Differential Freezing Tolerance of Rat Pancreatic Islets Depending on Their Size Variation

Iwao SAKONJU, Yasuho TAURA, Kouichi MAMBA\(^1\), Tatsuyuki SUZUKI\(^2\), Koichi TAKIMOTO\(^3\), Munekazu NAKAICHI, and Sanenori NAKAMA

Departments of Veterinary Surgery and \(^1\)Veterinary Anatomy, \(^2\)United Graduate School of Veterinary Medicine, \(^3\)Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753, Japan

(Received 9 March 1995/Accepted 6 June 1995)

ABSTRACT. We have investigated the freezing tolerance of rat pancreatic islets. Freshly isolated rat pancreatic islets were divided into three groups based on their longest diameter (small; 100–200 μm, medium; 201–300 μm, large; >300 μm). They were then cryopreserved at a slow cooling rate (-0.3°C/min) in the presence of dimethyl sulfoxide (Me\(_2\)SO) or ethylene glycol (EG). After storage at -196°C for 1–4 weeks, they were thawed and their ability to secrete insulin in response to fluctuations in glucose concentration was examined during three consecutive static incubations in vitro (1st; 2.8 mM, 2nd; 16.7 mM, 3rd; 2.8 mM). Morphological examination of the beta-granule population was determined by image analysis, and correlation with islets size was analyzed. The amount of insulin released from large-sized islets was significantly suppressed in EG (p<0.05) and Me\(_2\)SO (p<0.01) groups compared to unfrozen islets. However, the mean volume of the large-sized islets isolated from one rat accounted for 43.0% of the total volume. On the other hand, the amount of insulin released from small- and medium-sized islets did not differ from those of unfrozen islets, and their mean volumes were 13.2 and 43.8%, respectively. The percentage of cells with beta-granules was significantly correlated with size in both EG (r= -0.52) and Me\(_2\)SO (r= -0.35) groups, but no significant correlation was observed in the unfrozen islets groups. These findings suggest that large-sized islets are more susceptible to freezing injury than small- or medium-sized islets. Moreover, the volume distribution of isolated islets indicated that it may be important to retain the ability of insulin secretion from the large-sized islets.—KEY WORDS: cryopreservation, freezing tolerance, image analysis, rat pancreatic islet.


A number of reports indicate that frozen/thawed isolated islets secrete insulin normally in response to fluctuations in glucose. However, several other groups [6, 14] and ourselves [12] have demonstrated a reduction in glucose-stimulated insulin release from frozen/thawed islets in vitro. In addition, larger numbers of frozen/thawed islets are required to reverse diabetes as compared with freshly isolated islets [7, 9]. This undoubtedly would be due to the damage to islet cells during the cryopreservation process.

Although the viability of frozen/thawed islets is often assessed by analysing the data from “randomly selected islets”, pancreatic islets consist of small aggregates of cells, and have a considerable size variation typically 40–400 μm in diameter [15]. Because of their morphological characteristics, islets rarely show uniform equilibration with cryoprotective additives, and it is therefore possible that the size of islets can affect freezing tolerance.

In this study, we investigated the relationships between the freezing tolerance and islet size focusing on the loss of insulin secretory function and the beta-granule population in frozen/thawed rat pancreatic islets.

MATERIALS AND METHODS

Islet isolation and culture: Pancreatic islets were isolated from ad libitum-fed female Wistar rats (Kyudo, Co., Ltd, Japan) weighing 200–250 g as previously described [11, 12]. After several washes, islets were viewed with an inverted microscope and categorized quickly as small (100–200 μm), medium (201–300 μm) or large (>300 μm), depending on their longest diameter using an eyepiece ocular. Islets were then cultured for 2 days in the RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% (v/v) fetal calf serum (FCS) (Biomedicals Japan Co., Ltd., Australia) and antibiotics.

Exposure to cryoprotectant: Dimethyl sulfoxide (Me\(_2\)SO, Wako Pure Chemical Industries Ltd., Japan) and ethylene glycol (EG, Wako Pure Chemical Industries Ltd., Japan) were used as cryoprotectants. Categorized and cultured 100–120 islets were suspended in a sterilized plastic tube containing 400 μl of TCM 199 supplemented with 10% FCS. In the EG-cryopreservation group, 400 μl of TCM 199 containing 3.0 M EG was added directly, and then the sample was allowed to equilibrate at room temperature for 20 min (22°C) [12]. The concentration of Me\(_2\)SO was raised stepwise (0.67M; at 22°C for 5 min, 1.0M; at 22°C for 25 min, 2.0M; at 0°C for 15 min) [8]. After equilibration, islets were loaded quickly into the freezing straws.

Freezing and thawing: The straws were placed into a programmable freezer maintained at 0°C for 2 min. Then they were cooled from 0°C to -5.5°C at a rate of -1°C/min and seeded at -5.5°C. The straws were cooled to -40°C at a rate of -0.3°C/min, and then transferred to the liquid nitrogen for storage at -196°C.

After storage at -196°C for 1–4 weeks, the islets cryopreserved with EG were thawed by placing the straws in a 30°C water bath, and the contents were directly drained into 10 ml of culture medium. In the Me\(_2\)SO-cryopreserved
group, cryoprotectant was removed using the 0.75 M sucrose method of Rajotte et al. [8]. Thawed islets were cultured for another 4 days before being used for assay. Unfrozen control islets were cultured for 6 days without any further treatment. Culture medium was changed every two days.

Insulin release: Groups of 10 islets were selected from each group, and transferred into a 1.5 ml capped incubation vial (MS-4501W, Sumitomo Co., Ltd., Japan). Subsequently, the islets were incubated consecutively three times in the 0.5 ml of well gassed modified Krebs-Ringer bicarbonate buffer (KRB) [5] supplemented with various concentration of D-glucose (1st; 2.8 mM for 30 min, 2nd; 16.7 mM for 60 min, 3rd; 2.8 mM for 60 min). The supernatants from the second and third incubation periods were stored at -40°C until used for insulin assay.

The insulin concentration in the samples was determined by radioimmunoassay with rat insulin (Novo, Bagsvaerd, Denmark) as the standard and 125I-labeled insulin (Novo, Bagsvaerd, Denmark) as a radiotracer.

Morphometric measurement of sectioned islets: On the day of the insulin release test, the unfrozen or frozen/thawed islets were fixed in Bouin’s solution, and embedded in paraffin. Several 4-μm-thick serial sections were cut from the block, in order to determine the number of sections which could be cut from one block. One section located in the middle of the block was stained. The sections were stained with an indirect immunoperoxidase method employing guinea-pig anti-insulin antibody (Dako, Denmark) and peroxidase-conjugated rabbit anti-guinea pig IgG (Dako, Denmark).

After staining, only morphologically intact islets were photographed at 120X magnification under a microscope (BH-RFK, Olympus Co., Ltd., Tokyo) with a 530 nm green filter (G 530, Olympus Co., Ltd., Tokyo). The longest (LD) and shortest (SD) diameters of each islet was then measured on the back-lighted negative film using a micrometer scale which was photographed at the same magnification. The radius of each islet was obtained from (LD + SD)/4, and values for the area were subsequently calculated by the formula; (LD + SD)/4)² π [10].

Image analysis: A personal image analysis system (PIAS, LA-525, PIAS Co., Ltd., Osaka) was used, and all processing was carried out using the standard PIAS software.

All sectioned islet images captured from negative films using a CCD camera (Fujivision FV7, Fujifilm Co., Ltd., Japan) were recorded on disk. The video signal from the TV camera was digitized into a regular array of 512 × 512 pixels. Images of individual islets were duplicated to calculate both the number of pixels in insulin-positive areas (IP) and in whole islet (WI). The total number of pixels of both image sets were counted in the binary images.

Statistic analysis: In the insulin release test, data were expressed as mean ± SD. Groups of data were compared using the unpaired Student’s t test. Differences were considered to be statistically significant if t values corresponded to a probability of 0.05 or less. In the morphological study, the correlation coefficient r between the percentage of the insulin positive area (IP/WI) and the area of sectioned islets was calculated, and the corresponding regression lines drawn. The significance of r was estimated by t analysis.

RESULTS

Characterization of isolated pancreatic islets: Table 1 shows the mean longest diameter, number and volume distribution of islets which were isolated from one rat. Islet volumes were calculated using the formula 4/3πr³ (r=longest diameter/2). The number of islets smaller than 300 μm in diameter represented 83.3% of the total number, whereas their volume accounted for 57.0% of the total volume. On the contrary, although the number of large-sized islets occupied only 16.7% of the total number, their volume accounted for 43.0% of the total volume.

Insulin release: The amounts of insulin secreted by frozen/thawed and unfrozen islets are shown in Table 2. In comparison with the third incubation phase, an increased insulin secretion, ranging from a 1.76 to a 3.09 fold increase, was observed during the second incubation phase in all groups. The amounts of insulin secretion and the stimulation factors, in the small- and medium-sized islet groups, did not significantly differ from that of unfrozen islets. However, the amount of insulin secretion from large-sized islets during the second incubation phase was significantly less than that of unfrozen islets (Me₃SO; p<0.05, EG; p<0.01), and stimulation factors (2nd/3rd) were also significantly lower.

<table>
<thead>
<tr>
<th>Table 1. Characterization of freshly isolated rat islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size category (range)</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Small (100–200 μm)</td>
</tr>
<tr>
<td>Medium (201–300 μm)</td>
</tr>
<tr>
<td>Large (&gt;300 μm)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

a) Volume of individual islets was calculated using the formula 4/3πr³ (r=longest diameter/2).
Table 2. Insulin release of cryopreserved islets versus unfrozen islets

<table>
<thead>
<tr>
<th>Cryoprotectant</th>
<th>Incubation Phase</th>
<th>Small (100–200 μm)</th>
<th>Medium (201–300 μm)</th>
<th>Large (&gt;300 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfrozen</td>
<td>Second</td>
<td>1.05 ± 0.26</td>
<td>1.73 ± 0.36</td>
<td>2.43 ± 0.37</td>
</tr>
<tr>
<td>Control</td>
<td>Third</td>
<td>0.38 ± 0.13</td>
<td>0.75 ± 0.14</td>
<td>1.13 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>2nd/3rd</td>
<td>2.91 ± 0.88</td>
<td>2.32 ± 0.28</td>
<td>2.23 ± 0.48</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>Second</td>
<td>0.96 ± 0.18</td>
<td>1.61 ± 0.38</td>
<td>1.95 ± 0.35*</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>0.34 ± 0.10</td>
<td>0.80 ± 0.13</td>
<td>1.29 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>2nd/3rd</td>
<td>3.02 ± 0.84</td>
<td>2.03 ± 0.39</td>
<td>1.55 ± 0.33**</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>Second</td>
<td>1.11 ± 0.25</td>
<td>1.68 ± 0.37</td>
<td>1.87 ± 0.28**</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>0.37 ± 0.07</td>
<td>0.82 ± 0.25</td>
<td>1.04 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>2nd/3rd</td>
<td>3.09 ± 0.79</td>
<td>2.20 ± 0.78</td>
<td>1.76 ± 0.36*</td>
</tr>
</tbody>
</table>

Note. Values are means ± SD (ng/islets/hr) for eight experiments.

*, ** denote p<0.05 and p<0.01, respectively, when comparing unfrozen islets.

than that of unfrozen islets in both cryoprotectant groups (Me2SO; p<0.01, EG; p<0.05).

**Image analysis:** The decrement of the IP/WI ratio of the islets frozen with EG or Me2SO correlated significantly with the increment in the area of the sectioned islets (EG; r= -0.52, p<0.001, Me2SO; r= -0.35, p<0.001) (Fig. 1). On the other hand, no significant correlation was observed between the IP/WI ratio and area in unfrozen islets.

The distribution of the percentage of insulin-positive areas over the sectioned islets areas is shown in Fig. 2. The insulin-positive areas of sectioned islets which had areas larger than 20 x 10^3 μm^2 were significantly decreased as compared with unfrozen islets in both cryoprotectant groups.

In the light microscopic observations, the necrotic cells were often observed at the center of large-sized islets. This frequency of the unfrozen islets and islets cryopreserved with EG or Me2SO were 28, 52 and 45%, respectively.

**DISCUSSION**

Long-term cryopreservation potential of pancreatic islets adds significant flexibility to clinical trials [1, 4, 8, 13]. However, the problem of functional loss, which is a result of the freezing protocols, has still not been resolved. Recently, we first reported the use of EG as a cryoprotectant of low toxicity for the cryopreservation of islets, and found that insulin release from frozen/thawed islets in either the presence of EG or Me2SO decreased to 60% as compared with the unfrozen islets [12]. Our present study was designed to enhance the procedure in order to optimize the freezing protocols.

Reaven et al. [10] have shown that the amount of insulin released from islets in a single concentration of glucose is directly proportional to their size. As shown in Table 2, our results are consistent with this. In terms of the number of islets which could be isolated from donors, Wolters et al. [15] reported that the major part of the isolated islets volume is formed by a subpopulation of large islets, and that even a large number of small islets contributes little to the tissue volume of the total islets. Also Bonner-Weir [2] described that islets smaller than 160 μm in diameter represents 75% of the islets number but only 15% in volume, whereas islets larger than 250 μm in diameter represented only 15% of the number but 60% of the volume. Our present findings are in good agreement with this (Table 1). These observations indicate that the successful cryopreservation of large-sized islets is necessary to minimize the loss of functional islets. In other words, it is possible that the optimum freezing conditions for large-sized islets will differ from that of small- or medium-sized islets.

Hellman [3] reported that the mean relative area of beta cells in an islet was 79.8 percent in a 100-day-old rat. Although the percentage of insulin-positive areas presented here was slightly low even for unfrozen islets, this might be due to the difference of measurements, i.e., we measured the size of insulin positive areas but not the number of beta cells. However, it must be noted that the size of sectioned islets cannot be directly compared with that of freshly isolated islets because of contraction during the fixation and embedding procedures. Hence, in our study, we must consider freshly-isolated islets and sectioned islets separately in relation to their size.

The morphological evidence that the insulin-positive area on sections of frozen/thawed islets decreased along with their size (Figs. 1 and 2), taken together with the data concerning the release of insulin from the islets, lead us to believe that the large-sized islets were more susceptible to freezing injury. Moreover, the frequency of necrosis in the center of the frozen/thawed large-sized islets group was apparently higher in the unfrozen islets (Fig. 3). It is likely that the difficulty of proper penetration of cryoprotectant into the core of large-sized islets resulted in necrosis.

Further experiments will be needed to find optimal freezing conditions for large-sized islets, and which will therefore lead to a more successful islet cryopreservation protocol.
Fig. 1. Correlation between the percentage of insulin-positive areas and sectioned-islets areas.

Fig. 2. Distribution of the percentage of insulin-positive area over the sectioned-islets area. Values are shown as mean ± SD. * † ‡ represent p<0.05, p<0.01 and p<0.001, respectively, when compared to the unfrozen control.

Fig. 3. Light microscopy of islets cryopreserved with EG after culturing for 4 days after thawing. Four μm thick paraffin sections were stained by the indirect method using guinea-pig anti-insulin antibody and peroxidase-conjugated rabbit anti-guinea pig IgG. Immunohistology confirmed the presence of beta granules, but necrotic cells (arrow) were observed at the center of islets. ×33.
REFERENCES


