Pancreatic islet transplantation: studies on the technique and efficacy of islet isolation and transplantation

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4 Cell preservation in University of Wisconsin solution during isolation of canine islets of Langerhans*

Introduction

Long-lasting insulin independence after allogeneic islet transplantation in type-1 diabetic patients has been achieved recently in single cases, but requires a variable number of donors [6]. The islet mass and purity of the graft are essential for successful clinical transplantation, and complete purity is desirable to reduce or manipulate the immunogenicity of the graft [5,6,8,9]. Density gradient separation of isolated islets is now the most efficient purification technique — the variable outcome, however, usually necessitates the choice between a low yield of highly purified islets or a high yield of impure islets.

Little attention has been paid to the composition of the isolation solution and the impact of the conditions before density purification on outcome [9]. After collagenase digestion of the pancreas, islet isolation is traditionally performed in physiological salt based solutions such as Hanks' balanced salt solution (HBSS) and RPMI culture medium, under hypothermic conditions — to inhibit the action of collagenase and slow down degenerative processes. Potentially detrimental effects of hypothermia when the cell is exposed to a physiological milieu — such as cell swelling, and eventually loss of integrity of the cell membrane — are well documented in studies designed for the hypothermic preservation of whole organs [2,10]. Swelling of isolated cells is — unlike the situation for native cells of the intact organ — not limited by the volume of the extracellular compartment [2,23]. Moreover, collagenase isolation of cells may increase the permeability of the cell membrane, resulting in accelerated swelling and irreversible damage to the isolated cells [10,18].

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The UWS has been primarily designed to minimise the side-effects of hypothermia during cold storage of the pancreas [18,23]. We hypothesised that the UWS would be also more appropriate for islet isolation. We, therefore, compared the outcome after islet isolation in either the UWS or RPMI, and studied the impact of the isolation solution on final outcome after density gradient separation. First, conventional hyperosmotic gradients of dextran in HBSS were used — because hyperosmolality of the density solution has been shown essential for islet purification. Next, we further delineated the impact of the UWS — in the absence of the confounding effects of hyperosmolality — by purification in a novel normosmotic density gradient of Percoll in UWS.

Materials and methods

Design of the study

We used the splenic segment of the dog pancreas for 29 consecutive islet isolations (Fig. IV.1). Isolation was performed by collagenase digestion and dispersion of the tissue at 4°C in either RPMI (n = 8) or the UWS (n = 21). After using RPMI as the isolation solution, islets were purified in a hyperosmotic step gradient (densities 1.045-1.075-1.085-1.095 g/ml) of dextran in HBSS. After using UWS as the isolation solution, islets were purified by centrifugation in either the dextran gradient (n = 8) or an isodense step gradient (1.045-1.075-1.085-1.095) of Percoll in UWS, of near-normal osmolality (n = 13). Samples taken from the cut end of the splenic pancreas, the digest, and from the gradient layers, were compared by morphometry and amylase extraction to assess the recovery of islet and acinar tissue — and purity. Islet viability was studied by electron microscopy, in vitro during perifusion, and in vivo after total pancreatectomy and intrasplenic islet autotransplantation in another four dogs.

Density solutions

The 1.095-density solution (470 mOsm/kg H_2O) of dextran (D3759; Sigma, St. Louis, MO) was prepared in HBSS supplemented with 20 mM Hepes (pH 7.4), and autoclaved for 5 min at 120°C. The 1.095-density solution (340 ± 6 mOsm/kg H_2O) of Percoll (Pharmacia, Uppsala, Sweden) was made in a basal UWS containing regular amounts of potassium lactobionate, raffinose, potassium phosphate, magnesium sulphate, and 4% (w/v) Pentastarch (DCC-607; Du Pont Critical Care, Waukegan, IL). Final pH of the basal UWS was adjusted to 7.4 with NaOH; and diluted density solutions were made with

regular UWS (320 ± 5 mOsm/kg H_2O, density 1.045 g/ml; ViaSpan, Du Pont Pharmaceuticals, Wilmington, DE).

Islet isolation

After a 24-h fast, female, outbred beagles weighing 8–18 kg (Harlan, Zeist, The Netherlands) were anesthetised as described previously [4]. The splenic segment of the pancreas was mobilised and the pancreas was divided, where it overlies the portal vein (Fig. IV.1). After taking a specimen from the cut end for assessment, the duct was cannulated, and the gland was removed, weighed, and within 90 s from the onset of ischemia, perfused (flow 10 ml/min) for 5–10 min with 150 ml of a recirculating, ice-cold collagenase solution — containing 1633 U/ml collagenase (type XI; Sigma), 5% Pentastarch, 20 mM Hepes (pH 7.5), 4.5 mM sodium bicarbonate, and 90% of the regular Hanks' balanced salts. The gland was incubated in the remaining collagenase solution at 38°C for 20 min in a water bath. The solution was decanted, and tissue was immersed in ice-cold isolation solution — either UWS or RPMI-1640 supplemented with 10% newborn calf serum. The digest was stripped from large ducts, and dispersed by gentle aspiration and flush through a 14-gauge needle, and filtration through a 400 μm steel mesh. Trapped tissue was syringed and sieved again for 1–2 times. The finally trapped tissue — which largely consisted of ductal and vascular fragments — and the earlier removed ducts were blotted, weighed, and discarded. The digest was pooled by centrifugation at 200 *g* and 4°C for 2 min in 50-ml graduated tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) for measurement of the packed tissue volume in a single tube — using a ruler for subdivision of the graduation. After resuspension, samples were taken for assessment, and the digest aliquoted in tubes (packed tissue < 2 ml/tube) for density separation. Tissue was pelleted and resuspended in 12 ml of either a dextran or Percoll 1.095-density solution, and overlaid with 6 ml each of 1.085, 1.075, and 1.045 density solutions respectively (Fig. IV.1). Gradients were centrifuged at 4°C and 40 *g* for 4 min, and 500 *g* for 12 min. Purified islets were aspirated from the first layer (1.045/1.075) and second layer (1.075/1.085) of the gradients. After washing of the purified fractions and the combined other fractions of the gradients, aliquots were taken for assessment. Purified islets were cultured at ambient atmosphere in bacteriological petri plates with RPMI, containing 4.2 mM sodium bicarbonate, 20 mM Hepes, and supplemented with 10% fetal calf serum.

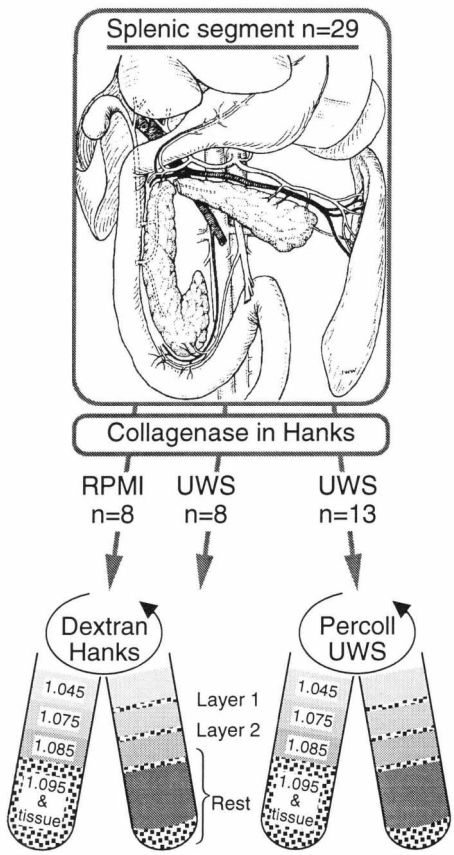


Fig. IV.1

Schematical representation of the design of the study

Perifusion studies

After overnight culture, aliquots containing 1 µl islets (range 0.3–3.3) were transferred to four 0.5-ml microchambers to conduct two duplicate tests in the Acusyst-S system (Endotronics, Coon Rapids, MO). Islets were perifused at 0.25 ml/min and 36°C with Krebs-Ringer-Hepes containing 2.5 mM glucose during a 90 min equilibration period, and 2.5 or 7.5 mM glucose thereafter. From 30 min prior to stimulation, fractions — as indicated in the figures — were stored at –20°C pending insulin assay [4]. Data from duplicate chambers were averaged, and basal secretion was defined as the mean value from –15 to 0 min.

Autotransplantation.

In another four dogs total pancreatectomy was performed for islet isolation from the whole pancreas in RPMI (n = 2) or the UWS (n = 2). Islets were purified in dextran gradients, washed in RPMI, and autotransplanted by venous reflux into the spleen of the dog. After surgery, meals were supplemented with protease-lipase-amylase pellets (Organon, Oss, The Netherlands). Fasting and 2-h postprandial plasma glucose (Glucose Analyzer II, Beckman Instruments, Palo Alto, CA) were assessed before, and weekly for 3 months after transplantation.

Morphometry, extraction, and purity

After Bouin fixation and paraffin embedding of pancreatic specimens, ten serial 5-µm sections were taken at 150-µm intervals, and stained with hematoxylin-eosin. The fractional volumes of islet and fibrous tissue were measured using a 400-point and 25-point grid respectively, by the point counting method as described previously [20], and the remainder was considered acinar tissue. Duplicate 50-µl aliquots of the islet suspensions were stained with dithizone (diphenylthiocarbazone; Sigma). Using an ocular micrometer at x100 magnification, the mean diameters of islets ≥ 40 µm were recorded for determination of the total volume of isolated islets per gram processed pancreas. A total number of 196 ± 25 islets were examined, and the coefficient of variation for duplicate measurements of islet volume was 17%.

For amylase extraction, pancreatic specimens and aliquots of the islet suspensions were homogenised and sonicated in HBSS buffered with 20 mM Hepes, incubated overnight at 4°C, and centrifuged. The supernatant was assayed by the Phadebas α-amylase test (Pharmacia, Uppsala, Sweden).

Table IV.1

Comparison of the effects of canine islet isolation in either UWS or RPMI, on islet and amylase recovery, acinar cell volume, and amylase concentration in acinar cells

	RPMI	p*	UWS
Islet yield (% islet volume in pancreas)	54±6	NS	50±4
Amylase recovery (% content pancreas)	72±4	<.02	95±5
Volume of isolated acinar tissue†			
Expected (ml)	7.7±0.4		8.5±0.4
Observed (ml)	8.1±0.5‡		7.2±0.3§
Observed (% expected)	106±7	<.0001	85±1
Amylase per µl acinar tissue			
Native tissue (IU)	10.1±1.8	NS	7.9±0.8
Isolated tissue (IU)	7.5±1.1‖	NS	8.2±0.8¶
Isolated (% native)	80±9	<.01	105±5

* Statistical analysis of differences between mean values after isolation in UWS *vs* RPMI. † Details for estimation of the expected and observed acinar volume are given in the Results section. ‡ Observed *vs* expected acinar volume in RPMI, NS. § Observed *vs* expected acinar volume in UWS, p <.0001. ‖ Amylase concentration before (native tissue) *vs* after isolation in RPMI, p = .06. ¶ Amylase concentration before (native tissue) *vs* after isolation in UWS, NS

Purity was expressed as the fractional islet volume. The volume of acinar tissue in the gradient fractions was estimated from the amount of amylase, by extrapolation from the ratio of amylase and packed tissue volume of the corresponding digest, assuming absence of fibrous tissue. For microscopy, 1–4% aliquots of the islet suspensions were pelleted, captured in plasma clots, and fixed in Bouin's solution. Paraffin sections were immunoperoxidase stained for insulin [4], and purity was estimated from the ratio of islet and acinar tissue.

Electron microscopy

Pancreatic specimens or isolated-islets captured in plasma clots were fixed in 1.5 % glutaraldehyde - 1% paraformaldehyde in 0.1 M cacodyl buffer (pH 7.4), postfixed in 1% OsO_4, and embedded in Epon. Ultrathin sections (50-70 nm) were stained with uranyl acetate and Reynold's lead citrate and examined in a Philips CM 10 electron microscope operating at 60 kV.

Statistical analysis.

Results are expressed as mean ± SE. Logarithmic transformation of data, and

arcsine transformation of percentage data were used when appropriate. Differences between means were tested by analysis of variance and multiple comparisons were made by the Tukey test and Scheffé's procedure for multiple contrasts. *P* values less than .05 were considered to be significant.

Results

Islet isolation.

The islet volume in the splenic pancreas amounted to 15.8 ± 1.0 µl/g (range 8.4 to 27.3), and the acinar volume amounted to 903 ± 3 µl/g. Because of the variability of the islet volume in the pancreas, the yield of isolated islets was expressed and analysed as a percentage of the native islet volume.

Table IV.1 summarises the recovery of islet tissue and amylase, and the effects of the isolation solution on the volume and amylase concentration of acinar tissue, before density gradient centrifugation. The isolation solution did not affect islet yield, but did affect the recovery of amylase and the volume of acinar tissue. Dispersed tissue in RPMI contained 72 ± 4% of the total amount of amylase in the pancreatic segment ($p<.01$). In contrast, no significant loss of amylase was observed during islet isolation in the UWS. Because ductal and vascular tissue had been largely discarded during isolation, we assumed the volume of isolated acinar tissue to equal the observed packed tissue volume, and we estimated the expected volume of isolated acinar tissue by subtraction of the weight of tissue discarded during isolation (9 ± 1% of the weight of the processed pancreatic segment) from the weight of the pancreatic segment. The volume of acinar tissue in the UWS amounted to 85 ± 1% ($p<.0001$) of the expected volume. In contrast, acinar tissue volume in RPMI was rather larger than expected (NS). Because the volume of isolated acinar tissue would be affected both by loss of acinar tissue during isolation, as well as by swelling or shrinkage of the acinar cells in the isolation solution, we calculated the amylase concentration in native and isolated acinar tissue as an indirect measure of the cell volume of the acinar cells. The amylase concentration of acinar tissue was not significantly affected by isolation in UWS, but had decreased ($p = .06$) to 80 ± 9% of the concentration in native acinar tissue after isolation in RPMI ($p<.01$ *vs* UWS). These results — indicating differential effects of the isolation solution on cell volume and amylase concentration of the acinar cells — were corroborated by microscopic observations of freshly isolated acini. Acinar cells in RPMI demonstrated a dark-brownish apical area and a large translucent basal area, whereas compact, uniform dark-brown cells were evident in the UWS.

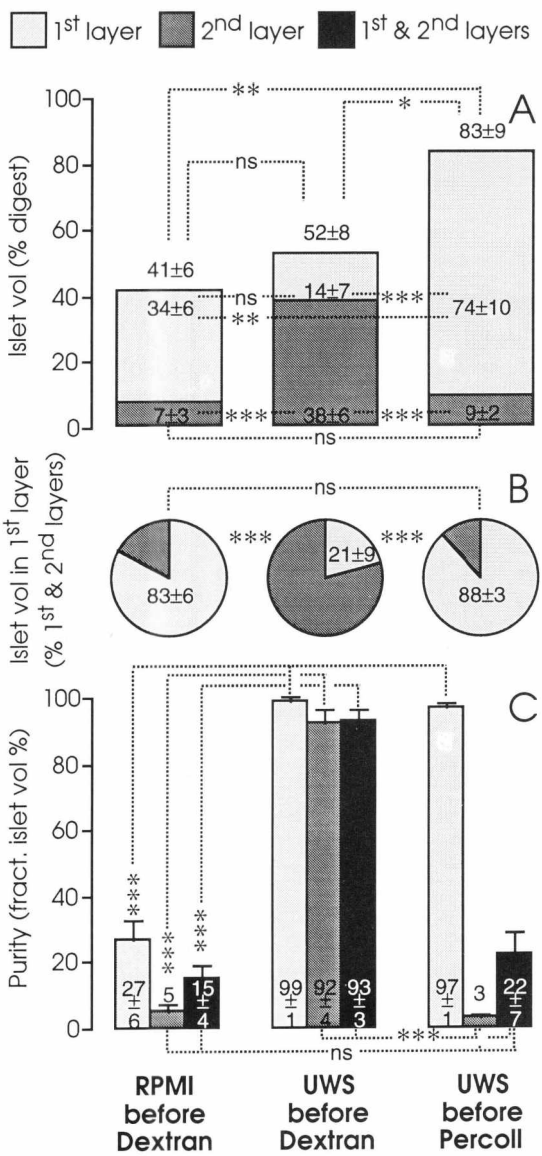


Fig. IV.2

*Islet recovery (**A**), distribution (**B**), and purity (**C**) at the first layer (density 1.045/1.075) and second layer (1.075/1.085) of isodense step gradients (1.045-1.075-1.085-1.095) of dextran in Hanks' balanced salt solution or Percoll in UWS, after islet isolation in RPMI or the UWS.*
** p<.05; ** p<.01; *** p<.001.*

Purification

Results after purification in the dextran and Percoll gradients are presented in Fig. IV.2. To minimise variability due to differences in the islet yield before purification, islet recovery after purification was expressed and analysed as a percentage of the volume of isolated islets before purification.

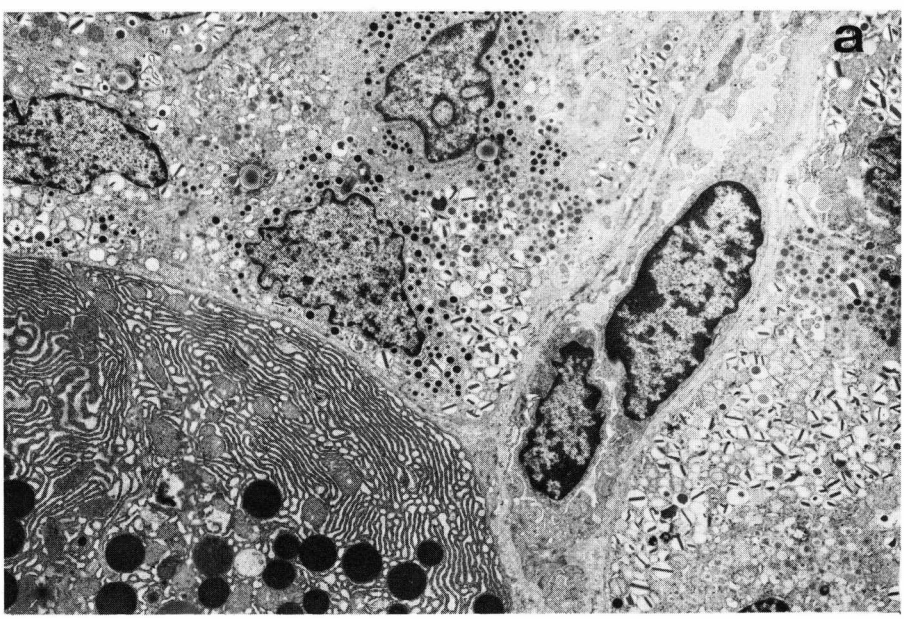
Comparison of the outcome of dextran purification after isolation in RPMI or the UWS, demonstrated no significant effect of the isolation solution on the total volume of islets collected from both the first and second layers of the gradient, which averaged 41 and 52% respectively of the volume of isolated islets before purification (Fig. IV.2A). However, islet recovery in the remainder of the dextran gradient (density > 1.085; not shown in figure) had increased ($p<.02$) from 5 ± 2% after isolation in RPMI ($n = 6$) to 20 ± 5% after isolation in the UWS ($n = 5$). The isolation solution affected the density of both islet and acinar tissue in the gradients. The apparent density of the major islet fraction had increased from 1.045/1.075 (first layer) after isolation in RPMI to 1.075/1.085 (second layer) after isolation in UWS ($p<.001$). Thus — expressed as a percentage of the total islet volume at both the first and second layers (Fig. IV.2B) — the islet volume at the first layer decreased from 83 ± 6% after isolation in RPMI to 21 ± 9% after using the UWS ($p<.001$). What is more important, the density of acinar tissue had also increased after using UWS. Contamination with acinar tissue at the first and second dextran layers was conspicuous after isolation in RPMI, but virtually absent after isolation in UWS. Amylase contamination of the purified islets after isolation in UWS, amounted to only 0.02% of the amylase content of the digest — corresponding to a mean 93% purity of the islet preparation (Fig. IV.2C). Comparison by electron microscopy of the intact pancreas and dextran-purified islets isolated in RPMI or the UWS demonstrated disappearance of the islet capsule, and a similar well-preserved endocrine cell ultrastructure, except for slightly swollen mitochondria after isolation in RPMI (Fig. IV.3).

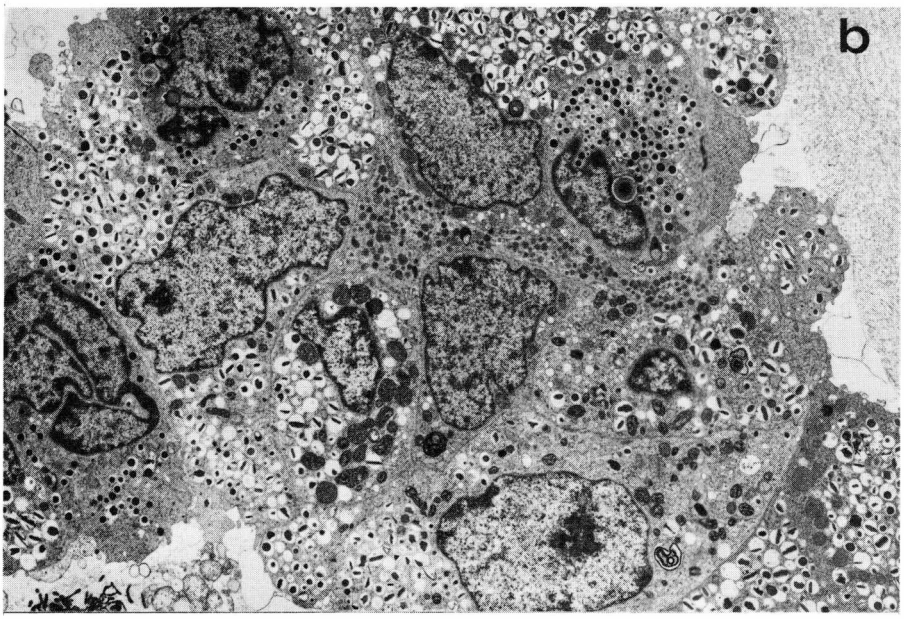
Because the outcome of dextran gradient separation would be affected both by the composition of the isolation solution before purification, as well as by the composition and osmolality of the density solution, we next studied purification in a normosmotic, isodense gradient of Percoll in UWS. Compared to the outcome of dextran separation of islets isolated in UWS, islet recovery at both the first and second Percoll layers had increased ($p<.05$) to a mean 83% of the digest islet volume (Fig. IV.2A). In fact, the volume of islets at these Percoll layers did not differ significantly from the islet volume in the corresponding digest suspensions. Islet recovery in the remainder of the Percoll gradient (density > 1.085; not shown in figure) was 5 ± 2% ($p<.01$ *vs* matching dextran

fractions after isolation in UWS; n = 10). The islet distribution in the two uppermost layers of the Percoll gradient demonstrated a lower islet density compared to the density in the dextran gradients after using the UWS during isolation (Fig. IV.2B). Acinar tissue contaminated the second layer of the Percoll gradient. Thus, the density of acinar tissue was also lower in the Percoll gradient. However, virtually no amylase and usually no acinar tissue were present at the first layer (Fig. IV.2C). Microscopy of the dithizone stained fresh preparations containing approximately 200 islets (Fig. IV.4), and histology of pelleted islets immunostained for insulin, demonstrated 1% acinar contamination — at most. Both the recovery (74 ± 10%) and purity (97 ± 1%) of islets at the first layer of the Percoll gradient had improved compared to the recovery and purity in the combined first and second layers of the dextran gradient after using RPMI (p<.01 and p<.001, respectively) or UWS (NS).

Fig. IV.3

*Electron micrographs of (**A**) the normal canine pancreas, (**B**) islets isolated in RPMI, and (**C**) islets isolated in the UWS, demonstrating a well-preserved ultrastructure after isolation in both solutions, except for slightly swollen mitochondria after isolation in RPMI. x 2200.*

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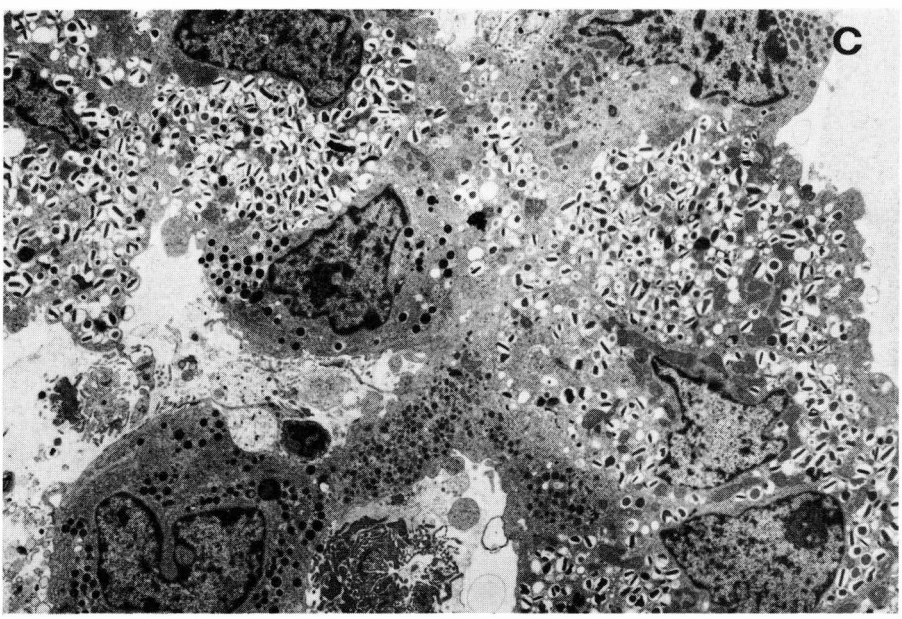
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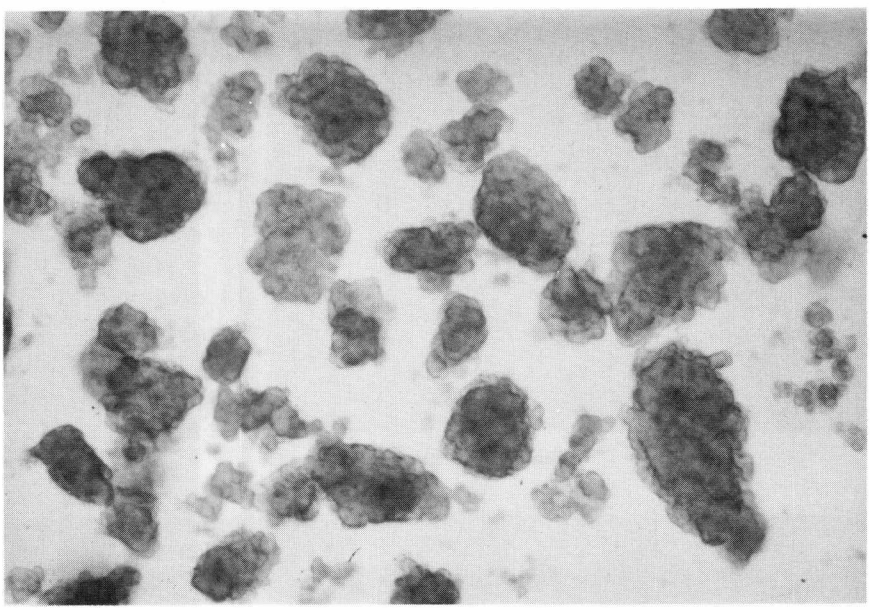
Fig. IV.4

Percoll purified canine islets stained with dithizone, demonstrating absence of exocrine tissue after using UWS as the islet isolation and density gradient medium.



Viability

Insulin release during perifusion (Fig. IV.5A) of dextran purified islets was considerably higher after isolation in RPMI (n = 4) compared to UWS (n = 6). After isolation in RPMI basal release was 0.27 ± 0.06 pmol/min per µl islet volume, compared to 0.02 ± 0 after isolation in UWS (p<.001). Islets isolated in UWS demonstrated a biphasic insulin response to 7.5 mM glucose, amounting to approximately five times basal secretion (Fig. IV.5B; p<.001). Only a transient, acute, response amounting to approximately 2 times basal release (p<.01) was observed after isolation in RPMI.

After intrasplenic autotransplantation of >6.0 µl islets per kg body weight in each of the total pancreatectomised dogs (mean dose 8.5 ± 2.5 µl/kg), normal fasting glucose levels and moderate postprandial hyperglycemia (< 11 mM) were observed from 2 weeks in all animals.

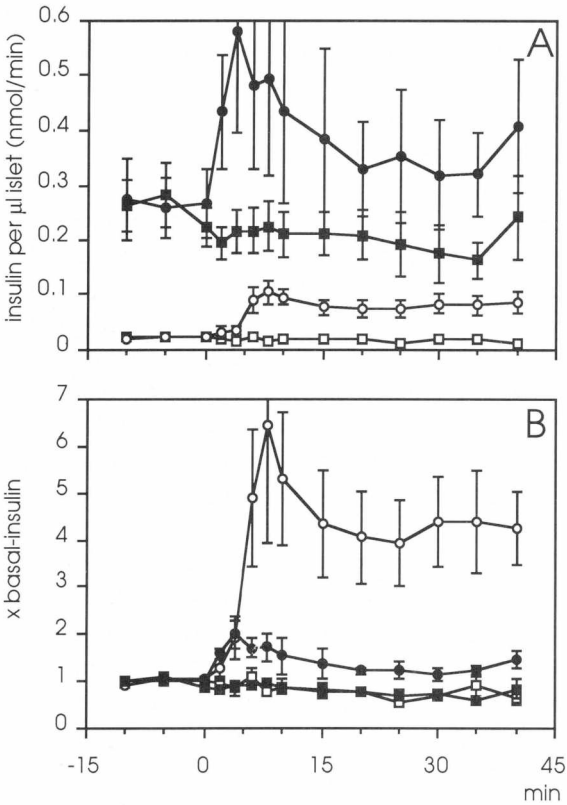


Fig. IV.5

*In vitro perifusion studies. (**A**) Insulin release, and (**B**) insulin release times basal release (mean value from –15 to 0 min) from islets isolated in RPMI (filled symbols) or the UWS (open symbols). Aliquots of the islet preparation were perifused in parallel, initially with 2.5 mM glucose and from 0 to 40 min with 2.5 (squares), or 7.5 mM glucose (circles).*

Discussion

We previously presented preliminary evidence that acinar contamination after density separation of islets can be prevented by substitution of UWS for conventional isolation solutions — and speculated, that cell swelling during hypothermic isolation in conventional solutions might explain the overlapping densities of islet and acinar tissue, while prevention of cell swelling in the UWS and, consequently, conservation of the density difference between islet and acinar tissue, would account for the dramatically improved purity after density separation [19]. This study confirmed this hypothesis, delineated the impact of the UWS on the regulation of cell volume during islet isolation, and further demonstrated an improved yield and viability of islet and acinar tissue after isolation in UWS.

We first studied the effect of substitution of the UWS for RPMI prior to islet purification in conventional hyperosmotic density gradients of dextran in HBSS. The isolation solution did not affect islet yield before purification, nor did it significantly affect the recovery of islets in the purified fractions (density ≤ 1.085) of the dextran gradient. However, the total recovery of islets in all fractions of the gradient had increased after isolation in UWS — which indicated an improved viability, e.g. increased resistance to hyperosmotic shock or sheer forces during centrifugation in this viscous density solution. Immediate effects of the isolation solution on the acinar tissue volume were reflected in the packed tissue volume before purification. Because ductal and vascular structures had been largely discarded during isolation, the volume of acinar tissue was assumed to equal the packed tissue volume. A reduction of the acinar tissue volume to 85% of the expected volume after isolation in UWS, and a rather increased amylase concentration in isolated acini — amounting to 105% of the concentration in native acinar tissue — indicated that the UWS not only prevented swelling but, moreover, extracted some of the intracellular water of acinar cells. The 95% recovery of amylase indicated that the reduction of tissue volume can be partially attributed to loss of acinar tissue. We, therefore, estimate that slight dehydration of acinar tissue in UWS resulted in a 5–10% reduction of the acinar cell volume. Changes in cell volume before purification were reflected by the apparent density of islets and acini in the density gradients. The densities of islet and acinar tissue were higher in the dextran gradient after isolation in the UWS compared to RPMI. However, near complete absence of acinar tissue in the major islet fractions of the gradient after isolation in UWS, and overlapping densities of islets and acini after isolation in RPMI, demonstrated the acinar cell to be more susceptible to cell swelling in the physiological solution. Swelling of acinar tissue in RPMI, causing damage to the cell membrane and loss of part of the acinar tissue, may also account for the lower amylase recovery. Tentatively, loss of amylase might be explained by release of amylase from viable cells. However, no significant loss of amylase was observed after isolation in the UWS. Thus, the decreased amylase concentration in isolated compared to native tissue, rather indicated a 20% increase of cell volume due to the uptake of extracellular water during isolation in RPMI. Recently, several groups have started to study human islet isolation in UWS (see Chapter 9), and a significantly improved outcome after density separation of islets isolated in UWS has been confirmed [12].

Cell density and the outcome of density separation will be affected also by the conditions during exposure of tissue to the density solution [15]. Hyperosmolality has emerged as an important criterion for islet purification.

Hyperosmolar solutions increase the density difference between the islet and acinar cells — probably, due to a differential dehydrating effect on the islet and acinar cells [9,21]. Thus — to clearly delineate the impact of the UWS on the regulation of cell volume and the apparent density of islet and acinar tissue — the hyperosmotic dextran gradient was replaced by a Percoll gradient in UWS with an osmolality of 320–340 mOsm/kg H_2O, which would be close to the osmolality of pancreatic tissue [22]. Only the impermeant components of the UWS known to be important to prevent cell swelling — i.e. lactobionate, raffinose, and Pentastarch — were included in the basal UWS of the Percoll gradient. Percoll consists of inert polyvinyl-pyrrolidone coated silica particles, which are also impermeable across the cell membrane. The low apparent density of islet and acinar tissue in the normosmotic Percoll gradient can be attributed to the absence of the dehydrating effect associated with the hyperosmotic dextran gradient. However — in contrast to previous reports [14,17] of poor purification of large mammal islets in conventional Percoll gradients — near 100% purity was observed at the major islet layer. Thus, our findings demonstrated the impermeant nature of the components of the UWS to be both necessary and sufficient to prevent cell swelling and obtain complete purity in the absence of hyperosmolality. Small differences between our microscopic findings and amylase recovery may probably be explained by contamination with free, extracellular, amylase during construction of the gradients, and incomplete washing of the islet fractions. The improved recovery of purified islets and decreased proportion of islets in the high density fractions (≥1.085 g/ml) of the Percoll gradient, may be attributed to a combination of the low viscosity, low polymer concentration, and the low osmolality of this density solution. Viscous solutions, and a high concentration of polymers — such as dextran and Ficoll — promote tissue aggregation and entrapment of islets in the acinar fraction of density gradients [9,15,21]. Less mechanical trauma due to the low viscosity of the Percoll gradient, and prevention of osmotic trauma [21] by using an iso-osmotic solution before and during purification, may have contributed to the almost complete recovery of islets in both the purified and non-purified Percoll fractions. The 74% recovery and consistent near 100% purity after Percoll purification in our study, compared favourably with previous reports of the variability and efficacy of canine and human islet purification [1,7,8,11,12,14,16,17,24].

The low basal insulin release and sustained fivefold insulin response to physiological glucose stimulation during perifusion of islets isolated in UWS compared favourably both with our observations of a tenfold higher basal release and transient response to glucose-stimulation from islets isolated in

RPMI, as well as with previous in vitro studies of canine and human islets, reporting a two to 40-fold higher basal insulin release and a zero to fourfold increase over basal release during supraphysiological glucose stimulation [3,8,13,14,17,24]. The viability of the final islet preparation — both after isolation in RPMI and the UWS — was evident by an intact ultrastructure and successful intrasplenic autotransplantation in pancreatectomised dogs. Therefore, high in vitro insulin release after islet isolation in the physiological solution may, at least partially, be attributed to adverse effects of exocrine contaminants during culture and perifusion. We conclude that the density and viability of islet and acinar tissue were best preserved in the UWS during hypothermic isolation and purification. Prevention of cell swelling during islet isolation in UWS should facilitate the analysis of other variables — such as predonation events and organ preservation conditions — that may affect the outcome in man. Consistent near complete separation of canine islets in the normosmotic, low viscous, density gradient of Percoll in UWS, clearly demonstrated the impermeant nature of the components of the UWS to be both necessary and sufficient to prevent cell swelling and obtain complete purity in the absence of hyperosmolality. These findings further suggest that purification in a density solution of impermeants will facilitate the adjustment of osmolality and viscosity to obtain similar results in man.

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