Hypothesis: Mechanical injury and oxidative stress caused by reoxygenation of isolated porcine islet cells result in their unresponsiveness to glucose stimulation.

Design: Adult pigs (weighing 25-30 kg) were anesthetized, and following intra-arterial infusion of ice-cold University of Wisconsin solution, a complete pancreatectomy was performed. The pancreatic duct was cannulated for infusion of digestion medium containing collagenase type P, 1.5 mg/mL; deoxyribonuclease I, 10000 U; and a water-soluble analogue of vitamin E (Trolox), 1 mmol/L. After 20-minute incubations on ice, and at 37°C, the pancreas was hand shaken for 1 minute, followed by filtration and separation on an automatic cell separator (COBE 2991). Islet cells, identified by dithizone staining, were perifused at 37°C.

Results: The mean±SEM yield of intact purified islet cells (50-200 µm in diameter), and mostly present in clusters, was 2398±143 cells per gram (n=12). Glucose stimulation caused a significant increase in biphasic insulin secretion in the perifusion experiments.

Conclusion: We have developed a simple, reproducible, and reliable procedure for isolating intact and viable porcine islet cells suitable for xenotransplantation.

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DIABETES mellitus is an enormous drain on the economy, as its management constitutes a large proportion of health care expenditures in the United States.1 A significant amount of the expenditures on diabetes results from the treatment of its secondary complications. The Diabetes Control and Complications Trial showed that the development of diabetic complications and their progression can be significantly slowed by strict glycemic control during intensive insulin therapy.2 However, it is stressful and difficult to achieve glycemic control by the methods of intensive insulin therapy used in the Diabetes Control and Complications Trial. Moreover, intensive insulin administration imposes a significant danger of hypoglycemia and unwanted weight gain to patients receiving the treatment.3,3 A better degree of glycemic control, than was seen in the Diabetes Control and Complications Trial, has been observed in diabetic patients who received islet transplants,4 without the adverse hypoglycemic episodes associated with intensive insulin therapy. Furthermore, in contrast to intensive insulin therapy, islet transplantation can prevent the development of diabetic complications because of the role played by C-peptide, a by-product of insulin processing.3 Hence, biological replacement of destroyed or functionally impaired β cells with normal islet transplants remains the best option to achieve glycemic control and prevent the development of disabling complications.4,9 Still, there are major obstacles to routine use of islet transplants as a treatment option. These include the shortage of pancreas from human donors and the need to prevent transplant rejection by the use of immunosuppressive drugs, which are often toxic to β cells and cause other adverse effects in transplant recipients.6,7,10 The ultimate goal in transplantation is the unlimited availability of organs or tissues to be transplanted in a simple procedure that requires no use of immunosuppressive drugs.

For decades, patients with diabetes have been treated with porcine insulin, and the pig is regarded as the ideal source of islet cells for clinical xenotransplantation.11,12 However, isolation of intact and viable porcine islet cells has become an enigma because of their highly fragile nature. Methods for the isolation of porcine...
MATERIALS AND METHODS

The protocol used for this study was approved by the Animal Care Committee, Duke University Medical Center, Durham, NC. Young adult male Yorkshire pigs (weighing 25-30 kg) were purchased from a local breeder (Shenise and Sons, Henderson, NC). Collagenase type P and deoxyribonuclease I were purchased from Boehringer Mannheim, Indianapolis, Ind. Crystalline pork insulin was a gift, and moniodinated iodine I 125 was obtained from New England Nuclear, Boston, Mass. RPMI 1640 medium was obtained from Gibco Co, Grand Island, NY. Pig serum was procured from the Duke University Medical Center Culture Facility. A water-soluble analogue of vitamin E (Trolox) was obtained from Aldrich Chemicals, Milwaukee, Wis. Hanks balanced salt solution, antibiotics, highly purified bovine serum albumin (fraction V), and all other chemicals were purchased from Sigma-Aldrich Corp, St Louis, Mo.

SURGERY AND ISOLATION OF PORCINE ISLET CELLS

The pigs were anesthetized with isoflurane. Following intraarterial infusion of ice-cold University of Wisconsin solution, a complete pancreatectomy was performed with sterile techniques. The pancreas was weighed and placed in a dish for cannulation of the pancreatic duct and infusion of digestion medium, comprising Hanks balanced salt solution containing collagenase type P, 1.5 mg/mL; deoxyribonuclease I, 10,000 U; and a water-soluble analogue of vitamin E, 1 mmol/L. After 20 minutes of incubation on ice, the pancreas was incubated at 37°C for 20 minutes before being hand shaken for 1 minute in the covered dish. The digested tissue was filtered using a nylon mesh with a porosity size of 1000 µm, and the islet cells were purified on a discontinuous iso-osmolar Ficoll gradient using an automatic cell separator (Cobe 2991). The islet cells were then washed in Hanks balanced salt solution and identified by dithizone staining. Islet size and numbers were assessed by examining aliquots of islet preparations on an inverted microscope (model CK-40; Olympus Co Ltd, Tokyo, Japan) fitted with a digital camera (Pixera Corp, Los Gatos, Calif), and linked to a computer program (UTHSCSA Image Tool for Windows Software; University of Texas Health Sciences Center at San Antonio) for determination of the number and size of particles.

CULTURE OF ISOLATED PORCINE ISLET CELLS

Following purification of islet cells, they were placed in sterile culture dishes containing RPMI 1640 medium, supplemented with 20% pig serum and a 1% mixture of antibiotics (penicillin and streptomycin sulfate) before overnight (18-24 hours) culture. The culture dishes were incubated at 37°C, in an atmosphere of humidified 95% air and 5% carbon dioxide, as previously described.

GLUCOSE STIMULATION OF INSULIN SECRETION

The islet cells were tested either immediately after isolation or following an overnight (18-24 hour) culture in RPMI 1640 medium using a perifusion procedure. Islet cells were preperfused at the rate of 1 mL/min for 1 hour at 37°C with a Krebs-Ringer bicarbonate buffer, which was gassed with 95% air–5% carbon dioxide, and maintained at pH 7.4. The Krebs-Ringer bicarbonate buffer contained 1% purified albumin and basal glucose, 3.3 mmol/L (60 mg/dL). Following preperfusion, basal samples were collected at 5-minute intervals for 20 minutes before the glucose concentration in the perfusate was raised to 16.7 mmol/L (300 mg/dL), and effluent samples were also collected for 30 minutes. The islet cells were finally perfused with the basal glucose perifusate for 20 minutes with sample collection. Solutions were changed using a stopcock system, and all samples collected on ice were stored frozen until radioimmunoassay for insulin.

DATA ANALYSIS

Data are presented as mean±SEM, and were statistically evaluated using a 1-way analysis of variance computer program (GraphPad, San Diego, Calif) to compare insulin secretion before, during, and after glucose perifusion, 16.7 mmol/L; depending on the outcome of the analysis of variance, the Bonferroni correction was used to assess the significance of difference between the mean rates of insulin secretion. P<.05 was judged to be statistically significant.

RESULTS

Typically, the harvested porcine islet cells, measuring between 50 and 200 µm in diameter, were intact and occurred mostly in clusters. Figure 1A shows islet clusters taken immediately after purification, and Figure 1B represents porcine islet cells after an overnight culture. In a series of 12 different preparations, the mean of the following variables was determined:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas weight, g</td>
<td>53.6 ± 3.7</td>
</tr>
<tr>
<td>Islet yield*</td>
<td></td>
</tr>
<tr>
<td>Before automatic cell separator use</td>
<td>3115 ± 325</td>
</tr>
<tr>
<td>Purified</td>
<td>2398 ± 243</td>
</tr>
<tr>
<td>Purified islet clusters, µm</td>
<td>644 ± 85</td>
</tr>
</tbody>
</table>

*Data are given as the islet yield per gram of tissue.
Glucose stimulation of freshly isolated islet cells caused biphasic insulin secretion, which increased from a mean basal rate of 1415±61 to a peak of 3423±506 pg per 6 islet cells per minute (n=8) (P<.01) and promptly returned to the basal rate on reduction of glucose to the basal level, as shown in Figure 2. When islet cells were tested after overnight culture, they also had an appropriate response to glucose stimulation (Figure 3), which was comparable to that of freshly isolated islet cells, shown in Figure 2.

**COMMENT**

It is particularly exciting that islet cells harvested with this procedure are predominantly intact, and occur mostly in clusters, thereby obviating the need for measurement of fragments of islet cells for transplantation as islet equivalents. This observation suggests that the avoidance of continuous mechanical agitation during the digestion of pancreatic tissue is necessary to prevent fragmentation of isolated porcine islet cells. In the present study, we have shown that the intact porcine islet cells respond appropriately to changes in glucose concentration. It was previously reported that isolated mouse and porcine islet cells were prone to reoxygenation injury. It is well established that hypoxia and reoxygenation, to which isolated islet cells are inherently subjected, cause the generation of toxic-free radicals that impair cellular function. Based on these observations, in the present study, we incorporated a chemical antioxidant, a water-soluble analogue of vitamin E in the digestion medium, to provide protection against reoxygenation injury for the isolated islet cells. It is possible that the avoidance of continuous mechanical agitation, combined with the presence of antioxidant in the digestion medium, may be responsible for the appropriate response of freshly isolated porcine islet cells to changes in glucose concentrations in the perifusate.

Islet cells can be stored by culture, which is also a step that precedes storage by cryopreservation of islet cells. Although we only examined porcine islet cells after an overnight culture in this study, the observation that porcine islet cells isolated by our procedure also respond appropriately to glucose stimulation after culture suggests that these is-
let cells may be suitable for storage by culture or cryopreservation, which would diminish their antigenicity.24 Also, in spite of its manual nature, the new procedure we have developed is simple, with an islet cell yield that is comparable to those reported from the semiautomated methods of isolation of porcine islet cells.13,16 In summary, we have developed a simple, reliable, and easily reproducible procedure for the isolation of functionally viable porcine islet cells suitable for routine use in xenotransplantation.

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