Technical challenges in human islet isolation

MAGNUS STÅHLE
Abstract


Transplantation of islets of Langerhans is an effective treatment option for patients with brittle type 1 diabetes mellitus. This treatment restores glucose control and also reduces hypoglycemia. Unfortunately, the outcome from islet isolations is variable, and many preparations do not yield sufficient islet number or islet quality.

The aim of this thesis was to improve the isolation procedure, thereby making more preparations available for clinical transplantation.

A well-established method for pathogen inactivation was applied to human serum used in the islet isolation process. Evaluation of isolated islets stored in medium supplemented with pathogen-inactivated serum showed that pathogen inactivation did not have negative effects. These findings will enable the use of human serum in clinical cell transplantation programs, while simultaneously increasing patient safety.

Pre-incubation of islets prior to gradient separation is an established standard in the field of islet isolation. Through a reduction in the pre-incubation step, isolation time could be reduced by almost an hour without affecting the isolation outcome.

A commercially available protease enzyme, clostripain, was added to the enzyme blend used in islet isolation. Addition of clostripain was found to increase the number of islets isolated as well as the purified tissue volume and fulfillment of transplant criteria. Use of clostripain should help to increase the number of successful isolations.

A newly developed pancreas-specific preservation solution, I-Let protect, was evaluated. As compared to standard preservation solutions, it can be used in situations of prolonged cold ischemic time without affecting the isolation outcome or islet functionality. I-Let protect can also be used in establishing a protocol that would eliminate the need for night-time isolations.

Through the work in this thesis, several key elements in human islet isolation have been optimized, and further knowledge has been gained.

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The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity.

Albert Einstein (1879-1955)

To my family
List of Papers

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


II  Ståhle, M., Honkanen-Scott, M., Ingvast, S., Korsgren, O., Friberg, AS. (2013) Human islet isolation processing times shortened by one hour: minimized incubation time between tissue harvest and islet purification. Transplantation, Dec 27;96(12):e91-3


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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ATMP</td>
<td>advanced-therapy medicinal products</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BAEE</td>
<td>Nα-benzoyl-L-arginine ethyl ester</td>
</tr>
<tr>
<td>CIT</td>
<td>Cold ischemic time</td>
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<tr>
<td>DCCT</td>
<td>The Diabetes Control and Complications trial</td>
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<td>EUTCD</td>
<td>European Union Tissues and Cells Directives</td>
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<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
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<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin (A1c)</td>
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<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<td>IA</td>
<td>Islet alone</td>
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<td>IAK</td>
<td>Islet after kidney</td>
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<td>IE</td>
<td>Islet equivalents</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HTK</td>
<td>Histidine tryptophan ketoglutarate</td>
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<tr>
<td>NDDG</td>
<td>National Diabetes Data Group</td>
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<tr>
<td>NNCIT</td>
<td>Nordic Network for Clinical Islet Transplantation</td>
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<td>NP</td>
<td>Neutral protease</td>
</tr>
<tr>
<td>PA</td>
<td>Pancreas alone</td>
</tr>
<tr>
<td>PAK</td>
<td>Pancreas after kidney</td>
</tr>
<tr>
<td>PI</td>
<td>Pathogen inactivation</td>
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<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
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<td>SIK</td>
<td>Simultaneous islet kidney</td>
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<td>SPK</td>
<td>Simultaneous pancreas kidney</td>
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<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<td>TLA</td>
<td>Tryptic like activity</td>
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<td>TLM</td>
<td>Two-layer method</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>WNV</td>
<td>West Nile virus</td>
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<tr>
<td>UVA</td>
<td>Ultraviolet A</td>
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<tr>
<td>UW</td>
<td>University of Wisconsin</td>
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Introduction

Diabetes is a widespread disease with increasing prevalence. For Type 1 diabetes, replacement therapy with transplantation of islets of Langerhans is a viable treatment for a selected group of patients. Islet transplantation restores blood glucose regulation, which leads to the avoidance of hypo- and hyperglycemic episodes. The process of islet isolation suffers from variability resulting from insufficient knowledge concerning key elements of the isolation process. Improvements in organ preservation, knowledge of enzyme function and enzyme use, as well as improvements in storage conditions are needed not only to improve islet quality but also to comply with regulatory demands and increase patient safety.

This thesis addresses and evaluates variables that are critical for both islet isolation outcomes and patient safety. Use of a new organ preservation solution has enabled us to prolong cold ischemic time while retaining islet viability and function, thus rendering late-evening and night-time isolations obsolete. It offers a central isolation facility the possibility of handling several organs at the same time, since it can prolong the cold ischemic time from 8-10 hours to 16-24 hours without a negative effect on the isolated islets. Furthermore, such flexibility may be accomplished without compromising islet functionality. A newly available enzyme component, clostripain, has been evaluated and found to dramatically increase isolation outcome and thus increase the number of transplantations to patients. Expanded knowledge of enzyme component integrity and stability has confirmed our way of handling enzymes. The removal of the islet incubation step prior to islet separation has been found to decrease islet isolation time by almost one hour while providing the same level of isolation outcome. A new treatment of human serum used in islet storage has been found to have no negative effect on islet quality and function, while increasing the safety of the patients receiving the transplants.

Through these improvements, islet-processing facilities will be able to improve isolation outcome, shorten isolation times, improve the flexibility of their facilities, and improve patient safety.
Aims

General aims
The work presented in this thesis was performed with the intent of improving islet isolation outcome and patient safety while still complying with regulatory demands.

Specific aims
Paper I
• To evaluate whether pathogen inactivation of human serum using Intercept technology could be applied for safety improvement without a negative effect on islet functionality

Paper II
• To shorten the isolation procedure while retaining islet function and quality

Paper III
• To evaluate how clostripain, a newly available commercial enzyme, would affect the digestion of the pancreas when added to the enzyme blend

Paper IV
• To evaluate whether a new organ preservation solution could improve islet isolation outcome
• To develop a procedure in which pancreata arriving at the laboratory late in the day could be oxygenated and processed the following day without affecting isolation outcome or islet functionality
Background

Regulation of blood glucose
Glucose is the main source of energy in human and mammalian cells. It is regulated and balanced by glucose intake (in the form of carbohydrate) in the intestines and by the utilization of glucose in metabolism. Glucose is stored as glycogen in the liver and muscles. When the body is in a fasting state, glycogenolysis (the conversion of glycogen to glucose) in the liver is followed by the release of glucose into the blood. Other nutrients such as fatty acids and proteins can also be converted into glucose by gluconeogenesis [1].

Two hormones are responsible for the main regulation and fine-tuning of glucose homeostasis: insulin and glucagon. Both hormones are produced and released by cells located in the islets of Langerhans. The function of these cells is controlled both by paracrine mechanisms and by central stimuli. Glucagon is a catabolic hormone produced by the alpha-cells in the islets. Its main function is to increase the blood glucose by stimulating glycogenolysis in the liver. Insulin, on the other hand, is an anabolic hormone produced by the beta-cells in the islets of Langerhans. Insulin enhances glucose utilization by facilitating glucose metabolism by muscle and fat cells, which decreases blood glucose concentrations [1].

Diabetes

History
Knowledge of diabetes as a disease extends back several thousand years. One of the first descriptions of diabetes, in Egypt, is dated to 1550 BC. In India, two Hindi physicians, Charac and Sushrut, were perhaps the first to describe the sweetness of diabetic urine, somewhere between 400 and 500 BC. A description of what is today called type 1 diabetes mellitus, given by Aretaeus of Cappadocia, dates back to the 2nd century AD [2].

In 1674, the Latin term *mellitus*, which means “sweet honey,” was added to the term “diabetes,” named by Thomas Willis [3]. The organ affected in diabetes mellitus was unclear for a long period of time. The first description of round cell clusters that were spread out in the pancreas was made by the medical student Paul Langerhans in his doctoral thesis in 1869 [4]. He did
not know the function of these groups of cells at the time, and he died in 1888, the year before Mering and Minkowsky published their work in which they made dogs diabetic by removing the pancreas. In doing so, Mering and Minkowsky were repeating similar experiments performed 200 years earlier by Johann Brunner. In Dr. Brunner’s experiments, partial pancreatectomies rendered the dogs initially diabetic, but because the head and part of the body of the pancreas remained, the dogs did not develop long-term diabetes; also, Dr. Brunner did not make the connection between the observed symptoms and findings and the pancreatectomies [5]. In recognition of Paul Langerhan’s thesis work, the name “islets of Langerhans” was given to these pancreatic structures by Edouard Laguesse in 1893 [4].

The name “insulin” for the secretion of the islets of Langerhans was coined by Meyer in 1909 from the Greek word *insula*, which means “island” [6].

During the summer of 1921, the pancreas extract project at the University of Toronto was testing crude extracts on diabetic dogs when they managed to isolate insulin. Within the next 2 years, 25,000 diabetic patients were insulin-treated in Canada and the USA [7]. Four men involved in the pancreas extract project were vital to its success: the surgeon Banting, the physiologist professor Macleod, the biochemist Collip, and the medical student Best. Banting and Macleod received the Nobel Prize in medicine in 1923 for the discovery of insulin, splitting the prize money with Collip and Best.

**Diabetes epidemiology**

Diabetes is a disease with increasing prevalence that has enormous effects on health systems worldwide. It currently affects more than 200 million people, and projections estimate that diabetes will affect approximately 5% of the world’s population by 2025 [8]. These estimates are based on the assumption that the levels of obesity and physical activity will remain constant.

Diabetes mellitus is not a single disease but is instead comprised of a group of metabolic diseases that are all characterized by the hyperglycemic state the patients are experiencing. The hyperglycemia results from defective insulin production, insulin action or, in some cases, both [9].

Until 1979, when The World Health Organization (WHO) published the National Diabetes Data Group (NDDG) classification system [10], there existed no generally accepted categorization for diabetes. This classification system included evidence showing the heterogeneity of diabetes mellitus, which is vitally important for the treatment of patients with diabetes. In 2003, a revision in the classification was published ([11], in which the terms insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus were eliminated and the terms type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus were adopted. Approximately 10% of all diabetes patients suffer from T1DM.
Type 1 diabetes mellitus

T1DM is characterized by a more or less complete destruction of the β-cells in the pancreas (insulitis), leading to a complete deficiency of insulin. The insulin deficiency causes life-threatening hyperglycemia in the patients. Disease onset is usually in childhood or early adulthood. Clinically, patients typically display weight loss, polyuria (excessive passage of urine), and polydipsia (extreme thirst). The destruction is believed to be immune-mediated, showing genetic predispositions, and may be triggered by environmental factors that are still poorly understood and defined. It has been demonstrated that overweight and obesity in pregnant women, particularly during early pregnancy (first trimester) are associated with increased risk for the offspring of developing T1DM, regardless of parental ethnicity [12]. Parental diabetes also increases the risk of developing T1DM in the offspring. It has been demonstrated that, regardless of ethnicity, parental type 1 diabetes can increase the risk of diabetes in offspring to up to 9 times that of the offspring of parents without diabetes.

Because the etiology of the disease is still unknown, no cure is available. The incidence of T1DM is increasing worldwide, with a generally higher incidence in developed countries. In Europe, the average yearly increase is 3-4%, but with periods of less rapid and more rapid increases in incidence [13]. One popular environmental factor candidate is viral infections, since human enteroviral (HEV) antigens, RNA, and occasional isolated virus have been found in samples from T1DM patients [14]. Another suggested explanation is that children who develop T1DM have different bacterial intestinal flora than do control children that do not develop T1DM [15].

Type 2 diabetes mellitus

T2DM is dominated by insulin resistance and relative insulin deficiency. It is often associated with metabolic syndrome. T2DM typically occurs in adults and is associated with physical inactivity, age, and obesity. The treatment requirements are highly varied as a result of the variability in the factors involved in the etiology of T2DM. Treatments are based on changes in the patient’s lifestyle, with the focus often placed on diet and exercise. The treatment is ideally individually based and should include monitoring of blood pressure, adiposity, behavioral aspects, and pathophysiological age, combined with drugs that stimulate insulin release [16].

Complications and long-term effects of T1DM

Patients with T1DM have a higher mortality that that of the appropriate reference population. The most acute and life-threatening consequence, acute death from ketoacidosis and hyperglycemia, can generally be prevented as a result of the discovery of insulin (1921). T1DM is now a treatable chronic disease, but hyperglycemia with ketoacidosis and hypoglycemia [11] still remain life-threatening throughout the life of diabetic patients. During the
early phases of the disease, mortality is dominated by diabetic ketoacidosis and hyperglycemia [17].

Increased levels of blood glucose initiate the destruction of the microvasculature, which leads to retinopathies, nephropathies, and neuropathies. Decreased blood flow also results in impaired wound healing and chronic ulcers; in some cases, it leads to blindness or peripheral gangrene, and eventually necessitates amputation [18]. The most common long-term complication causing death is cardiovascular disease. One recent Swedish study of more than 30,000 patients with T1DM showed that T1DM patients with an optimal glycemic control had more than twice the risk of cardiovascular disease of healthy controls. With suboptimal glycemic control and increased levels of glycated hemoglobin A1c (HbA1c), the risk of death from any cause, and from cardiovascular disease in particular, was increased by up to 8 and 10 times, respectively, when compared to the general population. These results strongly indicate the importance of keeping HbA1c levels normalized as much as possible, since poor glycemic control drastically increases mortality [19, 20].

### Insulin treatment, hyperglycemia, and hypoglycemia

The standard treatment for T1DM today is long-acting insulin combined with rapid-acting insulin taken in association with intake of a meal. Continuous subcutaneous insulin infusion via pumps has many advantages over multiple daily injections in terms of blood glucose regulation. It is costly, however, and therefore not offered to all potential patients. It also requires that the patient is always connected to the pump, and it has a risk of incidental non-delivery if, e.g. the motor in the pump stops working. Improved blood glucose monitoring can be achieved with continuous glucose monitors, eliminating the need for manual peripheral blood sampling. Combined continuous glucose monitoring with insulin pumps in a closed-loop system, sometimes referred to as an artificial pancreas, is being developed. Randomized controlled clinical trials combining continuous glucose monitoring with insulin pumps have been conducted since 2010, but a finished product is not yet clinically available [21].

Hypoglycemia does not usually occur in healthy people, because lowering the blood glucose concentration stimulates secretion of adrenalin and inhibition of insulin release. Glucagon release activates glycogenolysis in the liver, followed by release of glucose into the blood.

As demonstrated in the Diabetes Control and Complications Trial (DCCT), tight glucose control that lowers the glycated hemoglobin A1c (HbA1c) to near-normal levels is correlated with a reduction in long-term complications for diabetics, especially macrovascular complications [22].
Hypoglycemia unawareness and difficult-to-control diabetes

Lowering of the HbA1c, however, seems to increase the number of episodes of hypoglycemia. The regulatory mechanisms for hypoglycemia become impaired over time, and all T1DM patients will eventually lose their ability for counter-regulation [23]. Autonomic responses to hypoglycemia seem to be further reduced during sleep, resulting in both a heightened fear of severe hypoglycemic events during the night and actual nocturnal hypoglycemia in T1DM patients [24].

Repeated hypoglycemic episodes lower the glycemic thresholds for sympatho-adrenal responses, leading to hypoglycemia unawareness. Hypoglycemia unawareness is a major risk factor for severe hypoglycemic episodes. A clinical syndrome called hypoglycemia-associated autonomic failure (HAAF) is defined as the unawareness of hypoglycemic episodes and defective counter-regulation. Frequent episodes can lead to patients’ reduction in the intensity of their insulin treatment, which leads to acceleration of long-term complications. These patients also have higher mortality and complication rates than do other T1DM patients [25, 26].

Difficult-to-control diabetes is characterized by poor metabolic control and severe instability of blood glucose despite all available treatment. Patients have unpredictable and frequent hypoglycemic episodes, often requiring emergency hospital care [27]. Because of these frequent acute complications, the quality of life of both the patient and the patient’s family is severely compromised.

Beta-cell replacement

Despite all the methods available for regulating blood glucose levels, the only way to restore physiological function in diabetes is by replacing the β-cells via transplantation. There are currently only two types of transplantations that can be considered: pancreas transplantation and islet transplantation.

Pancreas transplantation

Whole-pancreas transplantation is a well-established clinical treatment that has been performed for more than half a century. The first report of clinical transplantation came in 1967, after Lillehei and Kelly performed the first transplantation at the University of Minnesota in 1966 [28]. The outcome was not good, as a result of complications related to the pancreatic surgery. At present, the pancreas is either transplanted simultaneously with a kidney from the same donor (simultaneous pancreas-kidney; SPK), alone (PA), or after a previous kidney transplant (pancreas after kidney; PAK). SPK is the
most common procedure and has the highest graft survival rate, 70% after 5 years [29]. Pancreas transplantsations are surgically complicated procedures and were previously associated with high mortality. As techniques have improved and technical failures decreased, the procedure has now become relatively safe, with patient survival above 95% after 1 year, and above 88% after 5 years [29].

Islet transplantation
The islets of Langerhans constitute approximately 1-4% of the entire pancreatic volume and are distributed throughout the pancreas [30]. In order for islets to be transplanted, they need to be separated from the rest of the pancreatic tissue. For patients with brittle diabetes and hypoglycemia unawareness, islet transplantation has proved to be an effective approach for restoring normoglycemia and eliminating episodes of hypoglycemia [31-33].

Three different patient groups with T1DM are suited for islet transplantation: 1) patients who have already received a kidney transplant and are on immunosuppressive therapy (islet after kidney; IAK); 2) patients with end-stage renal disease scheduled for kidney transplantation, who are getting islets and kidney from the same organ donor (simultaneous islet-kidney; SIK); and 3) patients suffering from brittle diabetes and hypoglycemia unawareness (islet alone; IA). Islet transplantation is a minimally invasive procedure in which the islets are transplanted into the liver via the portal vein. Patients are under local anesthesia, there are low risks of side effects, and the hospital stay is short when compared to whole-pancreas transplantation [34].

Islet isolation and transplantation

History of islet isolation and transplantation
The first documented attempt at islet transplantation was performed in 1893, when a British doctor subcutaneously transplanted minced pancreatic sheep tissue into a dying 13-year old diabetic boy [35].

One of the first to successfully isolate islets was Moskalewski [36], who in 1965 successfully combined enzymatic digestion, using collagenase, with mechanical digestion. Paul E. Lacy improved this procedure further, infusing the collagenase into the pancreatic duct. He and his group also evaluated different implantation sites, with the portal vein becoming a favored site [37, 38]. The transition from murine islet isolation to the human setting was, however, complicated. The step from experimental animal studies to human islet transplantation was not considered feasible until in the beginning of 1980s, when patients with chronic pancreatitis underwent near-total pancreatectomies. The patients were auto-transplanted with dispersed pancreatic tissue into the portal vein and achieved excellent metabolic glucose control. However, when diabetic patients were transplanted with allografts of dis-
persed pancreatic tissue, they were only occasionally cured of their diabetes. It was concluded that rejection was the probable cause of the failures [39]. The procedure also posed a risk of portal hypertension and intravascular coagulation [40]. Improvements were needed, and the field turned to large-animal models.

Insufficient numbers of islets still hindered clinical results until the laboratory in St. Louis under lead of Drs. Lacy, Sharp, and Ricordi developed the “semi-automated” digestion method in the mid-1980s, first in an animal setting and then for human isolation [41-43]. By using a stainless steel chamber with an inlet in the bottom and an outlet in the top, the pieces of pancreas were gently digested, and the islets could be collected as they were being freed, thus reducing the physical damage they sustained.

Meanwhile, in the UK, the group in Leicester [44] introduced a crucial step when they found a way to separate the islets from exocrine tissue by using a discontinuous gradient in a COBE 2991 centrifuge.

In 1990, Dr. Paul Lacy and his group in St. Louis were the first to report a case of insulin independence following islet transplantation, even though this independence did not last more than a month [45]. During the following decade, many centers in Europe and the rest of the world performed islet isolations and transplantations, with only sporadic success in patients with autoimmune T1DM; the results were much more encouraging for patients with surgically induced diabetes as a result of pancreatectomy. For these patients, up to 50% of the patients were insulin-free up to 1 year post-transplant [46, 47]. The disappointing results for TIDM patients hindered the growth of the field, and it was not until the development and publication of the so-called Edmonton protocol in 2000 that islet transplantation became a viable alternative to whole-pancreas transplantation [48]. The Edmonton group had developed a new steroid-free immunosuppression protocol that involved the administration of tacrolimus, a monoclonal antibody against interleukin-2 receptor, and sirolimus. They also transplanted freshly isolated islets from several donors [48].

The publication of the Edmonton protocol led to a vast expansion of the field of islet isolation and transplantation. Hand-in-hand with improvements in transplantation outcome, funding increased, and an international trial involving 10 centers in Europe, the USA, and Canada was initiated [33]. The aim of the trial was to improve islet isolation methods, to improve techniques for administering transplanted islets, and to develop new approaches for immunosuppressive therapies in order to minimize the toxic effects of the drugs required.
Regulatory guidelines and laws

While organ procurement and shipment in Sweden are regulated by The National Board of Health and Welfare SOSFS2009:30 (Socialstyrelsen), the process of islet isolation and storage has been regulated by the European Union Tissue and Cells Directive (EUTCD) since 2004 [49]. Islets are considered cell therapy, and regulations are dictated by two technical directives instituted in 2006 that provide the detailed requirements of the EUTCD [50, 51]. They specify, among other things, the traceability, coding, preparation, culture, and distribution of human cells and tissue. The three directives cover all academic and hospital activities, as well as commercial products involving human cells and tissue. These directives were implemented into Swedish law in 2008 [52], and regulations regarding donation, processing, and the use of tissue and cells within medical care and clinical research were published in 2009 [53-55]. The guidelines and laws state that all open-system work must be performed in a controlled environment according to good manufacturing practices (GMP). In order to guarantee the highest levels of cleanliness and safety, strict standards need to be applied to GMP facilities.

Organ donors and organ transport

By creating networks with a central islet isolation center and multiple procurement centers, improvements have been achieved in isolation outcomes and efficacy. Centralization benefits from the accumulation of knowledge and experience at a single center. It reduces costs and can be used to maximize the use of available donated organs. Several multicenter networks around the world have been created, including the Nordic Network for Clinical Islet Transplantation (NNCIT) [56-58]. Presently, all organs available for islet isolation within the Nordic countries come from brain-dead, cadaveric donors.

Within the networks, organs are transported between different hospitals and sometimes also between different countries, as in the Nordic Network. Transport increases the cold-ischemic time of the organ (CIT: the time from organ procurement to initiation of the isolation process), and it is therefore important to preserve the organ during transport. The standard protocol for most organ transports is static, cold preservation, known as hypothermic preservation. As the pool of organ donors is expanded via widening of donor criteria, new preservation methods and an improvement of current methods are needed [59]. Because of the reduction in cell metabolism as well as nutrient and oxygen consumption, hypothermic preservation is usually performed at 4°C. The purpose of the hypothermic preservation solutions is to make the organ tolerant of hypothermia and ischemia. However, even at 4°C, approximately 10% of the cells’ metabolic activity remains. Therefore, perfusion of the organs and immersion in preservation solution does not
completely protect against irreversible injury, if a critical time point is exceeded during CIT [60, 61]. The importance of efficient pancreas preservation prior to islet isolation has been reported [62].

The most commonly used preservation solution over the past 20 years has been University of Wisconsin (UW), which has been used for the preservation of heart, kidney, liver, pancreas, hepatocytes, and pancreatic islets [63]. Histidine tryptophan ketoglutarate (HTK) has been used as an alternative to UW for organ preservation since the end of the 1970s, but recent studies have found a negative correlation with isolation outcome as compared to UW when HTK is used, if the CIT exceeds 10 hours [64]. Efforts have been made to supply procured pancreata with oxygen by using a two-layer method (TLM), in which the pancreas is placed at the interface of the UW preservation solution and an oxygenated oxygen carrier. The pancreas is then transported to the islet isolation facility. While some studies have shown improvement in islet isolation outcome when TLM is used for marginal donors [65, 66], other and larger studies have found no beneficial effect of TLM use on the outcome of islet isolation [67, 68].

Enzymes used for islet isolation

In order to separate the islets from the rest of the pancreatic islet tissue, the interface between islets and exocrine tissue needs to be digested. Collagen is the main component of the interface. Collagen is used in humans and other mammals as the main structural component of the tissues of the body and is also used to repair tissue damage. There are several different forms of collagen. The collagen molecule is a triple helix and has a tight structure. This structural tightness makes native collagen difficult to degrade by enzymes in general. Collagenases are proteolytic enzymes, which break the long chain-like molecules of proteins into peptides. Proteolytic enzymes are classified based on their target site of cleavage. There are two major groups, exopeptidases, which target protein terminal ends, and endopeptidases, which target sites within a protein. Proteolytic enzymes are present in mammals, bacteria, and some viruses and plants.

Collagenases bind specifically to, and cut, amino acid sequences containing Pro-X-Gly-Pro, where X is usually an amino acid that is neutral, such as arginine or lysine. The combination of these sequences is unusual in proteins other than collagens. Therefore, collagenases are highly specific in their activity and are also the only enzymes that are able to degrade native collagen [69-71].

Commercially available collagenase used in human islet isolation is not human in origin but is instead produced by the bacteria Clostridium histolyticum; it is comprised of two isoforms of collagenase, class I and class II, also called ColG and ColH, respectively. Two homologous but separate
genes, colG and colH, encode the respective isoforms [72]. The two classes have both endopeptidase and exopeptidase activity. They have similar specificity but complement each other in terms of their mode of attack on the triple helix of collagen. Class I collagenase starts by hydrolyzing loci at the ends of the triple helix and then continues by cleaving all three strands simultaneously close to the C-terminus. Class II collagenase, on the other hand, begins to cleave in the interior of the helix [73-75]. There is also diversity of form (i.e., different isoforms) within each collagenase class. Not much is known about how these isoforms function [76], but they seem to be cross-reactive [77]. The ratio between collagenase classes I and II also seems to affect the efficacy of the enzyme blend in digesting the pancreas. No definite conclusions can be drawn because some studies have indicated an increased success in human isolations by reducing class I activity [78, 79], while the opposite has been found in rodents, where only collagenase class II is needed for pancreatic digestion if the collagenase is supplemented with neutral protease [80, 81]

Earlier collagenases from *C. histolyticum* are commonly called crude collagenase, because *C. histolyticum* produces several different collagenases that are able to degrade different types of collagen. In addition to producing collagenases, the bacteria also produce other proteases such as aminopeptidase, clostripain, phospholipase, and neutral protease, all of which have been present in the collagenase batches commercially available. Taken together, these factors make for huge variability in enzyme activity between different batches of collagenase, and sometimes even from vial to vial because of the instability of the enzymes [79] and dosing of product in the vials.

Over time, the collagenase purification has improved. Using purified collagenase alone without supplementing the enzyme blend, however, can result in insufficient digestion of the pancreas. The presence of these other proteases seems to be essential for proper release of the islets from the pancreatic parenchyma, and these additional enzymes synergize with collagenase to produce effective pancreas digestion. Caution is needed in the digestion, since excessive exposure to proteases can disintegrate and fragment islets and thus reduce islet yields [82, 83]. Collagenases containing higher concentrations of trypsin-like activity seem to facilitate significantly higher islet yields than do batches with lower amounts of this activity, an observation that may be true both in rodents and humans [84].

**Incubation prior to gradient purification**

Intact islets have a lower density than that of exocrine tissue, making a density-based separation possible. As an additional step prior to gradient separation of islets from exocrine tissue, standard protocols in many islet isolation facilities require a period of time in which all the digested tissue is incubated
in UW [85-87]. Because the islet isolation process is harmful for the tissue, it can affect cell integrity, and the resultant cell swelling is thought to be one of the reasons for the observed mixing of exocrine tissue with islets that can prevent proper separation. Therefore, a pre-incubation step is considered necessary to reduce the cell swelling while simultaneously increasing the density of the exocrine tissue. Increasing the differences in density between endocrine (islets) and exocrine tissue does appear to improve the purification of islets from exocrine tissue [85].

**Islet storage – disadvantages and benefits**

The beneficial effects of islet storage (culture) have been debated ever since the publication of the Edmonton protocol, in which freshly isolated islets were used for transplantation [48]. Several publications indicate that storage of isolated islets has detrimental effects on the islets. One factor of concern is islet survival, with several studies documenting deteriorating islet numbers in storage for both non-clinical preparations [88, 89] and clinical preparations [90-92]. Another aspect of islet storage is islet quality: Freshly isolated islets seem to be of higher quality than islets exposed to a period of storage [93-95]. These comparisons with freshly isolated islets were made for islets stored in culture flasks, which makes it difficult to extrapolate these results to today’s more sophisticated storage in semi-permeable bags, as described in the next section.

Nevertheless, there are advantages to islet storage. First, there is a logistical advantage. A short period of storage allows time for pre-transplantation quality assessment, induction of immunoregulatory protocols, and transport of patients and islets to transplant centers [96, 97]. Second, in vitro storage seems to be able to reduce the immunogenicity of islets when compared to that of freshly isolated islets [98-100]. It also can replenish islet ATP stores that have been depleted during CIT and the islet isolation procedure. When human islets are transplanted into diabetic mice, a short storage period improves post-transplant function over that of freshly isolated islets [101-104]. A short storage period may also reduce the expression of tissue factor (TF) and other inflammatory mediators and thus subsequently reduce problems associated with the instant blood-mediated immunological reaction (IBMIR) [99, 105-107]. Some studies even report beneficial effects in reducing the immunogenicity of the isolated islets, depending on which medium is used for storage [90, 108].
Methods of islet storage

Isolation disrupts the nervous and vascular connections of the islets, making the isolated islets fully dependent on the diffusion of oxygen and nutrients from the surrounding medium for their survival until revascularization is completed. Isolated islets are susceptible to anoxic damage because of their large size when compared to single cells and because of their high rate of oxygen consumption. Oxygen solubility is low in storage medium when compared to normal air. Islets have low levels of enzymes needed for energy production during anaerobic conditions, and their ability to eliminate oxygen radicals is also limited [109]. This situation creates pO₂ gradients in the islets, and central necrosis is often found in larger islets after a storage period [110]. Excess medium can decrease the oxygen availability in islets stored in standard culture T-flasks or culture dishes. The density at which the islets are stored also affects the oxygen consumption rate in the storage vessel. Most centers take samples, count the islet numbers, and then seed out the islets at a fixed number of islets per milliliter of medium. In this way, impure fractions still have more or less the same number of islets per milliliter even though these fractions may also sometimes contain large amounts of exocrine tissue, creating very high tissue concentrations in the storage container. The Nordic Network differs in its seeding of tissue in that the seeding is based on total tissue volume per milliliter of medium, independent of the ratio of islets to exocrine tissue. The rationale behind this decision is that the exocrine tissue will also consume oxygen and nutrients, so that regardless of purity of the fraction, the tissue concentration will not affect either islet recovery or functional viability.

Traditionally, islets have been kept in culture flasks, but the storage of islets in bags has also been shown to be feasible [111]. The advantages of using bags include their high sterility and safety. Bags offer a convenient and rapid way to handle large tissue volumes while at the same time reducing the risk of contamination. Bags also provide better oxygenation of the islets, since they permit gases to pass through the bag walls, including the surface on which the islets reside [112]. As compared to flasks, bags more easily provide opportunities to develop closed-system and GMP-processing protocols, making it easier to comply with regulatory demands.

Many clinical islet protocols include a storage period of 2-4 days prior to transplantation. The protocol for the Nordic Network essentially requests that the islets be kept at 37°C overnight after isolation. After the first medium change, the islets are kept at 25°C until transplantation. Reduction of the temperature can be of great importance, since the reduced temperature affects the survival of the tissue as well as the immunogenicity. Paul Lacy’s group first demonstrated the importance of reduced temperature in 1979 where they observed that prolonged islet allograft survival could be obtained by the use of in vitro storage at 24°C. The functional and morphological integrity of the islets was maintained for 1-4 weeks, and lowering the tem-
perature provided better preservation of functional integrity than at 37°C [113, 114]. Other studies have also indicated that a lower storage temperature may improve the outcome after transplantation [115-117].

Serum has long been regarded as an essential supplement to isolated cells from different tissues and mammalian species [118]. Serum seems to prevent the fragmentation of the islets in storage and preserves the secretory capacity of β-cells [119, 120]. It has been demonstrated that serum is preferred to preserve functional islet integrity and to prevent islet attachment to the storage vessel [119, 121, 122]. Some studies have reported that serum-supplemented medium is inferior to serum-free medium for preserving the function of stored human islets [123-125]. Serum-free media have to be supplemented with growth factors and nutrients to preserve the survival and function of human islets in storage [126, 127]. Human serum albumin (HSA) is also considered an alternative supplement to whole human serum [128, 129]. More recent studies, however, clearly emphasize the importance of using human serum for supplementing the storage medium, since it is better than HSA at improving human islet survival, function, and viability [130, 131].

Transfusion-transmitted diseases

Transfusion of blood components is a mode of entry for a number of pathogens. Bacterial contamination of blood components can result from exposure to skin bacteria at the time of donation. It can also be caused by asymptomatic bacterial infection of the blood donor or by contamination during blood processing. By using questionnaires assessing the risk of blood disease transmission, some unsuitable donors can be excluded. Testing donations for evidence of infection or potential infectivity is also used for pathogens that can be detected by available validated tests.

A brief glance at the history of transfusion-transmitted diseases gives a frightening perspective on how diseases have been transmitted through blood products, and some of them have had major impact on the general population. A few examples of diseases that have been transmitted:

**Syphilis** – It has been known for quite some time that syphilis can and has been transmitted by blood transfusions [132]. Today, this disease is treatable with antibiotics.

**Hepatitis** – Hepatitis has been known for several thousand years, but it was not until World War II that hepatitis emerged as an epidemic as a result of the major blood demands during the war. Several decades later, in the late 1960s, hepatitis B virus (HBV) was identified. The first antigen detected, hepatitis B surface antigen (HBsAg), became the basis of the first blood bank test for hepatitis [133]. The problem was that only 10% of the post-transfusion hepatitis was caused by HBV. Another type, hepatitis A, was
discovered, but it was not transmitted through transfusions, so one or more additional agents had to be responsible. Hepatitis C was not discovered until 1989 [139]. It is estimated that in developed countries, approximately 1 of every 50 blood units was infected with hepatitis C by the end of the 1980s. This led to development of chronic hepatitis in almost all patients receiving clotting factor concentrates, as well as in many chronic transfusion patients [134-138].

**Human immunodeficiency virus (HIV)** – In 1981, the first case of a new disease, acquired immunodeficiency syndrome (AIDS), was observed, and the disease spread rapidly. The pathogen responsible was difficult to identify, and AIDS cases continued to increase at alarming rates. In 1983-1984, HIV was discovered, and within a year an assay for anti-HIV was developed. It has been estimated that in 1987, the total number of transfusion-related HIV infections was 29,000 in the United States alone [139].

Despite screening for infectious diseases, pathogen transmission is still possible if a donation is made within the period between the time of infection and when the pathogen can be detected by a screening test. Historically, the first tests had quite low sensitivity, resulting in a relative long period for infection. For instance, the first test for HBsAg was based on gel diffusion. It took several years until the next version of tests against HBV was established [133]. The safety of blood products has improved markedly over the last two decades, at least in developed countries [140]. There are a number of serologic and nucleic acid tests that, in combination, provide a high level of safety in terms of known viral and bacterial transfusion-transmitted diseases [141]. Even so, the transmission of disease during transplantation remains a potential risk in public health care [142]. For many diseases, screening tests are not available. Transfusion-transmission of bacteria, viruses, protozoa, and prions is still a risk for patients in need of blood and blood products.

Newly emerging infections can be attributed to several different factors. One form of emergence is the discovery of previously unknown pathogens causing human diseases. A new pathogen may arise by mutation or transfer of an animal pathogen to humans. Examples of this are HIV, severe acute respiratory syndrome (SARS) in Asia, and West Nile virus (WNV) in North America [143, 144]. Known human pathogens may mutate and thus change their pathogenicity, as is the case for drug-resistant strains of bacteria and viral mutations such as that of the chikungunya virus. The virus mutation resulted in its ability to change its preferred vector to *Aedes albopictus* instead of *Aedes aegypti*, leading to the possibility of spreading the virus to new geographical areas [140].

The constant need for the improvement of safety in blood bank work has generated new techniques and equipment. Screening of blood donors and post-donation testing are reactive methods, whereas pathogen inactivation (PI) is a proactive approach to blood product safety. There are several differ-
ent pathogen inactivation methods, all with different levels of effectiveness and shortcomings [145]. PI can be efficient for many of the pathogens, including, for instance, malaria, Chagas disease, babesiosis, and bacterial contamination. PI is approved for platelets and plasma in Europe and has been in use for several years [146]. It has recently been approved by the United States’ Federal Drug Administration (FDA) for use on plasma but the Intercept system for platelets (described below) is still under regulatory review.

One specific form of PI is the Intercept® process, in which a synthetic photoactive compound, amotosalen (S-59), is used. It has been known for several thousand years that certain plants can be used to treat skin conditions such as psoriasis and vitiligo. There have been many studies that have examined the photobiology of these plants. It was discovered that the active substances in the plants consist of a group of photoactive compounds called psoralens. Psoralens and phototherapy are used in various kinds of clinical treatments for diseases such as psoriasis [147, 148], T-cell lymphoma [149, 150], and others [151]. Amotosalen HCl (S-59; Figure 1) is a synthetic psoralen compound developed by Cereus Corporation and Baxter Healthcare Corporation and used in the Intercept method. Amotosalen used in the Intercept process has received CE Mark registration as a Class III drug device. It is used clinically in many European countries [152] and has been in phase III clinical development for plasma and platelets in the United States [153]. Amotosalen has been reported to be safe in preclinical studies [154-156] and in healthy volunteers [157], as well as to be well tolerated in a wide range of patients [158-162]. It has also been used to prevent transmission of chikungunya virus during an epidemic [163]. The Intercept technology has proven to be effective toward bacteria, viruses, and protozoan parasites [164-171]. It is, however, not effective against prions, since prions are abnormally folded proteins that do not multiply in the patient. Prions can cause diseases such as bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease in humans. Some non-enveloped viruses, such as hepatitis A virus, hepatitis E virus, and poliovirus, as well as *Bacillus cereus* spores have all been demonstrated to show resilience to the Intercept process.
Materials and Methods

Please see the respective papers for a detailed outline of the specific experimental settings. Given below is a description of some of the methods used that are not commonly applied in the discipline.

Pathogen inactivation of human serum with the Intercept process (Paper I)

The Intercept® system was specifically developed to block the replication of DNA and RNA by irreversible crosslinking molecules of amotosalen HCl (a psoralen) onto RNA and DNA and thus inhibiting the ability of the pathogens to proliferate. Amotosalen does not kill the pathogens immediately, but only prevents their proliferation. Since transcription and replication are blocked, both the function and survival of the pathogens are affected, with the end result being the eventual killing of the pathogens. Amotosalen HCl molecules are small and able to penetrate cell membranes in order to reach their targets, DNA and RNA. Human serum should not be affected by this crosslinking.

There are two properties of the psoralens’ interaction with nucleic acid that make them particularly suitable for use as pathogen-inactivation agents for blood products: 1) They do not have significant sequence specificity, which means that they are not selective for the genomic material from any specific organism. 2) The cross-linking to nucleic acid and subsequent replication inhibition occur only in the presence of UVA light, and not in its absence. These properties allow for compound control through the use of UVA light; furthermore, they provide impressive potency encompassing the inactivation of any pathogen containing nucleic acids [172].

Psoralens have a three-ring structure and can be found naturally in foods such as celery, parsnips, and limes.
In our experiments, a pool of human serum from 6 blood donors was mixed and split equally into two portions. One portion was kept untreated as control, and the other was transferred into the Intercept Processing Set for Plasma. The serum was passed through an amotosalen HCl-filled container into an illumination bag and mixed. The illumination bag was exposed to UVA light and then transferred through a filter to remove the amotosalen residues. Both the amotosalen-treated and control plasma were then complement-inactivated. The details of the pathogen inactivation using the Intercept technology (Figure 2) are as follows:
Figure 2. Targeting of DNA and RNA by amotosalen molecules. Intercalation and monoadduct formation followed by crosslink formation. Used with permission from Cereus Corporation.

1. Intercalation – The first step in the pathogen inactivation process starts when the psoralens dock or insert into a helical nucleic acid strand. The process requires a rapid intercalation. The intercalated psoralen rings lies crosswise in the helical structure and parallel to the base pairs. The positioning of the ring structure in relation to the amino group is not essential for activity enhancement.

2. Monoadduct formation – When the reaction bag is placed into a UVA illumination apparatus and activated by UVA light, the intercalated psoralen undergo a photo-addition with a pyrimidine base to form a covalent monoadduct. Any ring of the psoralen’s three-ring structure can react.

3. Crosslink formation – The photo-addition process forming the monoadduct can be repeated so that the psoralens undergo another photo-addition with the opposite strand of the nucleic acid, creating a crosslink between the strands of the nucleic acid.

The psoralen can also form crosslinks in single-stranded genomes of DNA and RNA viruses. There are secondary structures in single-stranded nucleic acids, such as hairpin turns and loops, that allow for intercalation and crosslink formation by psoralens [172, 173].
Results and discussion

Pathogen inactivation of human serum used in islet storage (Paper I)

In human tissue culture and storage, human serum is widely considered to be critical for the wellbeing of cells in culture. However, given the history of transfusion-transmitted disease, there is considerable evidence to support the decision many clinical laboratories have taken to eliminate the use of human serum. Ongoing efforts through screening of blood donors and development of new tests have markedly increased patient safety. New technologies, such as PCR tests, are both more sensitive and quicker than older techniques and have reduced the time window from donation to analytic results. The combination of nucleic acid and serological tests provides a high safety level against known bacterial and viral pathogens [141]. All these efforts, however, are reactive and dependent on our knowledge of the pathogens.

Despite all our efforts, transfusion-transmitted diseases are still a risk for patients in need of blood and blood components. For many pathogens, there are no tests available, and there is always a risk from yet-unidentified pathogens.

Pathogen inactivation could offer a convenient, safe and proactive approach to the problem, since it can inhibit transcription and replication by all pathogens containing DNA and RNA.

The aim of this study was to evaluate whether pathogen inactivation with the Intercept system by Cereus, which already in use in many European blood banks for plasma, could be applied to human serum used in human islet isolations without negatively affecting the islets.

Human islets from 12 preparations were kept in storage for 12-24 hours. Following the release of the islets for research, the medium was changed and aliquots of 1200 islets were put into untreated Petri dishes, then suspended in CMRL-1066 supplemented with either pathogen-inactivated serum or control serum. The islets were kept in storage for 3-4 days, after which quality assessment was performed.

When challenged in a dynamic glucose perfusion, the islets kept in culture medium supplemented with pathogen-inactivated serum displayed an insulin release that was similar to that of the control islets. There was also no difference in stimulation index between the two groups.
Expression on the islets of the immunoregulatory cytokines TF (0.034 vs. 0.033 fmol/µl DNA), MCP-1 (0.064 vs. 0.0454 fmol/µl DNA), IL-6 (0.0024 vs. 0.0024 fmol/ml DNA), and IL-8 (0.058 vs. 0.0023 fmol/µl DNA) was not altered by using pathogen-inactivated serum, when compared to islets kept in medium supplemented with control serum. Similarly, no difference was found in the number of apoptotic/necrotic cells, as measured with an ADP/ATP ratio kit.

When in vivo functionality was evaluated by transplantation of islets under the kidney capsule of nude mice with streptozotocin-induced diabetes, islets kept in medium supplemented with PI serum and control islets displayed a similar potency in curing mice. Islets kept in PI serum cured 7 out of 9 mice, as compared to 8 out of 9 mice in the control group. Nephrectomy performed after 35 days post-transplant induced diabetes again, and thus confirmed the transplanted islets’ function in both cases.

Serum supplementation of culture medium is considered to be important for the preservation of functionality and the morphology of isolated islets, both in humans and other species [119-121]. Some studies have indicated that serum-supplemented medium is inferior to serum-free medium [123, 124] but the medium must then be supplemented with growth factors and nutrients to compensate for the missing serum components. More recent studies have strongly emphasized the importance of serum as a supplement to the medium [130, 131].

The results generated in this study support pathogen inactivation using Intercept technology to treat human serum used in human islet storage. PI did not affect either the functional integrity or viability of the islets. By implementing this technique routinely on human serum, prevention of pathogen transmission would potentially enable the use of human serum for clinical cell therapy.

Shortening of the isolation time by one hour (Paper II)

Almost all islet isolation protocols utilize density gradient centrifugation for separation of the islets from exocrine tissue, relying on the differences in density between the two. Improved results have been obtained when a pre-purification incubation step is used in which the digested tissue is incubated in UW for 30-60 minutes.

Because of the harm caused by organ retrieval, cold ischemia during transport, and damage from the islet isolation process, the goal has always been to reduce the time from procurement to finished islet isolation. We asked whether the islet isolation time could be shortened by removal of the pre-purification step without affecting the purification step or the isolation outcome. The reduction or removal of pre-purification incubation is im-
portant, because this approach to incubation should be considered some form of semi-CIT instead of properly controlled CIT.

The outcomes from preparations with a shortened incubation time included no differences in terms of total islet equivalents obtained, the total purity, or the transplantation rate between standard preparations and those with a shortened incubation time. In terms of quality assessment, the intracellular insulin content/DNA and the expression of cytokines IL-6, IL-8, MCP-1, and TF were not altered by the shortened incubation time. The islets’ ability to release insulin in response to dynamic glucose challenge was also not affected by the shortened incubation time.

As measured from the start of the enzyme infusion into the pancreas to when the purified islets were placed in the incubator, the total isolation time had been reduced by almost an hour when compared to standard incubation isolations.

By reducing the incubation time to a minimum, the purification step could be immediately performed, shortening total islet isolation time. Time reduction is important because it allows the tissue to start recovering sooner. It also reduces costs and might enable multiple organs to be handled within the same day.

Addition of clostripain to the enzyme blend (Paper III)

One of the most important steps in the isolation process is the enzymatic denaturation/digestion of the collagens, which constitute the major component of the interface between the islets and exocrine tissue. In order to achieve release of the islets, collagenase enzymes are used. Commercially available collagenases are produced using *Clostridium histolyticum* and, because of the nature of their production, multiple isoforms and classes of collagenases are mixed together with proteases in the product called crude collagenase. Historically, this situation has created great variability between different batches and sometimes even within different vials in the same batch [79, 174, 175], perhaps as a result of degradation occurring during storage prior to use.

Collagenase batches containing higher levels of tryptic-like activity (TLA) have been found to be more efficient than batches with lower levels of TLA. TLA was previously considered a by-product of the production process and, with improved purification, it has been almost entirely removed from the new, high-purity collagenases. TLA is thought to originate from clostripain, which is one of the latest commercially available supplemental enzymes. The aim of this study was to evaluate the potential beneficial effects of adding clostripain to the enzyme blend used for islet isolation.

One third of a vial of clostripain, 141 BAEE Units, was added to the enzyme blend of collagenase and thermolysin to give a final dose of 8% TLA
in 12 clinical-grade isolations; these preparations were then compared to 24 matched controls. Since donor matching was performed, there was no difference in terms of donor factors between the groups.

When isolation characteristics were compared, no difference could be found in terms of dissection time or pancreas weight. Digestion time was the same, and after digestion, the remaining undigested tissue in the chamber was similar in both groups, as was the harvest time and total pellet volume prior to purification. Likewise, the collagenase activity was similar in both groups; however, for the control pancreata, the amount of thermolysin used was higher (median, 2,999,333 U; range, 830,000-3,974,000 U) than that for the clostripain group (median, 1,502,500 U; range, 1,485,000-1,506,000 U; P=0.0030 between the groups).

The outcomes of the isolations were significantly different, with the use of clostripain in the study group yielding higher IE numbers (median, 391,565; range, 223,368-657,609 IE vs. median, 254,765 IE; range, 24,130-588,696 IE for the control group), higher IE/g pancreas (median, 3,598; range, 2440-6246 vs. median, 2,498; range, 233-6,086), and higher purified tissue volumes (median, 2,235 µl; range, 1,100-6,025 µl vs. median, 1,333 µl; range, 200-3,100 µl) for the clostripain group than for the control group. As for other outcome parameters, there were no differences in the total purity, recovery after 1 day of storage or size distribution between the two groups.

Even though extra proteases in the form of clostripain were added, the functionality of the islets was not affected, as demonstrated by both the similar stimulation index and levels of released insulin in response to dynamic glucose challenge. Size distribution showed that the addition of an extra protease did not affect the islet size.

Because of the increased number of isolated islets, there was a significant increase in the number of preparations fulfilling standard transplant release criteria in the clostripain group as compared to the controls. In the clostripain group, all 12 preparations fulfilled the transplantation criteria, versus 11 out of 24 control preparations.

Since no detrimental effects on functional viability could be detected for the islets isolated with clostripain, and the isolation outcome was markedly improved, the addition of clostripain to the enzyme blend has now been implemented in clinical standard protocols in the NNCIT isolation facilities.
Expanding cold ischemic time using I-Let protect preservation medium (Paper IV)

The importance of keeping the cold ischemic time as short as possible cannot be overstated. For practical reasons, however, cold ischemia is difficult to avoid completely and eventually leads to tissue damage.

The most commonly used preservation solutions are UW and HTK, both successfully used for the preservation of multiple organs. HTK, however, has been found to have negative effect on the isolation outcome and should be avoided for pancreatic islet preservation [64]. Two-layer preservation is also used for the preservation of pancreata intended for islet isolation, but its possible beneficial effect is not settled, because conflicting results have been presented [60, 66-68].

This study aimed to evaluate whether a newly developed preservation solution, F6H8S5, known as I-Let protect, would preserve pancreata intended for islet isolations at least as well as the standard preservation solutions. In a follow-up study, I-Let protect was evaluated as to whether it could be included in a protocol prolonging CIT prior to islet isolation without affecting islet functionality or isolation outcome. For ethical reasons, we could not take clinical grade pancreata and increase the CIT to >18 hours to create a control group for the follow-up study. Therefore, the control group in the follow-up study was comprised of clinical-grade pancreata with a CIT of <10 hours.

In the first part of the study, no differences were found in the trimmed pancreas weight, dissection or digestion time, total harvest time, remaining tissue in the chamber post-harvest, or total packed tissue pellet volume between pancreata preserved in I-Let protect and control pancreata. Donor and procurement factors also showed no differences between groups. An analysis of isolation outcome also did not find any differences in terms of islet yield or purity. Also, the fulfillment of transplant release criteria was similar in both groups.

Functionality, as measured by insulin secretion in response to glucose stimulation, did not differ between the two groups. Because recovery after overnight storage was similar, we concluded that I-Let protect did not have any detrimental effects on preserved pancreases. Disappointingly, it also had no beneficial effects similar to those found in a previous pre-clinical study [176]. The reason for the differences in results when compared to the pre-clinical study might be attributed to differences in the handling of the pancreas prior to its immersion in I-Let protect. In the pre-clinical study, the duodenum was removed and a rough trimming of the pancreas was performed, whereas in the present clinical study, the pancreas was immediately immersed together with duodenum, fat, and blood vessels. The presence of duodenum, fat, and blood vessels may have affected the accessibility of the pancreas to the preservation solution, thus impairing penetration into the
pancreatic tissue. It may also be that a CIT of <10 hours was an insufficient amount of time to yield any differences with good-quality clinical organs when compared to standard preservation solutions.

In the follow-up study, donor and organ procurement factors did not differ between pancreata with prolonged CIT preserved in I-Let protect and control organs, except for the CIT, with the controls having a significantly shorter CIT as a result of the study setup. In the prolonged CIT group, the trimmed pancreas weight was lower, the digestion time shorter and the total packed tissue volume prior to separation was lower than that of the control group. Even so, the isolation outcome did not differ between the groups in terms of IE or purity.

Prolonged CIT combined with I-Let protect preservation also preserved the capacity of the islets to release insulin in response to dynamic glucose challenge, resembling control organs with a CIT of almost half that in the study group.

Fulfillment of transplant release criteria did not differ between the two groups: 5 out of 6 organs in the control group fulfilled the criteria, as compared to 6 out of 6 organs preserved in I-Let protect with prolonged CIT.

We concluded that I-Let protect could indeed be used in situations of prolonged CIT without affecting functionality or isolation outcome. It can be used for establishing a protocol in which pancreata arriving in the late evening or at night can be roughly trimmed, placed into oxygenated I-Let protect, and subjected to islet isolation the following day. Such a system would eliminate the need for night-time isolations and reduce costs related to working outside office hours. The increased flexibility would also enable a central isolation center to handle organs arriving at the same time, while still retaining meaningful islet functionality and isolation outcome.

If donor acceptance criteria are expanded and pancreata from non-heart-beating donors are procured, I-Let protect could be used to preserve the pancreas during transport.
Conclusions

Specific conclusions

Paper I
- Pathogen inactivation can be applied to human serum used in islet storage without any negative effects on the islets
- Pathogen inactivation will enable the use of human serum in clinical cell transplantation programs without endangering patient safety

Paper II
- Isolation time can be shortened by one hour, thereby reducing the ischemic time associated with the islet isolation process

Paper III
- Addition of clostripain to the enzyme blend will increase the number of successful islet isolations and transplantations
- Use of clostripain does not detrimentally affect the isolated islets

Paper IV
- I-Let protect preserves islets as well as standard preservation solutions for CIT <10 hours
- I-Let protect can be used to prolong CIT, with similar isolation outcomes and islet functionality to those obtained with high-quality pancreases and short CIT

General conclusions

Through the results presented in this thesis, improvements in the islet preparation process can be performed to increase the number of successful preparations, shorten the isolation time, and increase patient safety.
Critical considerations regarding research design and methods

It is difficult to evaluate islet numbers. There are no standardized methods that are objective and consistent, making it very difficult, if not impossible, to make comparisons between different centers. Isolation centers take what are hopefully representative samples, and manual counting and size classification usually follow. This is the standard method used in most isolation centers. The results are highly subjective and depend heavily on the experience of the investigators. Even though a validated technique based on digital image analysis, with less variation than that seen with manual counting, was used for all evaluations of islet numbers in this thesis [177, 178], sampling was still a source of variation and uncertainty.

The standard clinical islet isolation protocol in the NNCIT does not call for samples of the digested tissue pool to be obtained prior to purification. As a result, no IE numbers or estimation of percentage of embedded islets are determined, thus making it difficult to evaluate whether the addition of clostripain to the enzyme blend would result in fewer embedded islets and possibly explain the increased IE numbers in the clostripain group in Paper III.

In Paper I, the Intercept method was used for pathogen inactivation of human serum. The rationale for choosing this technology was based on the results obtained for plasma and experience from the blood bank in Uppsala. There are, however, other technologies available that were not used. The results and outcome might have been different if another method had been used.
Future perspectives

Islet isolation and subsequent transplantation have been, and still are, limited by multiple factors that affect the isolation and transplantation outcome, from organ shortage, CIT, and enzyme variations to immunosuppression and destruction of islets at the time of transplantation [179, 180].

Organ preservation is very important, since even the most efficient isolation protocol cannot save tissue damaged by cold ischemia during transport. Newly developed mechanical technological solutions have had promising results, but the costs are high, and thus far they have also required specially trained personal to operate them. Improvements in the technology and ease of use will probably make new technical methods more commonly adopted in the future. Until then, static oxygenation is still the most convenient way to preserve the pancreas.

Because of the way they are produced, batch-to-batch variation in commercially available enzymes is inevitable. Well-purified and well-defined enzymes make it easier to tailor enzyme combinations and thus to counter variation. Recombinant produced enzymes could be the future, since variation should be eliminated, at least in theory.

Macro-encapsulation and nano-encapsulation of islets could be a promising way of preventing rejection in the future. Macro-encapsulation has already been used in phase I clinical studies, but nano-encapsulation is still in preclinical models. Covering the islet surface is a similar model that can be used for the prevention of the IBMIR. The islets could, for instance, be covered with heparin to prevent blood clotting. Mesenchymal stem cells or endothelial cells could also be used to promote revascularization. From a regulatory point of view, these treatments will classify the products as advanced-therapy medicinal products (ATMP) with more complex regulatory systems.

Using pluripotent human embryonic stem cells to differentiate into insulin-producing cells is no longer a theoretic model. Phase I clinical trials have been initiated, and at least four patients have received transplants thus far. Also, with the implementation of pathogen inactivation, human serum may prove safe for use in this therapy.
Type 1 diabetes is a disease with increasing prevalence. The disease is characterized by destruction of the only insulin-producing cells in the body, the \( \beta \)-cells. \( \beta \)-cells are located in cell clusters called islets of Langerhans in the pancreas, where they constitute 1-4\% of the organ; the rest of the pancreatic tissue is composed of exocrine tissue that produces enzymes used by the body for digesting food. Standard type 1 diabetes treatment is based on multiple daily injections of insulin. For some patients, however, insulin treatment is ineffective, and these individuals suffer from fluctuations in blood sugar, from extremely high to life-threateningly low.

One of two options for normalizing blood sugar levels in diabetics is islet transplantation. In order to transplant islets, they first need to be released from the rest of the pancreatic tissue, in a process known as islet isolation. The outcome of islet isolation is highly variable, and many preparations unfortunately never reach patients because of their insufficient islet numbers or islet quality. This thesis addresses some of the variables in this process, with the goal of improving isolation results in order to be able to treat more patients.

One critical variable is preservation of the pancreas during transport from procurement hospital to isolation center. We evaluated a newly developed preservation solution, I-Let protect, and compared it to standard preservation solutions. By using I-Let protect, we found that the cold ischemic time (time from organ procurement to the start of islet isolation) could be prolonged to more than 20 hours without any negative effect on the isolation outcome, while preserving islet function when compared to standard solutions that have a cold ischemic time of less than 10 hours. This new preservation solution will enable isolation centers to handle multiple organs arriving on the same day and also to avoid the necessity for night-time isolations.

One vital step in the isolation procedure is enzymatic digestion of the pancreas via the addition of collagenase and other proteases that disrupt collagen in the interface between the islets and exocrine tissue. Unfortunately, because of the process for producing collagenases, there have been and still are great variations between different batches of enzymes, and sometimes even within the same batch. We have evaluated the addition of a newly available protease enzyme, clostripain, to other enzymes used to digest the pancreas. The addition of clostripain increased the number of islets isolated...
and consequently the number of preparations that could be transplanted into diabetic patients.

Prior to separation of islets from other pancreatic tissue, standard protocols place enzyme-digested tissue in preservation solution at +4°C for approximately 1 hour to increase the difference in density between islets and exocrine tissue. By evaluating reduced incubation times, we found that the total isolation time could be reduced by almost one hour. This time reduction will get islets more quickly into incubator, where they can recover, and isolation costs are reduced.

Isolated islets can either be transplanted immediately or kept in storage (culture) for a few days. Storage has the advantage of allowing for quality testing, the starting of immunosuppressive treatments, and the transport of both patients and islets to a transplant center. It is considered beneficial to add human serum to the medium during storage, but as with all blood products, it brings a risk of transfusion-transmitted diseases. In one paper, we evaluated a way of treating human serum to inactivate pathogens and found that pathogen-inactivated human serum worked as well as did untreated serum. Treated serum can therefore be used in clinical cell therapies without risking patient safety.

Some of the results of these studies have already been implemented in our clinical work. Through a shortened incubation time, we now routinely achieve isolation times of 3 hours. We have also added clostripain to our standard isolation protocol. Pathogen inactivation of human serum has also been implemented. We will start to use I-Let protect as described earlier, which will lead to the avoidance of night-time work as well as more flexibility in our islet isolation facility.
Typ 1 diabetes är en sjukdom där de insulinproducerande cellerna i kroppen, β-cellerna, av oklar orsak förstörs av kroppens egna immunförsvar. β-cellerna finns i cellklumpar, så kallade Langerhanska cellöar, i bukspottkörteln där cellöarna utgör 1-4% av hela organet. Resten av bukspottskörteln består av vävnad som producerar matsmältningsenzym. Injektion av insulin flera gånger per dag är standardbehandling för typ 1 diabetes men för vissa patienter fungerar detta inte utan de har stora svängningar i sitt blodsocker från högt till så lågt att de kan hamna i insulinkoma och dö. För dessa patienter kan transplantation av Langerhanska cellöar vara en livräddande behandling.

Det finns två sätt att normalisera blodsockret och o-cellstransplantation är ett av dem. Innan man kan transplantera de Langerhanska öarna så måste de först frigöras från övrig pankreasvävnad genom o-cellsisolering. Resultaten från isoleringarna varierar och tyvärr så är det många preparationer som inte blir transplanterade till patienter på grund av för få eller dålig kvalitet. Denna avhandling behandlar några av variablerna som påverkar resultatet med målsättningen att förbättra isoleringsresultaten så att fler patienter kan bli behandlade.

En kritisk faktor är hur pankreas bevaras under transport från uttagssjukhuset till o-isoleringslaboratoriet. Vi utvärderade en ny preservationslösning, kallad I-Let protect, och jämförde med standardlösningar som används vid transport av pankreas. Genom att använda I-Let protect kunde vi förlänga den kalla ischemitiden (tid från organuttag till start av o-cellsisolering) till mer än 20 timmar utan att det hade någon negativ påverkan på mängden öar vi fick från isoleringarna jämfört med isoleringar där standardlösningar använts och med en kall ischemitid på mindre än 10 timmar. Detta gör att isoleringslaboratorier kan hantera flera organ som kommer samma dag och det gör även att nattarbete kan undvikas.

Ett annat kritiskt moment i isoleringsprocessen är enzymatisk nedbrytning av pankreas med hjälp av kollagenas och andra proteasenzymer som bryter ner kollagenet som finns vid gränsytan mellan öar och exokrin vävnad. På grund av tillverkningsprocessen av kollagenas har det, ända sedan de började användas, funnits stora variationer i effektivitet mellan olika batcher och ibland till och med inom samma batch. Vi utvärderade tillsats av ett nytt
proteas enzym, clostripain, till enzymblandningen som används vid isoleringar. När clostripain användes fick vi signifikant fler öar jämfört med isoleringar utan clostripain, vilket i sin tur gjorde att vi kunde transplantera fler patienter.

Innan gradientseparation där öarna skiljs från övrig pankreasvävnad, är det vanligt att den digerade vävnaden preinkuberas, d.v.s. placeras kallt i kylskåp i ca 1 timme. Detta görs för att öka skillnaden mellan öarna och den exokrina vävnaden. Resultaten från det tredje arbetet visar att även om denna preinkubering tas bort, fungerar separationen lika bra ändå. Denna tidsförkortning gör att öarna kommer in snabbare i inkubator där de kan börja återhämta sig och hela isoleringsprocessen blir cirka en timme kortare.


Från resultaten i dessa arbeten har vi kunnat förkorta isoleringsprocessen med nästan en timme. Vi har också rutinmässigt börjat använda clostripain i vårt kliniska arbete. Patogeninaktivering av humanserum har även detta börjat användas kliniskt. Vi kommer inom kort börja använda I-Let protect enligt beskrivning ovan vilket kommer reducera nattarbete samtidigt som det öka flexibiliteten vid vårt isoleringslabb.
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