ELSEVIER

Isolation of Viable Porcine Islets by Selective Osmotic Shock Without Enzymatic Digestion

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ABSTRACT

Islet transplantation is a potential cure for type 1 diabetes, but clinical results have been disappointing. Currently, islet isolation is by enzymatic digestion of the pancreas which has significant pitfalls: warm ischemia exposure, collagenase-induced damage to the islet mass and viability, poor reproducibility, high cost, a relatively low number of islets obtained per whole pancreas, and selection of islets for collagenase resistance rather than for glucose responsiveness. In the present study we performed a series of experiments in a porcine model to demonstrate the feasibility of a new isolation method based on selective osmotic shock (SOS) using very high glucose solutions, doubling or tripling physiological osmotic strength. The SOS method can be carried out at room temperature or in the cold eliminating warm ischemia time which damages the islets. The SOS method does not depend on the texture of the pancreas so all pancreases can be processed identically and the process can be fully automated. The SOS method isolates all the islets of the pancreas regardless of size and shape allowing a greater number of islets to be harvested. The SOS method avoids exposure to toxins in collagenase solutions, is inexpensive and selects for islets with high concentrations of Glut 2 transporters, representing the best glucose responding islets. The SOS method showed a comparable recovery of islets from young pig pancreas and the islets showed improved viability. We conclude that the selective osmotic shock (SOS) method of separating islets from the pancreatic tissue is superior to the collagenase method.

TYPE 1 diabetes mellitus is a worldwide disease primarily among pediatric patients. It is characterized by the selective immune-mediated destruction of insulin-producing beta cells by the pancreatic islets of Langerhans. The patients require life-long treatment with exogenous insulin. Despite intensive therapy, these patients are susceptible to a variety of complications that are responsible for increased morbidity and mortality rates. In a subset of metabolically unstable patients, acute hypoglycemia secondary to insulin therapy is a characteristic, devastating complication. During the past decades, islet cell transplantation has been considered to be a viable option.[4,32,43,45] Even though insulin independence is rarely achieved for more than 2 years, patients who have received islet transplants are protected against severe hypoglycemia and show improved metabolic parameters.[32,43]

One of the major challenges in islet transplantation is to automate and improve the islet isolation method, seeking to reproducibly extract a large number of viable[19,26] islets from a donor pancreas.[15,44] Islet isolation and purification for transplantation therapy is presently based upon enzymatic digestion of the pancreas with subsequent semi-automated purification using a physical density gradient as

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Supported in part by grants from National Science and Technology Fund (FONDECYT), Chile #1030596 (I.A.), #1030611 (E.R.), #1030661 (C.R.), and #1050571 (D.M.) and from the Juvenile Diabetes Research Foundation #5-2008-796 (I.A.).

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0041-1345/10/$–see front matter
doi:10.1016/j.transproceed.2009.11.030

developed by Ricordi.[27,37,42,47,53] The Ricordi method, sometimes with slight modifications, is used universally, but it has several significant pitfalls: collagenase-induced damage to the islets, low reproducibility, relatively low numbers of islets per whole pancreas, and high cost. The collagenase damage has been attributed to an inherent variability of collagenases,[41] even among purified Liberase preparations,[3] to contamination of Liberase preparations with endotoxins,[17] to variable collagen structure in individual islets,[22,23] and to warm ischemia during processing at 37°C to activate the enzyme.[11] One study has shown that islet cells actually internalize exogenous enzymes, causing decreased function and subsequent apoptosis.[2] Significant research efforts have focused on improving islet isolation techniques using collagenase or Liberase.[1,7,8,10,11,17,21,22,23,27,28,34,41,53]

Furthermore, it is known that individual islets of Langerhans show enormous variability in size, micro-architecture, and function.[9,35,52] With the collagenase method, the isolated islets are most likely selected for size, position in the pancreas, and physical resistance to the digestion process, not necessarily for responsiveness to glucose or for robust insulin secretion, making the process even less efficient. Several improvements have been developed, such as the use of a two-layer method with perfluorocarbon, or another method for oxygenation, improved preservation with cold University of Wisconsin solution before islets isolation,[21,46] addition of nicotinamide to the processing medium,[25] and use of a short-term culture period,[33] sometimes in association with caspase inhibitors or other agents that inhibit apoptosis. However, islet yield continues to be a major stumbling block to the procedure.[19,34] Thus, the relatively low success in this procedure has kept islet transplantation as an experimental procedure of limited clinical application.[24,36]

Pancreatic islet cells are endowed with glucose transporters (GLUT 2) in their cell membranes.[12,31,49,51] These transporters equilibrate glucose from the external medium with the cytoplasm in a matter of seconds, playing a central role in the extracellular glucose-sensing mechanism and being a hallmark of "glucose-responding" islets.[12,16,20,39,40] Furthermore, these transporters are not saturated, even at high glucose concentrations. Thus, glucose is not osmotically active in cells from glucose-responding islets. Very high concentrations of glucose, added on top of osmotically balanced salt solutions or culture media, cause cells without GLUT 2 transporters to shrink almost instantaneously through a net water efflux. After several minutes, most cells restore their initial volume[5,18,38] by activation of ion[14,29,30] and urea fluxes in a process called regulated volume increase, which leaves the cytoplasm relatively hypertonic (an increased concentration of internal ions balancing the external glucose concentration). When these cells are again exposed to physiological solutions, ie, media without glucose, the opposite occurs in terms of water fluxes, and the cells without Glut 2 swell and burst (Fig 1). In this manner, in the pancreatic tissue exposed first to a solution enriched in glucose and subsequently to an osmotically normal solution, we expect to see the selective destruction of pancreatic acinar cells and other non– glucose-responding cells.

We performed a series of experiments in a porcine model seeking to demonstrate the feasibility of a new experimental islet isolation and purification method, the Selective Osmotic Shock (SOS) method (patent pending), based on the "selection" of glucose-responsive islets by treatment with a high-glucose solution, followed by a zero-glucose solution. We evaluated morphological and functional parameters of the isolated islets in the culture, comparing islets isolated with the SOS method to those using the traditional collagenase method. We observed that the SOS method was feasible, showing recovery of abundant viable islets. We also noted that the process is faster and cheaper than the traditional method using collagenase. It can be performed at room temperature or in the cold. Also, this new technique is potentially suited to be fully automated.

MATERIALS AND METHODS

Islet Isolation, Purification, and Culture Conditions

Young Landrace swine of 26–35 kg underwent total pancreatectomy under general anesthesia, without perfusion using a cold preservation solution. Immediately after extraction, the pancreas which was cleaned under sterile conditions, was weighed, submerged in povidone- iodine solution, and washed with 2 L of sterile saline solution, all at room temperature (approximately 18°C).

Under a sterile laminar flow hood, the pancreas was chopped finely and divided into sterile 40-mL conical tubes, according to the experimental protocol. After weighing the content, the islets were isolated by enzymatic digestion with Liberase CI (Roche Cat. No. 11814435001, Basel, Switzerland) for 20 to 23 minutes at 37°C. The recovery rate was more than 60% of the islets as free islets. Islets isolated by SOS were obtained as follows: samples of chopped tissue were exposed to RPMI 1640 media supplemnted with either 300 mmol/L of glucose or 600 mmol/L of glucose for 20 or 40 minutes at room temperature (approximately 22°C). All samples were then centrifuged for 2 minutes at 1000 rpm and the solution rapidly replaced by a zero-glucose RPMI medium. The tissue was gently mixed, centrifuged, and washed again with fresh zero-glucose medium; the washing procedure was again repeated. The resultant mixture was either placed in culture or further purified as follows. Each of the portions of tissue was then processed by gentle mechanical disruption by suction and expulsion from a sterile syringe without a needle for approximately 15 minutes. The resulting mixture was centrifuged through a density gradient of Histopaque 1077 (Sigma-Aldrich no. 1077, St. Louis, Mo). The islet-enriched fraction was placed in RPMI 1640 culture media without bicarbonate, but with 5.6 mmol/L glucose, 20 mmol/L HEPES, 1% of streptomycin, 1% of penicillin, and 20% of inactivated horse serum at 37°C in a 5% CO_2 incubator. The culture media were replaced the first day after isolation and thereafter every 48 hours. The same purification and culture conditions were used for the Liberase-treated samples.

Islet Quantification and Purity

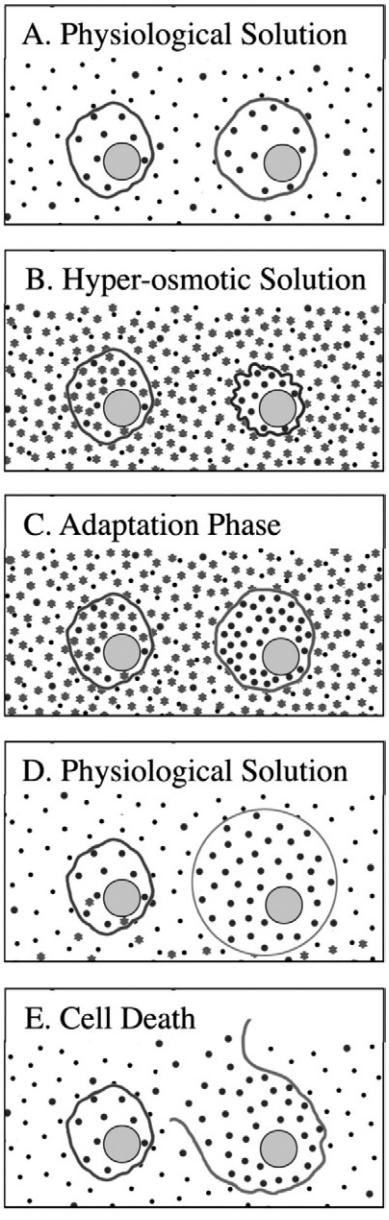
To evaluate islet recovery after isolation, we counted the total number of islets larger than 100 μm in diameter within an aliquot

of 50 μL and multiplied it by the total volume. The procedure was performed on day 4 of culture after purification to evaluate the number of intact islets. The number of islets per gram pancreatic tissue was also calculated.

Samples of the islet preparations were photographed using an Olympus (Center Valley, Pa) digital camera and a Wild (Colorado Springs, Colo) dissecting microscope. Photographs were processed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, Md, United States).

Assessment of Islet Function

Islet viability and function were evaluated using insulin secretion tests on day 4 after the isolation. Washed islets isolated under various experimental conditions were exposed to RPMI 1640 culture media with 5.6 mmol/L glucose and 20% horse serum for 90 minutes at 37°C in a 5% CO_2 atmosphere. Then the samples were centrifuged at 2000 rpm for 15 minutes and the supernates were collected and frozen at −20°C. The islets were resuspended in media as above but with 16.7 mmol/L glucose and cultured for a further 90 minutes. The supernate was analyzed using an immulite/immunlite 1000 insulin determination assay (Diagnostic Products Corporation, Los Angeles, Calif) with results expressed as μIU/mL. Finally, insulin secretion was normalized to basal secretion values and also to the number of islets in each dish. All protocols were approved by the Animal Ethics Committee of the University of Chile.



RESULTS

Islet Recovery and Purity

After SOS treatment, the number of isolated islets was highest using 600 mmol/L glucose for 20 minutes, as counted after 4 days in culture. In a representative experiment, 4089 islets per gram of tissue were isolated from the portion of pancreas exposed to a 300 mmol/L glucose solution, whereas 13,423 islets per gram of tissue were obtained from the portion exposed to 600 mmol/L glucose. From the same pncreas, Liberase isolation yielded 8543 islets per gram of tissue.

Figure 2 compares the purity and quality of the islets isolated by 600 mmol/L glucose (Fig 2B, 2D and 2F) with those isolated with Liberase (Fig 2A, 2C and 2E). It can be appreciated from the photographs that both the purity of the preparation (Compare Figs 2A and 2C with Fig 2B and 2D) and the capsular integrity of islets (Compare Figs 2E with Fig 2F) isolated by SOS were superior to islets isolated by Liberase in the traditional fashion.

Insulin Secretion

We evaluated insulin secretion from islets isolated by SOS in response to a culture medium supplemented with 5.6 mmol/L glucose or 16.7 mmol/L glucose after 4 days in culture. Table 1 shows the results of islets isolated after 20

Fig 1. Representation of selective osmotic shock hypothesis. Cells on left side of illustrations represent islet-cells containing Glut 2 transporters in the membrane; cells on the right side illustrate pancreatic acinar cells. In **A,** both cell types are exposed to physiological solutions, small dots representing sodium ions, mostly in the extracellular space, and large dots representing potassium ions, mostly in the intracellular space. In **B,** both cell types are exposed to very high glucose concentrations, approximately tripling the osmotic strength of the external solution. Large stars represent glucose. Islet-cells take up the glucose via Glut 2 transporters in the membrane in order to equilibrate the osmotic strength of the external solution whereas acinar cells rapidly loose water in order to equilibrate internal and external solutions, and this causes the cells to shrink. In **C,** acinar cells take up potassium ions to recover cell volume and to adapt to the extracellular osmotic strength. In **D,** the very high glucose solution is removed and the cells are again exposed to physiological solutions. Islet cells loose the extra glucose, again through Glut 2 transporters in the membrane. However, acinar cells rapidly take up water to dilute the hypertonic intracellular solution, and this causes the cells to swell. In **E,** the acinar cells swell so fast that they burst.

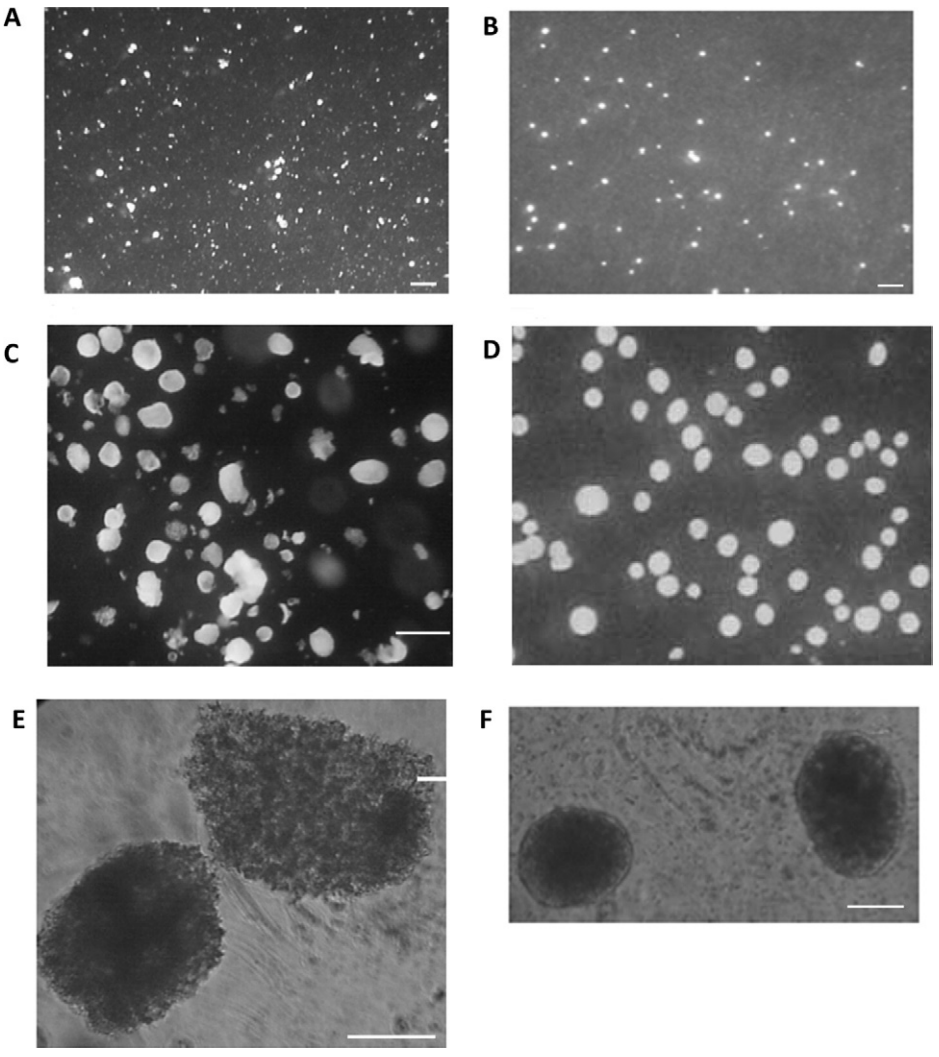


Fig 2. Light microscopy photographs comparing islets purified by SOS to enzymatic digestion. Figures **A**, **C**, and **E** represent typical islets isolated with Liberase. Figures **B**, **D**, and **F** represent islets isolated with the SOS method. Scale bars: A,B: 500 μm. C,D: 200 μm. E,F: 50 μm.

minutes of exposure to high, 300 or 600 mmol/L, glucose. After 4 days in culture, islets responded to a physiological glucose stimulus showing an increment in insulin secretion of about 3-fold over basal values.

Islets isolated by exposure to high glucose for 40 minutes showed lower levels of basal insulin secretion and responded poorly to glucose (results not shown). Islets isolated with Liberase showed no detectable insulin secretion, although the number of purified islets was similar.

Table 1. Insulin Secretion from Islets Isolated by SOS

Experimental Condition	Baseline Insulin Secretion/g of Pancreas Average (SD)	Stimulated Insulin Secretion/g of Pancreas Average (SD)
300 mmol/L glucose	39.59 (10.54)	97.46 (47.19)
600 mmol/L glucose	173.4 (33.2)	384.9 (83.44)

Note: Insulin secretion in response to the culture media supplemented with 5.6 mmol/L glucose (baseline) and 16.7 mmol/L glucose (stimulated) after 4 days in culture.

DISCUSSION

The SOS technique to isolate islets of Langerhans represents a novel approach in the field of islet cell transplantation. The elimination of enzymes from the islet isolation procedure has several advantages: enzymatic damage to islets is avoided, preparation purity is improved, and processing can be performed at cooler temperatures, is less costly and, most importantly, has the potential for full automation.

In recent years, there has been an explosion of interest in purified collagenase and Liberase preparations. Different preparations have been advocated for each type of pancreas seeking to leave insular capsule undigested and preferentially digest the connective tissue of the acinar pancreas,[1,7,8,10,11,21,22,23,27,34,53] thereby reducing endotoxins,[17] and minimizing variability among enzyme batches,[41] and even within a particular lot.[24,28] Some of these concerns are inherent to enzymatic digestion. Addressing some of these

concerns has made the ultra-pure Liberase preparations expensive.

Collagenase digestion of young pig pancreata yields islet-like cell clusters, which tend to fall apart upon culture with few large or robust islets.[6] In the experiments reported herein, we confirmed that islets isolated from 30-kg pigs by the Liberase method lacked integrity; however, those isolated by the SOS method contained intact capsules. Furthermore, with the SOS method, a greater number of islets were isolated per gram of tissue (13,423) than that reported from adult pigs (4210)[7] or (12,363[53]) or from human cadaveric donors (2279).[47] We propose that the collagen sheath of the young porcine islets may be particularly sensitive to collagenase treatment and that for this reason young pigs have not been recommended for islet isolations.[6] With the present method, however, young pigs are satisfactory islet donors.

Most researchers believe that cooling the pancreas and the islets of Langerhans during isolation and preparation is important to maintain long-term islet function.[7,48,50] Because collagenase digestion requires physiological temperatures of about 37°C for enzyme activation, islet tissue is exposed to a period of warm ischemia, with potential for damage. The SOS method was successful at cooler temperatures, thus protecting islets from damage.

The islets of Langerhans are known to be variable in size, shape, and function within a given pancreas.[9,35] Using the traditional method of islet isolation by collagenase digestion of the pancreas, some or many islets are damaged,[34] which probably relates to the type of collagen surrounding each islet, the size of the islet (larger ones may be destroyed while the smallest ones remain trapped in acinar tissue), and the particular state of the pancreatic tissue surrounding the islets. None of these parameters have been correlated with islet function. The density of GLUT 2 glucose transporters has, however, been identified as a positive index of islet function and responsiveness to glucose.[12,16,20,39,40] The method presented herein to isolate islets depends on GLUT 2; thus, it positively selects functional islets.

There have been several reports that the traditional method of islet isolation causes cells swelling. We propose that this is due to the use of glucose as an osmotic substitute for ions in the preservation or isolation media, thus causing cells with Glut 2 transporters to be exposed to effectively hypotonic solutions. This observation is compatible with our hypothesis that islet cells containing functional GLUT 2 transporters are osmotically blind to glucose in the media. Alternatively, there is evidence showing that a moderately high extracellular glucose medium induces swelling in alpha and beta cells, a phenomena abolished by using a GLUT inhibitor, phloretin, or a nonmetabolized sugar such as 3-O-methyl glucose, indicating that swelling by moderate concentrations of glucose may depend on metabolism, and may also indicate functional islets.[13] With the SOS method, glucose metabolism is inhibited by the cooler temperatures during the process.

Finally, the SOS method does not depend on the size of the islets or the texture of the pancreas from which they are to be extracted. Thus, the method is appropriate for full automation of human pancreata to obtain islets for transplantation. Automation of islet retrieval from donor pancreata may achieve greater success in islet transplantation because islets could be isolated on site immediately after pancreas procurement without the requirement for a specialized isolation team. Furthermore, isolated islets have been shown to remain viable for several days and can be easily transported to a transplantation site at room temperature.

In summary, we have described the development of a new islet isolation method without the use of enzymatic digestion by collagenase or Liberase. This SOS method is based on the addition of very high concentrations of glucose to the extracellular medium for a brief period to separate cells containing GLUT 2 transporters from others, thus selecting islets with glucose sensitivity. The method is faster and cheaper than the collagenase technique and avoids damage to the islets by excessive enzymatic digestion, by endotoxins, and by warm ischemia. Furthermore, the method can be fully automated.

ACKNOWLEDGMENTS

The authors wish to acknowledge the generous participation of veterinarian students Gail Alfsen, Bernardita Silva, and Rodrigo Valenzuela for assistance during the surgical procedures. We also thank the valuable cooperation of Pedro Martinez, Juan Carlos Soto, and Juan Carlos Soto, Jr. for their excellent technical support.

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