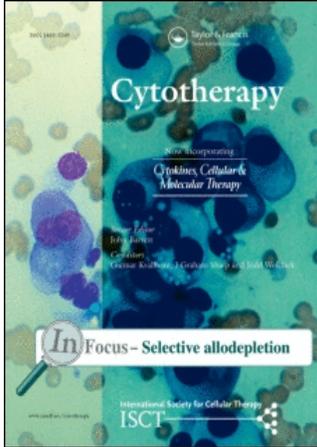


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# Utilization of a test gradient enhances islet recovery from deceased donor pancreases

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## Background

Islet transplantation is a viable treatment alternative for a select group of patients with type 1 diabetes. However, variables unique to the donor pancreas, such as age, fibrosis and edema, can influence the number and purity of the isolated islets. Thus isolation of a sufficient number of islets for transplantation from the pancreas remains challenging because of the lack of methods enabling reproducible isolation.

## Methods

Islets were isolated from 38 consecutive deceased donors using the semi-automated Ricordi method of islet isolation, and purified on a COBE 2991 cell processor using Ficoll-based continuous density gradients. Three different gradient protocols were used. These included a pre-defined gradient using different densities of Ficoll (1.100 g/mL and 1.077 g/mL) mixed with HBSS (group 1), a pre-defined gradient using single-density Ficoll (1.100 g/mL) mixed with University of Wisconsin solution (UW) (group 2) and a variable gradient using

single-density Ficoll (1.100 g/mL) with UW and densities selected based on the results of test gradients (group 3).

## Results

Group 3 yielded a better recovery of islets (74%) than groups 1 (43%) or 2 (37%) ( $P = 0.0144$ ). Viability was significantly higher in groups 2 and 3 ( $P = 0.0115$ ). Purity was not significantly different among the groups.

## Discussion

This method, using a simple test gradient, is a significant process improvement that can improve islet recovery without loss of viability or purity and increase the number of islet products suitable for transplantation.

## Keywords

human islet isolation, islet purification.

## Introduction

Islet transplantation is a viable treatment option for a select group of patients with type 1 diabetes [1]. There are several limitations to the wider application of islet transplantation, including the availability of donor organs. Isolation techniques continue to be improved, but obtaining a sufficient number of islets from a single pancreas for transplantation remains challenging. Individual differences in donor characteristics, along with variability in enzymes, contribute to inconsistent isolation results, even in an environment of strict adherence to islet isolation protocols required for clinical cell processing [2].

Islet purification is performed to minimize the amount of tissue implanted as part of the graft. Some reports support the presence of at least some exocrine tissue in the

transplanted product but, in general, higher islet purity is desired [3]. Benefits of increased purity may include decreased immunogenicity as a result of a smaller tissue volume being infused, possibly resulting in a decreased inflammatory response during transplantation. Infusion of larger volumes of tissue may also increase the risks of portal hypertension in recipients, particularly those that may undergo multiple islet infusions. Unfortunately, the purification process results in the loss of some portion of the islets present following pancreatic dissociation. Identifying a technique to purify islets completely from exocrine tissue has been an elusive goal. At present, most efforts have focused on maximizing islet recovery, with the understanding that there will be at least some, and sometimes significant, exocrine contamination. To this

end, many variations of gradient purification have been performed with varying degrees of success.

Huang *et al.* [4] recently introduced a gradient consisting of Ficoll (density 1.100 g/mL) and University of Wisconsin (UW) solution. UW is a cold-storage solution routinely used for organ preservation prior to solid organ transplantation. The use of UW as a component in the purification solutions resulted in increased gradient capacity, islet yield and islet viability [4]. This gradient is appealing because of its simple formulation as well its ability to purify a larger tissue volume on a single purification run. Unfortunately, we found no improvement in islet recovery with 12 consecutive isolations. Most often, islet recovery was diminished because of large amounts of exocrine tissue being present in the layers that contained islets. This is consistent with previous descriptions of variation in the density of exocrine tissue from preparation to preparation because of intrinsic acinar differences, tissue size and/or degranulation [5]. Exocrine density is usually higher than that of islets, but tissue damage or stress can cause a decrease in the difference between exocrine and endocrine tissue densities. This can result in suboptimal separation between the two tissues when using a pre-defined density gradient. New techniques to improve the consistency of islet recovery are needed.

One recent approach to improving islet recovery has been the use of 'rescue' gradients to maximize islet recovery [6]. This technique appears to be effective in increasing islet yield but also subjects the tissue to additional processing. Another method for enhancing islet purification is the use of test gradients. However, little is published about the specifics of attempts at employing test gradients, regardless of their formulation, in the context of large-scale islet purification with continuous gradients. We report our experience with islet purification using Ficoll-based continuous gradients, as well as a novel test gradient, to determine the optimal density of gradient purification solutions.

## Methods

### Organs

All protocols were reviewed and approved by the Mayo Clinic Institutional Review Board. All pancreases accepted for processing were from brain-dead multi-organ donors that had been declined for clinical use in whole organ or islet transplantation. Organs were procured for research use following *in-situ* vascular perfusion with UW solution

(Viaspan, DuPont Pharma, Wilmington, DE, USA;  $n = 37$ ) or histidine-tryptophan-ketoglutarate solution (HTK; Custodiol, Odyssey Pharmaceuticals, East Hanover, NJ, USA;  $n = 1$ ) and transported to the islet isolation laboratory. Our local procurement team recovered 14 of the 38 organs, with the remainder being procured by distant hospitals/transplant centers. The first 20 organs were processed as group 1, the next 12 as group 2 and the last six as group 3.

### Isolations

Islet isolations were performed using the automated method described by Ricordi *et al.* [7] with minor variations. Briefly, organs were trimmed of duodenum, fat and connective tissue and perfused with collagenase (Roche Molecular Biochemicals, Indianapolis, IN, USA) using controlled rate perfusion [8]. The pancreas was cut into several pieces and transferred to a stainless steel chamber containing several hollow stainless steel marbles (Altira Inc., Miami, FL, USA) and a 500- $\mu$ m mesh screen separating the top and bottom portions of the chamber. Fluid was warmed and maintained at 37°C ( $\pm 1$ ) for the duration of digestion. When appropriate digestion had been observed, enzyme activity was slowed by lowering the temperature and diluting the digest with modified RPMI-1640 (Mediatech, Herndon, VA, USA). Tissue digest was collected in conical tubes, centrifuged at 245 g for 1 min, and washed with modified M199 (Mediatech, Herndon, VA, USA) containing 5% albumin. The tissue was washed, combined, resuspended in UW solution and incubated on ice, with frequent inversion until purification [9].

### Gradient preparation

All gradient solutions were prepared using Biocoll (Biochrom AG, Berlin, Germany), a Ficoll-based cell separation solution. Gradient solutions in group 1 were prepared according to the standard islet gradients utilized in the Edmonton Protocol [1], using different densities of Ficoll (1.100 g/mL and 1.077 g/mL), along with HBSS (10 $\times$ ), sodium hydroxide (NaOH), hydrochloric acid (HCl) and HEPES, to create the heavy and light layers. Gradient solutions in group 2 were prepared by mixing 1.100 g/mL Ficoll and UW solution in varying pre-defined ratios, as described previously [4]. Gradient solutions in group 3 were prepared as in group 2 but with ratios of UW/Ficoll not determined until evaluation of a test gradient.

To prepare test gradients, a series of 5-mL solutions was prepared in 15-mL conical flasks by varying the percentages of UW solution and Ficoll (Table 1). Following a 30-min incubation of the tissue in UW solution, 100- $\mu$ L aliquots of tissue suspension were top-loaded on to each of the test gradients. Test gradients were centrifuged for 5 min at approximately 750 *g*. The test gradients were evaluated to determine the density that would be used for the heavy layer of the gradient. This was selected as the highest density that still had tissue suspended. The light density layer (1.061 g/mL) was routinely prepared using a UW/Ficoll ratio of 70/30. If the optimal density of the heavy layer was determined to be less than 1.071 g/mL, the light layer was adjusted to maintain a density difference of at least 0.011 g/mL. For example, if the optimal heavy layer density was determined to be 1.065 g/mL based on our test gradient, the light layer density was adjusted to be 1.054 g/mL.

### Purification

Purification was performed as previously described using a COBE 2991 cell processor (COBE BCT, Lakewood, CO, USA) modified with a core cooling system [10]. Continuous gradients were prepared and loaded at 25 mL/min onto the spinning COBE (2400 r.p.m.). Tissue suspended in

UW was top-loaded onto the COBE followed by a 50-mL rinse (RPMI-1640). After 5 min, gradient fractions were collected in 50-mL aliquots, washed and evaluated for islets. Selected fractions were retained for further evaluation.

### Islet evaluation

Islet counts were performed by staining 100- $\mu$ L aliquots of tissue with dithizone and evaluating under an inverted light microscope. Islets were sized in 50- $\mu$ m increments using an eyepiece micrometer and converted to islet equivalents (IE) as described previously [11]. Percentage recovery was calculated by dividing the post-purification count by the pre-purification count. Viability was performed via fluorescent staining using Syto-13/ethidium bromide [12]. A minimum of 50 cell clusters was assessed and counted as viable if both the core of the cell cluster and at least 50% of the total cluster stained for Syto-13. Purity was assessed by visual estimate or sizing of exocrine tissue similar to counts for IE.

### Statistics

Statistical analysis was performed using JMP 6.0 and SAS software (SAS Institute Inc., Cary, NC). Continuous data were analyzed using Kruskal–Wallis tests for comparison among the three groups. When this test showed a significant difference, Mann–Whitney tests were performed to analyze data between groups. The influence of variables on recovery was analyzed using Spearman's correlation. As data were approximately normal in distribution, multivariate linear regression analysis was performed to analyze the relationship between multiple variables and the islet product. Nominal data were analyzed using Fisher's exact test.  $P < 0.05$  was considered statistically significant.

## Results

### Demographic characteristics

Donor demographics are shown in Table 2. The body mass index (BMI) was significantly associated with group assignment ( $P = 0.0174$ ). Group 3 had a significantly higher BMI than groups 1 and 2 ( $P = 0.0115$  and  $0.0106$ , respectively).

### Results of islet isolation

Islet equivalents (IE) were compared among the three groups. Purification results are summarized in Table 3. The

**Table 1.** Test gradient preparation

Average measured density at 20°C (four replicates)	UW (mL)	Ficoll 1.100 (mL)	% UW/% Ficoll
1.047	4.7	0.3	94/6
1.051	4.4	0.6	88/12
1.054	4.1	0.9	82/18
1.058	3.8	1.2	76/24
1.061	3.5	1.5	70/30
1.065	3.2	1.8	64/36
1.068	2.9	2.1	58/42
1.071	2.6	2.4	52/48
1.075	2.3	2.7	46/54
1.078	2	3	40/60
1.082	1.7	3.3	34/66
1.085	1.4	3.6	28/72

*Using a standard UW/Ficoll gradient as a starting point, we varied the concentrations of the gradient components to create a series of solutions with distinct densities.*

*All density measurements were performed using a Densito portable densitometer (Mettler, Toledo, Ohio, USA) and are reported in g/mL.*

Table 2. Donor demographics

Group	No. organs	No. organs obtained from local OPO (%)	Age (years)	BMI*	Total ischemic time (h)	Amylase (U/L)	Lipase (U/L)	Max. glucose (mg/dL)
1	20	9 (45)	48.5 (42.5–54.5)	28.0 (23.8–29.8)	14.0 (8.9–17.9)	56 (38–88)	58 (21.5–123.5)	247.5 (195–305)
2	12	4 (33)	51.5 (46.5–58.8)	25.4 (23–30.8)	14.3 (11–17)	53 (30–121)	27 (12.3–77)	209.5 (189–294)
3	6	1 (17)	51.0 (38.8–60.3)	40.9 (30.9–46.5)	14.6 (10.3–17.3)	126 (37–238)	16 (12.5–129.5)	213 (186.5–327)

Data are shown as median with interquartile range (25th–75th).

\* $P = 0.0174$ . None of the other variables showed a difference among groups.

percentage recovery and viability were both significantly associated with group assignment ( $P = 0.0144$  and  $0.0115$ , respectively). Group 3 had a significantly higher percentage recovery than groups 1 and 2 ( $P = 0.0109$  and  $0.0308$ , respectively) while group 1 had a significantly lower viability than groups 2 and 3 ( $P = 0.0079$  and  $0.0486$ , respectively). The difference in purity among the three groups was not statistically significant (Figure 1).

One-hundred per cent (6/6) of isolations in group 3 resulted in recovery greater than 60%, compared with 30% (6/20) in group 1 and 17% (2/12) in group 2. Standard gradient densities (as utilized in group 2) were selected in only 2/6 isolations where test gradients were performed (Table 4).

### Relationship of baseline characteristics to characteristics of isolated islets

The influence of individual variables on recovery was analyzed using Spearman's correlation. We showed a strong correlation of islet isolation to BMI (correlation  $0.3722$ ,  $P = 0.0214$ ). After accounting for the method of isolation, BMI and other baseline variables did not correlate with the characteristics of islet product. The method of isolation showed a relationship with the percentage recovery of islets ( $P = 0.008$ ).

### Discussion

We used three Ficoll-based gradient methods for islet purification, including a novel test gradient, and found that the percentage recovery of islets was significantly higher in isolations for which we employed the test gradient compared with isolations that used a pre-defined gradient. In their original study, the gradient formulation proposed by Huang *et al.* [4] was shown to be at least as effective as other Ficoll-based gradients in terms of recovery and functional analysis. Our initial results confirmed an increase in viability with the use of this gradient formulation (Figure 1) but no increase in islet recovery. We enhanced our islet recovery using this gradient formulation with the application of a test gradient to determine optimal gradient densities. The gradient densities could be rapidly adjusted as there were only two constituents. It should be noted that our measured densities of solutions were quite different from the previous report using the same ratios of UW and Ficoll [4].

While we observed an increase in overall islet recovery, of more importance was the increased consistency between

**Table 3.** Gradient recoveries

	Tissue volume/ COBE run (mL)	Pre-purification (IE)	Post-purification (IE)*	% recovery†	Recovery > 60%‡
Group 1	20.0 (15.8–20)	312 986 (192 217–403 033)	112 194 (60 007–169 313)	41 (25–64)	6/20
Group 2	24.7 (19.8–29.8)	320 736 (225 244–404 436)	113 600 (75 929–174 860)	37 26–49)	2/12
Group 3	26.1 (24.9–31.0)	377 716 (328,996–415 645)	271 105 (236 669–281,847)	74 (69–76)	6/6

Data are shown as median with interquartile range (25th–75th).

\* $P = 0.0076$ , † $P = 0.0144$ , ‡ $P = 0.0013$ .

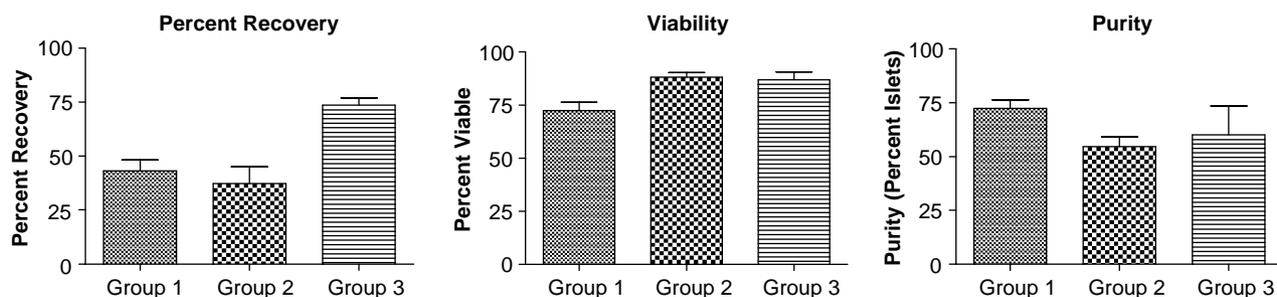
isolations in group 3. By optimizing the gradient densities, we were able to recover consistently greater than 60% of the islets following purification and, in most cases, more than 70%. In four of six isolations, we selected a gradient with a density range that was different from our standard gradient. Although the numbers were limited, this appeared to be in line with our observation that only 2/12 isolations in group 2 (using a standard pre-defined gradients) resulted in recovery greater than 60%. The addition of the test gradient reduced the variability in the purification portion of the islet isolation process.

The UW/Ficoll gradient also allowed us to process larger tissue volumes per COBE run, which reduced operating expenses, personnel requirements and processing time. Huang *et al.* [4] reported an ability to purify up to 60 mL of tissue on a single COBE run, while the recommended tissue volume for the standard islet gradient (group 1) is 20–25 mL. We have performed successful purification with up to 39 mL of tissue on a single COBE run using the UW/Ficoll gradient (Table 3). We have found that resuspending tissue volumes of greater than 40 mL in a total volume of 100 mL for COBE runs can

make pre-purification counts difficult because of the concentration of tissue.

The increased BMI in group 3 is difficult to ignore as a contributing factor in increased islet recovery, given the well-established relationship between BMI and overall islet yield [2]. For several reasons, we believe that the impact of a test gradient is not limited by BMI of the donor. First, stepwise multivariate regression analysis identified the method of isolation as the only reason for improved islet recovery in group 3. Second, BMI did not correlate with percentage recovery in isolations where test gradients were not performed. Third, analysis of only the donors with a BMI greater than 30 showed that group 3 recovery was consistently higher (Figure 2).

Poor quality organs is a possible explanation for our results with the standard UW/Ficoll gradient (group 2) being lower than previous reports. While we have limited *in vitro* assessment of islet viability, we do not have *in vivo* assessment of islet function for our isolations, limiting our ability to infer clinical utility of islets isolated in this manner. Predicting the potency of isolated islets is an ongoing challenge for the field, although enhanced methods for islet assessment are being reported [13–15].



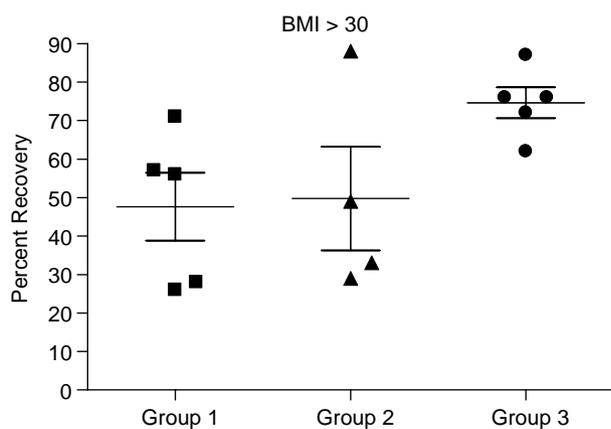
**Figure 1.** Percentage recovery in group 3 was significantly higher than in groups 1 and 2 ( $P = 0.0144$ ). Viability in groups 2 and 3 was significantly higher than group 1 ( $P = 0.0115$ ). The purity in group 2 was slightly lower than the other groups; however, the difference in purity among the three groups was not statistically significant ( $P = 0.0770$ ). Results are shown as mean  $\pm$  SEM.

**Table 4.** Gradient density selected following test gradients

Isolation using test gradient	Heavy layer density	Light layer density	% recovery
1	1.075	1.061	68
2	1.071	1.061	76
3	1.061	1.051	72
4	1.071	1.061	87
5	1.065	1.054	62
6	1.078	1.061	76

The application of this novel test gradient system to 'fine-tune' the gradient densities results in increased islet recovery, as well as more consistency between islet isolations, without loss of viability or purity. As the quality/functionality of islets from pancreata with shifts in exocrine density is not well described, the ability to purify these islets may be useful for future research studies. Because we have used our method on a small number of organs, we are currently using it in every isolation to confirm its utility. The best design to test improvements to the islet isolation process would be with randomization but, to our knowledge, so far no such study has been undertaken.

We have demonstrated that the use of a simple test gradient can improve islet recovery with minimal additional time and effort added to the isolation process. This step has the potential to increase the frequency of obtaining sufficient islets for transplantation. The improved islet recovery through the use of this test gradient is immediately applicable to clinical processing.



**Figure 2.** In isolations with donor BMI > 30, group 3 showed increased percentage recovery and more consistency in isolations. Results are shown as mean  $\pm$  SEM.

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