Islet transplantation a technical challenge

Studies on human pancreas preservation and enzymatic digestion

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Abstract

Islet transplantation has found its niche in diabetes treatment. It has contributed to a better quality of life and better glycemic control of patients with diabetes suffering from severe hypoglycemia that are not eligible for vascularized pancreas transplantation. Islet isolation is a technically challenging procedure. The different studies within this doctoral thesis aim to improve and standardize different steps in the isolation procedure. They are in particular looking to improve human pancreas preservation during cold storage, to optimize islet release from the exocrine tissue and to assess whether the isolated islet yield can be predicted from a biopsy.

We found that pancreas preservation with pre-oxygenated perfluorodecalin (two-layer method) did not improve the ischemic tolerance of the human pancreas as compared to cold storage with the University of Wisconsin (UW) solution. Furthermore, in pancreas with long cold ischemia time (CIT) (>10 hours), Histidine-Tryptophan-Ketoglutarate (HTK) had a limited preservation capacity as compared with the UW solution with respect to isolation outcome. We also found that during enzymatic pancreas digestion, Vitacyte HA was able to provide a similar islet yield and quality as Serva NBI with less collagenase activity and shorter digestion time. We further describe the first experience with a new GMP manufactured enzyme called Liberase MTF-S for successful human islet isolation. Finally, we found that the isolated islet yield could not be predicted from a biopsy taken from the head of the pancreas concerning solely morphological parameters of the islets tissue.

The improvement of pancreas preservation will allow for marginal organs with prolonged cold ischemia time to expand the donor pool. Better knowledge of how the pancreatic extracellular matrix is digested by collagenase will lead to a fast and predictable islet release from the exocrine tissue. By standardizing the isolation procedure and improving organ selection we will increase the success rate in human islet isolation, thereby making islet transplantation available for more patients.

Keywords: Islet transplantation, Human islet isolation, Pancreas preservation, Two-layer method, Perfluorocarbon, Cold storage, Cold ischemia, University of Wisconsin solution, Histidine-tryptophan-ketoglutarate, HTK, Pancreas preservation, Prediction, Collagenase

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“You can’t transplant islets unless you know how to isolate them”

Paul E. Lacy (1924-2005)

A Fernando, mi maestro, mentor y amigo.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Aims</td>
<td>12</td>
</tr>
<tr>
<td>General Aim</td>
<td>12</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>12</td>
</tr>
<tr>
<td>Background</td>
<td>15</td>
</tr>
<tr>
<td>Diabetes Mellitus in 2011</td>
<td>15</td>
</tr>
<tr>
<td>Blood glucose regulation</td>
<td>16</td>
</tr>
<tr>
<td>Insulin treatment in diabetes: hyperglycemia and hypoglycemia</td>
<td>16</td>
</tr>
<tr>
<td>Brittle diabetes and hypoglycemia unawareness</td>
<td>17</td>
</tr>
<tr>
<td>Beta-cell replacement therapy: pancreas vs. islet transplantation</td>
<td>18</td>
</tr>
<tr>
<td>Transplanting the endocrine pancreatic islet mass</td>
<td>19</td>
</tr>
<tr>
<td>Organ procurement for human islet isolation and transplantation</td>
<td>19</td>
</tr>
<tr>
<td>Ischemia and organ preservation in islet transplantation</td>
<td>19</td>
</tr>
<tr>
<td>Islet isolation: a technical challenge</td>
<td>23</td>
</tr>
<tr>
<td>Pancreas dissociation: a physical-chemical process</td>
<td>23</td>
</tr>
<tr>
<td>Pancreas histology: WYSIWYG? (What you see is what you get?)</td>
<td>27</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>28</td>
</tr>
<tr>
<td>The two-layer method (UW/Perfluorodecalin) (Paper I)</td>
<td>28</td>
</tr>
<tr>
<td>Characterization of islet morphology by insulin immunostaining (Paper V)</td>
<td>29</td>
</tr>
<tr>
<td>Statistics</td>
<td>29</td>
</tr>
<tr>
<td>Results and Discussions</td>
<td>30</td>
</tr>
<tr>
<td>The ischemic tolerance of pancreatic tissue with the Two-Layer Method (Paper I)</td>
<td>30</td>
</tr>
<tr>
<td>Lower islet yields using HTK during prolonged pancreas preservation (Paper II)</td>
<td>31</td>
</tr>
<tr>
<td>Vitacyte HA decreases digestion time without damaging islets (Paper III)</td>
<td>33</td>
</tr>
<tr>
<td>Mammalian Tissue-Free Liberase in successful human islet isolation (Paper IV)</td>
<td>34</td>
</tr>
<tr>
<td>Prediction of isolation outcome in the human pancreas (Paper V)</td>
<td>36</td>
</tr>
</tbody>
</table>
Conclusions................................................................................................................. 38
Paper I....................................................................................................................... 38
Paper II .................................................................................................................... 38
Paper III................................................................................................................... 38
Paper IV................................................................................................................... 38
Paper V.................................................................................................................... 39
Future Perspectives................................................................................................. 40
Summary in Spanish ................................................................................................. 41
Acknowledgements................................................................................................. 43
References................................................................................................................ 46
Abbreviations

AEC 3-amino-9-ethylcarbazole
CC1 Collagenase class I
CC2 Collagenase class II
CIT Cold ischemia time
CP/G C-peptide to glucose ratio
DCCT Diabetes Control and Complication Trial
ECM Extracellular matrix
EDIC Epidemiology of Diabetes Interventions and Complications
ESRD End-stage renal disease
GMP Good manufacturing practice
HAAF Hypoglycemia associated autonomic failure
HbA1c Glycated hemoglobin
HES Hydroxyethyl starch
HTK Histidine-tryptophan-ketoglutarate
IDF International Diabetes Federation
IE Islet equivalent
IN Islet number
MODY Maturity-onset diabetes of the young
NP Neutral protease
NS Not significant
PAK Pancreas after kidney
PBS Phosphate buffered saline
PFD Perfluorodecalin
PTA Pancreas transplant alone
SEM Standard error of the mean
SI Stimulation index
SIK Simultaneous islet kidney
SPK Simultaneous pancreas kidney
TLM Two-layer method
UNOS United Network for Organ Sharing
UW University of Wisconsin
WHO World Health Organization
(PZ)-U 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine
Introduction

The treatment of type-1 diabetes has been revolutionized by the introduction of innovative ways of insulin delivery and formulations. However, insulin is able to delay hyperglycemia-related complications but cannot prevent them. Hypoglycemia is a dangerous complication following insulin treatment, leading to physical and psychological morbidity in the diabetic patient. The ultimate goal is to achieve blood glucose homeostasis in a physiological manner. Islet transplantation has contributed to a better quality of life and better glycemic control of diabetics suffering from severe hypoglycemia, hypoglycemia unawareness and/or advanced complications that are not eligible for whole pancreas transplantation.

Several travails hinder the widespread clinical application of the procedure. Among others, the low efficacy of the islet isolation procedure is determinant in the fact the islets from multiple organs are needed to achieve insulin independence. The work within this doctoral thesis aims to improve different steps in the isolation procedure: pancreas preservation, enzymatic pancreas dissociation and prediction of the isolation outcome. Several preservation solutions have been compared to optimize the protection of the human pancreas against ischemia. We have also studied the outcome of isolations procedures with different enzyme blends for clinical islet isolation aiming at minimizing the time that islets are exposed to the harmful environment during the dissociation process. We further investigated whether isolation outcome could be predicted from a biopsy taken from the head of the pancreas, showing that islet morphology does not correlate to the isolated islet yield.

The improvement of the quality of the pancreas and the islets throughout the isolation and transplantation procedures is a prerequisite for successful islet engraftment and functioning over time in the transplanted diabetic patient.
Aims

General Aim
The work within this thesis aims to increase the success of human islet isolation, in particular by improving human pancreas preservation, optimizing enzymatic pancreas dissociation and predicting isolation outcome.

Specific Aims

Paper I
- To investigate the effect of the perfluorodecalin-based two-layer method in human pancreas preservation compared to the standard organ preservation method using the University of Wisconsin solution.
- To investigate the effect of pancreas preservation on islet isolation outcome of pancreata from elderly donors (>60 years).
- To study the effect of pancreas preservation with the two-layer method on post-transplant islet function by means of the C-peptide to glucose ratio.

Paper II
- To study whether histidine-tryptophan-ketoglutarate is effective as a static preservation solution for prolonged human pancreas cold storage before islet isolation.
- To compare histidine-tryptophan-ketoglutarate with the University of Wisconsin preservation solution.
- To study whether tissue edema is induced in the human pancreas during preservation with histidine-tryptophan-ketoglutarate and whether this influences islet isolation outcome.

Paper III
- To compare the efficacy of a new clinical-grade collagenase blend called Vitacyte CIzyme HA with the commonly used Serva NB1 for human islet isolation.
Paper IV
- To evaluate the efficacy of a new GMP-graded, mammalian tissue-free collagenase blend called Liberase MTF-S for human islet isolation.

Paper V
- To study whether human islet isolation outcome can be predicted from morphological parameters in a biopsy taken from the pancreatic head.
Background

Diabetes Mellitus in 2011

Diabetes Mellitus is a chronic disease characterized by an endocrine pancreatic dysfunction, which results in a deficient blood sugar counterregulation. Traditionally, diabetes has been classified into two main groups: type 1 (T1DM), previously named as insulin-dependent or juvenile-onset, and type 2 (T2DM), previously referred as non insulin-dependent or adult-onset (1). T1DM is characterized by an absolute insulin deficiency as a result of the beta-cells destruction, most often autoimmune mediated. T2DM, the most common, results from a progressive insulin secretory defect on the background of peripheral and hepatic insulin resistance (2). Other less common types of diabetes include gestational diabetes, pancreatogenic (when a patient is subjected to a sub-total or total pancreas resection), inherited monogenic forms such as maturity-onset diabetes of the young (MODY), etc. (3).

90 years after its discovery, insulin is still not available on a regular basis in developing countries, leading to a poor outcome in life expectancy of children with newly diagnosed T1DM (4). It is the major cause of blindness and kidney failure, being responsible for a million lower limb amputations every year (5) In 2008, diabetes mellitus led to 1.26 million deaths, being the 9th more common cause of death in the world (6). Despite major efforts for raising the awareness about the importance of the disease (7, 8), diabetes is one of the most common non-communicable diseases, accounting for one of the main global burden of disease.

The World Health Organization estimated the prevalence of diabetes to rise from 2.8% in year 2000 to 4.4% in 2030 (9), but recent data from the International Diabetes Federation (IDF) increases this figures to a global prevalence of 7.8 % or 438 million adults by 2030 (10). New emerging economies such as China present a rapid increase in the prevalence of the disease. The diabetes epidemic in this country has reached 92.4 million adults by 2010 (11), and it is estimated to be close to half a billion by 2030 (12). Scandinavia has the highest incidence of T1DM among children (10). Furthermore, diabetes nephropathy is the primary cause of end-stage renal disease (ESRD) among new patients receiving dialysis in Sweden (13).
Blood glucose regulation

Glucose is the main source of energy in mammalian cells. Its narrow regulation results from the balance of glucose intake (carbohydrates) in the intestine and glucose utilization (basal metabolism, exercise). After eating, glucose is stored directly as glycogen in the liver and in the muscles (glyconeogenesis). In the fasting state, the liver plays a key role in endogenous glucose production by releasing the glucose into the blood from the previously stored glycogen (glycogenolysis). Glucose is also produced from other nutrients such as proteins or fatty acids (neoglucogenesis) (14).

Two hormones mainly regulate the fine-tuning of glucose homeostasis: insulin and glucagon. Insulin is an anabolic hormone that enhances glucose utilization by facilitating glucose metabolism by fat and muscle cells (14), which results in a decreased blood glucose concentration. On the contrary, glucagon is a catabolic hormone that increases blood glucose mainly by stimulating glycogenolysis in the liver. Both hormones are produced in the pancreatic islets of Langerhans, small endocrine organs within the pancreas. The total islet mass accounts for 1-2% of the volume of the pancreas and is spread throughout the whole organ. They are controlled by central stimuli as well as paracrine mechanisms.

Insulin treatment in diabetes: hyperglycemia and hypoglycemia

Diabetes is characterized by a relative or absolute lack of internal insulin secretion. As a result, glucose cannot be metabolized by muscle and fat cells and accumulates in the blood (hyperglycemia). Thus, cells starve in the absence of insulin and the patient enters a catabolic state leading to death if left untreated.

The high amount of circulating glucose affects the blood vessels of different organs (15). Intracellular hyperglycemia occurs in tissues not able to downregulate their glucose uptake, affecting the smaller blood vessels (microangiopathy) in the eyes (retinopathy), nerves (neuropathy) and kidneys (nephropathy). Other blood vessels may as well be damaged due to atherosclerosis (macroangiopathy), leading to a higher risk of cardiovascular disease.

Exogenous insulin replacement has been the standard of care since the discovery of insulin in 1921. Intensive insulin treatment and its capacity to delay the onset and slow down the progression of diabetes-related complications were extensively studied in T1DM patients in the early 1990’s in the Diabetes Control and Complication Trial (DCCT) (15). In this trial, conventional therapy (1-2 daily insulin injections) was compared to intensive therapy (3 or more insulin injections or insulin pump). It was found that HbA1c correlates directly with the appearance of macro and microvascular complications. Furthermore, the Epidemiology of Diabetes Interventions and Com-
plications trial (EDIC), with 90% of the patients from the DCCT trial, showed a further reduction in diabetes complications and, moreover, a 42% reduction in overall cardiovascular risk (16). On the other hand, these trials did not study patients with severe complications and/or frequent hypoglycemia.

The DCCT trial showed that intensive insulin therapy leads to a 3-fold increase in iatrogenic severe hypoglycemia(17). Severe hypoglycemia is often described as a “hypoglycemic episode requiring intervention from another person”. It has been estimated to account for 1.3 episodes per patient per year, affecting one third of all patients with type 1 diabetes (18).

Brittle diabetes and hypoglycemia unawareness

As the body of a diabetic patient is unable to control the glycemia, blood sugar levels can vary extremely, leading to both hypo- and hyperglycemia. In insulin-treated patients, iatrogenic hypoglycaemia (low blood sugar levels) is the result of insulin excess (due to differences in the pharmacokinetics between exogenous and endogenous insulin and in relation to the carbohydrate intake and the level of physical exercise) and the malfunction of the hypoglycaemia counterregulation mechanisms. The fear for hypoglycemia hinders the patient from the maintenance of euglycemia (19), thereby increasing the risk for diabetes complications. It implies both physical and psychological morbidity for the patient, being often identified as the limiting factor in the management of diabetes (20).

In healthy individuals, the falling blood glucose concentration stimulates adrenalin secretion and inhibits insulin release thereby increasing insulin concentration in the beta cells of the pancreas (21). As a result, alpha-cells will be enhanced via a paracrine signal to secrete glucagon, which in turn activates glycogenolysis in the liver and the release of glucose into the bloodstream (22).

All type 1 diabetes patients will loose their ability to counteract hypoglycemia in a physiological manner at a certain point after diagnosis, which directly correlates with the remaining pancreatic beta-cell function (23). Being unable to activate a normal response, diabetics are rendered dependent on an adrenaline-mediated response to rise their blood sugar and recover from hypoglycemia (24). Furthermore, frequent hypoglycemia lowers the glycemic thresholds for sympatho-adrenal responses (25) and for the activation of neuroglycopenic symptoms (hypoglycemia unawareness). These two features are described in a clinical syndrome called Hypoglycemia-associated autonomic failure (HAAF).
Beta-cell replacement therapy: pancreas vs. islet transplantation

The avoidance of hypoglycemic episodes restores the ability to sense hypoglycemia (26). Today a number of technical applications are available for more physiological insulin delivery, providing better metabolic control and fewer hypoglycemic episodes (27), e.g. insulin pumps (subcutaneous, intraperitoneal), sensor-augmented pumps and close-loop devices (28, 29). Even though these devices are a step closer to an “artificial pancreas”, the restoration of the blood glucose homeostasis has been only achieved by replacing the insulin-producing beta cells (30).

Pancreas transplantation as a mean of treating brittle or unstable diabetes has been explored since the end of the 19th century, even before insulin was discovered and linked to the beta cells within the pancreatic islets (31). The first clinical report comes from 1966, with rather poor results due to the complications related to the pancreatic surgery (32). Over the years, the technique has improved and, thanks to modern immunosuppressive drugs and effective infection control, this therapy offers today long-term graft survival comparable to other transplanted organs, especially in combined pancreas-kidney transplantation (SPK) (33). The number of patients receiving a pancreas after kidney (PAK) or only a pancreas (PTA) has also increased (34) thanks to the possibility of achieving long-term metabolic control and reduction of diabetes complications (35). However, the stringent donor requirements and the high co-morbidity limit this therapy to relatively young patients with advance kidney disease and/or severe hypoglycemia but no history of cardiovascular disease (34).

If the patient suffers from brittle diabetes and/or hypoglycemia unawareness and has already a transplanted kidney or preserved kidney function, islet transplantation may be a better option (36). For islets to be transplanted, they need to be separated from the surrounding exocrine tissue, a procedure known as islet isolation. (37).

After organ procurement, isolation of human pancreatic islets takes place in a GMP-certified laboratory. Islet viability and functionality is controlled before purified islet fractions are transplanted into the liver by infusion via a catheter into the portal vein system. Although not free from potential complications, modern islet transplantation technique offers a low risk for side effects and is associated with short hospital stay as compared to vascularized pancreas transplantation (38).

It has long been discussed how to allocate organs for pancreas vs. islet transplantation, as both treatment modalities compete for organs of good quality (39). In fact, pancreata from old and obese donors are considered to be not suitable for vascularized organ transplantation (40, 41) while they frequently provide high yields in islet isolation (42, 43). At present, pancre-
ata from young and slim donors are usually prioritized for whole-organ transplantation while pancreata for islet isolation are mostly retrieved from old and obese donors. The reason that long-term graft survival is inferior for islet transplantation compared to whole-pancreas is still under debate. Islets from older “extended criteria” donors have been shown to have a decreased functional capacity compared to younger donors (44). Whether this influences current clinical human islet isolation results remains to be elucidated (45).

As both therapies require lifelong immunosuppression, the decision whether the patient is a candidate for either pancreas or islet transplantation or both relies on a careful evaluation of the risk-to-benefit ratio.

Transplanting the endocrine pancreatic islet mass

Beta-cell replacement therapy by means of islet transplantation is a paradigm of cell transplantation and a promising option for the treatment of patients suffering from brittle diabetes and life-threatening hypoglycemia unawareness. With the introduction of new immunosuppressive protocols and the improvement of the isolation technique (46), the success rate of the procedure is today comparable of whole pancreas transplantation (47, 48). Recent analysis of the Edmonton Trial has shown that even if insulin independence is not sustainable after transplantation, persistent islet function provides both protection from severe hypoglycemia and improvement of glycated hemoglobin (49).

Despite this promising appraisal, the low efficacy of the isolation procedure in relation to the shortage of suitable donors is up to now one of the biggest disadvantages. Islets from multiple donors are needed to treat one single recipient and there is a loss of graft function over time. These facts provide evidence of the destruction and/or low engraftment of the majority of the implanted cells and the subsequent malfunction of the remaining ones over time (50).

Organ procurement for human islet isolation and transplantation

Ischemia and organ preservation in islet transplantation

Regionalization of islet-processing facilities is being explored as a solution to maximize utilization of donor pancreata. It has shown to improve isolation efficiency and reduce costs, leading to the realization of clinical trials in islet transplantation (49, 51, 52). To date, several multicenter networks have
been established in Europe and the United States, among them the Nordic Network for Clinical Islet Transplantation in Scandinavia (53).

Due to logistical reasons, organs are usually transported between hospitals and sometimes between countries to optimize pancreas allocation. Transportation of a vascularized organ unequivocally implies ischemia due to the lack of oxygenated blood and the accumulation of metabolic end products within the ischemic tissue. As a time-dependent mechanism, ischemia progressively damages the endothelial cells in the transplantable organ, making it unable to recover its function after transplantation and reperfusion.

Cold static organ preservation is the current standard for organ transplantation (54). During organ retrieval, blood vessels are perfused with a cold preservation solution to remove the blood and prevent intravascular thrombosis, accelerate the cooling of the organ and counteract the ischemic and hypothermic damage to cells. Hypothermic preservation solutions are therefore used to make the organ tolerant to ischemia and hypothermia.

During hypothermia (usually 4°C), cell metabolism decreases leading to a reduced oxygen and nutrient consumption. Since 10–12% of the normal metabolic activity is still operative in the ischemic tissue at 4°C, hypothermic organ perfusion and subsequent immersion in various preservation solutions such as University of Wisconsin solution (UW) or Histidine-Tryptophan-Ketoglutarate (HTK) do not completely prevent irreversible pancreas injury once a critical period of cold ischemia is exceeded (55). The temperature-related reduction in the activity of Na/K ATPase pump leads to an intracellular accumulation of sodium and the resulting osmotic influx of water (56). To prevent this, colloids are added to the preservation solution. Other common components in preservation solutions are antioxidants, enzyme inhibitors and vasoactive substrates. The overall aim of all these compounds is to prevent the deterioration of the organ during hypothermic storage and the so-called ischemia-reperfusion injury once the circulation to the organ is restored in the recipient.

Efficient preservation of the pancreas prior to islet isolation has been reported to be of high importance for achieving a high islet yield (57, 58). In the present thesis, papers I and II explore two different solutions to preserve the pancreas prior to the isolation procedure.

**University of Wisconsin solution**

The UW solution has been the most frequently used preservation solution for static cold storage in abdominal organ preservation for the past 20 years. It has been successfully applied for preservation of kidney, liver, pancreas, heart and small bowel as well as pancreatic islets and hepatocytes (59).

It was originally designed for pancreas preservation with a similar electrolyte composition as the intracellular microenvironment (high K+ and low
Na\(^{+}\)) and the use of lactobionate and raffinose to prevent hypothermia-induced cell swelling (59). In addition, it contains hydroxyethyl starch (HES) as a colloid to prevent interstitial edema. Phosphate buffer is included in the UW solution to counteract acidosis as well as glutathione and allopurinol as free-radical scavengers. ATP depletion and the subsequent malfunction of the ion pumps will lead to a rise in intracellular calcium (60). This has been reported to be important for activation of endogenous pancreatic enzymes resulting in extensive and rapid autolysis of the ischemic pancreas (61-63). In the UW solution, it is assumed that this can be partly prevented by the supply of adenosine as energy substrate as well as lactobionate as Ca\(^{2+}\) chelator, thus hampering hypothermia-induced mitochondrial damage.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>UW (Belzer)</th>
<th>HTK Custodiol</th>
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<tbody>
<tr>
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<td></td>
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<tr>
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<tr>
<td>Dexamethasone</td>
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All units are in mmol/L unless otherwise stated. Adapted from reference (64).

**Table 1.** Comparison between components in UW and HTK preservation solutions.

Finally, penicillin, insulin and dexamethasone as well as fresh glutathione (to compensate oxidation during cold storage) may be added prior to use as recommended by the manufacturer (65).

Before the flushing of the organ, the solution should be filtered to remove potential particles and adenosine crystals that may form under cold storage (66). While perfusing the organ, the high amount of HES makes the UW solution highly viscous and it has been reported to stimulate red blood cell aggregation, thereby preventing adequate perfusion of the organ (67). Furthermore, UW solution must be flushed out of the organ before transplantation due to the high K+ concentration, which otherwise will
lead to vasoconstriction (68) and cardiac arrest. The UW solution has been used for pancreas preservation prior to whole organ transplantation but, when used before human islet isolation, it seems to be suitable only for a limited period of time (55).

**Two-Layer Method**

The inability to isolate a critical mass of viable islets from UW-preserved pancreata with prolonged CIT encouraged the concept of supplying ischemic pancreatic tissue with oxygen utilizing the hyperoxygen carrier perfluorodecalin (PFD) (69). By storing the organ in the interface between an oxygen-saturated oxygen carrier and UW, it was hypothesized that both oxygen and nutrients are provided to maintain ATP production (70).

Evidence in the canine model (69, 71) led to several pilot studies indicating the benefit of preserving the human pancreata with the TLM during cold storage (72, 73). In addition, it was suggested that pancreata from marginal donors could be recovered for clinical islet transplantation (74) after additional preservation with the TLM (75).

Despite initial enthusiasm, several studies from others (76) and us (paper I) could not show a significant positive effect on isolation outcome or post-transplant islet function in a large cohort of pancreata. These disappointing results could be explained by experiments in pig pancreata demonstrating that the TLM can oxygenate only a small fraction of the surface of the pancreas, but is not able to reach the hypoxic core (77).

**Histidine-Tryptophan-Ketoglutarate**

Originally designed as a cardioplegic solution in the 1970’s (78), HTK solution has been increasingly used as an alternative to the UW solution for abdominal organ preservation. Compared to UW (Table 1), HTK uses histidine instead of phosphate as a buffer, tryptophan as a membrane stabilizer, ketoglutarate instead of adenosine as energy substrate and mannitol as impermeant instead of raffinose and lactobionate. The low Na⁺ levels resemble an intracellular fluid while the low K⁺ makes HTK safe for release into the circulation of the recipient.

Due to the lower viscosity, it was suggested that HTK accomplish a better perfusion and faster cooling during organ procurement (79). Even though a greater volume of solution was used, HTK perfusion remained less costly than UW solution (80). To date, there is no general consensus over its suitability for organ preservation, with a series of late conflicting reports rising concerns about the efficiency of the solution for different indications (81-84).

In vascularized pancreas transplantation, several studies have shown no difference between HTK and UW in terms of early graft function and patient survival (83, 85, 86). However, pancreata flushed with HTK have been asso-
associated with a higher incidence of postoperative complications including pancreatitis and decreased rate of insulin-independence at hospital discharge (81). In a recent study on the UNOS (United Network for Organ Sharing) database including over 4000 patients, HTK preservation was associated with increased early graft loss (84).

In islet transplantation, HTK solution has been reported to be similar to UW in terms of islet yield, viability and in vitro insulin secretion (87). Furthermore, the percentage of preparations suitable for clinical transplantation increased by two fold in HTK-preserved pancreata (88). However, the high flushing volume may induce tissue edema, affecting pancreas viability and subsequent islet isolation in a negative way (paper II).

Islet isolation: a technical challenge

The islet isolation procedure essentially consists of two main consecutive steps: first, the dissociation of the pancreatic tissue by a combination of enzymatic digestion and mechanical disruption and second, the separation of the islets from the exocrine tissue by means of density gradient centrifugation. Islet isolation has been attempted since the beginning of the 20th century, but the combined mechanical and enzymatic approach was first described by Moskalewski in 1965 (89). Subsequent discoveries in terms of enzymatic digestion (90, 91) and tissue purification (81, 92) paved the way for successful large-scale human islet isolation (93, 94) (for review see ref. (37, 95)).

As stated by Dr. Paul E. Lacy, a pioneer of islet transplantation, in his last public speech in December 2004 (96), the discovery made by the young Camillo Ricordi was to set a new reference for enzymatic pancreas dissociation that still today is the standard in most laboratories (93). This, however, means that neither the main steps in human islet isolation nor the results have substantially developed since the 1980’s, which reflects nothing but the complexity of the technique and the need for further research.

Pancreas dissociation: a physical-chemical process

Islet isolation starts with the trimming of the peripancreatic fat and connective tissue under aseptic conditions. The pancreas is distended with the enzyme solution via the pancreatic duct in order to distribute the enzyme in the acinar tissue. The distended organ is then cut into several pieces and transferred to the digestion chamber. To enhance the pancreas dissociation, the chamber is agitated so that the perfused tissue progressively disintegrates while incubating at 37°C. Complete removal of exocrine tissue from the surface of the islet is crucial for the subsequent density-based separation procedure.
Islets of Langerhans are highly vascularized mini-organs spread throughout the pancreas. The separation and release of the islets from the acinar tissue is performed by enzymatic degradation of the extracellular matrix (ECM) in the endo-exocrine interface. The pancreatic ECM is rich in collagen (97), an essential structural component of the connective tissue. Because of its structural function, collagen fibers are only degraded by a few proteases. The current protocols for human islet isolation use collagenase from the bacterium Clostridium histolyticum.

**Clostridial enzyme blends for human pancreas dissociation**

The crude bacterial collagenase product obtained from fermentation of *Clostridium histolyticum* contains different active components, such as collagenase, the most essential one, phospholipase, clostripain, elastase, aminopeptidase, galactosidase and other proteases (98). Crude collagenase is therefore a mixture of enzymes working together to disaggregate the pancreatic tissue. Collagenase is characterized into six different isoforms (α, β, γ, δ, ε, ζ), which are further divided into two subclasses1: Class I and Class II (99).

In the past, islet isolation was performed utilizing crude collagenase (100). However, the introduction of purified enzyme blends during the 1990’s improved isolation outcome in the clinical setting. As a result, crude collagenase was abandoned for human islet isolation (101, 102). On the other hand, the purification of crude collagenase did not eliminate batch-to-batch variability, contributing to unpredictable isolation results (103).

Collagenases are highly specific enzymes that hydrolyze the native collagen molecules. Collagenase class I (CC1) and II (CC2) are the main components of purified enzyme blends for islet isolation. We know that they have different roles in collagenase digestion: CC1 (α, β and γ) is more stable and has a greater activity toward native collagen, whereas CC2 (δ, ε and ζ) has a moderate collagen activity, being characterized by the ability to digest a broader range of peptide substrates as compared to CC1 (104, 105). The ratio between collagenase classes has been reported to be of importance for optimal islet cleavage as proven in the rat and human pancreas (106, 107). Furthermore, both CC1 and CC2 act synergistically on collagen degradation (108).

Purified collagenase blends are unable to fully dissociate the pancreatic ECM due to the lack of non-collagenolytic enzymes and other macromolecules as compared to the crude collagenase (104, 109). As a result, additional collagenolytic proteases are needed to achieve adequate tissue digestion (109). Neutral protease (NP) from Clostridium histolyticum has been extensively used in clinical islet isolation (110). Thermolysin, a thermostable neutral protease produced by the gram-positive bacteria Bacillus thermopro-

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1 Based on their activities toward native collagen and the synthetic peptide FALGPA.
teolyticus, has been previously used to replace clostridial neutral protease (101) and today is present in several efficient collagenase blends (Paper III and IV). Based on our experience (110) and that of others (111), neutral proteases should be carefully titrated as to avoid islet damage (112). This exemplifies the complexity of islet isolation by means of enzymatic pancreas dissociation (113, 114).

**Commercial enzymes for islet isolation: what do we get?**
The current policy of collagenase manufacturers is to purify enzyme blends to the highest possible extent and to obtain a product that consists solely of collagenase and a neutral protease. The first available purified enzyme blend called Liberase HI was introduced in 1994 and became the gold standard for human islet isolation (115, 116). This product contained collagenase class I and II as well as the neutral protease thermolysin, with a low endotoxin level (101). Several years after its release, it became obvious that a strong intra- and inter-lot variability was present in Liberase HI (123).

Although recombinant collagenase was developed in parallel, the final product did not reach the market, even though it provided similar isolation results as Liberase (117). Nevertheless, the variable enzymatic activity of different batches is a persisting problem that exists among product from other enzyme manufacturers (89, 118), being a major obstacle in achieving reproducibility in islet isolation. As a result, a most recent study has shown the efficiency of crude collagenase for human islet isolation, concerning also the commercial background of islet isolation research (119).

Collagenase degradation during storage (114) or during isolation (120) due to the auto-digestion of the different components is suggested to be responsible for the large inter- and intrabatch variability in efficiency of enzymes blends. However, contamination from non-collagenolytic enzymes has recently been reported in protocols for islet isolation without the addition of NP (121). On the other hand, production of highly purified enzyme blends may remove toxic components (122) but also enzymatic activities such as clostripain, which may be crucial for isolation success (118). Another detrimental byproduct of *C histolyticum* fermentation is endotoxin. It has been shown that this toxin is present in crude collagenase blends and can easily contaminate other reagents (123), resulting in deleterious effects on islet viability and transplantation outcome (124-126).

Currently, most manufacturers offer both collagenase and protease as separate vials, allowing for a customization of the digestion step (127). It also prevents collagenase degradation and increases stability of the enzyme products. However, this customization requires vast isolation experience as well as the utilization of human donor pancreata for enzyme testing, which can be a technical and financial handicap for those centers with limited resources or organ supply.
Enzymes for clinical islet isolation

In 2007, Liberase HI was withdrawn from the market because of the risk of potential transmission of bovine spongiform encephalopathy due to the use of bovine neural tissue in its manufacture procedure (128). As a result, the islet transplant activity worldwide decreased (129) while all active centers performing clinical islet isolation had to switch to alternative enzymes such as Serva collagenase NB1 (120) or Vitacyte collagenase HA (130) (Paper III).

The discontinuation of Liberase HI from the market remains a plausible although not absolute explanation for the decline of islet transplant activity in the second half of the last decade (131). Nevertheless, this was a clear signal for the worldwide islet community that clinical islet transplantation programs depend on an oligopoly of enzyme manufacturers.

Shortly after Liberase HI was precluded from clinical islet isolation, most of the active transplantations centers switched to collagenase blends provided by Serva. Stimulated by the growing demand for effective enzymes for islet isolation in clinical trials, Vitacyte collagenase HA was introduced in the market in 2007 as an alternative to Serva NB1 (131). Similar to Serva, Vitacyte offered the possibility of tailoring pancreas digestion by commercializing collagenase and thermolysin in different vials.

Further development within the Roche enzyme production led to the commercialization of a new enzyme blend free from exposure to mammalian tissue (Liberase MTF-S). Initial experience with this new combined enzyme blend has proved its efficacy in human islet isolation (Paper IV). Moreover, chromatographic analysis of this new GMP-grade (Good Manufacturing Practice) Liberase confirms the stability of the different collagenase fractions as compared to the NB1 enzymes by Serva (132).

The introduction of GMP standards in enzymatic islet isolation has improved the documentation and traceability of the islet production process as well as patient safety. This, however, has not resulted in an improved or more homogenous islet isolation results. To date it is still unclear how the different components in the collagenase blend accomplish the dissociation of the ECM or which of them are essentially needed for islet release. Novel insights into the crude collagenase product suggest that the native enzyme composition is equally effective as the combination of purified enzyme fractions, once the toxic components are removed, but for a fraction of the price of the GMP-compliant enzymes (119).

During decades, the development of enzyme products for islet isolation has been limited by the commercial application of these biological products. New clinical trials in islet transplantation and the availability of new enzyme blends for human islet isolation stimulates both islet researchers and enzyme manufacturers to improve standardization of enzymatic pancreas dissociation and to increase human islet isolation outcome.
Pancreas histology: WYSIWYG? (What you see is what you get?)

Human islet isolation outcome is characterized by a high variability, making the procedure unpredictable and difficult to standardize. As a result, only 50% of the isolated islet preparations fulfilled the criteria required for clinical transplantation: ≥ 5000 IE per kg body weight of the recipient or 300,000 IE, islet purity >30%, packed tissue volume ≤5 mL and a biphasic insulin response to dynamic glucose perifusion corresponding to a SI of ≥2.0.

Several studies suggest that donor body mass index (BMI), age and CIT are critical for successful islet isolation (43, 57). Additional efforts have been made to predict isolation outcome through metabolic testing of the beta-cell mass in the donor (133).

It has been discussed whether islet number and morphology in the native pancreas determines isolation success (134). Characterization of the pancreatic ECM has provided evidence for correlation of islet yield with the islet morphology in the human pancreas (135). In the pig model, pre-digestion screening of pancreatic biopsies has proven to increase isolation success by selecting the most suitable organs for processing (136). A biopsy taken during pancreas procurement may help us to decide whether to process a pancreas for islet isolation or not based on the islet morphological features and the weight of the organ.
Material and Methods

Please refer to the appropriate section in the respective papers for descriptions of materials and methods. Methods of particular interest are described in this chapter.

The two-layer method (UW/Perfluorodecalin) (Paper I)

Pancreas shipment was performed utilizing a validated and certified organ transport system that consisted of two plastic jars, four cooling aggregates and an isolated transport container. During preservation with the TLM, explanted pancreata were perfused with UW solution and immediately placed in the inner plastic jar immersed in 200 ml of cold clinical-grade perfluorodecalin pre-charged for 30 – 45 min with 100% oxygen at a flow rate of 2000 ml/min. During oxygenation, the PFD was covered with a 100 ml-layer of UW (Figure 1). This procedure resulted in a partial oxygen tension of 750 – 800 mm Hg that remained constant for at least 30 h (137). Afterwards, the organ was fixed at the interface between the PFD and UW in a 600-ml container that was filled completely with UW and placed into the transport system for shipment (Figure 2).

*Figure 1.* Preservation container and oxygenation device.

*Figure 2.* Preservation container with the pancreas in a metal grid holder. The UW and PFC levels are indicated in the right.
Characterization of islet morphology by insulin immunostaining
(Paper V)

The human biopsies used in this study were taken from the head of the pancreas in a routine fashion after removal of the duodenum, peripancreatic fat and connective tissue and before intraductal enzyme injection. The biopsies were immediately fixed in 4% paraformaldehyde and subsequently dehydrated in increasing alcohol concentrations (70%, 80%, 95%, absolute alcohol and xylene) and finally embedded in paraffin. Afterwards they were sectioned on a microtome at 4 µm and air-dried at 37°C overnight. The sections were kept at room temperature until further processing. Prior to staining, the tissue slides were deparaffinized to remove the embedding media and were rehydrated with decreasing concentrations of alcohol. The sections were stained for insulin using the Envision system, which comprises the following steps: blocking of endogenous peroxidases with 0.3% H₂O₂ in PBS (Phosphate buffered saline) for 15 min; incubation with a primary guinea pig anti-human insulin antibody at 37°C over night, followed by goat anti-rabbit Envision antibody, detection in AEC (3-amino-9-ethylcarbazole) substrate-chromogen, counterstaining with hematoxylin and finally embedding in glycerol gelatin.

The pancreas slides where digitalized using the digital scanner Aperio SCAN scope XT. The digitalized slides where further analyzed with software provided by the same company. Islet basic morphological features such as perimeter, area, and diameter were measured directly on the slides. Islet volume was calculated assuming the islets to be perfect spheres. Insulin staining was analyzed utilizing a built-in macro in the Aperio software. Islets were evaluated by determining islet particle number (IN/mm²), islet equivalent calculated according to a standard islet diameter of 150 µm, (IE/IN) and percentage of islet (insulin positive) area in the biopsy. Islets were divided into 50 µm diameter ranges as previously described (138).

Statistics

All results are presented as mean ± standard error of the mean (SEM) (Papers I, III, IV and V) or mean ± standard deviation (SD) (Paper II). Comparisons of continuous variables were carried out by Mann-Whitney test. Categorical variables were analyzed using Fisher’s exact test. In paper I, the comparison of post-transplant CP/G values after each islet infusion between UW and TLM preserved organs was carried out by Wilcoxon test. In the same paper, correlation between CIT and islet yield was calculated by Spearman’s rank test considering a confidence interval of 95%. In Paper V, correlation between morphology parameters and isolation variables is expressed as Pearson correlation coefficient. Differences were considered significant at \( P<0.05 \). \( P>0.05 \) was termed as not significant (NS).
Results and Discussions

The ischemic tolerance of pancreatic tissue with the Two-Layer Method (Paper I)

The approach of supplying explanted human pancreata with oxygen utilizing the TLM for an additional preservation period has demonstrated to increase the percentage of islet isolations suitable for clinical islet transplantation after prolonged CIT. Furthermore, it has been shown to improve the use of marginal donors for successful islet isolation (74, 75). These promising results motivated the introduction of the TLM in the Nordic Network for Clinical Islet Transplantation in 2004 (53).

To study whether the TLM has contributed to an improvement in isolation outcome in our clinical islet transplantation program, we have retrospectively analyzed the outcome of 200 human islet isolations performed after either cold storage in UW or preservation by TLM. The decision to store the organs by the TLM was arbitrarily met by the local procurement team. The two experimental groups were categorized into three subgroups according to duration of CIT: A) 0 to <6 hours, B) 6 to <12 h and C) 12 to 18 h. To investigate the effect of pancreas preservation on isolation outcome for pancreata from elderly donors, subjects older than 60 years of age were selected from the different CIT categories and pooled together according to preservation solution (UW vs. TLM). No differences in terms of donor and isolation variables where found between the different CIT categories in the respective preservation solution or between TLM and UW preservation in the sub-analysis for elderly donors.

The present analysis did not reveal any significant differences between pancreata stored in either oxygenated PFD or UW alone with respect to isolation outcome and islet posttransplant function as measured by the CP/G ratio. Our results are in agreement with a similar study regarding islet yield, graft function and tissue ATP content (76), an important parameter to assess the effects of oxygenation on oxidative energy generation.

Whether histomorphological variables such as tissue firmness and texture influence pancreatic oxygen supply is yet unknown, but oxygen determinations in human tissue indicated that short-term TLM cannot sufficiently oxygenize more than 20% of the entire pancreatic volume (77). In contrast, in the smaller canine pancreas a positive correlation between pancreas oxygenation, tissue oxygen pressure, ATP generation, and graft function of auto-
transplanted pancreata was found (139). Another prospective study has demonstrated a protective effect of the TLM on yield and in vitro function of islets isolated from ischemically predamaged canine pancreata (71). However, experiments in pigs have revealed that TLM-induced restoration of islet function in vitro does not correspond to improved islet function after transplantation (140). A study in ischemically predamaged rat pancrea indicates a clear benefit of TLM preservation on subsequent islet isolation and transplantation (141). Nevertheless, since oxygen diffusion into the pancreas depends on the thickness of the tissue, the rat pancreas has been considered to be unsuitable as a model of oxygenation of the human pancreas due to the difference in the size of the organ (142).

Our data indicate no beneficial effect of the TLM on islet isolation outcome from elderly donors. Surprisingly, we found a negative correlation between CIT and in vitro function exclusively in TLM-stored pancreata. Moreover, a tendency toward a reduction in the transplantation rate in TLM-stored pancreata (29% vs. 50%) was observed in this donor population.

Another important donor factor that significantly affects human islet isolation outcome is donor BMI. Pancreata from obese donors are generally covered by significant amounts of fat (143). Obstructive quantities of fat and connective tissue can interfere with oxygen penetration into the pancreatic core. It seems to be important that pancreata from slim donors are selected for efficient pancreas oxygenation during prolonged storage (72). Alternatively, pancreata could be trimmed before preservation with the TLM, thus facilitating oxygen penetration into the tissue, which is usually the case for the short-term oxygenation following cold storage (144, 145).

In conclusion, our analysis of 200 islet isolations from human donor pancreata that had been stored in UW or preserved by TLM has clearly shown that TLM-mediated oxygenation has no beneficial effect on islet isolation outcome and post-transplant function in type 1 diabetic recipients, when compared to cold storage in UW solution alone. In addition, no protection of marginal pancreata, characterized by increased donor age and/or prolonged CIT, was found after TLM storage. These data indicate that the ischemic tolerance of the human pancreas cannot be extended by oxygenation through the use of TLM.

Lower islet yields using HTK during prolonged pancreas preservation (Paper II)

In human islet transplantation, HTK has been proposed as an alternative to UW, providing similar isolation outcome. However, these studies assessed only pancreata preserved for a relatively short period of CIT (87, 88). Currently, efforts are ongoing to centralize clinical islet isolation in highly experienced units to maximize resources and reduce costs (52, 146). On the other
hand, this may result in prolonged transportation, thus increasing CIT. The purpose of this study was to investigate whether islets can successfully be isolated from HTK-preserved pancreata after prolonged CIT within the Nordic Network for Clinical Islet Transplantation.

In a retrospective analysis including 64 human islet isolations, HTK-perfused pancreata with a CIT of more than 10 hours were matched to organs with a CIT of less than 10 hours according to age, BMI, gender, and pancreas weight. Finally, a similar number of UW-perfused organs were selected according to the variables mentioned above.

None of the isolation- and culture-related parameters differed significantly among HTK- or UW-perfused pancreata when the effect of CIT (<10 vs. >10 h) was compared. When pancreata were pooled in preservation groups independent of CIT, the trimmed pancreas weight was significantly higher in HTK organs as compared to UW (P=0.039). This finding corresponded to a dry-to-wet weight ratio that was approximately 16% lower after HTK perfusion (P=0.007). Regardless of duration of CIT, the volume of HTK utilized for flushing was approximately twice as much compared to UW perfusion volume (P<0.001).

The reduction in islet yield caused by prolonged CIT appeared to be quite marked when HTK was used for preservation. On the other hand, quality assessment did not reveal any significant differences between HTK or UW-perfused organs or between CIT categories within the same preservation group.

The reasons for replacement of UW by HTK can be partially related to economic considerations (80) and the improvement of organ cooling thanks to the high perfusion volume and relatively low pressure (79). Moreover, the specific composition and high viscosity of UW can result in harmful formation of adenosine crystals during cold storage (66). The presence of HES can also lead to stimulation of red blood cell aggregation, thereby preventing adequate perfusion of the organ (67). Crystal formation and deposits has not been found in HTK (66, 147). As reviewed in the introduction of this thesis, UW solution must be removed before implantation of the organ because of the high K+ content bearing the risk of undesirable cardiovascular events (68).

The low viscosity of HTK may benefit the clearance of the perfused organs compared with UW (148). In this context, it has been speculated whether the adverse effect of HTK on graft survival is related to pancreas edema caused by using high perfusion volumes as recommended by the producer (86, 148, 149). Although obvious edema was not seen during inspection of the HTK-preserved organs, the dry-to-wet weight ratio clearly indicated a higher fluid content in the tissue, probably caused by the higher perfusion volume of preservation solution. This may explain why islet yield
was significantly reduced after HTK perfusion when calculated per gram pancreas but not per whole pancreas as compared to UW storage.

Several islet studies so far did not find any difference between HTK and UW with regard to isolation and post-transplant outcome (87, 88, 150). This is in agreement with numerous studies evaluating the effect of HTK on pancreas (82, 83, 86), kidney (151), and liver transplantation (152). In contrast, a reduced efficacy of HTK compared with UW was particularly noticed in pancreas (84) and liver transplantation (153) after prolonged cold storage.

The present study suggests that HTK has certain limitations for pancreas preservation prior to human islet isolation in comparison to the UW solution. Although the functional integrity of islets isolated after CIT of >10 h was not affected by HTK preservation, prolonged cold storage resulted in a reduced islet yield. Nevertheless, no significant difference was found with respect to isolation outcome when CIT was <10 h. Our findings indicate that UW preservation should be preferred when clinical islet transplantation is intended after prolonged CIT.

Vitacyte HA decreases digestion time without damaging islets (Paper III)

Islet isolation outcome depends significantly on the efficiency of the enzyme blend utilized for pancreas dissociation. With the withdrawal of Liberase HI, Serva collagenase became the standard enzyme blend utilized for clinical purposes in most centers. Since 2008 a new enzyme named Vitacyte CLzyme collagenase HA (Vitacyte HA) has been available for clinical human islet isolation. This study investigates whether Vitacyte HA can be considered as a significant alternative to Serva collagenase NB1 (Serva NB1) regarding isolation outcome and islet quality.

For that purpose, the outcome of 18 human islet isolations performed with Vitacyte HA was compared to 18 donor-matched pancreata processed with Serva NB1 using identical procedures for isolation and quality assessment. In the study, Serva NB1 was complemented with Serva neutral protease NB, whereas Vitacyte HA was complemented with Vitacyte CLzyme thermolysin.

Vitacyte HA and Serva NB1 provided similar islet yield and purity ($P=0.076$). Although the amount of collagenase activity infused per gram pancreatic tissue (PZ)-U/g was significantly lower for Vitacyte HA compared with Serva NB1 ($P<0.01$), the average digestion time required for sufficient islet release was significantly shorter ($P<0.01$). Furthermore, the average packed tissue volume per gram pancreas tended to be slightly larger using Vitacyte HA ($P=0.058$) whereas enzyme selection did not affect the percentage of undigested tissue.
The percentage of islets belonging to the different size categories was nearly identical in both experimental groups, indicating that islet integrity was not affected by the choice of enzyme blend. Islet recovery and post-culture purity were also similar. Although the intracellular insulin content was significantly lower in islet isolated with Vitacyte HA ($P<0.05$), the stimulation index and the profile of dynamic glucose-stimulated insulin release indicated a similar insulin secretory capacity in both groups. The ADP/ATP ratio was significantly lower when islets were isolated using Vitacyte HA ($P<0.01$).

Among other donor and isolation variables analyzed, only digestion time correlated significantly with the ADP/ATP ratio ($P<0.01$). No significant differences were found between Vitacyte HA and Serva NB1 regarding the cytokine and tissue factor expression.

The rapid liberation of islets during pancreas digestion is important to minimize the tissue damage induced by the harmful environment characterized by anoxia and hyperosmolarity (154). This is supported by the finding that islet ADP/ATP ratio correlates with digestion time. Most importantly, the higher efficiency of Vitacyte HA did not deteriorate islet integrity compared with Serva NB1 as expressed by similar islet size distribution, survival after culture, insulin secretory capacity, and cytokine expression. Whether the potency of Vitacyte HA is related to a preserved integrity of collagenase class I, as indicated by a slightly increased islet purity (99, 130), or to the application of a complementary protease different from that of Serva NB1 remains to be clarified.

From this study, we conclude that the combination of Vitacyte collagenase and thermolysin is an efficient enzyme blend for human islet isolation, providing isolation results similar to those obtained with Serva NB1. Furthermore, it reduces the digestion time and facilitates a more cost-effective isolation with the use of a lesser amount of enzyme.

**Mammalian Tissue-Free Liberase in successful human islet isolation (Paper IV)**

After the withdrawal of Liberase HI from the market due to safety reasons, the manufacturing company developed a new enzyme blend with similar components, where no mammalian tissue was used in the manufacture process, hence giving the name of the new product: Liberase MTF-S (mammalian tissue-free)

Together with the islet transplant centers of the University of Leiden (The Netherlands) and Lille (France), we described our first experience with the new GMP-grade enzyme blend Liberase MTF-S. In this multicenter study, 14 human pancreata were processed according to similar isolation protocols (37, 155).
Liberase MTF-S was provided by the manufacturer as a premixed blend containing 1570 (PZ)-U of collagenase and 79,328 caseinase units of thermolysin per vial. In 10 of 14 cases, one vial was used for processing of one pancreas whereas 1.5 vials were administered in four cases. The amount of collagenase and thermolysin was adapted to the pancreas weight due to the large variability in organ weight. The average recirculation time required for islet release was 18.1±1.8 min.

After digestion, the percentage of undigested tissue remaining in the digestion chamber was 14.5%±2.1%. Packed tissue volume per gram pancreas was 0.46±0.04 ml/g. Islet yield pre-purification was 416,570±61,840 IE, equivalent to 3660±510 IE/g. Purification failed in 2 of 14 preparations because of technical problems. These preparations were excluded from the study. Of the remaining 12 pancreata, three were considered unsuccessful as they yielded less than 100,000 IE. Islet recovery post-Ficoll reached 65.9%±6.2% with a purity of 59.0%±3.8% and 13.8%±3.2% of embedded islets. After culture, islet survival was 73.9%±4.9%, with similar purity to freshly isolated islets. Islet fragmentation index (islet count/IE) was determined pre-Ficoll (1.0±0.1), post-Ficoll (1.2±0.2), and after culture (1.4±0.2, \( P=0.05 \) vs. post-Ficoll).

Quality assessment showed a viability of 86.7%±2.8%, an SI of 2.8±0.7 and intracellular insulin content of 376±91 U/ng DNA. Two of the processed preparations did not respond adequately to glucose stimulation. However, 5 of 12 preparations fulfilled all criteria for clinical islet transplantation.

This data demonstrates that Liberase MTF-S reduces digestion time required for complete human pancreas dissociation and for efficient islet release. The increase of the fragmentation index reflects a certain extent of islet disintegration during islet separation and culture, indicating the aggressive character of the MTF-S enzyme. This is additionally stressed by an islet recovery of 74±5%, which is inferior as compared to results obtained with the use of Liberase HI or Serva NB1 (120).

Liberase MTF-S is characterized by a low percentage of degraded collagenase class I, the collagenase isoform that seems to be responsible for the lot-to-lot variability observed in Liberase HI (130, 156-158). The variability observed in our study reflects most likely a learning curve with this new product, which was characterized by a higher thermolysin to collagenase ratio compared with Liberase HI (158, 159). Indeed, most of the isolations that did not meet the criteria for clinical transplantation were performed during the initial phase of the study.

High doses of non-collagenolytic activities seem to be beneficial for rapid islet release but can deteriorate islet viability, the reason why they should be administered carefully (110). Notably, these results were obtained using a premixed enzyme blend. The efficiency of Liberase MTF-S could be further
optimized if it is applied as a two-component product facilitating the individual adjustment of thermolysin.

Prediction of isolation outcome in the human pancreas (Paper V)

The ability to predict isolation outcome could dramatically improve the success rate of the isolation procedure, save the costs spent for failed islet isolations and increase the chance for an islet preparation to be transplanted into a type 1 diabetes recipient.

A previous study observed a positive correlation between the islet morphology and isolation outcome, suggesting a possible role of the native islet mass as a determinant factor for isolation success (135).

The current retrospective study was performed to correlate the outcome of strictly standardized isolation procedures with morphometric evaluation of pancreatic biopsies. After insulin staining and digitalization of the pancreatic slides, islet morphology from 29 human pancreatic routine biopsies was correlated to the outcome of islet isolations performed with validated batches of Serva collagenase NB1. Isolations were further categorized as successful or unsuccessful if they yielded more or less than 2000 IE/g, respectively.

Donor and isolation variables were similar in successful and unsuccessful isolations except for the digest pellet volume \( (P=0.0083) \) and purity \( (P=0.019) \) that were higher in the successful group. The mean biopsy area measured 27.61±2.55 mm\(^2\) and contained 91.7±8.2 islets or 9.94±0.43 islets/mm\(^2\). Beta cells (positive insulin staining) covered 1.53±0.2% of the biopsy surface. In average, sectioned islets had a perimeter of 188±8.13 µm, a diameter of 63.55±2.63 µm and an area of 3303±835 µm\(^2\). The mean volume of an individual sectioned islet was 429,677±74901 µm\(^3\).

On average, almost 50% of the islets were smaller than 50 µm in diameter whereas 15% were larger than 100 µm and only 4.5% larger than 150 µm. The different size categories were equally distributed in both experimental groups except for the percentage of islets with a diameter between 100 and 150 µm, being higher in biopsies from successful isolations \( (P=0.051) \). In our hands, none of the morphological parameters included in this study correlated with isolated islet yield.

One possible explanation for this finding is that our biopsies were taken from the head of the pancreas, which may not be representative for the whole gland. Previous studies in the native human pancreas demonstrated that the insulin content in the tail is higher as compared to the head (160). This is, however, in contrast to early histology studies suggesting that the human pancreas has a relatively constant islet size distribution throughout the gland with a slightly higher frequency of small islets in the head (134, 161)
A previous biopsy study found that islet size and number of islets per defined area correlates positively but not significantly with total isolated islet yield in the human pancreas. Nevertheless, in this study samples were taken both from the head and tail of the pancreas (135). Furthermore, the study focused on a semi-quantitative evaluation of the peri-islet tissue rather than on morphological analysis, showing that edema and low interstitial fat content affects negatively the isolated islet yield, which may have implications for enzymatic pancreas digestion (Paper II).

As reported in the pig model, isolations were successfully performed after identifying the presence of large islets in a biopsy as a predictive factor for successful isolation (136). Likewise, in a study by Anazawa et al. (162), the number of native islets in the splenic lobe of the pig pancreas significantly correlated with islet yield.

Since none of the morphological parameters assessed in the present study were predictive for the isolated islet yield, other factors might determine the suitability of a donor pancreas for human islet isolation. Several studies indicate that the composition of the collagen fiber matrix (163, 164) and its distribution within the pancreas (165) may be important for the dissociation process. The fact that the extent of islet encapsulation by the collagen matrix is extremely variable has implications for islet integrity and viability during isolation (166). Since CC1, CC2 and neutral protease have no specificity toward the peri-insular extracellular matrix and the intra-islet connective tissue, islets that are not fully covered by the collagen capsule can be damaged by the enzymatic digestion (105). Furthermore, total collagen, pancreatic edema and intraparenchymal fat show a remarkable heterogeneity between individuals, suggesting that along with donor-related factors, variations in the native pancreatic tissue determine isolation outcome (166).

Previous observations that in vitro collagen degradation differs from “real” enzymatic pancreas digestion indicate that there is no correlation between the collagen degradation capacity of an enzyme and its capacity to dissociate pancreatic tissue (105). Furthermore, detachment of other structures in the islet-exocrine interface such as cell-to-cell and cell-to-matrix contacts may be an additional requirement for dissociation of pancreatic tissue (167). Taken all together, this indicates that a morphological analysis alone is not a reliable method to predict effective pancreas dissociation and isolation success.
Conclusions

Paper I

- The two-layer method (PFD/UW) cannot extend the ischemic tolerance of the human pancreas as compared to the UW solution alone.
- The TLM is unable to improve preservation from pancreas from elderly donors.
- Preservation with the TLM does not benefit islet graft function as compared to UW storage.

Paper II

- HTK is effective for short-term pancreas preservation as compared to UW solution.
- Tissue edema under HTK preservation may lead to lower isolation yields from human pancreata with prolonged cold ischemia as compared to storage in UW solution.

Paper III

- Vitacyte HA is an efficient enzyme blend for isolation of human pancreata.
- Vitacyte HA provides similar isolation outcome as compared to Serva NB1, but with shorter digestion time and lesser amount of enzyme.

Paper IV

- MTF-Liberase is able to provide successful isolation results from human pancreata.
- The high neutral protease (thermolysin) content present in MTF-Liberase may damage islet morphological integrity, as seen by the higher islet fragmentation.
Paper V

- Human islet isolation outcome does not entirely depend on the native islet mass within the pancreas as evaluated in biopsies taken from the head of the pancreas.
Future Perspectives

Towards one-to-one equation in clinical islet transplantation

The outcome of human islet transplantation has been limited by several travails: pancreas donor shortage, unpredictable isolation outcome, impossibility to diagnose islet rejection, low islet engraftment and reduced long-term graft function. This thesis aimed to improve two of them: pancreas preservation and enzymatic pancreas dissociation. The implementation of the research findings described above will hopefully improve isolation outcome, bringing us closer to the achievement of normoglycemia and insulin independence by transplanting one single islet preparation.

Organ preservation

Improvement in the field of organ preservation has been achieved by the use of machine perfusion (168). Preliminary studies in islet isolation from the pig pancreas have shown promising results of machine perfusion as compared to cold storage (169). Due to the high cost of this technique, the use of lipophilic and biocompatible carriers for static cold preservation seems to be a reasonable alternative (170).

Enzymatic islet isolation and prediction of isolation outcome

Standardization of enzymatic pancreas dissociation is required to improve islet isolation outcome (103). However, it should be complemented with the characterization of the pancreatic tissue due to the inner variability of collagen composition in the ECM of the human pancreas (166). Prediction of isolation outcome will most likely depend on our ability to tailor the enzymatic and separation strategies to the current organ.
El trasplante de islotes de Langerhans consiste en restituir las células productoras de insulina que la diabetes ha destruido. Se prefiere al trasplante de páncreas por su carácter mínimamente invasivo y por el menor riesgo quirúrgico, aunque a día de hoy sus resultados a largo plazo son más limitados. Está indicado en pacientes con diabetes tipo 1 que sufren de un control inestable con hipoglucemias severas inadvertidas, así como en pacientes diabéticos trasplantados de riñón y/o con otras complicaciones graves.

El principal problema para su aplicación clínica es la dificultad para extraer los islotes del páncreas durante el proceso conocido como “aislamiento de islotes”. Además, la imposibilidad de diagnosticar el rechazo temprano y la inmunosupresión ineficiente condicionan la baja supervivencia del trasplante a largo plazo.

El trabajo recogido en esta tesis doctoral tiene como objetivos: 1. Optimizar la preservación del páncreas durante el transporte desde el quirófano hasta el laboratorio de aislamiento; 2. Mejorar la digestión enzimática del páncreas para liberar los islotes de forma rápida y efectiva, sin dañar su función; 3. Analizar la anatomía del páncreas como factor determinante en el resultado del aislamiento de islotes humanos.

El estudio sobre la tolerancia isquémica del páncreas previo al aislamiento de islotes muestran que el método de las dos capas (solución de la Universidad de Wisconsin (UW)/perfluorocarbono) no puede aumentar el tiempo de isquemia fría del páncreas humano. La preservación del páncreas en la solución de histidina-triptófano-ketoglutarato (HTK) previa al aislamiento de islotes es equiparable a la solución de referencia UW a corto plazo, aunque es limitada a largo plazo, posiblemente debido al edema producido durante la perfusión del páncreas del donante. Dos nuevas enzimas, Vitacyte HA y MTF-Liberase, han sido utilizadas para la digestión del páncreas humano con objeto de obtener islotes libres de tejido acinar exocrino. La enzima Vitacyte HA es una colagenasa rápida y eficiente, con unos resultados similares pero con tiempos de digestión más cortos y menor cantidad de enzima que la colagenasa Serva NB1. Además, los primeros aislamientos con la nueva enzima Liberase libre de tejido mamífero (MTF-S) han dado resultados prometedores en cuanto al número de islotes aislados y su viabilidad así como el porcentaje de trasplantes clínicos. Finalmente hemos demostrado que el estudio de la histología del páncreas y la morfología de los islo-
tes es incapaz de predecir la cantidad de islotes que se van a poder aislarn del páncreas humano.

El avance de la técnica de aislamiento contribuirá a la obtención de un número suficiente de islotes para conseguir la independencia insulínica con un solo páncreas. Este es el paso previo para la aplicación clínica del trasplante de islotes como tratamiento de la diabetes inestable con complicaciones graves.
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