Cryo-isolation: A Novel Method for Enzyme-Free Isolation of Pancreatic Islets Involving In Situ Cryopreservation of Islets and Selective Destruction of Acinar Tissue

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ABSTRACT

Background. A critical component of treating type I diabetes by transplantation is the availability of sufficient high-quality islets. Currently, islets can be obtained only by reliance on an expensive, inconsistent, and toxic enzyme digestion process. As an alternative, we hypothesize that cryobiologic techniques can be used for differential freeze destruction of the pancreas to release islets that are selectively cryopreserved in situ.

Methods. Pancreases were procured from juvenile pigs with the use of approved procedures. The concept of cryo-isolation is based on differential processing of the pancreas in 5 stages: 1) infiltrating islets in situ preferentially with a cryoprotectant (CPA) cocktail via antegrade perfusion of the major arteries; 2) retrograde ductal infusion of water (or saline solution) to fully distend the gland; 3) freezing the entire pancreas to −160°C, and stored in liquid nitrogen; 4) mechanically crushing and pulverizing the frozen pancreas into small fragments; and 5) thawing, filtering and washing the frozen fragments with RPMI 1640 culture medium to remove the CPA. Finally, the filtered effluent (cryo-isolate) was stained with dithizone for identification of intact islets, and samples were taken for static glucose- stimulated insullin release assessment.

Results. As predicted the cryo-isolated contained small fragments of residual tissue comprising an amorphous mass of acinar tissue with largely intact embedded islets. The degree of cleavage of the cryoprotected islets from the freeze-destroyed exocrine cells, was variable. Islets were typically larger than their counterparts isolated from juvenile pigs with conventional enzyme-digestion techniques. Functionally, the islets from replicate cryo-isolates responded to a glucose challenge with a mean stimulation index = 3.3 ± 0.7 (n = 3).

Conclusions. An enzyme-free method of islet isolation relying on in situ cryopreservation of islets with simultaneous freeze-destruction of acinar tissue is feasible and proposed as a novel method that avoids the problems associated with conventional collagenase digestion methods.

Today, the field of islet transplantation relies on enzymatic digestion processes that destroy the extracellular matrix of the tissue, releasing the entrapped islets for further processing and purification. This widely practiced procedure has drawbacks due principally to the difficulty of controlling the digestive process to yield an optimum quantity of viable cells. Moreover, the process is harsh and even toxic, causing some inevitable loss of valuable cells. Furthermore, the process relies upon the purest forms of the enzymes, which are very expensive and still subject to batch variations that have led to variability and inconsistency in attempts to optimize and standardize these processes. As a potential alternative, the present study explores the feasibility of a new differential freezing method, cryo-isolation, for separation of endocrine tissue from exocrine

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tissue, based on the known susceptibilities of cells to freezing injury.[1-3]. In this process, the pancreas is pretreated by differential perfusion of the endocrine and exocrine tissues in a way designed to maximize destruction of the exocrine tissue, while cryopreserving the islets in situ.

METHODS

Pancreases were procured from juvenile pigs (3–6 months old) with the use of approved procedures as described previously.[4,5] The concept of cryo-isolation is based on differential processing of the pancreas in 5 stages, as follows.

Step 1: Vascular Infusion of Islets in Situ with a Cryoprotectant Mixture

Antegrade perfusion of the celiac and superior mesenteric arteries with a cryoprotective solution was performed under controlled conditions of temperature and pressure to preferentially equilibrate the islets within the gland. Perfusion was maintained sufficiently long enough to allow equilibration of the islets, but not the whole gland, with dimethyl sulfoxide (DMSO) as a permeating cryoprotective additive (CPA). Thirty minutes perfusion with 2 mol/L DMSO in Unisol solution[6,7] on ice was selected for this feasibility study based on previous studies.[8-10] The rationable for this step is to deliver sufficient CPA to the islets to protect them against freezing injury during subsequent freezing of the pancreas.

Step 2. Ductal Infusion of Water (or Isolonic Saline)

Retrograde infusion of water into the pancreatic duct was initiated immediately after completion of step 1. This process was continued until the gland was visibly distended. The rationale for this step is to impregnate the exocrine tissue with water to facilitate extensive destructive ice formation in the noncryoprotected tissue during cooling and freezing.

Step 3. Freezing of the Pancreas

After completion of steps 1 and 2, the entire pancreas was cooled to subzero temperatures until frozen solid at −160°C. The rationale for this step is to maximize ice formation in the unprotected exocrine gland to facilitate tissue disruption for subsequent disintegration of the gland to release the cryoprotected islets.

Step 4. Physical Distruption of the Pancreas

The pancreas was disrupted into small fragments by mechanical crushing/grinding and pulverization while still frozen and then further homogenized with an ice blender. The resultant frozen tissue slurry was thawed by addition of cold RPMI 1640 culture medium, which also served to dilute the CPA.

Step 5. Separation and Purification of the Islets

The resultant product was filtered through a stainless steel mesh to remove large pieces of undisrupted pancreas and stringy waste. The filtered effluent (cryo-lysate) was washed by centrifugation and stained with dithizone for identification of intact islets. Samples were also taken for static glucose–stimulated insulin release assessment using previously published methods[4].

RESULTS AND DISCUSSION

Figure 1 shows various examples of the stained tissue in the cryoisolate. As predicted, the tissue was reduced to an amorphous mass in which clearly distinguishable islets were present. The degree of cleavage of the islets from the exocrine tissue was variable. Figure 1A shows intact islets embedded in residual exocine tissue with a vascular or ductal structure clearly apparent. In contrast, some discrete islets were more clearly cleaved with very little residual exocrine or ductal tissue (Fig 1D). In all cases, the acinar tissue appeared unstructured and amorphous, consistent with generalized destruction with an abundance of zymogen granules dispersed throughout the acellular debris (Fig 1B and 1C).

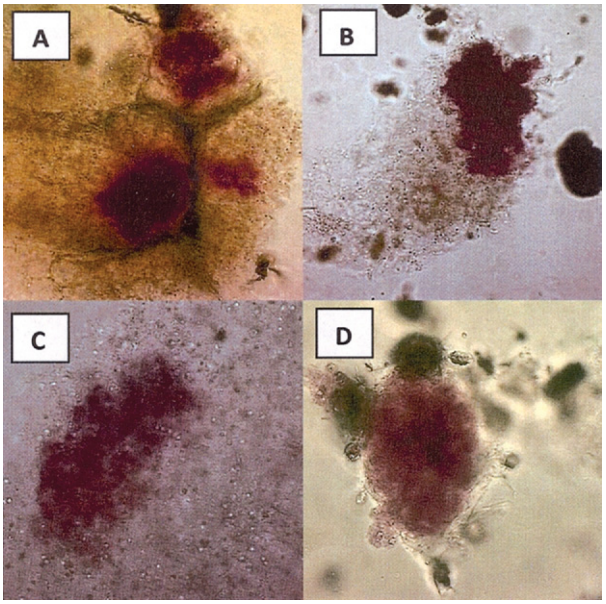

Fig 1. Dithizone staining of the cryo-isolate, showing intact islets and destroyed exocrine tissue. Magnification ×40.

The high abundance of zymogen granules observed in the islet preparations of these preliminary cryo-isolation trials is an excellent indication that our objective of selective acinar cell destruction was achieved. In contrast to conventional islet isolation, where lytic enzymes from damaged exocrine cells are present at the optimal temperature for their activity, processing in the cold was anticipated to attenuate detrimental enzymatic activity during islet purification.

Islets were typically larger than their counterparts isolated from juvenile pigs with the use conventional enzyme-digestion techniques.[4] Functionally, the islets from replicate cryo-isolates responded to a glucose challenge with a mean stimulation index of 3.3 ± 0.7 (n = 3). From this pilot study we conclude that an enzyme-free method of islet isolation relying on *in situ* cryopreservation of islets with simultaneous freeze destruction of acinar tissue is feasible and is proposed as a new method that avoids the problems associated with conventional collagenase digestion methods.

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