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# Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors Using Hydroxyethyl Starch and Dimethyl Sulfoxide as Cryoprotectants

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Although widely used, DMSO is toxic for pancreatic islets. We combined hydroxyethyl starch (HES) with DMSO to simplify the procedure of freezing and thawing, and to decrease the toxicity of DMSO. A preclinical study was performed using islets from beagle dogs. After storage for 4 weeks, the islets were thawed and examined. The islet structure was well maintained after thawing. Although the number of the islets decreased to 71.2 ± 20.1%, the function of the islets was evaluated by static incubation after thawing and showed a 1.80 ± 0.78 stimulation index. We have introduced this technique for the cryopreservation of human islets from non-heart-beating donors. Twelve cases of human islet cryopreservation were performed. The sample tube of each human cryopreservation was thawed to evaluate the morphology, contamination, and endocrine function. Although fragmentation was observed in five samples (41.6%), the other seven (58.4%) showed a normal structure when evaluated by microscopic and electron microscopic study. The stimulation index (SI) of static incubation deteriorated from 3.37 ± 3.02 to 1.34 ± 0.28 after thawing. We divided the thawed islets into two groups: group 1 ($n = 8$), SI >1.2; group 2 ($n = 4$), SI <1.2. The group 1 islets showed a higher rate of normal structure (87%) than did group 2 (25%). Moreover, the SI before cryopreservation was 4.01 ± 3.57 in group 1, which was higher than the SI of 2.11 ± 0.72 in group 2. Based on the good results from the preclinical study using a large-animal model, this method was introduced for clinical application. Even from the pancreata of non-heart-beating donors, a successful islet cryopreservation was achieved. However, the isolated islets with poor function should not be cryopreserved for transplantation.

Key words: Cryopreservation; Human islets; Hydroxyethyl starch; Islet transplantation

## INTRODUCTION

Pancreatic islet transplantation is considered to be the most physiologically advantageous procedure for the treatment of type 1 diabetes mellitus. The introduction of the Edmonton protocol, with a highly improved rate of insulin independency, encouraged us to promote clinical islet transplantation (18,20). In Japan, clinical islet transplantation was first performed by Kyoto University in 2004 (10). Thirteen type 1 diabetic patients, thus far, have undergone one or more islet transplantations. According to the social circumstances of Japan, the pancreata were able to be harvested only from non-heart-beating donors for islet isolation. The non-heart-beating donors in Japan are usually of higher age and the most frequent cause of death is a cerebrovascular disorder. Moreover, the withdrawal of respirator support is not commonly performed and, thus, a cannulation of the aorta before a cardiac arrest is not necessarily performed. Therefore, the viability of the pancreata may deteriorate due to various factors that include aging, a prolonged warm ischemia, and a damaged microcirculation. In Japan, the criteria for fresh islet transplantation have been determined based on the Edmonton protocol (20). According to the rules of the Japanese Islet Transplant Team, the islets must be cryopreserved even if the results (yield, purity) do not reach the criteria for fresh islet transplantation.

The cryopreservation of human pancreatic islets offers many advantages for clinical transplantation. Cryostorage allows for the accumulation of a large amount of donor tissue so that a sufficient number of islets with

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a desired HLA tissue type can be provided for transplantation. The frozen islets can be shipped to other institutions worldwide. In addition, an accurate pretransplant evaluation, in terms of safety and efficacy, is possible during cryopreservation. However, the major problem with the cryopreservation of islets is a decreased number and function of the frozen–thawed islets compared with the fresh islets. According to the Edmonton protocol, only fresh islets can be used for clinical islet transplantation.

In the present study, we have designed a simple technique of cryopreservation using hydroxyethyl starch (HES) and dimethyl sulfoxide (DMSO) as cryoprotectants for the purpose of protecting the islets from the toxic effect of DMSO. Based on the preclinical study using a large-animal model, this technique was introduced for the cryopreservation of human islets from the pancreata of non-heart-beating donors.

## MATERIALS AND METHODS

### *Preclinical Study Using Beagle Dogs*

*Animals and Islet Preparation.* Five beagle dogs, weighing from 7.5–12.5 kg, were used. The islets were isolated from the pancreata of the dogs by an automated two-step digestion method that we have developed, followed by a Ficoll purification as previously described (5,6).

*Cryopreservation and Thawing of the Islets.* After an overnight culture, a known number of islets were suspended in RPMI-1640 containing 5% DMSO, 6% HES, and 4% FBS on ice, then transferred into a 75-ml cryogenic storage container (7005-2, CharterMed Inc, Lakewood, NJ). The container was cooled using a programmed freezing system, Cryomed Model 1010 (Forma Med Inc., Marietta, OH). After 4 weeks of storage in liquid nitrogen, the container was rapidly thawed in a 37°C water bath. The islets were sedimented and resuspended with RPMI-1640 containing 10% FBS.

*Static Incubation.* To assess the function of the thawed islets, static incubation was performed. Briefly, five aliquots of 10 islets were placed into 12-well transwell microplates with 1 ml RPMI-1640 containing 3.3 mmol/L D-glucose and 0.1% BSA as the basal medium. After 60 min, the culture transwells were transferred into new 12-well microplates with RPMI-1640 containing 20 mmol/L D-glucose and 0.1% BSA (glucose stimulation). After 60 min, the culture transwells were transferred to new 12-well microplates to add basal medium again for an additional 60-min culture. Each medium was centrifuged and immediately frozen for a later assay of the insulin concentration by ELISA. The stimulation index was calculated by comparing the insulin content in the glucose stimulation medium with the second basal medium.

### *Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors*

*Harvesting of the Pancreata.* Twelve pancreata were harvested from the non-heart-beating donors under an informed consent from September 2003 to July 2006. The characteristics of donors are shown in Table 1. Half of the non-heart-beating donors were over 50 years of age and, thus, the major cause of death was a cerebrovascular disorder. Five of the cases were not permitted a cannulation of the aorta and a systemic heparinization before cardiac arrest, which resulted in the prolonged warm ischemic period. In addition, the durations of hypotension and anuria were extremely prolonged because the withdrawal of a respirator is not commonly performed in Japan.

*Islet Preparation.* The pancreata were preserved by the two-layer method (9,11) in eight cases and by simple cold storage in University of Wisconsin solution in four cases, and were transferred to the Cell Processing Center located in the Clinical Research Center of our hospital. The islet isolation was performed according to the Edmonton protocol with some modifications. Briefly, the pancreas was distended with cold Liberase solution (Liberase HI™, Roche Diagnostics, IN) by a ductal injection. Thereafter, the distended pancreas was cut into several pieces and put into a Ricordi chamber and digested using a closed automated system at 37°C. The shaking of the Ricordi chamber was performed either by hand or by a shaker. The pancreatic digests were collected in a flask on ice and were purified on a Euro-Ficoll discontinuous solution using a COBE 2991 cell processor.

*Cryopreservation and Thawing of the Islets.* After an overnight culture with serum-free medium [1% L-glutamine, 1% antibiotic antimycotic solution, 16.8 mM zinc sulfate, 1% ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 ng/ml selenious acid, 5.35 mg/ml linoleic acid, 1.25 mg/ml albumin; Collaborative Biomedical Products), CMRL1066], the islets were cryopreserved. The cryopreservation method used is the same procedure as described above in the preclinical study except for the use of human albumin in place of fetal bovine serum. In addition to the container, eight sample tubes containing 500–1000 islets were cryopreserved for the purpose of a sample check during cryostorage. From 2 weeks to 3 months after cryostorage, the sample tube was thawed using the rapid thawing technique as described above to evaluate the islet morphology, to check for the presence of bacterial or fungal contamination, and to evaluate the islet function.

**Table 1.** Characteristics of the Non-Heart-Beating Donors Used for the Cryopreservation of the Islets

| |
|---|
| Age (years): 43.2 ± 20.2 (10–69) (>50 years: 6 cases) |
| Male/female: 6/6 |
| Cause of death |
| Cerebrovascular disorder: 7 cases (58.3%) |
| Hanging (suicide): 6 cases (50%) |
| Brain tumor: 1 case (8.3%) |
| Cannulation into the aorta before cardiac arrest: Yes—7 cases (58.3%), No—5 cases (41.7%) |
| Warm ischemic time (min) 10.5 ± 10.5 (1–30) |
| Total ischemic time (min) 325 ± 54.3 (231–436) |
| Duration of hypotension* (min) 333 ± 332 (0–840) |
| Duration of anuria (min) 447 ± 620 (0–1800) |

*Maximum blood pressure: <60 mmHg.

*Evaluation of the Islets.* The frozen–thawed islets in the sample tube were cultured overnight in serum-free medium and were evaluated to determine if it was possible to use the islets for transplantation, by the following examinations. The morphology of the islets was microscopically examined by a staining with dithizone, and by an immunostaining with anti-insulin and antiglucogon antibodies. In addition, an electron microscope was used for further study of the islets' morphology. The supernatant of the cryopreservation solution was checked for contamination by bacteria, including the acid-fast bacteria and the tubercle bacillus, as well as fungal organisms, to assure the safe use for transplantation. As a functional assay, a static incubation was performed and the stimulation index was calculated as mentioned above.

## RESULTS

### *Preclinical Study Using Beagle Dogs*

The yield and purity of the islets of the five isolations, which were evaluated after an overnight culture and before cryostorage, were 80,349 ± 37,164 IEq and 87.0 ± 5.7%, respectively. Although the purities improved to 96.2 ± 1.6% after thawing, the number of the islets decreased to 57,595 ± 31,027 IEq (recovery rate: 71.2 ± 20.1%). In the morphologic study, however, the shape of the frozen–thawed islets was well maintained and the fragmentation or clumping of the islets was hardly observed.

The simulation index (SI) calculated from static incubation was 1.80 ± 0.78. In addition, the islets of isolate #2 were examined by a perifusion study and a prompt release of insulin with two peaks was observed (Fig. 1).

These preclinical data demonstrated the effective preservation of both the structure and the endocrine function of the frozen–thawed islets in our cryopreservation method.

### *Cryopreservation of Human Pancreatic Islets From Non-Heart-Beating Donors*

*Yield and Purity of the Islets.* The results of the islet isolation are shown in Table 2. Because the high-yield (5,000 IEq/kg recipient body weight) viable islets with over 30% purity are used for fresh islet isolation, the results are from isolations that did not fulfill the criteria for fresh islet isolation.

*Check for Contamination.* The supernatant of the cryopreservation solution of all sample tubes was examined by a direct staining, and by a culture, for the contamination check. The direct staining and the culture for bacteria, including the acid-fast bacteria and the tubercle

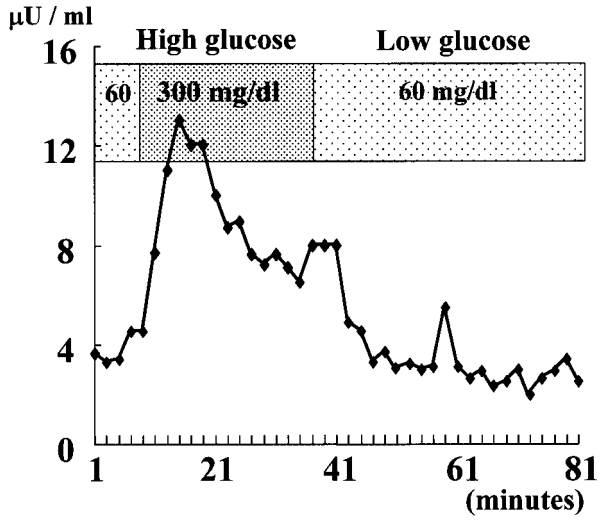


**Figure 1.** Insulin secretion from the frozen–thawed beagle islets was assessed by dynamic perifusion system. Prompt insulin release with two peaks showed the preservation of endocrine function of the frozen–thawed islets.

**Table 2.** Results of Islet Isolation From Non-Heart-Beating Donors*

| |
|---|
| Number: 12 |
| Yield (IEq†): 108,656 ± 45,803 (37,840–177,800) |
| Weight of pancreas: 75.3 ± 23.6 (37–108) |
| Yield/g pancreas (IEq†): 1,546 ± 699 (394–2857) |
| Purity (%): 40.0 ± 18.1 |

*Department of Surgery, Chiba-East National Hospital; the cases not used for fresh islet transplantation (September 2003 to July 2006).
†IEq: the number of the islet equivalent to 150 μm.

bacillus, as well as fungal studies, were all negative for the presence or the growth organisms. The data showed that the frozen–thawed islets in the present study were safe for transplantation.

*Morphological Study of Frozen–Thawed Islets.* A severe fragmentation was observed in five samples (5/12; 41.6%) and the other seven samples (7/12; 58.4%) showed a normal morphology evaluated by the microscopic examination with a dithizone staining and a hematoxylin-eosin staining (Fig. 2). The well-preserved islets showed a positive insulin staining and a normal fine structure by an electron microscopic study (Fig. 3).

*Static Incubation.* The SI of the static incubation deteriorated from 3.37 ± 3.02 at precryostorage to 1.34 ± 0.28 after thawing. We divided the thawing islets into the following two groups: group 1 ($n = 8$)—the SI after thawing was >1.2, and group 2 ($n = 4$)—the SI after thawing was <1.2. The group 1 islets showed a higher rate of good preservation of the islets' structure at the point of precryostorage (7/8; 87%) than did the group 2 islets (1/4; 25%) (Fig. 4). Moreover, the SI of the static incubation performed at precryostorage was 4.01 ± 3.57 in the group 1 islets, which was higher than the 2.11 ± 0.72 determined in the group 2 islets (Fig. 4).

## DISCUSSION

Islet transplantation is considered to be the most physiological treatment for type 1 diabetic patients. Although clinical islet transplantations have been successful in a limited number of patients (4) before 2000, the Edmonton protocol, which was introduced by an Alberta University group, drastically improved the results of clinical islet transplantation (18,20). In the Edmonton protocol, only fresh islets were used, immediately after isolation, for transplantation to achieve insulin independency in the diabetic patients. Cryopreserved islets were not used in this protocol in spite of the previous clinical experience with a cryopreserved islet allograft (19,22).

Encouraged by the successful results of the Edmonton protocol, the Japanese Islet Transplant Team prepared for the start of clinical islet transplantation, and the first human islet isolation was performed by our group on September 12, 2003. The first human islet transplantation for a type 1 diabetic patient was performed by the Kyoto group (10). In Japan, however, the pancreata from non-heart-beating donors are indicated for use in islet isolation because the pancreata from brain-dead donors are usually used for pancreas or pancreas/kidney transplantation.

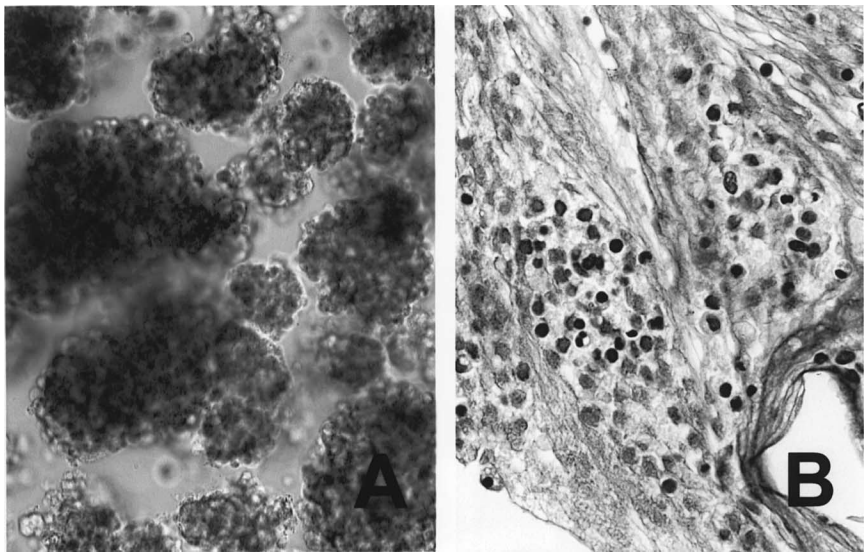


**Figure 2.** Microscopic findings of the frozen–thawed beagle islets stained with diphenylthiocarbazone (A) and hematoxylin-eosin (B). The normal morphology was preserved without fragmentation.

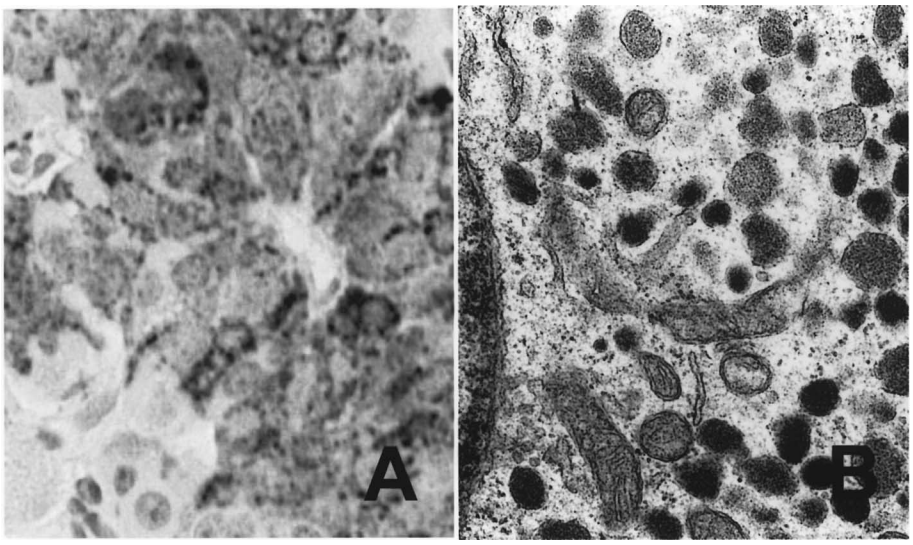


**Figure 3.** Frozen–thawed beagle islets stained with anti-insulin antibody (A) and electron microscopy (B). Positive staining of insulin as detected on the islets and the normal structure of organelles of the islet cell demonstrated the good preservation of the frozen–thawed islets.

In Japan, the withdrawal of a respirator is rarely performed even though the donor is diagnosed to be suffering from brain death. Moreover, the donors frequently are not given the examination to diagnose brain death, and thus, a cannulation of the abdominal aorta with a double balloon catheter via a femoral artery and a systemic heparinization are not indicated for the donors before cardiac arrest. Out of 12e non-heart-beating donors in the present study, we could not perform cannulation and heparinization before cardiac arrest in five cases (41.7%). Under the present conditions that exist in Japan, the viability of the pancreata may decrease before harvesting, thus often resulting in a poor yield and a low purity of the islet isolation. According to the rules of the Japanese Islet Transplant Team, the islets must be cryopreserved even if the results do not reach the criteria required for fresh islet transplantation.

Cryopreservation is thought to be an ideal method for

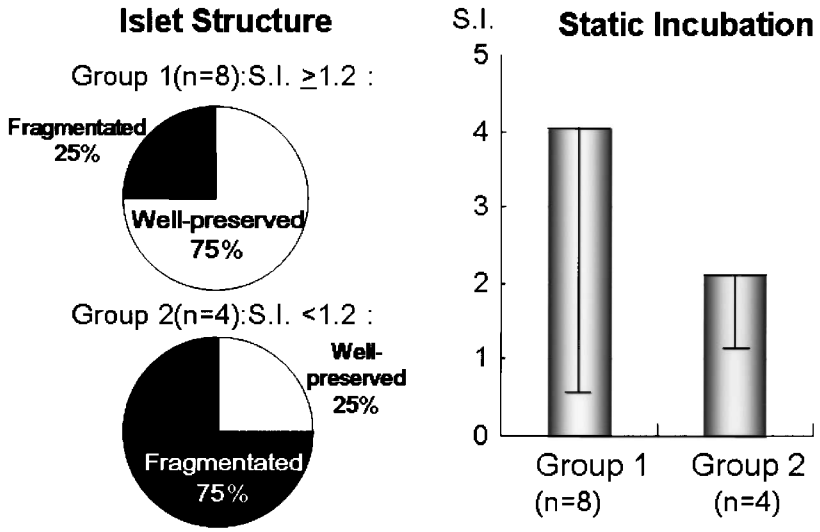


**Figure 4.** Human islet structure and stimulation index of static incubation at precryostorage. In group 1, islet structure was better preserved (75%) and the stimulation index was higher than those in group 2.

the long-term storage of human pancreatic islets, and many investigations concerned with the use of islet cryopreservation have been performed (3,7,12,15,16,21). The cryopreservation of islets may benefit many aspects of clinical islet transplantation. The islets are susceptible to contaminations by bacteria and fungi during the procedure of islet isolation and the check for contamination is impossible except for a gram staining in the fresh islet transplantation. The endocrine function does not necessarily correlate to the islet appearance immediately after isolation. The evaluation of the endocrine function, including a static incubation and a perfusion study, is also impossible in a fresh islet transplantation. For the safety and efficacy of the islets, contamination check and the evaluation of the endocrine function are primary, and these data can be obtained during the cryostorage of the islets. The cryostorage allows the accumulation of a large amount of donor tissue so that a sufficient number of islets with a desired HLA tissue type can be provided for transplantation. Furthermore, the frozen islets can be shipped to other institutions worldwide. The immunogenicity of the islets may deteriorate during cryostorage according to the reduction of the MHC antigen (2,13).

The major disadvantage of cryopreservation, however, is the deterioration of the number and function of the islets after thawing (17). One of the major causes is the toxicity of the DMSO, which is widely used for cryoprotection. DMSO has been used as an intracellular cryoprotectant. However, because of its toxicity against islet cells, the DMSO must be added stepwise and also diluted stepwise with sucrose. Several materials, including trehalose (1), ethylene glycol (8), and polyethylene glycol (14), were used as a cryoprotectant for the cryostorage of the islets in previous studies. In the present study, we combined hydroxyethyl starch (HES) as an extracellular cryoprotectant with the DMSO to simplify the freeze–thawing procedure. By adding HES, the final concentration of the DMSO can be reduced from 10–12% to 6%, which reduces the direct toxicity of the DMSO and simplifies the freeze–thawing procedure. This modification may contribute to a preservation of the islets during the cryopreservation procedure and result in a higher yield and an improved function after thawing. Furthermore, the simplification of the procedure is important to reduce the opportunity for contamination. In the present preclinical study, although the islet number decreased to 71.2% after thawing, the structure was well maintained and a positive insulin secretion against the glucose challenge was seen both in the static and the dynamic perifusion study, thereby demonstrating the ability of our method to achieve good preservation of the islets during cryostorage.

From the results of the present study using human islets isolated from non-heart-beating donors, the safety of our method was confirmed because no contamination was detected in any of the 12 cryopreservations. As for the function of the islets, the SI decreased by cryostorage from $3.37 \pm 3.02$ to $1.34 \pm 0.28$. It is questionable, however, that the levels of SI directly reflect the islet function, and the cutoff level of the stimulation index is used to decide the use for transplantation. In the present study, we divided the frozen–thawed islets into two groups using 1.2 as the cutoff level of the SI. The SI for group 1 was >1.2, which can be used for transplantation, and the SI for group 2 was <1.2, which should not be used for transplantation. A normal structure and positive insulin staining, as evaluated both by light microscopy and by electron microscopy, were observed in group 1, and the fragmentation of islets and a negative insulin staining were detected in group 2. These data may indicate that the cutoff level of 1.2 for the static incubation that we used is reasonable to decide the fate of the frozen–thawed islets (i.e., whether or not to use them for transplantation).

Another important finding from the present study is the possibility of the evaluation of fresh islets to determine which should be cryopreserved and which should not. In group 1, both the structure and function were already well maintained after isolation. Especially the fragmented islets immediately after isolation reflected the poor viability of the pancreas from the donors with poor conditions, including old age, a prolonged warm ischemia, and/or a cold ischemia. Therefore, we can select the fresh islets for cryopreservation if the criteria is determined, which is an important consideration from the both the economic and the academic points of view.

In conclusion, we developed a new cryopreservation method by the combined use of HES with DMSO to simplify the procedure of cryostorage of pancreatic islets and to decrease the toxicity of the DMSO. Based on the good results of our preclinical study using a large-animal model, our method has been introduced to clinical islet cryopreservation. The human islets were able to be cryopreserved using our method with good preservation of both the structure and the function. The islets with a poor viability, however, should not be used for cryopreservation and transplantation. Further studies are needed to accomplish a high recovery and a higher function of the frozen–thawed islets.

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