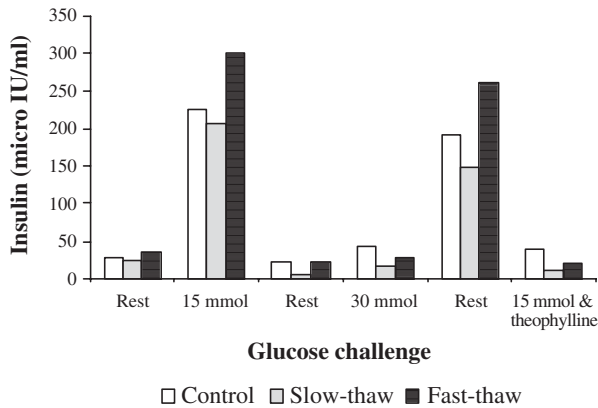


Fig. 2. Insulin release from control, slow and fast thawed islets, 2 days post-thawing. The protocol and time periods are described in Fig. 1.

islets (Figs. 1 and 2). It is noted that the response to 30 mmol/l glucose was late and the islets did not show a good response to the final challenge with theophylline and elevated glucose.

C-peptide release from slow thawed islets was significantly lower ( $P < 0.05$ ) than that of the control islets, 1 and 2 days after thawing (Figs. 3 and 4). Fast thawed islets, 1 and 2 days post-thaw, showed C-peptide secretion that was similar or apparently slightly less, but not significantly different, than that of the control islets (Figs. 3 and 4).

*Histological and immunohistochemical findings*

The control islets cultured for 4 or 5 days appeared as spherical or ovoid structures containing varying numbers of intact cells. The islet cells were arranged in anastomosing irregular cords separated by a rich network of capillary spaces (Fig. 5). The peripheral parts of the islets were mostly regular and formed a continuous layer. The diameter of most of the islets ranged from 80 to 230 μm. No sign of degeneration was detected in the islet cells, even in the fragmented islets; the cells appeared normal. The control islets showed positive staining with anti-insulin serum (Fig. 6).

The slow thawed islets, cultured for 1 day post-thawing, appeared with irregular periphery that did not form a continuous layer (Fig. 7). Few islets showed regular periphery

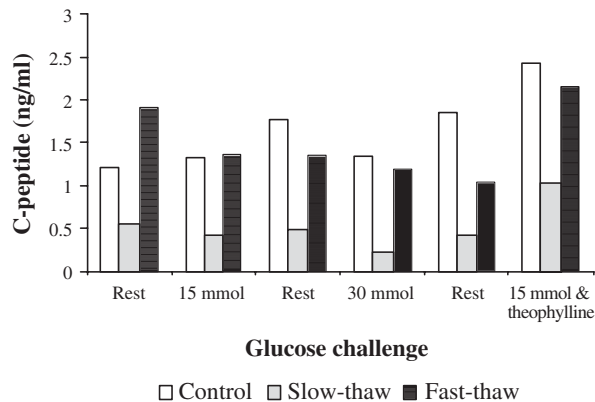


Fig. 3. C-peptide release from control, slow and fast thawed islets, 1 day post-thawing. The protocol and time periods are described in Fig. 1.

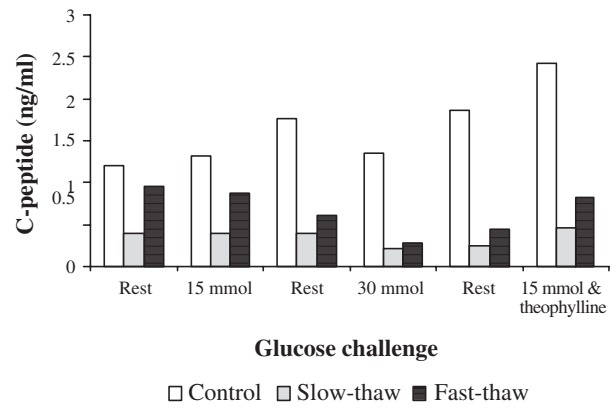


Fig. 4. C-peptide release from control, slow and fast thawed islets, 2 days post-thawing. The protocol and time periods are described in Fig. 1.

that formed a continuous layer. Two days post-thawing, most of the islets showed signs of degeneration in the form of fragmented cells, especially in the central part of the large islets (Fig. 8). The reaction of the slow thawed islets to the anti-insulin serum was variable, ranging from strongly positive reaction to markedly weak reaction.

The fast thawed islets appeared, 1 day after thawing, as spherical or ovoid structures containing anastomosing irregular cords of cells (Fig. 9). The islets appeared more or less similar to the control islets; with regular periphery, healthy cells, and no signs of degeneration. Similar histological appearance was seen in the fast thawed islets 2 days post-thawing. The reaction of the fast thawed islets to the anti-insulin serum was generally weaker than that of the control islets (Fig. 10). In many islets, particularly the large ones, the cells differed slightly in their degree of reaction to anti-insulin; the cells at the periphery of the islets showed strongly positive reaction, whereas the cells in the center showed somewhat weak reaction.

*Morphometric analysis*

The mean profile diameter of the isolated islets was generally lower than that of the intact pancreas (Table 3).

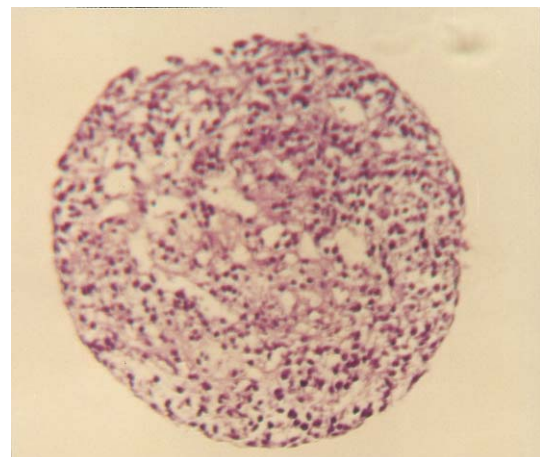


Fig. 5. An isolated islet of rat fetus, cultured for 4 days (control). Hematoxylin and eosin stain. ×400.

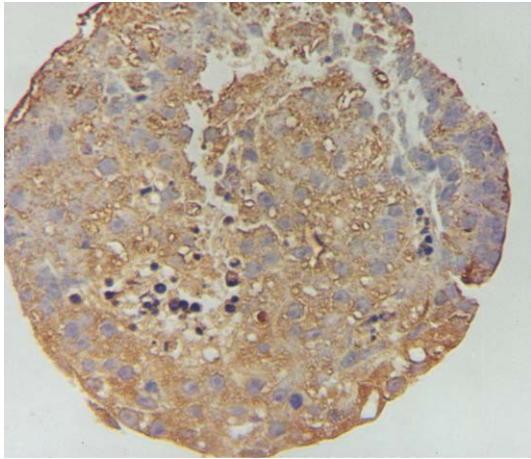


Fig. 6. An isolated islet of rat fetus cultured for 5 days (control) and stained for insulin. Most of the islet cells are positively stained (brown color). Immunoperoxidase stain for insulin.  $\times 800$ .

The mean profile diameters of the slow thawed islets were significantly lower ( $P < 0.05$ ) than those of the control islets. The mean profile diameters of the fast thawed islets were not significantly different from those of the control islets (Table 3).

### Discussion

In the present work, fetal rat islets were isolated from the pancreas by the collagenase digestion and culture technique of Hellerstrom et al. (1979), which is the most commonly used technique for isolation of the fetal islets. Our preparations of the isolated islets proved to be functionally intact and viable. This was assessed by measuring the insulin and C-peptide concentrations in the cultured islets, and repeated challenge of the cultured islets with 15 and 30 mmol/l glucose which resulted in highly reproducible responses of insulin release that were maintained for up to 5 h. Maximal response was elicited by theophylline, a standard test for measuring the functional integrity of the islets (Lacy et al., 1972; Malaisse, 1973; Henquin and Meissner, 1984).

The results of the present work showed that insulin and C-peptide contents in slow thawed islets were higher than those of the control, whereas the fast thawed islets showed results

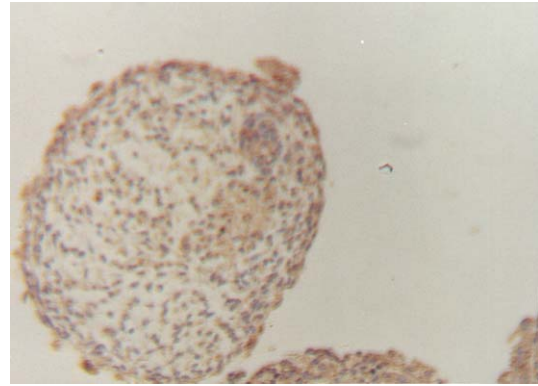


Fig. 8. An isolated islet of rat fetus cultured for 2 days after slow thawing and stained for insulin. Most of the islet cells showed weak positive reaction to anti-insulin serum (brown color). Immunoperoxidase stain for insulin.  $\times 400$ .

that were more or less similar to that of the control, except immediately following fast thawing. Insulin and C-peptide release from slow-thawed islets was lower than that of the control islets, and were nearly similar to that of the control in fast-thawed islets. Whereas extensive work has documented the effect of fast thawing (Sander et al., 1986; Simonsen et al., 1987; Dawidson et al., 1988; Kneteman et al., 1989; Beattie et al., 1993; Liu et al., 1993; Andereggen et al., 1996; Gonzalez-Clemente et al., 1997), few reports could be located in the literature comparing the effect of slow and fast thawing temperature on the cryopreserved islets (Taylor and Benton, 1987; Tze and Tai, 1990; Rich et al., 1993). Taylor and Benton (1987) found by physiological studies that survival of the isolated rat islets was greater than 50% with fast warming rate, compared to slow warming. Tze and Tai (1990) compared rapid (in 37 °C water bath) with slow (in air) thawing of the rat pancreatic endocrine cells (PEC) and insulinoma cells and reported up to 80% cellular viability (by trypan blue dye exclusion) with a rapid thawing. Rich et al. (1993) proved by transplantation studies that rapid warming of rat cryopreserved islets at 200 °C/min is much better than slow warming at 50 °C/min in maintaining the normal glycaemic state of the recipient rats.

The higher insulin contents and C-peptide contents at D-5 in the slow thawed islets may be attributed to the inhibitory effect of the thawing techniques on the insulin or C-peptide release

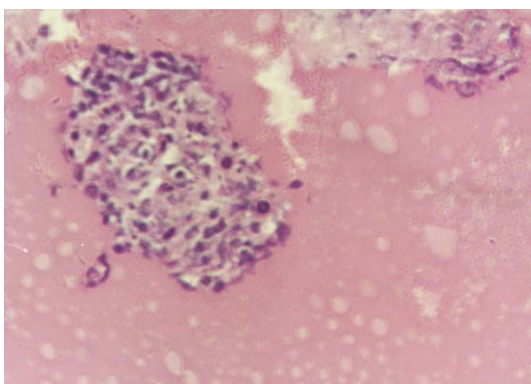


Fig. 7. An isolated islet of rat fetus cultured for 1 day after slow thawing. Hematoxylin and eosin stain.  $\times 600$ .

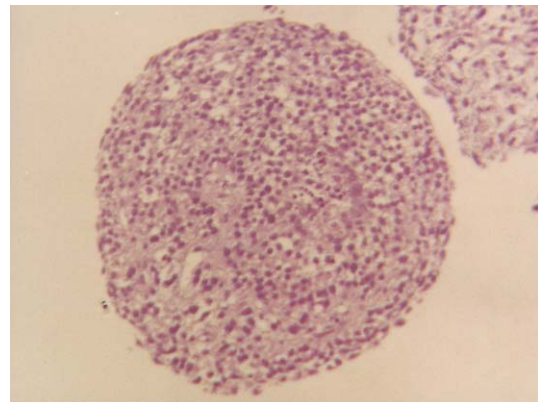


Fig. 9. An isolated islet of rat fetus cultured for 1 day after fast thawing. Hematoxylin and eosin stain.  $\times 400$ .

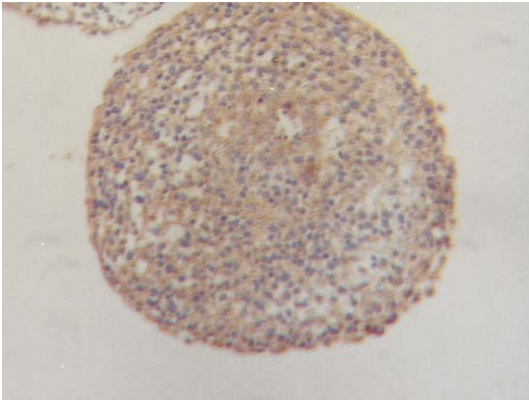


Fig. 10. An isolated islet of rat fetus cultured for 2 days after fast thawing and stained for insulin. Most of the islet cells are positively stained (brown color). Immunoperoxidase stain for insulin.  $\times 400$ .

from the secretory granules. The late response of the fetal islets to 30 mmol/l glucose and the improper response to the final challenge with theophylline could be attributed to the immaturity of the fetal islets, as it was noticed in the control islets as well as in the slow and fast thawed islets. Similar findings were also reported in the literature and were attributed to the immaturity of the B-cells (Dudek et al., 1984; Hegre et al., 1984; Mourmeaux et al., 1985; Masaki et al., 1987; Tuch and Zheng, 1993; Bergsten et al., 1998; Mendonca et al., 1998). However, the underlying mechanism is not exactly known. Some authors attributed it to low glucokinase activity (Taniguchi et al., 2000), lack of peptidergic stimulation by GH and TGF-Alpha (Tu et al., 1999; Sjöholm et al., 2000b), or inhibition by somatostatin through paracrine interaction (Sjöholm et al., 2000b). There is a proof that the fetal islets continue to differentiate into mature islets after culture or transplantation (Hegre et al., 1984; Dudek et al., 1984; Tuch and Zheng, 1993; Sjöholm et al., 2000a).

The value of tissue culture before cryopreservation or after thawing has been suggested by several authors (McKay and Karow, 1983; Sandler and Andersson, 1987; Foreman and Taylor, 1989; Vasir et al., 1989; Kneteman et al., 1989; Foreman et al., 1993; Lakey et al., 1997; Duvivier et al., 1999; Gatto et al., 2003). McKay and Karow (1983) have found that islet function can be significantly improved by increasing the duration of post-thaw culture. Sandler and Andersson (1987) reported that the percentage recovery of cryopreserved islets was increased with pre-thawing and post-thawing cultured for 3 days. Foreman and Taylor (1989) found that post-thaw culture (72 h) was essential for the retention of insulin

secretory function of the cryopreserved/thawed islets. Vasir et al. (1989) showed that the best results could be obtained when cryopreservation of adult inbred Lewis rat islets was followed by rapid thawing to 37 °C and then a 24 h culture. Kneteman et al. (1989) found that insulin release from the cryopreserved human islets could be improved from 79% to 87% with post-thaw 48 h culture period. Foreman et al. (1993) found that cryopreserved rat islets required 72 h of culture for a survival rate of more than 50%. Lakey et al. (1997) reported the recovery of islets after 48 h of tissue culture, following cryopreservation and thawing. Duvivier et al. (1999) found that increasing the culture time (3, 7, 14 or 21 days) before and after cryopreservation/thawing of pig islet cells, improved insulin release in response to glucose. Gatto et al. (2003) indicated that coculture of human islets with pancreatic ductal epithelial cells (DEC) represents a valuable tool to improve the survival and functional activity of islets, especially following cryopreservation/thawing.

Although functional studies of the cultured islets were reported by many authors (Slavin et al., 1977; Dudek et al., 1980; Georgiou and Mandel, 1984; Mourmeaux et al., 1985; Yang and Wu, 1989; Leduque et al., 1992; El-Naggar et al., 1993), we could not find, however, any comprehensive study combining the effect of cryopreservation/post-thaw culture with the morphological findings. Although a few authors (Dawidson et al., 1988; Von Mach et al., 2003) described some structural aspects of the cultured cryopreserved fetal islets, however, detailed comprehensive correlation of structure and function was not documented. Dawidson et al. (1988) found that frozen-thawed human fetal pancreata (HFP) appeared viable as judged by light and electron microscopy. Von Mach et al. (2003) found a negative correlation between islet size and viability that was significantly enhanced after cryopreservation ( $r < -0.8$ ); where the large islets represented a highly susceptible population concerning damage due to cryopreservation. The results of the present work showed that the fast thawed islets were morphologically intact, as proved by a continuous layer of regular periphery, absence of signs of degeneration, slight decrease in the mean profile diameters, and better staining with the anti-insulin serum. Our results are comparable to the findings of the cultured, non-cryopreserved islets described by Dudek et al. (1980), Georgiou and Mandel (1984), Mourmeaux et al. (1985), Yang and Wu (1989) and Leduque et al. (1992).

It could be concluded that the morphological findings strongly support the findings of the functional studies that fast

Table 3  
The mean profile diameters ( $\mu\text{m}$ ) of islets after slow and fast thawing

	Intact pancreas	D-1	D-2	D-3/thawed	D-4	D-5
Control	247 $\pm$ 17 (600)	196 $\pm$ 9 (600)	192 $\pm$ 10 (600)	193 $\pm$ 7 (200)	189 $\pm$ 8 (200)	191 $\pm$ 7 (200)
Slow-thaw				135 $\pm$ 8 (200)	124 $\pm$ 7 (200)	112 $\pm$ 10 (200)
( <i>t</i> -test)				( $p < 0.05$ )	( $p < 0.05$ )	( $p < 0.05$ )
Fast-thaw				174 $\pm$ 6 (200)	167 $\pm$ 7 (200)	169 $\pm$ 7 (200)
( <i>t</i> -test)				NS	NS	NS

Note: NS = non significant.

Data are presented as mean $\pm$ SEM (number of specimens).

thawing and post-thaw culture is much better than slow thawing. Post-thaw cultures for 1 or 2 days did not show any significant difference in the morphology of the islets after both fast and slow thawing.

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