

A Closed System for Islet Isolation and Purification Using the COBE2991 Cell Processor May Reduce the Need of Clean Room Facilities

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During the isolation of human islets of Langerhans the digest has repeated direct contact with the ambient atmosphere. In order to fulfill GMP requirements in clinical applications, the entire cell preparation must be performed in clean room facilities. We hypothesized that the use of a closed system, which avoids the direct exposure of tissue to the atmosphere, would significantly ease the preparation procedure. To avoid the direct atmosphere exposure we tested a modification of the isolation and purification process by performing all islet preparation steps in a closed system. In this study we compared the isolation outcome of the traditional open preparation technique with the new closed system. Pancreata from 6-month-old hybrid pigs were procured in the local slaughterhouse. After digestion/filtration the digest was cooled, collected, and concentrated in centrifugation containers and purified thereafter in the COBE2991 by top loading (control). In the control group 502 ± 253 IEQ per gram pancreas were purified. The total preparation time amounted to 12 h. In the closed system the digest was cooled and directly pumped into the COBE2991 for centrifugation followed by supernatant expelling. Bag filling, centrifugation, and expelling were repeated several times. Islets in pellet form were then purified by adding a gradient (bottom loading). Using this closed system 1098 ± 489 IEQ per gram pancreas were purified with a total cell viability of $67 \pm 10\%$ and a β -cell viability of $41 \pm 13\%$. The total preparation time reduced to 6 h. After 24 h of cell culture the viability of β -cells was still $56 \pm 10\%$ and was only reduced after the addition of proapoptotic IL-1 and TNF- α to $40 \pm 4\%$, indicating that freshly isolated islets are not apoptotic. In conclusion, the closed system preparation is much faster, more effective, and less expensive than the traditional islet preparation. The closed system may be applicable for human islets preparations to restrict the need of clean room facilities for islet preparations to a minimum and may open the way for islet preparations without clean room demand.

Key words: Porcine islets; COBE2991; GMP; Islet transplantation

INTRODUCTION

The isolation of islets of Langerhans from large animals or humans is routinely performed by using the continuous enzymatic digestion filtration method with small species-specific adaptations (11–14). During the isolation of human islets of Langerhans, the digest has repeated direct contact with the ambient atmosphere. This pertains not only to the initial organ preparation for enzyme injection but also to the repeated centrifugation steps to concentrate the digested tissue suspended in a large amount of elution medium. This concentration step is usually performed in a separate room centrifuge before density gradient purification of islets in the COBE2991 cell processor (14,15).

In order to fulfill the requirements of GMP guidelines

for clinical islet applications, the entire cell isolation must be performed in clean room facilities (3,16). One islet isolation by two persons occupies a clean room for at least 12 h. Thus, the use of clean rooms requires large economic and administrative efforts, which may hinder establishing a clinical islet isolation program. To ease the current protocol of islet preparation we attempted to modify the islet processing procedure. We hypothesized that the COBE2991 cell processor might be useful not only for tissue purification but also for tissue concentration. Such an arrangement would allow the tissue concentration and purification in the closed system of the COBE bag.

In order to test the closed system modification we coupled a flow-through cooling device between the Ricordi chamber and the COBE2991 cell processor. In

the COBE bag the digest concentration was immediately performed. Once the bag was completely filled, the digest was immediately centrifuged and the supernatant was expelled. During this procedure we took advantage of some technical features of the COBE2991 cell processor that are not used in routine islet isolation protocols. The closed system entirely omitted all tissue concentration and washing steps usually performed in a second room centrifuge. The similarity between the isolation protocols of islets from human or porcine pancreata allows to establish human islet isolation protocols by training with porcine organs (10). In this study we used the pig pancreas to evaluate the suitability of a new closed system for islet isolation and purification.

This procedure represents one step forward towards the development of a technical solution for a GMP-compliant islet processing to be performed outside clean rooms.

MATERIALS AND METHODS

Organ Procurement

All organs were taken from 6-month-old young market weight hybrid pigs (100 kg). Each organ was procured after a warm ischemic time of 20 min. The pancreas was immediately purified from fat, vessels, and connective tissue. The duodenal lobe of the pancreas was removed and stored in cold HBSS for biochemical analysis.

The splenic lobe of the porcine pancreas was injected via the pancreatic duct with 50 ml ice-cold UW solution to preserve the organ and to demonstrate organ integrity (2). The digestion started 3 h after organ procurement.

Pancreas Digestion

Liberase (Roche, 1.5 mg/g tissue, dissolved in 150 ml HBSS at room temperature) was injected into the splenic lobe via the pancreatic duct. Organs were digested in a hand-made Ricordi chamber (volume 600 ml, mesh size 500 μ m, 8 stainless steel marbles) connected to a heating device (400 ml). Before digestion, this system was prewarmed to 37°C with 1 L HBSS (Biochrom AG, Berlin, Germany) supplemented with HEPES (Biochrom AG, 25 mM).

Open System for Isolation and Purification

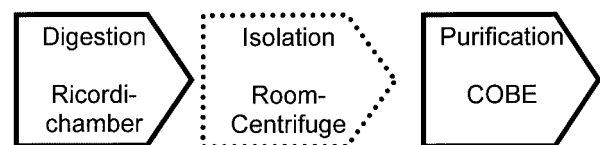
The open system for islet isolation consisted of the Ricordi chamber for continuous digestion/filtration, a cooling device made of stainless steel containers, centrifugation vials, and a COBE2991 cell processor. When the optimal organ dissociation in the chamber was observed after microscopic inspection the continuous digestion/filtration phase was stopped. New medium was added for elution and the digested tissue was flushed away (100 ml/min) via the cooling device into centrifu-

gation containers each accommodating a volume of 200 ml. Each container was preloaded with 4 ml albumin solution (20%). Four of these containers were immediately centrifuged (10 min at $400 \times g$, 6°C). The rest of the containers were filled with effluat (a total of 3 L) and stored on ice until centrifugation. The pellets were successively combined, resuspended in precooled HBSS (supplemented with albumin), and washed once (10 min at $400 \times g$, 6°C). The final pellet was resuspended in UW solution (50 ml) and stored on ice for 60 min under gentle agitation. For density centrifugation, the spinning COBE2991 was successfully loaded with a bottom layer (250 ml UW/Optiprep solution, density 1.2), a middle layer (UW/Optiprep solution, 100 ml density 1.09), and a top layer (120 ml UW solution, density 1.05). The digest was added by top loading the gradient (6). After centrifugation (1000 rpm/min, 5 min) fractions (20 ml) were pumped out and were screened for islets by dithi-zone staining. Islet yield is given as number of islet equivalent (IEQ) determined by areal density analysis.

Closed System for Islet Isolation and Purification

The closed system for islet isolation consisted of the Ricordi chamber for continuous digestion/filtration, the cooling device, and the COBE2991 cell processor. The collection of digest in centrifugation containers and the use of the second room centrifuge for tissue concentration was omitted. The differences in the experimental setup between the classical open system and the new closed system are summarized in Figure 1. When the optimal organ dissociation in the chamber was observed the continuous digestion/filtration phase was stopped,

a) Classical Setup



b) New setup

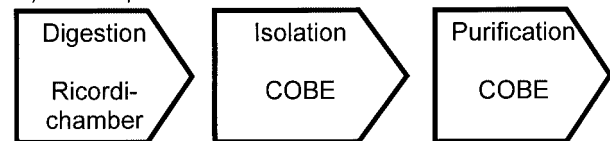


Figure 1. Comparison of the traditional and the new islet isolation and purification setup. Boxes drawn in solid lines denote operating steps in a closed system, and broken lines denote operating steps in an open system with contact to the ambient atmosphere.

new medium was added for elution, and the digest was flushed away (100 ml/min) via the cooling device directly into the bag of the COBE2991 cell processor. A maximum of 600 ml digest (10°C) was immediately centrifuged (1 min, 1500 rpm/min). The bag was completely emptied (super-out function, COBE2991) leaving a concentrated digest (50 ml) in the bag. The effluante (14°C) was checked by dithizone staining for the absence of islets. The Ricordi chamber was flushed again with fresh HBSS buffer and the cooled effluante was again centrifuged inside the COBE. This procedure was repeated until a total of 3 L of buffer had passed the COBE. Islets were purified from the concentrated digest in the bag by using the modified UW/Optiprep discontinuous gradient (6). The bottom layer was added and mixed for a period of 15 min to dissolve the concentrated tissue (mix-function, COBE2991). The middle and top layers were added as described above. After centrifugation (1000 rpm/min, 5 min) the tissue was pumped out and fractions (20 ml) were screened for islets after dithizone staining. Islet yield was determined by areal density analysis (7).

Biochemical Analysis of the Pancreatic Tissue and of Purified Islets

About 50 mg of pancreatic tissue taken from the duodenal lobe was homogenized in HBSS, filtered, and subsequently analyzed for protein and insulin content as well as amylase activity. Similarly, a fraction of the final islet preparation was suspended in HBSS, homogenized, filtered, and analyzed accordingly. A fraction of purified islets (50 IEQ/ml HBSS incubated with either 3 mM glucose or 16.7 mM glucose) was tested for glucose-induced insulin secretion in static incubations for a period of 1 h at 37°C. Glucose-induced insulin secretion was determined as stimulating index (SI) calculated as the ratio of insulin secretion in the presence of high or low glucose concentration.

Islet Cell Dissociation

Islets were dissociated into single-cell suspensions by incubating aliquots of approximately 1000–1500 IEQ in 1 ml accutase solution (PAA Laboratories, Pasching, Austria) at 37°C for 15 min and subsequently dispersed by gentle pipetting (4).

Determination of β -Cell Viability

Single cell suspensions were incubated with 1 μ M Newport Green PDX acetoxymethylether (NG; Molecular Probes) for 30 min at 37°C in PBS without Ca^{2+} and Mg^{2+} . Newport Green allows for the definition of cell subsets according to the zinc content (4,8). After washing, cells were stained with 7-aminoactinomycin D (7-AAD; Molecular Probes) or with propidium iodide (PI),

which both bind to DNA when cell membrane permeability is altered after cell death. Cell suspensions were analyzed using a FACSsort cytometer (Becton Dickinson) with CellQuest software (4).

Cell Culture

Islets (1500 IEQ/ml) were cultured for 24 h in HAMS-F12 supplemented with 10% FCS either in the absence or presence of cytokines (IL-1 and TNF- α at a concentration of 10 ng/ml each). After cell culture, islets were concentrated by centrifugation ($300 \times g$, 5 min) before they were treated with accutase as described above.

Statistics

Values are given as mean \pm SD. Mean values between the open and closed preparation groups were compared using unpaired, two-sided *t*-test with unknown variance. A significant difference was assumed for values of $p < 0.05$.

RESULTS

Digestion

A biochemical analysis of 10 porcine pancreata was performed (Table 1). When separated in the two groups of islet processing, there was no difference in organ weight, accommodated liberase volume, or insulin or amylase content. Using the closed system it was not possible to analyze the number of isolated islets, because the tissue was directly transferred into the bag of the COBE2991. In the control series of experiments performed with the classical open setup, we detect 821207 ± 424314 IEQ or 9426 ± 4585 IEQ/gP after isolation, indicating a complete organ dissociation. The digestion time and tissue residue in the Ricordi chamber at the end of

Table 1. Digestion of Porcine Pancreata in the Open or Closed System

Variable	Unit	Open System		Closed System	
		Mean	SD	Mean	SD
Pancreas	g	88.0	11.4	86.5	10.4
Load	g	118.0	15.6	112.1	12.8
Insulin	mU/mg Pr	5.6	0.5	12.6	10.0
Amylase	U/mg Pr	525	249	1883	2176
Digestion	min	27	3	32	4
Residue	%	15	4	11	5
IEQ	<i>n</i>	821206	424314	—	—
IEQ	<i>n</i> /g Pa	9425	4584	—	—

Values are means and SD in four experiments (open system) or six experiments (closed system). Abbreviations: g, gram; U, unit, mU, milliunit; mg, milligram; Pr, protein; Pa, pancreas weight in grams; IEQ, islet equivalents.

the digestion were similar in both groups. This indicates that the organ digestion in both processes had reached a comparable state and that the digestion in the closed system would be performed in a standardized and reproducible manner.

Purification

In both the open and closed system, purified islets were always observed in fraction number 6 with very little islets seen in the neighboring fractions. Compared with the classical open method, the number of purified islets using the closed system was significantly increased (Table 2). Also the insulin content in the islet fraction and the amylase residual activity were higher in the closed system group; however, none of these values reached statistical significance due to the larger variation of values in the closed group. No difference in islet metabolic activity immediately after purification was observed. There was a clear difference in the time needed to complete an entire preparation in either method. Employing two persons a total of 10–12 h was needed to complete an islet isolation using the old method, whereas only about 6 h for two persons are needed for the islet isolation using the closed system.

Islet Viability and Purity

Figure 2a shows purified islets obtained after using the closed system. This preparation contained mainly islets and no visible exocrine tissue contamination. The measured residual amylase activity indicated a purity of more than 99%. This preparation contained some ductal tissue fragments (Fig. 2a, arrows). This islet cell preparation was examined by FACS analysis. 7-AAD staining identified a distinct fraction of dead cells compared with

the weak fluorescence signal of living cells in the presence of 7-AAD. In this preparation, there was a fraction of about 67% living cells in the entire cell preparation (Fig. 2b). Addition of Newport green increased the fluorescence signal 10 to 300 times over the autofluorescence signal of unstained cells (data not shown). Again, a fraction of about 67% of all cells could be identified as NG positive. Analyses of six different islet preparations showed that NG identified only one cellular subgroup with a strong fluorescence signal (data not shown). It was not possible to subdivide the NG-positive cells for further analysis. The combined analysis of NG-positive and 7-AAD-negative cells identified a population of about 40% living β -cells (Fig. 2c). A total of six islet preparations in the closed system were accordingly analyzed and the results are summarized in Figure 2d. The data show $67 \pm 10\%$ vital and $41 \pm 13\%$ vital β -cells. Thus, using the closed system the observed islet quality can be reproducibly obtained.

For the sake of comparison, one analysis of islets taken from the open system preparation was analyzed as well. Although the absolute number of isolated islets was much lower, 7-AAD could identify vital cells (55.4%) and in combination with NG living β -cells (15.4%) in this preparation (data not shown).

Cell Culture

In order to analyze the survival of isolated islets taken from the closed system, cell culture experiments were performed either in the absence or presence of cytokines. If the process of islet isolation in the closed system effectively triggered apoptosis, a further addition of cytokines should be ineffective and all cultured β -cells should be dead after 24 h. On the other hand, if isolated islets are not apoptotic, cell death may be induced by cytokine addition. Two islet isolations were examined. The results of one preparation are shown in Figure 3. The addition of cytokines reduced the fraction of vital β -cells from 63% to 43%. In a second experiment performed with another islet cell preparation the addition of cytokines reduced β -cell survival from 49% to 38% (data not shown). This indicates that β -cells survived to a large extent a period of 24 h after isolation and remained sensitive towards the addition of cytokines. The closed system for islet isolation may thus produce islets that are not designated for apoptotic cell death.

DISCUSSION

For the purpose of clinical islet transplantations, clean rooms are mandatory to meet GMP requirements for cell preparations (3,16). Today the obligation to use clean rooms relates to the initial pancreas preparation, its distention with enzyme solution, the sampling of ef-

Table 2. Purification of Porcine Islets in the Open or Closed System

Variable	Unit	Open System		Closed System	
		Mean	SD	Mean	SD
IEQ	<i>n</i>	45241	24552	97201*	51258
IEQ	<i>n/g Pa</i>	502	253	1098*	489
Insulin	mU/mg Pr	176	82	4745	10044
Insulin	U	33.8	14.1	78.2	88.8
Amylase	U/mg Pr	24	10	158	367
SI		1.5	0.5	1.6	0.7

Values are means and SD in 4–10 observations. Units: g, gram; U, unit, mU, milliunit; mg, milligram; Pr, protein; Pa, pancreas weight in grams; IEQ, islet equivalents; SI, stimulatory index.

* $p < 0.05$ for comparison of mean values in the open system group and the closed system group.

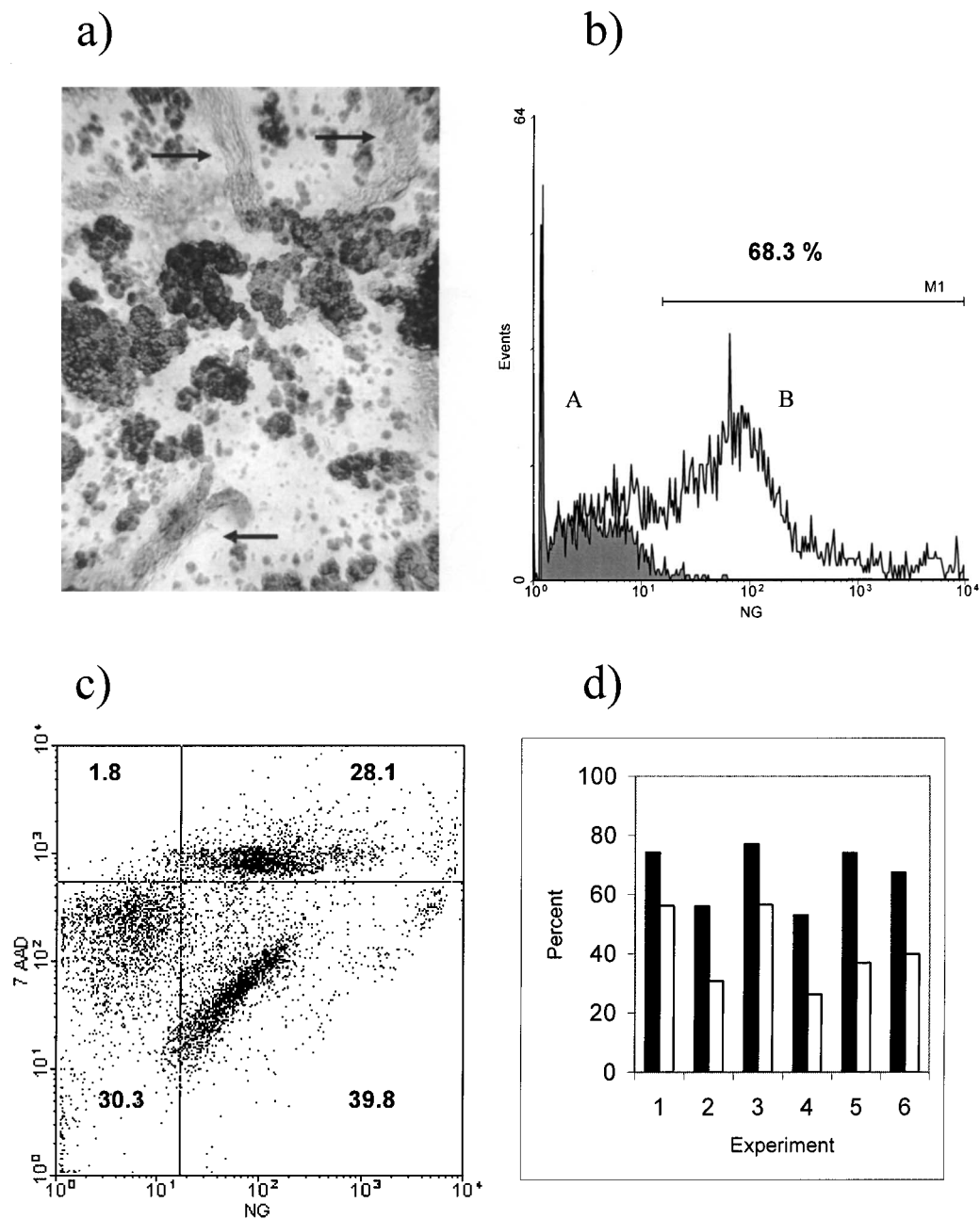


Figure 2. FACS analysis of purified porcine islet cells. (a) Purified islets after dithizone staining (original magnification 100 \times). Arrows indicate co-isolated ductal structures. (b) Identification of β -cells by Newport Green (NG, 68%) in this preparation. Dark shaded area denotes the autofluorescence of cells in the absence of NG. (c) Dot-plot of 7-AAD-negative (living) and NG-positive cells (β -cells) identifying a distinct population of living β -cells (39.8%). (d) Summary of six isolations performed in the closed system configuration. Black columns denote percentage of vital cells and white columns denote percentage fraction of living β -cells.

fluate, and the washing and packing of the final cell product. However, the necessity to use clean rooms may be abolished for all preparation steps following enzyme distention provided the preparation can be performed in a closed system. The presented study is an attempt to

realize such a procedure. Because unrestricted availability of clean rooms may hinder establishing clinical islet transplantation programs, the presented technique may be a useful technical improvement.

The essential step in realization of the closed system

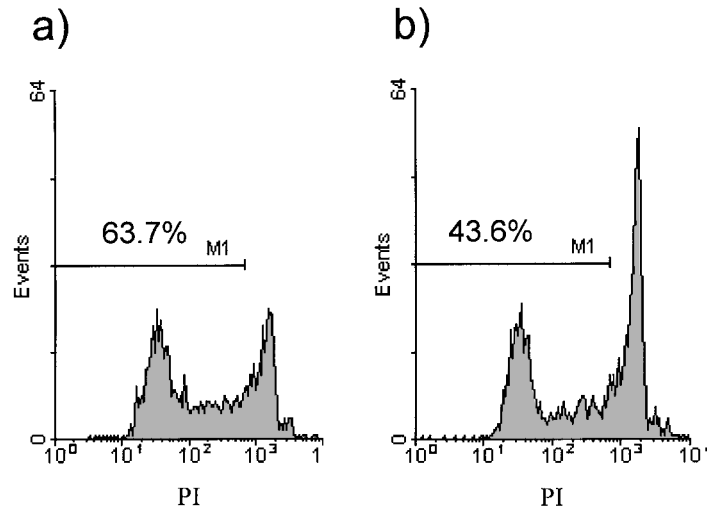


Figure 3. FACS analysis of purified porcine islet cells after cell culture. PI: propidium iodide. In a parallel analysis with PI and NG a gate was defined containing more than 95% of all β -cells in this preparation. This gate was used in the present analysis with PI only. (a) Fraction of living β -cells after cell culture for 24 h under control conditions. (b) Fraction of living beta cells after cell culture for 24 h in the presence of cytokines (IL-1 and TNF- α at a concentration of 10 ng/ml each).

for islet preparation is the intensified use of the COBE2991 cell processor. The COBE2991 cell processor has been primarily developed for producing blood cell concentrates in a nonsterile surrounding. The centrifugation of large volumes, the ease of generating density gradients, and the possibility of fractional emptying has made the COBE2991 a gold standard for islet purification (1,14,15). However, the COBE2991 comes into use only during the final islet purification step when the islets had already repeated contact with the ambient atmosphere. This study demonstrates the suitability of the COBE2991 to perform islet isolation and purification in an entirely closed system. It seems, therefore, possible to perform islet isolations also for clinical applications outside clean room facilities by exploiting the closed bag system of the cell processor in a similar manner as used for blood component preparations.

In the closed system for islet isolation there are some modifications of the classical setup described by Ricordi (11–14). First there is the use of a flow-through stainless steel cooling chamber in direct connection with the COBE centrifugation bag. Second, there is the use of the COBE2991 for repeated digest centrifugation leading to an accumulation of the digest in the closed COBE bag. At last there is the use of the COBE mix-function for resuspension of the pelleted digest after isolation. The repeated direct centrifugation of the digest speeds up the entire preparation procedure, which can now be finished by two persons within 6 h working time. This preparation avoids the collection of the digest in disposable centrifugation containers and the need of a second room

centrifuge. It has been demonstrated previously that a final collection of islets after purification directly into a cell suspension bag under sterile conditions is possible (18). Thus, an entirely closed system for islet preparation seems feasible.

Islets from young pigs are not entirely protected by a collagen capsule and therefore easily dissociate into fragments (17). Hence, efficient isolations of islets from young pigs has been difficult but can now be achieved after gradient modifications (6). The presented study demonstrates that the closed system, which takes advantage of the modified UW/optiprep gradient, allows an efficient purification of islets.

The comparison between the open and the closed system is based on entirely separated organ preparations without taking into account that there may be some organ variations. Analyzing the two series of organ preparations, no significant organ specific variations are observed. These separate preparations are necessary because it is technically not possible to divide one preparation in two parts that could be purified by either method. Compared with previous data, the number of purified islets in the open system is low, but similar to previously repeated values (6). Although it was not our intention to improve the existing techniques for islet isolation and purification, the comparison of the closed versus the open system shows a higher islet yield in the closed system. The obtained islet yield in the closed system correlates well with a previous study performed with young pigs in an open system using the same density gradient (73535 ± 20036 IEQ or 1336 ± 381 IEQ/gP) (6).

In the closed system the islets are exposed to repeated centrifugations in the COBE bag before they are purified by a procedure similar to COBE bottom loading. This procedure resembles the reported rescue gradient purification reported by Ichii (5). As demonstrated there, repeated centrifugation combined with bottom loading of islets does not have adverse effects on islet viability. We therefore assume that the islets isolated in the closed system are not stressed more than those islets exposed to rescue gradients.

A definite test of β -cell viability may be achieved by measuring apoptosis with TMRE using flow cytometry (4). This technique requires the presence of a NG^{bright} subfraction, which can be separated from a NG^{dim} fraction consisting of other endocrine cells. For human islets this procedure has been successfully applied to detect different degrees of apoptosis after cell isolation and culture. In all our analyses performed with apparently pure porcine islet fractions, we were not able to identify this subfraction in the NG-positive cell population. Purified porcine islet cells contain only a single fraction of NG-positive cells, which cannot be subdivided by any reasonable marker. With porcine islet cells we are thus unable to apply the complete flow cytometric analysis proposed by Ichii (4).

In the absence of a direct apoptotic marker for porcine islet cells we examined the survival of NG-positive cells after 24 h in cell culture in the absence or presence of cytokines. A cytokine induced β -cell death in conjunction with a high amount of apoptotic cells is detectable in human islets after 18 h of cell culture (4). Our cell culture experiments show that NG-positive islet cells survive cell culture with a stable percentage of vital cells whereas cytokine-treated cells die to a larger extent. This excludes the possibility of generating a large fraction of proapoptotic β -cells by applying the closed system.

In conclusion, the data show that the closed system for islet isolation may be a useful preparation technique to avoid an excessive need of clean room facilities. It remains to be tested whether the closed system performs well with human islets.

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