

Pancreatic Islets Isolation Using Different Protocols with *in Situ* Flushing and Intraductal Collagenase Injection

A. KINASIEWICZ^{1,2}, M. JUSZCZAK³, J. PACHECKA², P. FIEDOR³

¹*Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences,* ²*Department of Biochemistry and Clinical Chemistry, Faculty of Pharmacy, and* ³*Department of General and Transplantation Surgery, Medical University of Warsaw, Warsaw, Poland*

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Summary

Human islet transplantation seems to be a very promising clinical procedure for patients with type I diabetes mellitus. The aim of our study was to investigate the influence of *in situ* intravascular flushing with University of Wisconsin (UW) solution and intraductal collagenase injection at the time of pancreas procurement on the isolated islets and exocrine tissue injury. Our experiments indicated that *in situ* perfusion with the UW solution has a beneficial effect on pancreatic islets and intraductal distention results in an increase in the concentration of pancreatic enzymes released into the cold preservation solution during ischemic conditions. Cold ischemia reduced islet yield, but pancreas perfusion with the UW solution showed better ischemic tolerance of isolated islets during glucose static incubation. We conclude that intravascular pancreas flushing has a crucial effect on recovery and yield of pancreatic islets and protects against exocrine tissue injury.

Key words

Pancreatic islets • Amylase • Cold ischemia • Preservation

Introduction

Since the discovery of immunosuppressive drug the transplantation has become unique therapy for patients with heart, kidney, liver, pancreas and other organ failure. Organ harvesting, including pancreas, requires *in situ* flushing for rapid cooling and blood cell removal. Solutions used for this procedure are preservation solutions that are also used for organ storage. Every abdominal organ is characterized by its own biochemical profile and vascular properties. In case of multiorgan recovery procedure, the pancreas and other abdominal organs are treated in the same conditions. Pancreases are recovered for whole organ transplantation

and for islet transplantation following islet isolation. Because microvascular complications are most frequent after pancreas transplantation, the properties of vascular system seem to limit the final result. Vessel thrombosis and graft pancreatitis with the risk of acute graft failure, are still the major problems in pancreas transplantation. Graft thrombosis occurring in 10-30 % requires removal of the gland (Douzdjian *et al.* 1993, Fernandez-Cruz *et al.* 1993). In the case of islet transplantation, the limiting factor is the yield of islet recovery, strongly reduced by the duration and conditions of cold ischemia. Islet transplantation could be a remedy for type I insulin-dependent diabetes mellitus, providing a cure rather than treatment (Scharp *et al.* 1991, Shapiro *et al.* 2000). Islet

transplantation offers several advantages over whole organ transplantation, including the ability to store islets prior to implantation, resulting in islet banking (Kneteman *et al.* 1989). Other primary graft non-function is still not explained phenomenon that could be connected with cold storage islet damage. First, we try to find the best solution and conditions to preserve islet cell survival between removal of the donor pancreas and its processing in the laboratory, which occurs in clinical islet transplantation.

The aim of our study was to compare different islet isolation protocols with or without organ flushing and pancreas distention during organ procurement. In our experiments, we used UW solution whose properties seem to protect pancreas from ischemia injury.

Methods

Animals

Adult male WAG rats (n=48) weighing 200-250 g were used as organ donors. Donor rats were anesthetized by ether inhalation.

Experimental protocol

University of Wisconsin solution was used for all organ perfusions and pancreas preservations. To compare the role of pancreas *in situ* perfusion and distention with collagenase solution in the time of organ procurement, we created four groups: Group I – pancreases without flushing followed by collagenase distention and subsequently cold storage; Group II – pancreases without flushing followed by cold storage without collagenase solution distention; Group III – pancreases with UW-solution flushing, followed by collagenase solution distention and subsequently cold storage; Group IV – pancreases with UW-solution flushing, followed by cold storage without collagenase solution distention. Pancreases were perfused with stable flow rate of 0.3 ml/min for 10 min.

Pancreases were stored at 4 °C for 60 and 180 min (in each group, for 60 and 180 min of cold ischemia, n=6) in 50 ml plastic tube with 25 ml of preservation solution. The aliquots of solution were taken and frozen at –80 °C until assayed.

Rat islet isolation

Pancreatic islets were isolated using modified method of Lacy and Kostianovsky (1967). Pancreases were distended by intraductal injection of collagenase

solution (2 U/ml in HBSS) either before or after cold storage, depending on the experimental group (collagenase P, Boehringer-Mannheim, Germany) into the common bile duct after occlusion of the distal end, close to the duodenum. Digestion was performed in water bath at 37 °C for 16 min. Subsequently, the digestion was stopped by addition of cold HBSS and the suspension was washed twice from collagenase (HBSS, 550 x g, 5 min, 4 °C). Islet separation was done by centrifugation on Histopaque discontinuous gradients (HBSS/1.083 g/ml, 16 min, 800 x g, 4 °C). To ensure 100 % purity of the preparation, islets were handpicked and counted under an inverted microscope. The islets were cultured in Petri's dishes at 37 °C in humidified 5 % CO₂ in RPMI-1640 medium supplemented with 10 % of fetal calf serum and antibiotics.

Assessment of endocrine secretory function

Endocrine function was assessed by insulin secretion in glucose static incubation. Ten pancreatic islets were suspended three times for 45 min in 3 ml of RPMI-1640 solution supplemented with 10 % of fetal calf serum with addition of various glucose concentrations (basal I – 1.67 mM, stimulation – 16.7 mM, basal II – 1.67 mM, respectively). Medium samples were frozen and kept until assayed. The stimulation index (SI) was calculated as the insulin released into the stimulation medium divided by insulin released into the basal I medium.

Insulin radioimmunoassay

The concentration of insulin in the samples was determined by double antibody radioimmunoassay (RIA) (Morgan and Lazarow 1963) using guinea pig anti rat insulin, mono-¹²⁵I-human insulin, and rat insulin as standard.

Pancreatic enzymes analysis (amylase and lipase)

Amylase and lipase concentrations in preservation solutions were determined using kinetic assay available in commercial kits (Sigma-Aldrich).

MDA concentration

The measurement of malondialdehyde (MDA) was used to quantify the tissue lipid peroxidation process by the thiobarbituric acid method (Slater and Sawyer 1971, Ikeda *et al.* 1994). Pancreatic specimens (about 100 mg) taken after the preservation period were homogenized in 5 vol of 180 mM KCl, 50 mM Tris/HCl,

10 mM EDTA (pH 7.4) containing 0.02 % (wt/vol) butylated hydroxytoluene to avoid spontaneous oxidation of unsaturated lipids, the homogenate was then precipitated with 10 % (wt/vol) trichloroacetic acid (TCA) and the resulting supernatant was incubated at 100 °C for 45 min with an equal volume of 0.67 % (wt/vol) thiobarbituric acid (TBA). After cooling, the supernatant was extracted with 1 ml of n-butanol and absorption was measured using spectrophotometer at $\lambda = 535$ nm.

Protein concentration

Protein concentration in pancreatic specimens after homogenization was determined using modified Lowry method with Protein Assay Kit from Sigma (USA). Lowry reagent solution was added to the sample and allowed to stay for 20 min at room temperature. With rapid and immediate mixing, Folin & Ciocalteu's Phenol Reagent was added. After 30 min at room temperature, the absorbance was measured at 550 nm and protein concentration was calculated from the calibration curve.

Statistical analysis

For quantitative studies, the data were analyzed

by paired Students t-test. $P < 0.05$ value was considered to be significant.

Results

Islet yield

The highest islet recovery after 60 min of cold ischemia were obtained in group I (584±58 islets) in which pancreases were injected collagenase solution at the time of procurement without *in situ* flushing (Table 1). The lowest result of islet isolation was obtained in group II (413±51 islets) where the pancreases were injected collagenase solution without flushing after cold ischemia. Cold ischemia for 180 min reduced islet recovery, and the lowest number of islets was found in group II. For both 60 and 180 min of cold ischemia a comparison of groups II and IV showed that *in situ* flushing had a beneficial effect on islet recovery, when the pancreases were distended with collagenase solution after cold ischemia time. For pancreases distended during the time of procurement (groups I and III), we found similar trend, but there was no significant difference between the groups.

Table 1. Islet cell recovery from rat pancreas subjected to 60 min of cold ischemia in UW solution.

	Group I	Group II	Group III	Group IV
60 min of cold ischemia	584±58	413±51**	497±74*	523±91 [#]
180 min of cold ischemia	438±51	337±118	459±83	508±139 [#]

Data are mean value from one pancreas ± SD. Significantly different: *,** from group I ($P < 0.05$, $P < 0.01$), [#] from group II ($P < 0.05$).

Endocrine function

Using different glucose concentration in the incubation media, free islets were tested in their endocrine function by insulin secretion. Cold ischemia for 60 min did not cause any considerable changes in insulin secretion. Prolongation of cold ischemia time to 180 min caused the impairment in endocrine function in groups I and II characterized by a deterioration of reaction to glucose. Pancreases, which were flushed *in situ* (groups III and IV) revealed better cold ischemic tolerance observed in the glucose test. Stimulation index (SI) for these groups showed a value above 1, suggesting the ability of islets to respond to stimulation (Table 2). SI differences between group III and IV were not significant for both 60 and 180 min of cold ischemia. When we

compared the groups I vs. III and II vs. IV, we observed higher SI values for pancreases flushed with UW solution than for non-flushed (Table 2).

Estimation of exocrine tissue injury

To determine the exocrine tissue injury, we investigated the release of amylase and lipase into the preservation solution. The samples were taken after cold ischemia and analyzed. After 60 min of cold ischemia, there were no significant differences between investigated groups in both amylase and lipase concentrations. After 180 min of preservation, the concentration of pancreatic enzymes rose significantly and the highest value of lipase concentration was observed for group II (2694±475 IU/ml) and the lowest

for group IV (2105±315 IU/ml, vs. group II, $p<0.05$) (Fig. 1). A comparison of amylase concentration in particular groups yielded similar results as for lipase released to preservation solution. The highest value was

found in group II and reached 12230±4148 IU/ml, whereas the lowest values were observed in group IV (6867±3893 IU/ml, vs. group II, $p<0.05$) (Fig. 2).

Table 2. Insulin concentration (IU/ml/10 islets) and Stimulation Index in glucose static test of islets isolated after 60 and 180 min of cold ischemia.

60 min of cold ischemia Glucose concentration	Glucose Static Incubation			Stimulation Index
	1.67 mM	16.7 mM	1.67 mM	
Group I	126±13	197±15	68±13	1.580±0.184
Group II	140±32	191±36	65±35	1.437±0.463
Group III	115±12	222±17 *	86±12 *	1.949±0.293 *,#
Group IV	117±11	217±13 *	75±13	1.865±0.181 *

180 min of cold ischemia Glucose concentration	Glucose Static Incubation			Stimulation Index
	1.67 mM	16.7 mM	1.67 mM	
Group I	227±15	138±12	71±11	0.610±0.065
Group II	211±27	150±21	66±29	0.723±0.137
Group III	165±19 **	186±51 *	67±14	1.115±0.241 **,##
Group IV	152±26 **	177±32 *	56±13	1.188±0.304 **,##

Data are mean value ± SD. Significantly different: *,** from group I ($P<0.05$, $P<0.01$), #,## from group II ($P<0.05$, $P<0.01$)

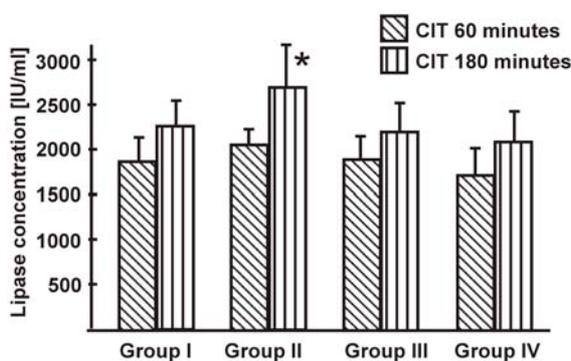


Fig. 1. Lipase concentration in preservation solution after 60 and 180 min of cold ischemia. * $p<0.05$ (from group IV).

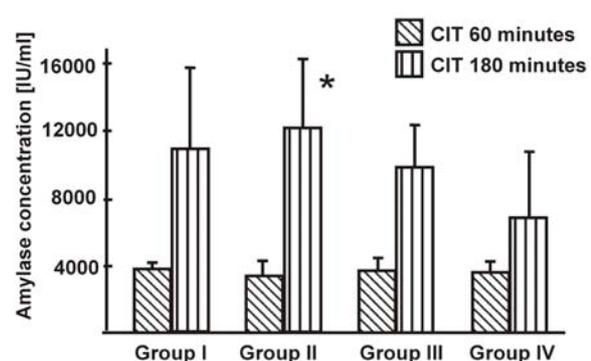


Fig. 2. Amylase concentration in preservation solution after 60 and 180 min of cold ischemia. * $p<0.05$ (from group IV).

Lipid peroxidation in pancreatic tissue

Malondialdehyde (MDA) was used as an indicator of peroxidative changes in the preserved pancreatic tissue. Specimens of the pancreas were taken after the exposure to 60 and 180 min of cold ischemia.

The concentration of MDA was expressed in terms of protein concentration in homogenized tissue. After 60 min of cold ischemia, we observed significant lower MDA concentration in groups III and IV (0.295±0.098 and 0.225±0.071 µg/mg of protein,

respectively), which were perfused with UW solution before pancreas procurement, than in groups I and II (0.472±0.170 and 0.441±0.158 µg/mg of protein, respectively, groups I vs. III $p<0.05$, groups II vs. IV $p<0.01$), which were harvested without *in situ* flushing. After 180 min of cold ischemia, the lowest value of MDA in the pancreatic tissue was observed in group IV, but the differences between particular groups were not significant.

Discussion

Islet isolation using collagenase solution to dissolve the pancreas began with the pioneer work of Moskalewski (1965). Lacy and Kostianovsky (1967) introduced an islet isolation technique in rats that involved the ductal distension of the pancreas and collagenase digestion. Success in islet isolation from the pancreas of large animal species was reached by Ricordi *et al.* (1989) when they introduced an automated method for large-scale isolation of islets from the human and porcine pancreas.

One of the most important parameters characterizing successful islet isolation is the number of free islets recovered from pancreas. Purity of islets affects the success in clinical islet transplantation. Islet number recovered in isolation process depends on many factors including cold ischemia time and solution used for organ preservation.

University of Wisconsin solution, which is the most advanced and commonly used vascular perfusion solution during multiorgan donation, is extensively used as cold preservation solution for storage of organs awaiting transplantation. Composition of the UW solution is similar to that of intracellular fluid and contains a low concentration of Na^+ and a high concentration of K^+ . The UW solution contains lactobionic anion and raffinose as impermeants, and hydroxyethyl starch (HES) to maintain colloidal pressure. This composition prevents cell swelling that occurs in cells stored in physiological solutions in the cold, when the cell-membrane Na^+/K^+ pump becomes less efficient. The role of HES in the UW solution is to maintain vascular networks when an organ is flushed out with cold preservation solution (Belzer and Southard 1988, D'Alessandro *et al.* 1989).

The duration of cold ischemia is crucial in pancreatic islet recovery and properties of the solution influence the outcome of the isolation process. Our results from previous experiments confirmed the superior properties of UW solution (Kinasiewicz *et al.* 2000) so that we also used this solution in the present study. *In situ* organ flushing, which is a standard protocol in clinical pancreas procurement process, is not always used in experimental investigations. In this paper, we proved the necessity of pancreas flushing before islet isolation for both the clinical procedure and experimental investigations. Comparison of the two methods of pancreas distention, before and after cold storage,

suggested to distend the gland with collagenase solution during the time of pancreas procurement (Munn *et al.* 1989, Casanova *et al.* 1994).

Intraductal injection of the collagenase solution following pancreatectomy had a beneficial effect on islet recovery, when the pancreases were perfused with preservation solution and the time of preservation did not exceed 60 min. The yield of isolated islets was markedly improved by using the procedure involving organ flushing. Intraductal injection of collagenase solution directly after procurement of the gland did not affect the recovery of islets after prolonged time of preservation.

We found that the use of *in situ* flushing significantly improved the endocrine properties of isolated islets. The cells were characterized by a better response to glucose in the static test which resulted in a higher SI. Prolonged time of cold ischemia affected the secretory reaction of islet cells and reduced the value of SI for all four groups. The use of *in situ* pancreas flushing with the UW solution ameliorated the decrease in stimulatory response. The preparation of viable islets depends on the ability to preserve cell survival between removal of donor pancreas and its processing in the laboratory.

We think that insufficient *in situ* flushing of the pancreas before its storage results in an inappropriate distribution of storage solution in some parts of the organ, causing reduction of the well-preserved islet tissue.

Antioxidative properties of UW solution were observed in our study. MDA is usually used as marker of lipid peroxidation in tissues subjected to ischemic conditions (Slater and Sawyer 1971, Slater 1984, Ikeda *et al.* 1994). After 60 min of ischemia, we observed significant differences in MDA concentration, revealing the beneficial effect of *in situ* flushing. Protracted cold ischemia equalized MDA values, although the lowest ones were observed in pancreases flushed without collagenase injection in the time of pancreas procurement. These results suggest that collagenase might interact with the tissue and cause the acceleration of the peroxidation process.

Exocrine tissue damage and interaction at the tissue level can influence the islet outcome and their secretory function. Intravascular *in situ* flushing that removes blood cells from the vascular system seems to have a beneficial effect on the interaction in the preserved exocrine tissue. Amylase and lipase levels in the preservation solution were lowered when pancreases were previously flushed with UW solution. These

findings confirm the protective effects of UW solution components. The increase in the concentrations of pancreatic enzymes after intraductal injection of collagenase solution can be explained as a pressure effect of distention, whereby the tissue is partially damaged by the solution tearing of some cells. Thus the amylase concentration could be a marker of pancreas damage during cold preservation (Brazda *et al.* 1997, 1998). Similar findings were obtained with lipase in our experiments and its concentration can indicate the biochemical changes in ischemically damaged pancreas.

These observations indicate that the intravascular *in situ* flushing with the UW solution has beneficial effect on pancreatic tissue. Intraductal injection of collagenase solution influences exocrine tissue integrity giving similar beneficial results in islet recovery and their function.

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Reprint requests

Andrzej Kinasiewicz PhD, Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Trojdena 4, Warsaw, Poland. E-mail: kinas@ibib.waw.pl